Induction and Repression of Steroid Hydroxylases and Dehydrogenases in Mixed Culture Fermentations

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SUMMARY

Some factors affecting induction and repression of steroid-transforming enzymes were studied in single and mixed cultures of *Arthrobacter simplex*, *Nocardia restrictus* and *Streptomyces roseochromogenes*. In single or mixed cultures induction and repression of 1-dehydrogenase, 16 α -hydroxylase, and 20-ketoreductase were found to be dependent on the type of steroid substrate and the composition of the medium. In mixed cultures patterns of growth as well as enzyme induction and repression were altered by culture interaction. An observation having practical significance was that 16 α -hydroxylation and 1-dehydrogenation of 9 α -fluorohydrocortisone to triamcinolone could be done, in a single fermentation operation, by a mixed culture of *A. simplex* and *S. roseochromogenes*. The practicality of this transformation derives from the fact that 20-ketoreductase, an enzyme responsible for the production of undesirable by-products in cultures of *A. simplex*, was completely repressed in the mixed culture system. Although growth of *A. simplex* was suppressed by metabolites of *S. roseochromogenes*, conversion of steroids with this mixed culture was reproducible and controllable within a wide range of ratios of organisms in the starting mixtures.

INTRODUCTION

Multiple steroid transformations by mixed cultures have been reported by McAleer, Dulaney & Dulaney (1959) and Spalla, Modelli & Amici (1962). However, they added different micro-organisms sequentially and did multiple conversions in stepwise operations without isolation of the intermediates. The first practical mixed-culture fermentation for steroid conversion was reported by Shull (1959). He mixed a diluted culture of *Curvularia lunata* (11 β -hydroxylator) with a fresh inoculum of *Mycobacterium phlei* (1-dehydrogenator) and did the conversion of cortexolone to prednisolone in a single operation. Kimura (1962) reported the transformation of cortexolone to prednisolone by mixed cultures of *C. lunata* and *Bacillus sphaericus*.

We have found that *Arthrobacter simplex* (1-dehydrogenator) and *Streptomyces roseochromogenes* (16 α -hydroxylator) can transform 9 α -fluorohydrocortisone (F2) to 1-dehydro-16 α -hydroxy-9 α -fluorohydrocortisone (triamcinolone, F4) in a one-step fermentation. In our mixed culture fermentation with *A. simplex* and *S. roseochromogenes*, F2 is first converted to the intermediate, 1-dehydro-9 α -fluorohydrocortisone (Δ^1 -F2), which is subsequently transformed to F4 (Fig. 1). 16 α -Hydroxy-9 α -fluorohydrocortisone (F3) is not detected. Unless the mixed culture fermentation is carried out under conditions in which 20-ketoreductase is repressed, however, all of the substrate (F2) and the intermediate (Δ^1 -F2) formed in the early stage of the fermenta-

tion process are reduced at the 20-keto position. Thus the success of carrying out the multiple conversion of F2 to F4 depends entirely upon providing conditions in which 1-dehydrogenase and 16 α -hydroxylase are selectively induced and 20-ketoreductase is repressed. We have found that the undesirable enzyme, 20-ketoreductase, which is induced when *A. simplex* is grown alone in soybean meal medium, is repressed when cultures of *A. simplex* are mixed with *S. roseochromogenes*.

Fig. 1. Conversion steps of 9 α -fluorohydrocortisone (F2) to 1-dehydro-16 α -hydroxy-9 α -fluorohydrocortisone (F4) by mixed culture of *Arthrobacter simplex* and *Streptomyces roseochromogenes*.

METHODS

Organisms. The organisms used were: *Arthrobacter simplex*-X^s (original Squibb Culture No. 6062-V3, which is sensitive to an antibiotic substance or substances (X) produced by *S. roseochromogenes*); *Arthrobacter simplex*-X^r (Squibb Culture No. 6216-VI, which is resistant to X); *Streptomyces roseochromogenes* (Squibb Culture No. 6186); *Nocardia restrictus* (Squibb Culture No. 2914). The *A. simplex* strains were maintained on yeast beef agar slopes, and the *S. roseochromogenes* and *N. restrictus* cultures on glucose yeast extract agar slants. Stock cultures were stored as suspensions in skimmed milk, frozen and held over liquid nitrogen at about -150° .

Culture growth. All cultures were grown in 100 ml. medium in 500 ml. Erlenmeyer flasks and shaken at 25° on a Gyrotory G-52 shaking machine (New Brunswick Scientific Co.) at 280 cycles/min. in a 2-inch diameter circle. Inoculum was developed in two 48-hr stages. For the fermentation stage 5 ml. portions of each vegetative culture were used to inoculate 100 ml. of the broth. Samples (5 ml.) were removed at intervals for colony counts and steroid measurement. Steroid substrate and organisms were added at zero time.

Media. Germination medium E33 contained (g.) 15, extracted soybean meal (Archer-Daniels-Midland, Minneapolis); 11, glucose; 2, CaCO₃; 2.2, soybean oil; in 1 l. distilled water. Fermentation medium E34 was made of (g.) 20, extracted soybean meal; 33, glucose; 7.5, CaCO₃; 2.2, soybean oil; 1, KH₂PO₄; 1, K₂HPO₄; in 1 l. distilled water.

Growth curves. Growth of each organism in pure culture and mixed culture systems was followed by colony counts of samples drawn, at intervals, from the fermentation broth.

Inoculum ratios. The ratios were determined on the basis of colony counts of samples drawn, at zero time, from the mixed culture fermentation broth.

Preparation of samples for steroid measurement. Five-ml. samples were drawn at intervals from the fermentation broth, and each sample extracted with 2 ml. 4-methyl-2-pentanone (MIBK).

Paper chromatography. Steroid samples (0.1 ml. each of the MIBK extracts) were spotted and developed by descending chromatography on Whatman No. 1 paper in benzene ethanol water (50 + 25 + 50, by vol.) solvent.

Steroid identification and measurement. Following development of the chromatograms, steroid spots were detected by ultraviolet (u.v.) scanning. For identification heavy reliance was placed on comparative mobility (R_f) values, established with authentic materials prepared in our laboratories in fermentations conducted with these organisms. One or more standards were placed on each papergram. For quantitative estimation the detected spots were cut out, eluted with 95 % (v/v) ethanol in water, and read at wavelength 240 m μ on a Beckman DU spectrophotometer. The concentration of steroid in each sample was then estimated by comparison with a standard solution equilibrated with MIBK and chromatographed as were the samples.

Enzyme identification. Qualitative identification of enzyme activities was made by noting the presence or absence of characteristic steroid transformation products. Positive identification of steroids was made by isolating and characterizing products in a few mixed culture experiments. The transformation products to be expected with *Streptomyces roseochromogenes* in single culture with these steroid substrates have been described by Smith, Foell & Goodman (1962). The transformation products to be expected with pure cultures of *Arthrobacter simplex* and *Nocardia restrictus* have been described by Goodman, May & Smith (1960).

RESULTS

Enzyme induction and repression in pure cultures of Arthrobacter simplex-X^s

In the transformation of F2 to Δ^1 -F2, 1-dehydrogenase and 20-ketoreductase play critical roles. Both 1-dehydrogenase and 20-ketoreductase were induced, when *Arthrobacter simplex-X^s* was germinated in soybean meal medium E33 and then fermented in soybean meal medium E34 containing F2 as substrate. As a result, the organism transformed some of the substrate F2 to Δ^1 -F2, and then simultaneously converted compounds F2 and Δ^1 -F2 to 20-dihydro-F2 and Δ^1 -20-dihydro-F2. Consequently, only 20-dihydro products were seen in fermentations lasting longer than 23 hr. In contrast, selective induction of 1-dehydrogenase and repression of 20-ketoreductase were observed when *A. simplex-X^s* was germinated in two stages, using peptone yeast extract (Difco, Detroit) medium and Yeastamin (Vico Products, Chicago) medium and then fermented in Yeastamin medium containing steroid substrate, F2.

The enzymic response of *Arthrobacter simplex-X^s* to a different steroid, 16 α -hydroxy-cortexolone 16,17-acetonide, was studied in a different series of media. Both 1-dehydrogenase and 20-ketoreductase were repressed, when *A. simplex-X^s* was germinated in two stages of soybean meal medium E33 and fermented in cornsteep liquor medium E24. However, selective induction of 1-dehydrogenase and repression of 20-ketoreductase were seen when *A. simplex-X^s*, germinated in two stages of cornsteep liquor medium E24, was fermented in the same cornsteep liquor medium.

Enzyme induction and repression in pure cultures of Streptomyces roseochromogenes

Induction of 16 α -hydroxylase and repression of 20-ketoreductase were observed when *Streptomyces roseochromogenes* was germinated in soybean meal medium E33

and fermented in soybean meal medium E34 containing F2 steroid substrate. Under these conditions the organism converted all of the F2 substrate to F3 in 97 hr, without producing any detectable 20-dihydro-product. When *S. roseochromogenes* was exposed to a different steroid, cortexolone, in the same sequence of soybean meal media (E33 $\rightarrow$ E34), however, both 16 α -hydroxylase and 20-ketoreductase were induced. On the other hand, the two enzymes were both repressed when the organism was germinated in two stages of peptone-yeast extract medium E6 and Yeastamin medium E2I, and fermented in E2I with F2 steroid substrate.

Fig. 2. Steroid conversion curves by mixed cultures of *Arthrobacter simplex* and *Streptomyces roseochromogenes*. The two organisms were grown separately in two stages of medium E33, and inoculated together into E34 containing F2 230 $\mu\text{g./ml.}$ (1) F2: 9 α -fluorohydrocortisone (substrate); (2) Δ^1 -F2: 1-dehydro-9 α -fluorohydrocortisone (intermediate); (3) F4: 1-dehydro-16 α -hydroxy-9 α -fluorohydrocortisone (product).

Patterns of enzyme induction and repression in the mixed culture system of Arthrobacter simplex-X^s and Streptomyces roseochromogenes

When the two organisms were grown separately in two stages of soybean meal medium E33, mixed and fermented in soybean meal medium E34 containing F2 steroid substrate, induction of 1-dehydrogenase by *Arthrobacter simplex*-X^s and 16 α -hydroxylase by *Streptomyces roseochromogenes* was seen. However, 20-ketoreductase which was induced in the pure culture system of *A. simplex*-X^s was repressed in the mixed culture system of this organism and *S. roseochromogenes*. Thus the mixed system allowed development of conditions that influenced a change from induction to repression of the 20-ketoreductase of *A. simplex*-X^s, the induction of which is not desirable in the transformation of F2 to F4. A time course experiment was done with this F2 to F4 conversion system. Substrate F2 (230 $\mu\text{g./ml.}$) was almost completely converted to Δ^1 -F2 intermediate within 48 hr. Conversion of Δ^1 -F2 to F4

was initiated early and continued after F2 had disappeared. The maximal titre of final product was achieved in 72 hr in this experiment (Fig. 2).

Enzyme induction and repression in pure cultures of Nocardia restrictus and in the mixed culture system of this organism and Streptomyces roseochromogenes

Nocardia restrictus (SC 2914) is known to possess both 1-dehydrogenase and 20-ketoreductase. For comparison with *Arthrobacter simplex*-X^s, this organism was therefore tested in a single culture system and in a mixed culture system with *Streptomyces roseochromogenes*. Induction of 1-dehydrogenase and repression of 20-ketoreductase was seen in the single culture system of *N. restrictus* when tested in soybean

Fig. 3. Effects of changing initial ratios of *Arthrobacter simplex*-X^s inoculum and *Streptomyces roseochromogenes* inoculum on growth in E34 medium. A: *A. simplex*-X^s; S: *S. roseochromogenes*.

meal media (E33 → E33 → E34). In the mixed culture system of the organism with *S. roseochromogenes*, on the other hand, selective induction of 16 α -hydroxylase and repression of both 1-dehydrogenase and 20-ketoreductase were seen. Thus the results illustrate a different type of altered enzyme induction and repression with a different mixture of organisms.

The effects of changing initial ratios of inoculum of Arthrobacter simplex-X^s and Streptomyces roseochromogenes on growth curves and steroid transformation

It was suspected that one of the factors controlling the success of the multiple steroid transformation by the mixed cultures might be the ratio in which the two organisms were mixed. By changing the volumes of inoculum, five different ratios of

viable organisms (colony count) were tested: (1) 5.0×10^{10} *Arthrobacter simplex*-X^s: 6.0×10^9 *Streptomyces roseochromogenes* (8.3:1) at 0 time; (2) 3.0×10^{10} : 7.0×10^9 (4.3:1) at 0 time; and 5.5×10^{10} : 0 at 23 hr; (3) 1.0×10^{11} : 8.5×10^8 (11.8:1) at 0 time; (4) 2.7×10^{11} : 8.0×10^9 (33.8:1) at 0 time; (5) 6.9×10^{10} : 1.6×10^{10} (4.3:1) at 0 time. For each condition, growth curves of *A. simplex*-X^s and *S. roseochromogenes* were followed up to 71 hr and F₄ product was measured after 71 hr of fermentation. In the range of mixture ratios covered, similar growth patterns were observed (Fig. 3). The patterns of steroid transformation were also alike in the different sets of conditions, and the overall yields ranged from 70 to 89 % (mole/mole) (Table 1). Thus the ratio of inocula in the range covered did not have any significant effect on the multiple steroid transformation.

Table 1. *Effect on steroid conversion of changing ratios of inocula of Arthrobacter simplex-X^s and Streptomyces roseochromogenes*

Sample*	Fermentation time (hr)	F ₂ input μ g./ml. broth	F ₄ found μ g./ml. broth	Conversion mole (%)
L6-1	0	234	—	—
	71	—	199	83
L6-2	0	226	—	—
	71	—	187	81
L6-3	0	264	—	—
	71	—	244	89
L6-4	0	286	—	—
	71	—	236	81
L6-5	0	224	—	—
	71	—	162	70

Inoculum size was determined by viable organism count.

* Samples. Ratios of *A. simplex* X^s organisms to *S. roseochromogenes* organisms at 0 time,

L 6-1 8.3:1
 L 6-2 4.3:1 and 5.5×10^{10} : 0 at 23 hr
 L 6-3 11.8:1
 L 6-4 33.8:1
 L 6-5 4.3:1

Growth of Arthrobacter simplex strains and Streptomyces roseochromogenes under mixed culture conditions

The characteristics of growth of each organism were studied during the mixed culture fermentation with *Arthrobacter simplex*-X^s or *A. simplex*-X^r and *Streptomyces roseochromogenes*. When *A. simplex*-X^s was cultured alone in soybean meal medium E34 growth reached a maximum within 24 hr and the colony count remained more or less unchanged up to 120 hr. On the other hand, the maximum growth of *S. roseochromogenes* in pure culture was attained at 96 hr and then decreased at 120 hr, probably because of exhaustion of nutrients. When the two organisms were mixed and grown together in soybean meal medium E34, *A. simplex*-X^s decreased in number and nearly disappeared in less than 48 hr. *A. simplex* resistant to *S. roseochromogenes* metabolites appeared after 72 hr.

The antibiotic-producing capability of *Streptomyces roseochromogenes* has been reported by several laboratories, since the earliest report by Ishida (1950). In these

studies, we have made no effort to identify the inhibitory substances produced by the strain of *S. roseochromogenes* used. Earlier we had recognized that an antagonism between these cultures existed and could be shown by conventional techniques, namely, cross-streaking on agar, or tube-dilution assaying of *S. roseochromogenes* culture fluid with *Arthrobacter simplex* as the test organism.

A resistant mutant (*Arthrobacter simplex*-X^r) was isolated from 240 hr fermentation broth of a mixed culture of the parent *A. simplex*-X^s and *Streptomyces roseochromogenes*.

Fig. 4

Fig. 5

Fig. 4. Growth curves of *A. simplex*-X^s and *S. roseochromogenes* in mixed culture fermentation during which *A. simplex*-X^r mutants are developed. (1) A-X^s: *A. simplex*-X^s, (2) A-X^r: *A. simplex*-X^r, (3) S: *S. roseochromogenes*.

Fig. 5. Growth curves of *A. simplex*-X^r and *S. roseochromogenes* in mixed culture fermentation in E34 medium. (1) A-X^r: *A. simplex*-X^r, (2) S: *S. roseochromogenes*.

The growth curve of the resistant *A. simplex* mutant was then compared with that of the original sensitive strain under mixed conditions with *S. roseochromogenes*. The mutant was found to be distinctly different from the parent strain in its response to *S. roseochromogenes* metabolites (Fig. 4, 5). The growth curve of the resistant mutant fluctuated with a period of about 48 hr, suggesting that the transmission of the resistance property to daughter organisms was not uniform. Under mixed conditions with the parent sensitive *A. simplex*, *S. roseochromogenes* grew without any interference and its pattern of growth was similar to that seen in the single culture of the organism (Fig. 4). When *S. roseochromogenes* was cultured with the resistant *A. simplex* mutant, a smaller number of viable *S. roseochromogenes* organisms (2×10^9 ml. vs. 3×10^9 ml.) was counted (Fig. 5). The *S. roseochromogenes* organisms may have been in competition for growth supporting substances with the resistant *A. simplex* mutant under the

mixed condition. In spite of the fact that the growth in the mixed culture system of resistant *A. simplex* (X^r strain) and *S. roseochromogenes* differed from the growth pattern seen in the mixed culture system of the *A. simplex* parent (X^s strain) and *S. roseochromogenes*, the amounts of steroid turnover in the two systems were alike, being 79 and 78 % (mole/mole), respectively.

DISCUSSION

The use of a mixed culture fermentation for steroid conversion has economic importance if, by its use, isolation of an intermediate can be eliminated or better control of the bioconversion steps can be achieved. One mixed-culture fermentation we have described has both advantages. In the conventional process for producing triamcinolone from 9 α -fluorohydrocortisone, the substrate is first 16-hydroxylated in a *Streptomyces roseochromogenes* fermentation and the intermediate, 16 α -hydroxy-9 α -fluorohydrocortisone, isolated. Substantial losses of the intermediate occur during the isolation step. The isolated intermediate is then converted by fermentation with *Arthrobacter simplex*, to triamcinolone, isolated and purified. The second advantage, better control of the bioconversion steps, is achieved by the complete repression in the mixed culture of the 20-ketoreductase enzyme. This enzyme accounts for considerable by-product formation in the single culture conversion of 16 α -hydroxy-9 α -fluorohydrocortisone to triamcinolone with *A. simplex*, and consequently close monitoring of the fermentation is needed to ensure that the fermentation is terminated when the conversion to desired product is at a maximum and yet the formation of undesired product, 20-dihydro-triamcinolone, is still at a minimum.

The phenomena of enzyme induction and repression have been recognized for many years (Monod & Cohn, 1952; Monod & Cohen-Bazire, 1953). We have found that enzyme induction and repression patterns relating to the transformation of steroids can be influenced markedly by medium composition, by steroid substrate and, unexpectedly, by the interaction of organisms in mixed culture systems. Two enzymes, 1-dehydrogenase synthesized by *Arthrobacter simplex*, and 16 α -hydroxylase produced by *Streptomyces roseochromogenes*, play critical roles in the multiple conversion of 9 α -fluorohydrocortisone to triamcinolone by mixed cultures of the two organisms. The multiple transformation of 9 α -fluorohydrocortisone to triamcinolone in the mixed culture system depends entirely upon repressing selectively a third enzyme, 20-ketoreductase, which can be synthesized by both organisms under conditions where induction is favoured. We found that the 20-ketoreductase enzyme, which was induced in the pure culture system of *A. simplex* grown in soybean meal medium with 9 α -fluorohydrocortisone present, was repressed in the mixed culture system of the organism with *S. roseochromogenes*. Changes in enzyme induction and repression patterns were also seen in the mixed culture system of *Nocardia restrictus* and *S. roseochromogenes*. Both 1-dehydrogenase and 20-ketoreductase, which were inducible in the pure culture system of *N. restrictus* grown in the same soybean meal medium, were repressed in the mixed culture system of the organism with *S. roseochromogenes*.

We postulate, therefore, that certain inducing substances present in the medium in our mixed culture system are rapidly metabolized to certain substances, through the action of the micro-organisms, in the very early stage of fermentation, and these metabolites then no longer inactivate specific repressors and therefore changes from induction to repression of certain enzymes are effected.

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