# Bile acid induction of 7α- and 7β-hydroxysteroid dehydrogenases in *Clostridium limosum*

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**Abstract** When grown in the presence of bile acids, two strains of *Clostridium limosum* were found to contain significant amounts of NADP-dependent 7α/7β-hydroxysteroid dehydrogenase and NAD-dependent 7α-hydroxysteroid dehydrogenase which were active against conjugated and unconjugated bile acids. No measurable activity could be found when deoxycholic acid (3α, 12α-dihydroxy-5β-cholan-24-oic acid) was used as substrate. No 7β-hydroxysteroid dehydrogenase activity and only a trace of 7α-hydroxysteroid dehydrogenase activity could be demonstrated when bile acid was deleted from the growth medium. If bile acid was added after the time of inoculation, the amounts of 7α/7β-hydroxysteroid dehydrogenase were greatly reduced. Enzyme enhancement was blocked by addition of rifampicin. The 7α/7β-hydroxysteroid dehydrogenase components had pH optima of approximately 10.5. Both the 7α/7β-hydroxysteroid dehydrogenase activities were heat-labile, with the 7β-component being the more stable of the two. When ranked according to the level of enzymes induced, the order in increasing bile acid induction power on an equimolar scale (0.4 mM) was: 7-ketodeoxycholic acid, cholic acid, chenodeoxycholic acid, and deoxycholic acid. Both 7-ketolithocholic acid and ursodeoxycholic acid were ineffective as enzyme inducers. Optimal induction was achieved with high concentrations of cholic acid (5 mM) and a harvest time of 24 hr. Addition of ursodeoxycholic acid to medium containing optimal concentrations of deoxycholic acid suppressed enzyme induction. With regard to the NADP-dependent 7α-hydroxysteroid dehydrogenase activity, a dihydroxy bile acid had a higher apparent $K_m$ value than a trihydroxy bile acid; chenodeoxycholic acid (0.42 mM) versus cholic acid (0.14 mM). In contrast, with the NADP-dependent 7β-hydroxysteroid dehydrogenase activity, a trihydroxy bile acid had a higher $K_m$ value; ursocholic acid (0.076 mM) versus ursodeoxycholic acid (0.018 mM). The NAD-dependent 7α-hydroxysteroid dehydrogenase activity with cholic acid as substrate had the highest apparent $K_m$ value of 0.62 mM. — **Sutherland, J. D., and C. N. Williams.** Bile acid induction of 7α- and 7β-hydroxysteroid dehydrogenases in *Clostridium limosum*. *J. Lipid Res.* 1985. **26:** 344–350.

**Supplementary key words** bile acids • enzyme induction

A number of anaerobes have been shown to participate in the formation of urso bile acids from primary bile acids. These include *Clostridium absonum* (1, 2), certain lecthinase-lipase-negative clostridia (3, 4), *Peptostreptococcus productus* (5, 6), and *Eubacterium aerofaciens* (6–8).

*Clostridium absonum* and the unidentified clostridia are examples of single organisms that are capable of 7α-hydroxyl group epimerization in pure culture (intraspecies epimerization, (9)). The 7α/7β-hydroxysteroid dehydrogenases responsible for the epimerization by *C. absonum* have been demonstrated and studied (10, 11). In contrast *P. productus* and *E. aerofaciens* contain only a 7β-hydroxysteroid dehydrogenase (6–8) and epimerization (5, 8) in whole cell cultures (interspecies epimerization, (9)) depends on co-culturing either of these organisms with a known 7α-hydroxysteroid dehydrogenase elaborating bacterium such as *Bacteroides fragilis* (12, 13) or *Escherichia coli* (14, 15).

*Clostridium absonum* 7α-hydroxysteroid dehydrogenases (NADP- and NAD-linked) and 7β-hydroxysteroid dehydrogenase (NADP-linked) are inducible by several bile acid substrates and a non-substrate, deoxycholic acid, and are repressed by the respective urso end product (10, 11). Up to this time little has been reported on the enzyme system in the lecithinase-lipase-negative clostridia isolated by Edenharder et al. (3, 4). The 7β-hydroxysteroid dehydrogenases in *P. productus* and *E. aerofaciens* are NADP-linked, constitutively synthesized, of low total activity, and are unstable (6, 8).

The primary significance of 7α-hydroxyl group epimerization appears to be the lower toxicity of ursodeoxy-

Abbreviations: TLC, thin-layer chromatography; HSDH, hydroxysteroid dehydrogenase; ATCC, American Type Culture Collection; VPI, Virginia Polytechnic Institute; NADP, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid); UC, ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid); 7-KDC, 7-ketodeoxycholic acid (3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid); CDC, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); UDC, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid); 7-KLC, 7-ketolithocholic acid (3α-hydroxy-7-oxo-5β-cholan-24-oic acid); DC, deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid); GCDC, glycochenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoyl glycine); GUDC, glycoursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoyl glycine); TCDC, taurochenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoyltaurine); TUDC, tauroursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoyl taurine).

cholic acid than chenodeoxycholic acid due to a lesser hydrophobicity of the former (16). With *C. absonum*, polyacrylamide gel electrophoresis has shown that no less than five new polypeptide bands, other than those associated with enzyme activity, are present in cultures induced by either chenodeoxycholic acid or deoxycholic acid (17). Thus induction of 7$\alpha$/7$\beta$-hydroxysteroid dehydrogenases may represent an entire shift in the synthesis of proteins to facilitate survival of an organism in a "hostile" bile salt environment.

Recent interest in ursodeoxycholic acid and bacterial organisms capable of synthesizing this bile acid has arisen from its oral administration for the dissolution of human cholesterol gallstones (18–24). Commercial microbiological synthesis of ursodeoxycholic acid remains a possibility, but *C. absonum* does not appear to be the best candidate due to its inability to grow in high concentrations of chenodeoxycholic acid (1). However, *C. absonum* 7$\beta$-hydroxysteroid dehydrogenase purified by Procion Red affinity chromatography (25) is now being used to spectrophotometrically quantify ursodeoxycholic acid in ursodeoxycholic acid-rich biles (26).

Recently we have demonstrated that a soil isolate of *Clostridium limosum* can convert primary bile acids to 7$\beta$-hydroxy and 7-keto products (27). It is the purpose of this communication to study the inducibility of both 7$\alpha$/7$\beta$-hydroxysteroid dehydrogenases in *C. limosum* and to preliminarily characterize these enzymes.

## MATERIALS AND METHODS

### Bile acids used

Cholic acid (CA) was from J. T. Baker Chemicals, Phillipsburg, NJ; deoxycholic acid (DC), glycochenodeoxycholic acid (GCDC), glycoursodeoxycholic acid (GUDC), taurochenodeoxycholic acid (TCDC), and tauroursodeoxycholic acid (TUDC) were from Calbiochemicals, Los Angeles, CA; chenodeoxycholic acid (CDC) and ursodeoxycholic acid (UDC) were from Sigma Chemicals, St. Louis, MO; ursocholic acid (UC) was kindly donated by Drs. R. A. De Pietro and A. F. Hofmann of the Division of Gastroenterology, School of Medicine, University of California, San Diego, CA; 7-ketodeoxycholic acid (7-KDC) and 7-ketolithocholic acid (7-KLC) were from Steraloids, Wiltshire, NH. All bile acids were of the highest grade commercially available. Thin-layer chromatography (TLC) (28) revealed that all bile acids were pure.

### Strains of bacteria

Strain F-14 was isolated from soil as previously reported (27). The isolate was further characterized by Dr. L. V. Holdeman of the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA as a strain of *C. limosum* (27). *C. limosum* strain VPI 2709 was kindly donated by Dr. L. V. Holdeman. Stock cultures were maintained in cooked meat medium under aerobic conditions. Cooked meat and brain-heart infusion broth were products of Difco Laboratories, Detroit, MI.

### Culture conditions

Bacteria were cultured aerobically (27) in 100 ml of freshly autoclaved brain-heart infusion broth containing various bile acids. Sterile 100-ml graduated cylinders were used as growing vessels and bacterial growth was monitored by measuring culture turbidity at 660 nm in an LKB Biochrom Ultrospec 4050. A 10% inoculum (v/v) was used throughout the study.

### Preparation of cell extracts for enzyme assays

Bacteria were harvested after 6 hr of growth by centrifugation at 6000 *g* for 15 min (4°C) unless otherwise stated. Whole cells were suspended in 3 ml of 100 mM sodium phosphate buffer (pH 7) containing 1 mM EDTA and 1 mM dithiothreitol. Cell suspensions were broken by two passages through a French pressure cell at a cell pressure of 14,000 psi, and cell extracts were centrifuged at 6000 *g* for 15 min (4°C). The supernatant fluid containing 7$\alpha$/7$\beta$-hydroxysteroid dehydrogenases (HSDH) was then rapidly frozen in liquid nitrogen and stored at −70°C.

### Assay for 7$\alpha$/7$\beta$-HSDH activities

The formation of NAD(P)H was measured at 340 nm and 25°C using an LKB Biochrom Ultrospec 4050 spectrophotometer. Each assay cuvette (1 ml) contained 300 mM glycine/NaOH buffer (pH 10.5), 1 mM NADP or 5 mM NAD, 1 mM bile acid, and 5–50 $\mu$l of thawed cell extract or 50 $\mu$l of reconstituted lyophile (10 mg/ml water). Enzyme activity was determined under conditions where reaction rates were linear with time and protein concentration. Specific activities are expressed as units HSDH per mg protein where 1 unit HSDH equals 1 $\mu$mol NAD(P)H transformed per min.

### Kinetic studies of 7$\alpha$/7$\beta$-HSDH activities

Michaelis-Menton constants ($K_m$) and maximum velocities (V) were calculated on the basis of Eadie-Hofstee plots using initial reaction rates (29).

### Assay for protein concentration

Total protein concentrations were estimated according to the method of Bradford (30) using commercially prepared reagent and globulin standard (Bio-Rad Laboratories, Richmond, CA).

## RESULTS

Bile acid-inducible NADP- and NAD-dependent 7α-HSDH activities and NADP-dependent 7β-HSDH activity were demonstrated in two strains of *C. limosum* which produced similar yields of 7α/7β-HSDH (**Table 1**). All subsequent experiments described in this report were performed with strain F-14, an established 7α-hydroxyl group epimerizing organism (27).

Optimal activity was recorded at approximately pH 10.5 for the 7α-HSDH enzyme components (**Fig. 1**). The 7β-HSDH activity was shown to be optimal between pH 9.8 and 10.5. On the basis of these findings a pH of 10.5 was chosen for the various 7α/7β-HSDH assay systems.

When a crude F-14 cell extract was maintained at 30°C and assayed for NADP-dependent 7α/7β-HSDH, the 7β-oriented activity was considerably more stable with time than the 7α-HSDH activity (**Fig. 2**). In fact, as much as 95% of the 7α-HSDH activity was abolished after 30 min of incubation at 30°C.

Time course studies on the growth of F-14 and the related production of 7α/7β-HSDH with 0.4 mM DC as an added inducer (**Fig. 3**) showed that at least 6 hr of growth is required before optimal levels of enzymes can be obtained. Further additional time did not result in improved enzyme yields except when CA was added as an inducer. Enzyme levels were maintained as long as sample cultures remained free of oxygen, thus ensuring the viability of the cells.

Both CDC (**Fig. 4A**) and DC (Fig. 4B) were effective in enhancing the production of NADP- and NAD-dependent 7α-HSDH activities, which were barely measurable when the bile acids were deleted from the growing medium. The 7β-HSDH was similarly enhanced, but no 7β-HSDH activity was measurable when bile acid was excluded from the growing medium. At concentrations above 0.5 mM CDC or DC, growth of the bacteria was inhibited and the enzyme yields were suppressed. When CA was added to the medium, much higher concentrations could be added without significant growth inhibition. At a harvest time of 6 hr, as much as 2 mM CA could be added, resulting in a yield of enzyme that was

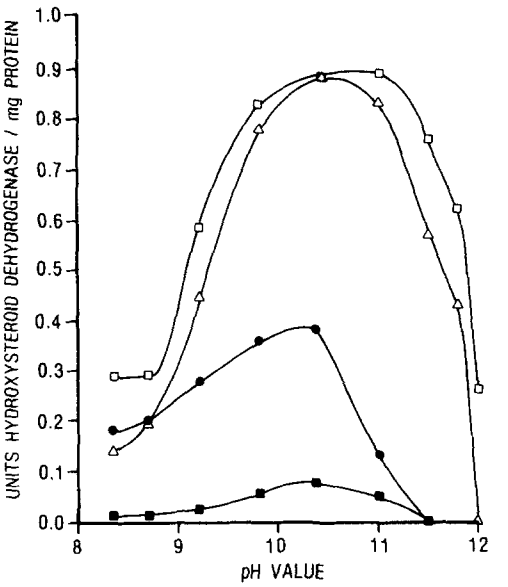


**Fig. 1** Effect of pH value on a crude cell extract of F-14 7α/7β-HSDH (freshly thawed enzyme extract, 0.4 mM DC as an inducer). Symbols: (□—□), Ca/NADP; (△—△), CDC/NADP; (●—●), UDC/NADP and (■—■), CA/NAD.

TABLE 1. 7α/7β-HSDH in two strains of *C. limosum*

| Addition Made | Strain No. | Units HSDH/mg Protein: Assay Systems (Substrate/Cofactor) CA/NADP | CDC/NADP | UDC/NADP | CA/NAD | Absorbance[a] (660 nm) |
|---|---|---|---|---|---|---|
| No bile acid | F-14 | 0.001 | 0.0007 | | 0.001 | 1.020 |
| | 2709 | 0.0008 | 0.0004 | | 0.001 | 1.065 |
| DC (0.4 mM) | F-14 | 0.76 | 0.64 | 0.33 | 0.052 | 0.592 |
| | 2709 | 0.56 | 0.46 | 0.28 | 0.034 | 0.760 |
| CDC (0.4 mM) | F-14 | 0.46 | 0.41 | 0.21 | 0.034 | 0.658 |
| | 2709 | 0.43 | 0.36 | 0.23 | 0.026 | 0.789 |
| CA (1 mM) | F-14 | 0.59 | 0.50 | 0.27 | 0.042 | 0.764 |
| | 2709 | 0.56 | 0.48 | 0.31 | 0.035 | 0.925 |
| 7-KDC (0.4 mM) | F-14 | 0.021 | 0.018 | 0.009 | 0.002 | 0.940 |
| | 2709 | 0.016 | 0.009 | 0.007 | 0.002 | 1.054 |
| UDC (0.4 mM) | F-14 | 0.005 | 0.004 | 0.003 | 0.002 | 0.861 |
| | 2709 | 0.001 | 0.0004 | 0.0004 | 0.001 | 1.052 |
| 7-KLC (0.4 mM) | F-14 | 0.002 | 0.002 | 0.001 | 0.001 | 0.834 |
| | 2709 | 0.0006 | 0.0003 | 0.0003 | 0.001 | 1.035 |

[a]Culture harvest time, 6 hr.

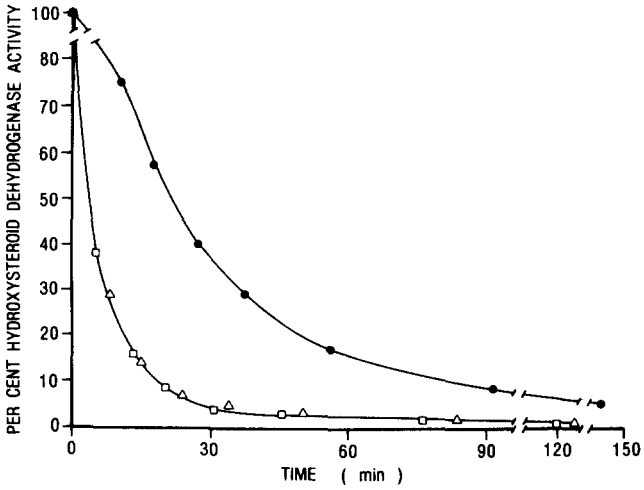


**Fig. 2** Effect of incubation at 30°C on a crude cell extract of F-14 7α/7β-HSDH (reconstituted lyophile, 10 mg/ml water; from pooled cell extracts induced with either CA, CDC, or DC). Symbols as in Fig. 1. CA/NAD was not measured.

2–3 times that produced with optimal concentrations of CDC or DC (results not shown). Even larger yields were obtained with higher concentrations of CA and a harvest time of 24 hr (**Fig. 5**). This was not the case with CDC or DC (results not shown). Other bile acids were tested as inducers, including 7-KDC, UDC, and 7-KLC (Table 1). As can be seen, 7-KDC was considerably less effective than CA, CDC, or DC while UDC and 7-KLC were ineffective as inducers. It is interesting to note that at low equimolar concentrations of bile acid, ie., 0.4 mM, CA is a poor inducer compared to CDC and DC (compare Figs. 4A, 4B, and 5).

When 1 mM CA was added to a culture 2 hr after the time of inoculation, approximately one-third of the enzyme activity was lost compared to addition at 0 hr (**Table 2**). Similar findings were obtained with CDC and DC as well as with other addition times at 0.5, 1, and 1.5 hr (results not shown). If rifampicin was also added at 0 or 2 hrs respectively, 7α/7β-HSDH were found in considerably reduced amounts (Table 2).

When DC was introduced into the growth medium at a concentration of 0.4 mM and increasing amounts of UDC were added to a series of cultures, a distinct suppression of enzyme levels was observed (**Fig. 6**).

Some kinetic parameters for the F-14 7α/7β-HSDH are summarized in **Table 3.** As can be seen, the F-14 7α/7β-HSDH were active against both conjugated and unconjugated bile acids. The highest $K_m$ was for the 7α-HSDH using CA as substrate and NAD as cofactor. It should be noted that for the NADP-dependent 7α-HSDH $K_m$ values, a difunctional substrate (CDC) was three orders of magnitude higher than a trifunctional substrate (CA). In contrast, for the NADP-dependent 7βHSDH $K_m$ values, a trifunctional substrate (UC) was approximately four orders of magnitude higher than a difunctional substrate (UDC). Michaelis-Menton kinetics were observed in all cases studied.

## DISCUSSION

The results confirm and extend the work previously done on this organism (27). Clearly, the ability of *C. limosum* to make urso bile acids lies in the fact that 7α/7β-HSDH working in sequence can convert primary bile acids to their 7β-hydroxylated derivative via a 7-keto intermediate.

The inducibility of the 7α/7β-HSDH renders this organism in the same class as *C. absonum* (10, 11). Thus far *C. absonum* and *C. limosum* are the only known 7α-hydroxyl group-epimerizing organisms in which there is evidence for the presence of bile acid-inducible enzymes. In both organisms enzyme induction can occur both with a *a*) substrate for 7α-HSDH (oxidative direction), i.e., CA and CDC; *b*) substrate for 7β-HSDH (reductive direction), i.e., 7-KDC; and *c*) non-substrates such as DC. Conditions for optimal induction, though, are strikingly different in the two organisms. In the case of *C. absonum,* optimal induction is achieved by addition of 0.4 mM CDC at approximately 2.5 hr after inoculation in a 6-hr incubation period (11). With *C. limosum,* addition of 5 mM CA at 0 hr of a 24-hr incubation period results in optimal induction. This result was surprising, since CA is a rather poor inducer for *C. absonum* (10). The reason, no doubt, lies partly in the ability of *C. limosum* to grow in high concentrations of CA (27).

The time of bile acid addition is important for optimal induction in the *C. limosum* system. The inducer (CA,

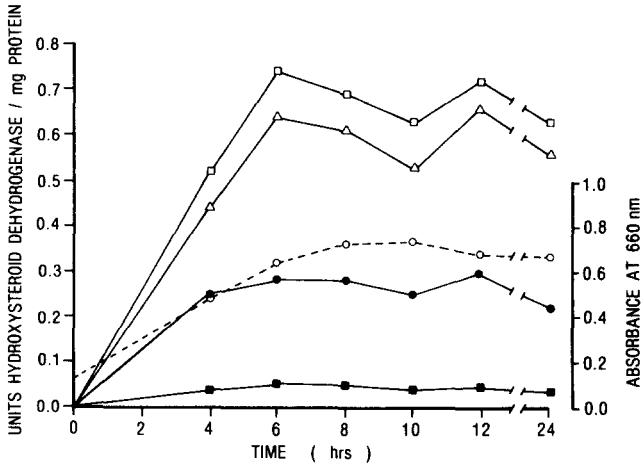


**Fig. 3** Effect of time on the induction of F-14 7α/7β-HSDH by 0.4 mM DC. Symbols: (□—□), CA/NADP; (△—△), CDC/NADP; (●—●), UDC/NADP; (■—■), CA/NAD; and (○- - -○), absorbance of culture at 660 nm.

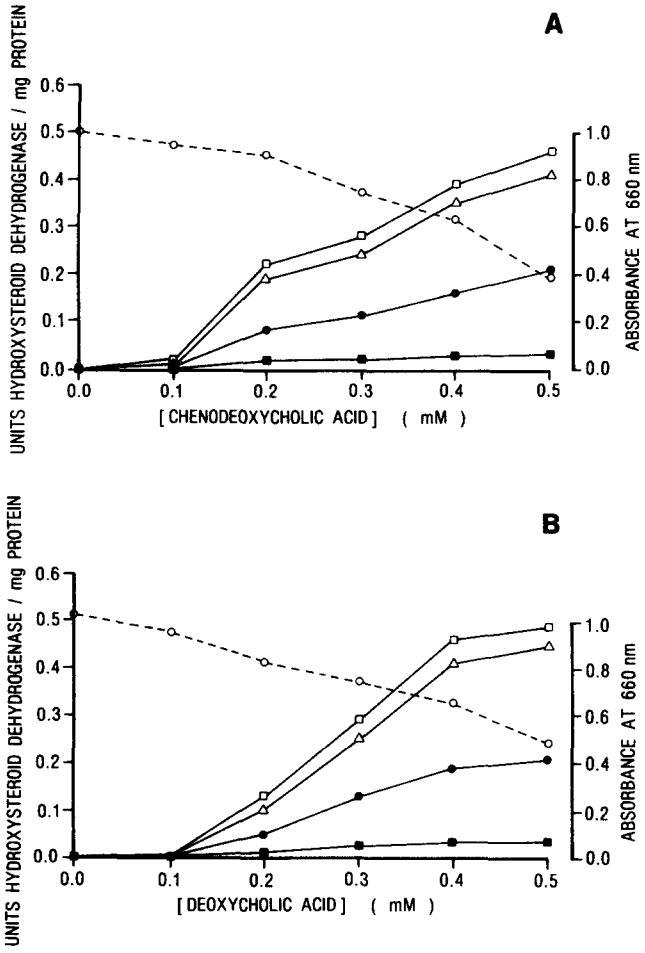


**Fig. 4** Effect of bile acid concentration on the induction of F-14 7α/7β-HSDH by (A) CDC and (B) DC. Symbols as in Fig. 3. Harvest time, 6 hr.

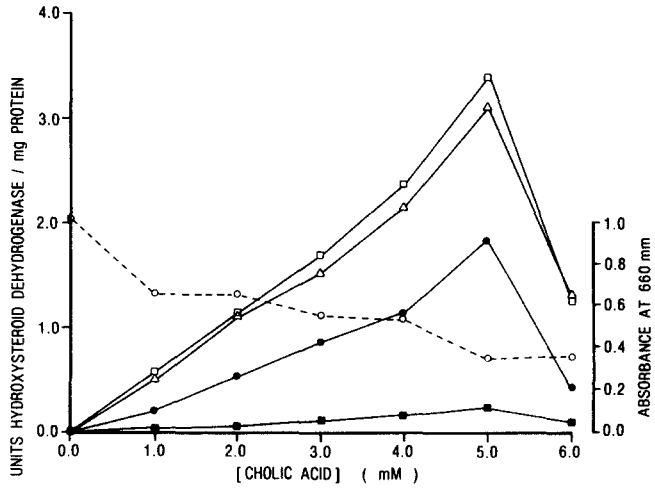


**Fig. 5** Effect of CA concentration on the induction of F-14 7α/7β-HSDH. Symbols as in Fig. 3. Harvest time, 24 hr.

DC, CDC, etc.) must be added at the time of inoculation or induction of 7α/7β-HSDH is dramatically reduced. This was not the case with *C. absonum*, where two different effects were observed depending on the class of bile acid inducer added (11). For *C. limosum* it appears that all generations of dividing bacteria must be exposed to the inducer for good induction.

The total lack of reactivity of *C. limosum* cell extracts with DC indicates the absence of any 3α- or 12α-HSDH activities. The only products visible on TLC after incubation of primary bile acids with the two strains of *C. limosum* are the 7β-hydroxy and 7-keto products (27, and J. D. Sutherland, unpublished results). Why a non-substrate such as DC should induce 7α/7β-HSDH in *C. limosum* (as well as in *C. absonum*) is not clear at this time.

It appears that UDC is capable of blocking the induction effect of DC (Fig. 6). Further investigation will be necessary to establish whether UDC is functioning as a specific end product repressor of the 7α/7β-HSDH. This observed effect has also been demonstrated with *C. absonum* (10, 11). In the case of *C. absonum*, however, there appears to be more UDC suppression of DC induction (10, 11).

Investigation of *C. limosum* 7α/7β-HSDH $K_m$ values

TABLE 2. Effect of rifampicin on *C. limosum* 7α/7β-HSDH production

| Addition Made | Units HSDH/mg Protein: Assay Systems (Substrate/Cofactor) CA/NADP | CDC/NADP | UDC/NADP | CA/NAD | Absorbance[a] (660 nm) |
|---|---|---|---|---|---|
| CA (1 mM), 0 hr | 0.77 | 0.59 | 0.29 | 0.044 | 0.737 |
| CA (1 mM) + 200 μg rifampicin, 0 hr | 0.18 | 0.14 | 0.040 | 0.010 | 0.158 |
| CA (1 mM), 2 hr | 0.23 | 0.20 | 0.073 | 0.015 | 0.789 |
| CA (1 mM) + 200 μg rifampicin, 2 hr | 0.023 | 0.016 | 0.0061 | 0.0023 | 0.630 |

[a]Culture harvest time, 6 hr.

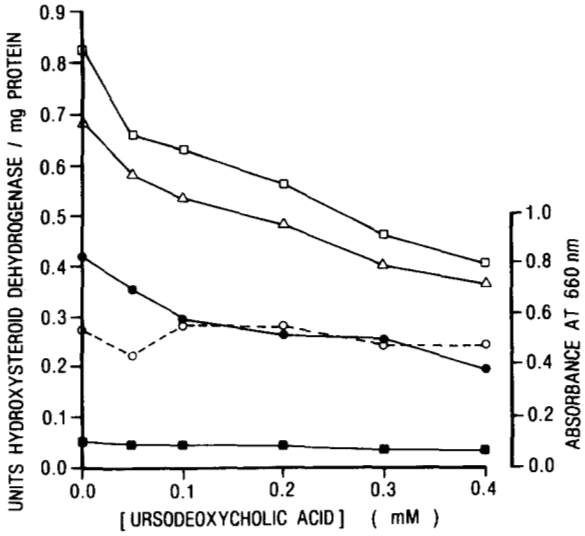


**Fig. 6** Effect of UDC on DC induction of F-14 7α/7β-HSDH. Cultures contained 0.4 mM DC. Symbols as in Fig. 3. Harvest time, 6 hr.

(Table 3) reveals that for the NADP-dependent 7α-HSDH, CA has a considerably higher affinity (lower $K_m$) for the enzyme than CDC. Thus, epimerization of CA should predominate in whole cell cultures of *C. limosum,* as was shown in the previous study (27). In the case of *C. absonum,* the findings are totally opposite (10).

It is interesting to note that while cell extracts of *C. limosum* F-14 were active against both conjugated and deconjugated bile acids, whole cell cultures were active only against deconjugated bile acids (27). This finding may suggest a selective transport of deconjugated bile acids across the cell membrane and an intracellular 7α/7β-HSDH enzyme system.

TABLE 3. Some kinetic parameters of *C. limosum* F-14 7α/7β-HSDH activities

| Assay System | | |
|---|---|---|
| Substrate/Cofactor | $K_m$ (mM)[a] | V[b] |
| CA/NADP | 0.14 | 0.57 |
| CDC/NADP | 0.42 | 0.69 |
| GCDC/NADP | 0.24 | 0.85 |
| TCDC/NADP | 0.29 | 0.75 |
| UC/NADP | 0.076 | 0.14 |
| UDC/NADP | 0.018 | 0.17 |
| GUDC/NADP | 0.093 | 0.074 |
| TUDC/NADP | 0.14 | 0.062 |
| CA/NAD | 0.62 | 0.072 |
| NADP/CA | 0.035 | 0.51 |
| NADP/UDC | 0.14 | 0.16 |

[a]Estimated from Eadie-Hofstee plots using a crude cell extract of *C. limosum* F-14 7α/7β-HSDH (reconstituted lyophile, 10 mg/ml water; from pooled cell extracts induced with either CA, CDC, or DC).
[b]Units HSDH/mg protein.

Whether the *C. limosum* 7α/7β-HSDH activities are one or several separate enzyme entities remains unanswered. Heat inactivation studies suggest separate components since the 7β-HSDH activity is more heat-stable than the 7α-HSDH. As a source of 7β-HSDH, *C. limosum* is potentially better than *C. absonum;* however, all methods to date have failed to separate the two enzyme activities in *C. limosum.* Purification of *C. limosum* 7α/7β-HSDH has been achieved using immobilized triazine dye affinity chromatography (25) with either Reactive Red 120-Cross-Linked Agarose or Reactive Blue 2-Cross-Linked Agarose (J. D. Sutherland, unpublished results).

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