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7α-Dehydroxylation of Bile Acids by Resting Cells of an Unidentified, Gram-Positive, Nonsporeforming Anaerobic Bacterium

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Transformation of bile acids by washed whole cells of strain HD-17, an unidentified gram-positive anaerobic bacterium isolated from human feces, was studied. 7α-Dehydroxylase was produced only during adaptive growth on medium containing 7α-hydroxy bile acids. Both the extent of hydroxylation and the state of conjugation of the bile acids had marked effects on the induction of the enzyme, and the order of the enzyme induction was conjugated cholic acid ⩾ cholic acid > taurochenodeoxycholic acid ≥ chenodeoxycholic acid. The addition of excess glucose to the growth medium appreciably reduced the enzyme level. The induced enzyme required strict anaerobic conditions for activity and had an optimal pH range of 6.5 to 7.5. In contrast with the induction of the enzyme, the induced enzyme showed a low degree of substrate specificity between cholic acid and chenodeoxycholic acid, with some preference for the former. In addition, the organism contained 3α-, 7α-, and 12α-hydroxysteroid dehydrogenases, and the addition of bile acids to the medium somewhat enhanced the production of the oxidoreductases. The dehydrogenations were obviously stimulated by oxygen as a terminal electron acceptor. The organism also contained bile salt hydrolase.

The chief bacterial transformation of bile acids is 7α-dehydroxylation, which results in the formation of the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) from the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA), respectively (9, 25). Although most bile acids excreted in the feces are the products of this reaction, 7α-dehydroxylase activity has been found only in certain strains of anaerobic lactobacilli (6, 23), *Clostridium leptum* (formerly *Bacteroides* 28 S) (8), *Clostridium bifermentans* (5, 10), *Clostridium sordellii* (10), *Bacteroides* spp. (2, 4), and *Eubacterium* sp. (14, 31). The proposed mechanism for the 7α-dehydroxylation involves an initial diaxial *trans* elimination of the 7α-hydroxy group and the 6β-hydrogen atom, and the resulting Δ^6-intermediate is subsequently reduced by *trans* hydrogenation at the 6α and 7β positions (26). Since the latter process especially seems to require a strict anaerobic environment and the enzyme has been reported to be labile even in the cell-bound state (1), in most cases the reaction has been demonstrated only in anaerobically growing cultures. Only a few attempts to show 7α-dehydroxylation of bile acids by washed whole cells have been successful (14, 27, 28, 31).

We have isolated from human feces 13 strains capable of 7α-dehydroxylating bile acids (12), and fortunately, we were able to detect the activity in whole-cell suspensions of one of these organisms (designated strain HD-17) prepared under atmospheric conditions. In this study we examined the inducibility and some other properties of the 7α-dehydroxylase by using resting cells of strain HD-17.

MATERIALS AND METHODS

Bacterial strain. Strain HD-17 is an unusually coiled anaerobic, gram-positive, nonsporeforming rod isolated from human feces (12). The organism morphologically resembles *Clostridium cocleatum* and *Clostridium spiroforme* but is clearly distinct from these species on the basis of gas-liquid chromatography (GLC) and biochemical reactions examined by the procedures of Holdeman and Moore (13). In addition, the organism is unique in that in the growth medium it exclusively 7α-dehydroxylates CA, whereas it biotransforms CDCA, preferably through 7α-dehydrogenation (11, 12). The organism was maintained in GAM semisolid agar (29; Nissui Pharmaceutical Co.) in a tightly stoppered tube at 4°C, and the inoculum came from an overnight culture in GAM broth.

Preparation of washed whole cells. Unless otherwise stated, 150 ml of buffered GAM broth containing 100 μg of taurocholic acid (TCA) per ml was inoculated with 7.5 ml of the inoculum culture. After 21 h of incubation at 37°C in an anaerobic jar under nitrogen, the culture was harvested by centrifugation at 6,000 × *g* for 30 min at 4°C. The cells were washed three times with 0.1 M sodium phosphate buffer (pH 7.0) contain-

ing 0.1% sodium thioglycolate. The final sediment was suspended in the same buffer solution at a density of 40 mg (wet weight) per ml. All these procedures were carried out under ordinary atmospheric conditions.

Assay of 7α-dehydroxylase activity in washed whole cells. The standard reaction mixture consisted of 3.0 ml of 0.1 M sodium phosphate buffer (pH 7.0), 0.1% sodium thioglycolate, 500 μg of bile acid, and 1.0 ml of washed cell suspension. The mixture was placed in a small test tube and held in a water bath at 37°C without agitation under atmospheric conditions. The reaction was stopped after the desired period of incubation by adding three drops of 6 N HCl.

Analysis of bile acids by GLC. Free bile acids extracted with ethyl acetate from acidified samples (spent culture medium or incubated reaction mixture) were methylated by the methanol-sulfuric acid procedure and were analyzed by GLC with a 3% QF-1 column as previously described (12). Individual bile acids were identified by relative retention time compared with methyl DCA. Quantities were calculated by measuring peak areas and were expressed as percentages of composition after confirmation that the recovery of sample bile acids was compatible with the quantity of the original substrate bile acid.

Bile acids. Bile acids are referred to by abbreviations of their trivial names, to which the following systematic names are given: CA, 3α,7α,12α-trihydroxy-5β-cholanoic acid; CDCA, 3α,7α-dihydroxy-5β-cholanoic acid; DCA, 3α,12α-dihydroxy-5β-cholanoic acid; 7-ketodeoxycholic acid (7KD), 3α,12α-dihydroxy-7-keto-5β-cholanoic acid; 7-ketolithocholic acid (7KL), 3α-hydroxy-7-keto-5β-cholanoic acid; 12-ketolithocholic acid (12KL), 3α-hydroxy-12-keto-5β-cholanoic acid; LCA, 3α-hydroxy-5β-cholanoic acid; glycocholic acid (GCA), 3α,7α,12α-trihydroxy-5β-cholanoyl glycine; TCA, 3α,7α,12α-trihydroxy-5β-cholanoyl taurine; taurochenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoyl taurine.

The free bile acids used as substrates were CDCA (99.9% pure), a gift from Tokyo Tanabe Pharmaceutical Co., and CA (99.1% pure), purchased from Sigma Chemical Co., both of which proved to be pure when tested by GLC. Glycine or taurine conjugates of bile acids were obtained from Calbiochem-Behring. The methyl esters of bile acids used as GLC references were all obtained from Gasukuro-Kogyo Co.

RESULTS

Reaction products produced by washed whole cells of strain HD-17. Figure 1 shows the separation by GLC of bile acids formed from CA and CDCA. CA (Fig. 1A) was almost exclusively metabolized through 7α-dehydroxylation followed by the oxidative modification of the resulting DCA (peak a) to 12KL (peak b) and the 3,12-diketo derivative of DCA (peak c). A small portion of CA was 7α-dehydrogenated to 7KD (peak d), and its further oxidation at C-12 also followed (peak e). CDCA (Fig. 1B) was moderately dehydroxylated to LCA (peak f), and dehydrogenations were observed in positions 3 and 7 (peaks g and i). These results show that whole cells contain 7α-dehydroxylase as well as the

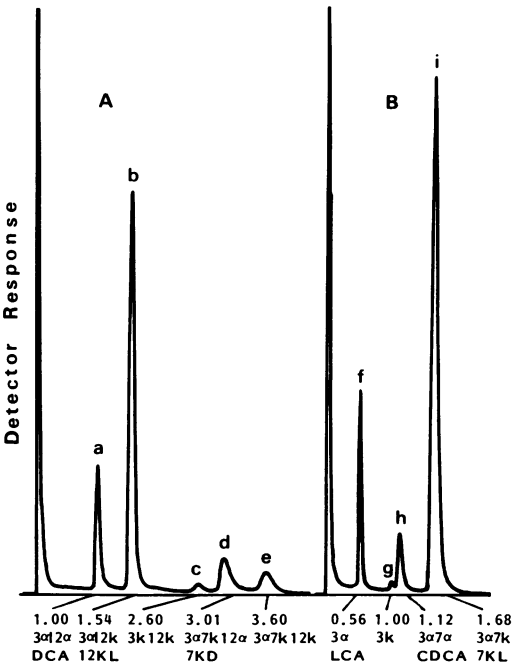

FIG. 1. Separation by GLC of methyl esters of bile acid products from CA (A) and CDCA (B) produced by washed whole cells of strain HD-17. Washed whole cells (40 mg) grown in the presence of TCA was incubated for 21 h with CA or CDCA in sodium phosphate buffer (pH 7.0) without agitation under ordinary atmospheric conditions. Bile acid extracts were separated on 3% QF-1. Individual bile acids are noted by relative retention times with the retention time of methyl DCA as 1.00 and also by substituents in 5β-cholanoate (α,α-hydroxy group; keto group).

3α-, 7α-, and 12α-hydroxysteroid dehydrogenases (HSDHs). Figure 2 also shows the 7α-dehydroxylated products from CA consisted of DCA, 12KL, and the 3,12-diketo derivative of DCA. The dehydroxylated metabolites from CDCA were LCA and the 3-keto derivative of LCA.

Induction of 7α-dehydroxylase activity by various bile acids. Table 1 and Fig. 3 show 7α-dehydroxylase activity in whole cells grown in the presence of various bile acids. The enzyme activity was detected only in whole cells prepared from cultures grown in the presence of 7α-hydroxy bile acids. Conjugated CA (TCA or GCA) was much more effective than free CA. In contrast, CDCA, regardless of the state of conjugation, did not induce significant amounts of the activity. The effects of various concentrations of TCA and CA on enzyme induction were then studied (Table 2). TCA in concentrations up to 100 μg/ml was very effective in enhancing the production of the enzyme, which was not formed in the absence of an inducer. At concen-

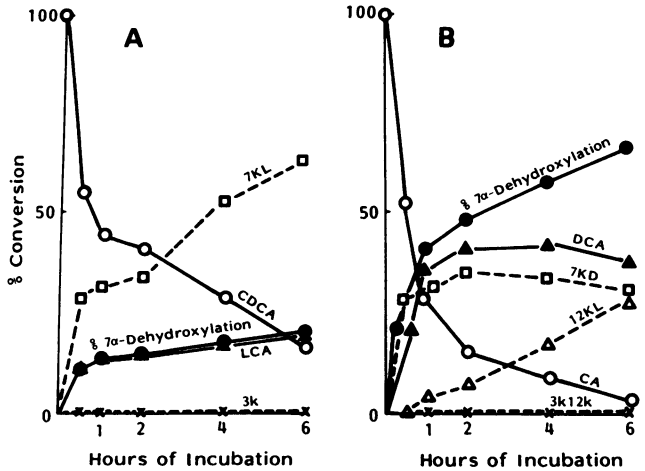

FIG. 2. Changes in percentage composition of metabolites from CDCA (A) and CA (B) during incubation by TCA-induced whole cells. The experiment was performed in the same way as that shown in Fig. 1 up to 6 h. Metabolites were quantified as methyl esters on QF-1, and the total of the percentage composition of 7α-dehydroxylated bile acids was expressed as the percentage of 7α-dehydroxylation. Although LCA (7α-dehydroxylated product) and 7KL (7α-dehydrogenated product) were formed from CDCA, 7α-dehydrogenation took preference over 7α-dehydroxylation. CA, on the other hand, was quantitatively converted into DCA through 7α-dehydroxylation, and portions of the resulting DCA were further oxidized to form 12KL. 7KD (7α-dehydrogenated product) progressively increased in concentration to reach a plateau after 2 to 4 h of incubation and then declined because of the reversible nature of dehydrogenation. Minor quantities of 3-keto derivatives were also produced from DCA and LCA. k, Keto group.

trations above 100 μg/ml, however, TCA did not further increase the enzyme yield. When CA was added to the growth medium as an inducer, a linear increase in the level of the enzyme activity was demonstrated at concentrations up to 1,000 μg/ml. However, CA was about 10 times less effective than TCA as an inducer. The inclusion of the two bile acids at concentrations up to 1,000 μg/ml did not affect the growth of the organism. All cultures generally reached maximum growth by 12 h. Optimal production of the enzyme occurred between 12 and 24 h; the activity then declined.

Effect of glucose on enzyme production. The enzyme production was also influenced by the composition of the medium (Table 3). The maximum production was observed when 0.02 M phosphate buffer was added to GAM broth. If glucose was added in excess, enhanced cell growth was observed, but the enzyme formation was almost completely inhibited.

pH optimum for 7α-dehydroxylation. The activity in whole cells was detected over a pH range from 4.7 to 8.4, with an optimum pH of 6.6 against CA. However, the optimum pH was 7.3 when CDCA was used as a substrate.

Comparison of activity against CA and CDCA. In the growth medium, CA was extensively dehydroxylated, whereas CDCA underwent little dehydroxylation; however, cells grown on TCA or GCA dehydroxylated not only CA but also CDCA to a fair extent (Table 1). These results show that the 7α-dehydroxylase is less specific in whole cells than in the growth medium with regard to the extent of hydroxylation of bile acids.

TABLE 1. Induction of 7α-dehydroxylase activity by various bile acids

Inducer	Concn (μg/ml)	% 7α-Dehydroxylation in:		
		Growth medium[a]	Whole cells incubated with[b]:	
			CA	CDCA
TCA	100	100	87	35
GCA	100	100	95	42
CA	100	94	7	2
	150	97	13	0
TCDCA[c]	150	9	1	0
CDCA	150	5	1	0
None	0	0	0	0

[a] Growth medium containing each of the indicated bile acids was analyzed after 21 h of anaerobic incubation.

[b] Washed whole cells grown in the presence of various bile acids were incubated with CA or CDCA for 21 h at pH 7.0.

[c] TCDCA, Taurochenodeoxycholic acid.

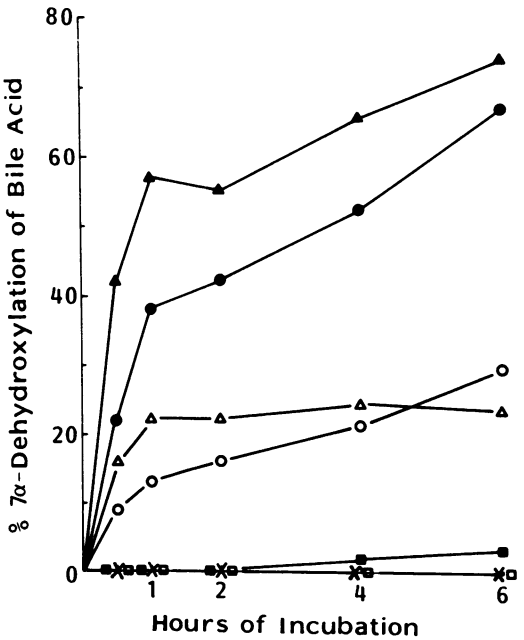

FIG. 3. Time course of 7α-dehydroxylating activity induced by various bile acids. Each bile acid was added to the growth medium at 100 μg/ml. The percentage of 7α-dehydroxylation was determined in cells grown in the presence of various bile acids with either CA or CDCA as the substrate at the indicated times under atmospheric conditions. Results are shown for TCA-induced cells against CA (●) and CDCA (○), GCA-induced cells against CA (▲) and CDCA (△), CA-induced cells against CA (■) and CDCA (□), and uninduced cells against CA and CDCA (×).

Transformation of CA under aerobic and anaerobic conditions. CA was gradually 7α-dehydroxylated to DCA, and the resulting DCA was further 12α-dehydrogenated to 12KL under anaerobic conditions (Fig. 4). Although 7KD was produced during the early phase of incubation, most of this compound disappeared by 8 h of incubation because of the freely reversible nature of the dehydrogenation. When the reaction mixture was shaken in air during incubation, the dehydroxylation was completely abolished, and only oxidative processes took place. That is, CA was converted to 7KD, and the resulting 7KD was further dehydrogenated to give the presumed 7,12-diketo derivative of CA.

7α-HSDH. When CA was included in the growth medium as an inducer, 7α-HSDH activity increased about 1.5-fold (Fig. 5). CA and CDCA were dehydrogenated to similar extents. In contrast with 7α-dehydroxylase, this oxidoreductase had no significant substrate specificity for the two primary bile acids.

12α-HSDH. The addition of bile acid to the medium caused about a twofold increase in 12α-HSDH activity, although there was measurable activity when bile acid was omitted from the medium. The efficacy as an inducer was in the order of TCA = GCA > CA (Fig. 6). As for the medium, the yield of the enzyme was optimal from cells grown in the buffered GAM broth (Table 3).

Finally, all detectable 7α-dehydroxylase activity was lost after the whole cells were broken by sonication.

TABLE 2. Effect of concentrations of conjugated and unconjugated CA on the induction of 7α-dehydroxylase activity[a]

Inducer	Concn (μg/ml)	% 7α-Dehydroxylation of CA in:						
		Growth medium	Whole cells at time (h):					
			0.5	1	2	4	6	21
None	0	0	0	0	0	0	0	0
TCA	10	100	1	4	2	4	4	10
	100	96	7	11	15	30	33	53
	500	94	8	10	15	18	40	62
	1,000	91	9	10	15	21	40	62
CA	10	100	1	1	2	2	3	8
	100	100	1	1	1	4	7	12
	500	96	1	1	1	6	9	32
	1,000	87	8	8	9	18	25	49

[a] For procedures, see footnotes to Table 1.

DISCUSSION

Aries and Hill (1) first stated that the 7α-dehydroxylase activity in cell extracts of various intestinal bacteria is almost completely inducible with CA. Stellwag and Hylemon (28) reported that the enzyme activity against CDCA appears to be inducible with CA, whereas the activity against CA is constitutive in whole cells of *C. leptum*. Furthermore, White et al. (31) stated that the induction of the enzyme is specific for bile acids containing a free carboxy group at C-24 in cells of *Eubacterium* sp. In contrast with these findings, conjugated CA (TCA or GCA) was a notably more effective inducer than free CA in our present study (Table 1). The strongest induction of the enzyme by TCA, which is not a substrate for 7α-dehydroxylase (24, 28), has not been reported and seems puzzling. However, depletion of the substrate from the medium may be more delayed for TCA than for CA because TCA must be deconjugated by bile salt hydrolase (the organism contains this enzyme) to be dehydroxylated. Therefore, it seems reasonable that cells grown on TCA would retain higher activity than those grown on CA if the decline in activity is related to the depletion of the inducing substance in medium as described by Watanabe et al. (30). Indeed, at concentrations of 1,000 μg of CA per ml, appreciable amounts of un-

TABLE 3. Effect of the composition of the medium on 7α-dehydroxylase production

Additives[a]	Bacterial growth[b]	Final pH of medium	% 7α-Dehydroxylation of CA in whole cells at time (h):						% 12α-Dehydrogenation of DCA in whole cells at time (h)[c]:					
			0.5	1	2	4	6	21	0.5	1	2	4	6	21
None	78	6.7	3	6	7	13	15	29	10	11	12	17	26	63
0.02 M phosphate buffer	85	6.8	13	20	19	28	31	51	16	16	19	25	30	71
0.02 M phosphate buffer + 0.7% D-glucose	43	6.0	0	0	0	0	0	2	8	8	9	11	12	39

[a] The basal medium was GAM broth containing TCA; the initial pH of the medium was adjusted with 1 N NaOH to 7.4.

[b] Percentage of transmittance of light at 660 nm.

[c] 12α-Dehydrogenated products from DCA were 12KL and the 3,12-diketo derivative of DCA.

changed CA still remained in the medium, so that cells grown with large amounts of CA showed high enzyme activity (Table 2).

In addition, White et al. (31) stated that CA induces about fourfold higher activity than CDCA in cells of *Eubacterium* sp. However, CDCA, irrespective of the state of conjugation, induced much less enzyme activity than CA did (Table 1 and Fig. 3). Although the difference between CDCA and CA is only that the former lacks the hydroxy group at C-12, the absence of this group has a significant effect on enzyme induction in this organism. Usually, dehydroxylating organisms transform CA and CDCA to similar extents, as reported by Midtvedt and Norman (24) for *Lactobacillus* strain II. But our organism exceptionally dehydroxylated CA almost exclusively and showed little action on CDCA in the growth medium (11, 12). This is because CDCA is far less effective an inducer than CA is. In contrast, cells induced with TCA dehydroxylated fair amounts of CDCA, although CA was the preferred substrate for 7α-dehydroxylase (Table 1). In this respect, the induced enzyme showed less difference in activity against CA and CDCA.

The enzyme activity occurred over a pH range from 4.7 to 8.4, with an optimum pH of 6.6 against CA. Similar pH curves were observed for whole cells of *C. leptum* (27) and *Eubacterium* sp. (14) when CA was used as a substrate. In contrast with the report of Stellwag and Hylemon (28) that the activity in cells of *C. leptum* against both CA and CDCA has the same optimum pH of 6.5 to 7.0, the optimum pH (7.3) for the dehydroxylation of CDCA was more alkaline than that of CA. The finding that cells have two different pH optima against CA and CDCA

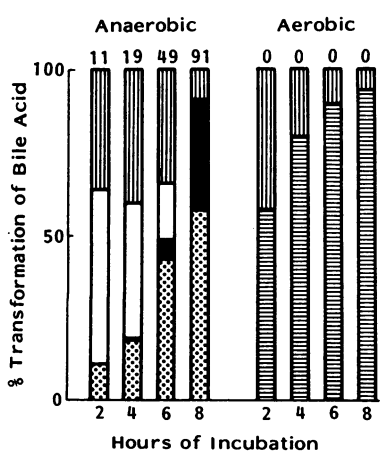

FIG. 4. Transformation of CA by whole cells under aerobic and anaerobic conditions. TCA-induced cells were incubated with CA at pH 7.0 in a Thunberg tube under N_2 (anaerobic) or in an L-shaped tube with continuous shaking in air (aerobic). The percentage of 7α-dehydroxylation is shown at the top of each column. Symbols: □, CA; ■, 12KL; ⊡, DCA; ▥, 7KD; ▤, presumed 7,12-diketo derivative of CA.

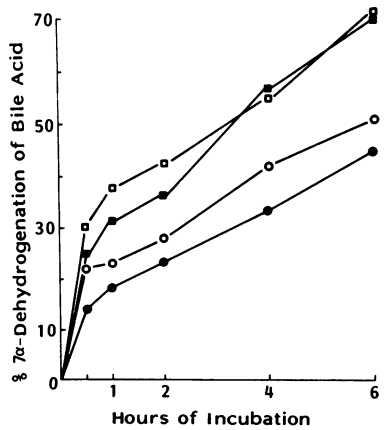

FIG. 5. 7α-Dehydrogenating activity of CA-induced and uninduced cells. Cells were grown in the presence or absence of CA. Metabolites formed by 7α-HSDH (7KD from CA and 7KL from CDCA) were quantified as methyl esters on QF-1, and the percentage composition of 7KD or 7KL was expressed as a percentage of the 7α-dehydrogenation of bile acid. Results are shown for CA-induced cells against CA (■) and CDCA (□), uninduced cells against CA (●) and CDCA (○).

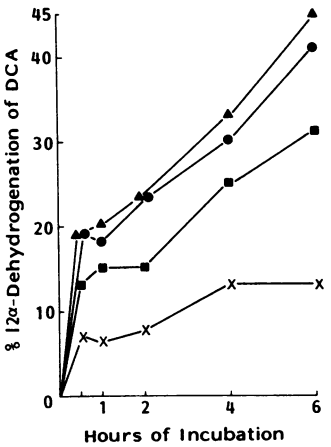

FIG. 6. Time course of 12α-dehydrogenating activity in cells induced with various bile acids. Washed whole cells grown in the presence of TCA, GCA, or CA were incubated with DCA at pH 7.0 for the indicated times in test tubes without agitation. Metabolites produced from DCA by 12α-HSDH (12KL and the 3,12-diketo derivative of DCA) were quantified as methyl esters on QF-1, and the total of their percentage composition was expressed as a percentage of the 12α-dehydrogenation of DCA. Symbols: ▲, TCA-induced cells; ●, GCA-induced cells; ■, CA-induced cells; ×, uninduced cells.

suggests the possible presence of two distinct enzyme entities, one specific for CA and the other specific for CDCA.

When the glucose concentration in the medium was increased from 0.3 to 1.0%, there was a complete loss of the enzyme activity with enhancement of cell growth (Table 3). The pH of the medium fell from 7.4 to 6.0 because of the fermentation of glucose. This final pH 6.0 was more acidic than the final pH 6.5 which was emphasized by Aries and Hill (1) as being critical for the 7α-dehydroxylase formation. This suppression of enzyme production may be explained as follows: first, the acidity of the medium may have a harmful effect on enzyme stability, so that the activity might fall rapidly with time even if large amounts of the enzyme are produced; second, glucose may repress the enzyme production, as many inducible enzymes are repressed by it (3).

7α-Dehydroxylase competes with 7α-HSDH for the 7α-hydroxy group. We agree with Aries and Hill (1) that only in an anaerobic environment does this competition result in the eventual removal of the substituent (Fig. 4). Since the rate of dehydroxylation was not decreased when incubation was in air without agitation as much as it was when incubation was in nitrogen, the degree to which atmospheric oxygen penetrated into the reaction mixture seemed to be limited, and fairly anaerobic conditions could be maintained in the mixture without agitation even under ordinary atmospheric conditions. Aeration of resting cells, in contrast, completely inhibited the dehydroxylation, whereas HSDH activities were favored by oxygen as a terminal electron acceptor (22) (Fig. 4).

HSDH production has been reported to be inducible (1, 15, 21), noninducible (7, 18–20), or suppressible (16, 17) by bile acids. The results obtained here (Fig. 5 and 6) show that 7α- and 12α-HSDHs are inducible. Therefore, it seems that the inducibility of HSDHs varies from species to species.

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