The metabolism of primary, 7-oxo, and 7β-hydroxy bile acids by *Clostridium absonum*

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Abstract *Clostridium absonum* was shown to metabolize primary bile acids to give rise to both 7-oxo bile acids and 7β-hydroxy (urso) bile acids. At relatively low redox potential (Eh) values, high yields of urso bile acids were achieved (60–75%). If, however, the Eh value of the culture was allowed to rise above approximately −100 mv, the 7-oxo bile acid would tend to predominate (more than 75%) and the "death phase" was accelerated. Growth of *C. absonum* in sterile graduated cylinders instead of in conventional Erlenmeyer flasks was effective in delaying the rise in Eh value with time (which appears largely due to diffusion of atmospheric oxygen into the medium) and in preserving a higher viable count of organisms. It is proposed that the formation of excess amounts of 7-oxo bile acid is a manifestation of oxygen toxicity and that it could be mediated by an increasing intracellular NADP:NADPH ratio. Additionally, the reaction: primary bile acid ⇄ oxo bile acid ⇄ urso bile acid was shown to be partially reversible. When the organisms were grown with [24-^{14}C]chenodeoxycholic, -cholic, or -7-keto-lithocholic acid, this reaction could be clearly demonstrated. The addition of an equimolar concentration of deoxycholic acid (which itself is not metabolized) effectively enhanced the rate of bioconversion of cholate and 7-keto-lithocholic, but not chenodeoxycholate (whose rate of bioconversion was the fastest of the three). When the organisms were grown with urso bile acids (ursocholic or ursodeoxycholic) or with 7-keto-deoxycholic acid, very little metabolism occurred unless deoxycholic acid was added which induced formation of primary and keto bile acids. In all cases, formation of oxo bile acid from primary or urso bile acid occurred as the Eh value of the medium rose with time and could thus be delayed by the use of a cylinder instead of a flask for growing the culture. These results were rationalized by demonstrating that induction of 7α- and 7β-hydroxysteroid dehydrogenase is strongly mediated by chenodeoxycholic and deoxycholic acids, weakly mediated by cholic and 7-keto-lithocholic acids, and ineffective with 7-keto-deoxycholic, ursocholic, and ursodeoxycholic acids.—**Sutherland, J. D., and I. A. Macdonald.** The metabolism of primary, 7-oxo, and 7β-hydroxy bile acids by *Clostridium absonum*. *J. Lipid Res.* 1982. **23:** 726–732.

Supplementary key words hydroxysteroid dehydrogenase • keto bile acids • urso bile acids

The possible metabolic fate of the 7α-hydroxyl groups of primary bile acids in the human intestine includes: 7α-OH dehydroxylation, oxidation (to the ketone), and epimerization (to the 7β-OH group). A number of fecal *Clostridia* and *Eubacterium* isolates have been shown as active for 7α-OH dehydroxylation (1–4), while facultatives such as *E. coli* (5) and anaerobes such as *B. fragilis* (6, 7) and *C. sporosphaeroides*-like organisms (8) can oxidize 7α-OH groups (but not 7β-OH groups) to the corresponding ketone. It is now well established that the third reaction, 7α-OH epimerization, can occur in mixed fecal cultures (9–11), and very recently, human fecal isolates responsible for this reaction have been described as lecithinase-lipase-negative *Clostridia* (12).

In a recent report, we have shown that a soil isolate, *C. absonum* a "*perfringens*-like" organism (13), can convert both primary bile acids into urso (7β-OH) bile acids and 7-oxo bile acids (14). Additionally, we have shown the presence of 7α- and 7β-hydroxysteroid dehydrogenases in cell-free preparations of *C. absonum* (15), strongly supporting the hypothesis that 7α-OH epimerization proceeds through a 7-oxo intermediate. It is the purpose of this communication to report further study on the mechanism by which primary bile acids can be converted to urso-bile acids and vice versa in *C. absonum*.

MATERIALS AND METHODS

Materials

Clostridium absonum strain VPI #6905 (14, 15) was used throughout the study. Brain heart infusion (BHI)

Abbreviations: BHI, brain heart infusion; TLC, thin-layer chromatography; Eh, redox potential; HSDH, hydroxysteroid dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; UC, ursocholic acid (3α-, 7β-, 12α-trihydroxy-5β-cholan-24-oic acid); 7K-DC, 7-keto-deoxycholic acid (3α-, 12α-dihydroxy-7-keto-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); 7K-LC, 7-keto-lithocholic acid (3α-hydroxy-7-keto-5β-cholan-24-oic acid); DC, deoxycholic acid (3α-, 12α-dihydroxy-5β-cholan-24-oic acid); ATCC, American Type Culture Collection; VPI, Virginia Polytechnic Institute.

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broth and cooked meat broth were products of Difco Laboratories, Detroit, MI. Cholic acid was from J. T. Baker Chemicals, Phillipsburg, NJ; deoxycholic acid (DC) was from Calbiochemicals, Los Angeles, CA; chenodeoxycholic (CDC) and ursodeoxycholic (UDC) acids were from Sigma Chemicals, St. Louis, MO; 7-keto-deoxycholic acid (7K-DC) and 7-keto-lithocholic acid (7K-LC) were from Steraloids, Wiltshire, NH; ursocholic acid (UC) was kindly donated by Drs. R. A. DiPietro and A. F. Hofmann of the Division of Gastroenterology, School of Medicine, University of California, San Diego; labeled [24-^{14}C]cholic acid and CDC were products of New England Nuclear, Lachine, Quebec. Labeled intermediates, [24-^{14}C]7KLC and 7K-DC, and labeled urso-bile acids, [24-^{14}C]UDC and UC, were made by growing *C. absonum* in the presence of [24-^{14}C]CDC or cholic acid in 10-ml BHI cultures (each at a concentration of $2 \cdot 10^{-4}$ M) and extracting the spent bacterial medium as described before (14).

Ether, methanol, chloroform, acetic acid, and parahydroxybenzaldehyde (Komarowsky's reagent) were from Canadian Laboratories, Montreal, Quebec. Liquid scintillation fluid (catalogue #R19229-82) was from British Drug House, Montreal, Quebec. Commercial sheep's blood plates were a product of Becton Dickinson, Cockeysville, MD.

Growth of *C. absonum*

Time course studies were performed with 50-ml cultures which were either grown in 125-ml flasks or 50-ml graduated cylinders at 37°C and outside an anaerobic chamber. Cultures were inoculated with 5 ml of a 10-ml "overnight" starter culture. Cultures contained $2 \cdot 10^{-4}$ M 24-^{14}C-labeled primary or 7-oxo bile acid, or $5 \cdot 10^{-5}$ M 24-^{14}C-labeled urso bile acid with and without the simultaneous addition of $2 \cdot 10^{-4}$ M DC (unlabeled). Approximately 0.01 μCi of label was used per study. Sampling volumes of 3.5 ml were withdrawn at various time intervals. The absorbance at 660 nm of each culture sample was measured in a Beckman DB-GT spectrophotometer equipped with a Beckman 10 inch recorder. Cultures were then frozen at −20°C until extraction.

Estimation of Eh values

Redox potential (Eh) values were estimated by an Orion Eh probe using a 0.05% cysteine standard which gave an Eh value of +25 mv.

Identification of products

Products of 7α-OH oxidation and epimerization of cholic acid and CDC were identified by their R_f values on TLC plates (14, 15), their color and fluorescence characteristics under a UV lamp after spraying with Komarowsky's reagent (16), as well as the ability of commercial standards to co-chromatograph with the *C. absonum* product in question. Extraction, thin-layer chromatography elution, and counting procedures were performed as described previously (14).

Viable count estimations

Samples of *C. absonum* broth cultures (at t = 24 and 72 hr) were diluted serially into ice-cold BHI broth and 0.1-ml aliquots were spread over blood agar plates that were incubated anaerobically in a Gas Pak system (BBL). Colonies were counted after 24 hr incubation.

Effect of various bile acid inducers on 7α- and 7β-HSDH activities

Ten ml of starter culture was added to 100 ml of BHI broth containing $4 \cdot 10^{-4}$ M bile acid "inducer" as before (15). Cells were harvested at 6 hr by centrifuging at 6000 *g* for 20 min at 4°C. The pellet was resuspended in 3 ml of 0.1 M sodium phosphate buffer pH 7.0 containing 10^{-3} M EDTA and 10^{-3} M dithiothreitol.

The bacteria were lysed in a French pressure cell and the cell-free preparation was centrifuged at 6000 *g* for 20 min at 4°C. The supernatant fluid was decanted from the pellet. A mucopolysaccharide-like substance that tended to float on the supernatant fluid was generally avoided in transferring the cell-free preparation. Cell-free preparations were immediately assayed. The formation of NAD(P)H was followed at 340 nm and 25°C using a Beckman DB-GT grating spectrophotometer and 10 inch Beckman recorder. Each assay cuvette (1.0 ml) contained 1.0 mM NAD(P), 0.3 M glycine/NaOH buffer, pH 10.5 or pH 9.5, $1.0 \cdot 10^{-3}$ M bile acid, and 10–50 μl of enzyme solution.

RESULTS

Time course studies with primary bile acids (Figs. 1 and 2), 7-oxo bile acids (Figs. 3 and 4) and urso bile acids (Figs. 5 and 6) are presented.

These reactions were performed in singlet. However, repeated experiments on two selected time course curves revealed that the metabolic curves were reproducible within a 10% difference margin. The addition of DC to the system was shown to be effective in promoting the formation of UC from cholic acid when the cultures were grown in flasks (compare **Fig. 1b** to Fig. 1a), and hastened the rate of formation of UC but did not affect the final yield when the cultures were grown in cylinders (compare Fig. 1d to Fig. 1c). However, DC had only a marginal effect on increasing the UDC yield from CDC (**Fig. 2b** compared to Fig. 2a, and Fig. 2d compared to Fig. 2c). Growth of the organisms in a cylinder instead

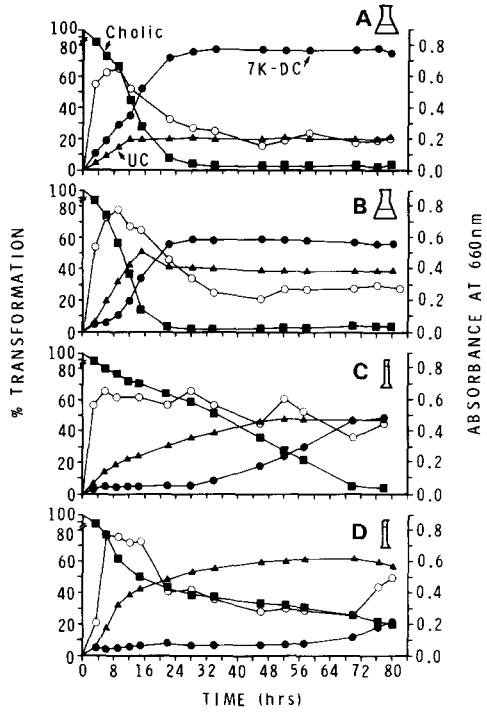

Fig. 1. Time course curve for the degradation of $2 \cdot 10^{-4}$ M [24-^{14}C]cholic acid by a 50-ml culture of *C. absonum* A) grown in a flask without DC, B) grown in a flask in the presence of $2 \cdot 10^{-4}$ M DC, C) grown in a cylinder without DC, and D) grown in a cylinder with $2 \cdot 10^{-4}$ M DC. Symbols: ■——■, cholic acid; ▲——▲, UC; ●——●, 7K-DC; ○——○, absorbance of culture at 660 nm.

of a flask was effective in sustaining a higher level of UC (giving as much as a 60% yield) from cholic acid (Fig. 1d compared to 1b). When CDC was a substrate, growth of the organisms in a cylinder was dramatically effective in maintaining the level of UDC after 8 hr (Fig. 2c and 2d). When the organisms were grown in a flask, most of the UDC generated in the first 8 hr was rapidly converted to 7K-LC and could not thereafter be recovered (Fig. 2a and 2b). The conversion of UDC to 7K-LC was actually more thorough in the presence of DC than in its absence. The explanation for this observation is not yet clear.

Similar results were obtained in **Fig. 3** which shows the metabolism of 7K-DC. The addition of DC to the flask was shown to be effective in promoting the formation of higher levels of UC (compare Fig. 3b with 3a). However, growth of the organisms in the cylinder plus the addition of DC was most effective in generating UC. When the substrate was 7K-LC (**Fig. 4**), the picture was similar, except that the addition of DC did not play a large role in increasing the yield of UDC (Fig. 4b compared to Fig. 4a). Again, it was only when the organisms were grown in a cylinder that a high yield of UDC was maintained in the culture (up to about 60 hr, Fig. 4c).

When the organisms were fed UC (**Fig. 5**) or UDC (**Fig. 6**) and grown in flasks, the results were in marked contrast with those for the primary or oxo substrates (Figs. 1–4); very little metabolism occurred (Figs. 5a and 6a). The cells could be induced to metabolize UC or UDC by the addition of DC to the culture which was effective when the ratio of DC:urso bile acid was 4:1 (Figs. 5b and 6b), or greater. However, the main metabolic products were oxo bile acids (7K-DC and 7K-LC). Only minor amounts of cholic acid and CDC were produced, and they leveled out to about 3% and 6%, respectively. When the same systems were studied using a cylinder (instead of a flask), the yields of cholic acid and CDC could be enhanced to about 12% and 15%, respectively (Figs. 5c and 6c). Moreover, the use of a cylinder was instrumental in delaying the metabolism of most of the UC and UDC to 7K-DC and 7K-LC. A further addition of DC to the cylinder cultures which gave a final DC concentration of $4 \cdot 10^{-4}$ M and a ratio of DC:UDC or DC:UC of 8:1 was also performed. Very similar results to the 4:1 (DC:urso bile acid) systems were obtained giving no improved yield of primary bile acid (results not shown).

In **Fig. 7**, the Klett units (absorbance at 660 nm), pH values, and Eh values of *C. absonum* cultures are represented for cultures with and without $2 \cdot 10^{-4}$ M CDC. As seen in Fig. 7a and 7b, cultures in cylinders (triangles)

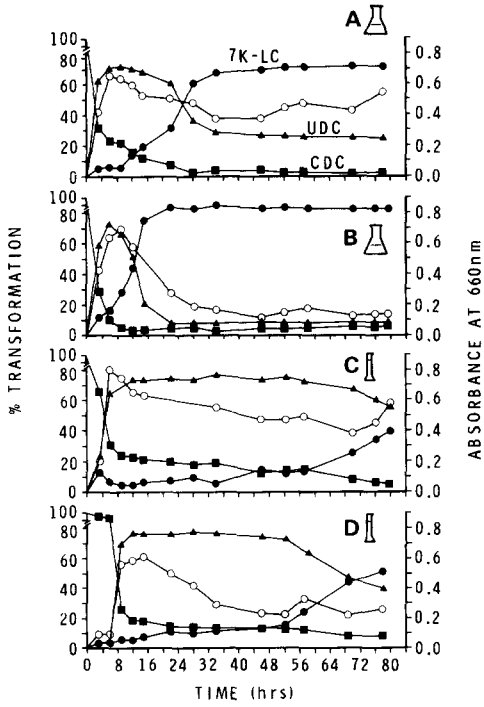

Fig. 2. Time course curve for the degradation of $2 \cdot 10^{-4}$ M [24-^{14}C]CDC by a 50-ml culture of *C. absonum*. Sequence A–D identical to Fig. 1. Symbols: ■——■, CDC; ▲——▲, UDC; ●——●, 7K-LC, ○——○, absorbance of culture at 660 nm.

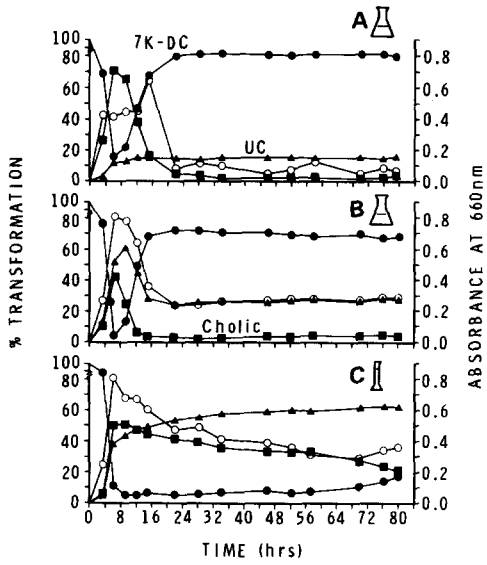

Fig. 3. Time course curve for the degradation of $2 \cdot 10^{-4}$ M [24-^{14}C]7K-DC by a 50-ml culture of *C. absonum* A) grown in a flask without DC, B) grown in a flask with $2 \cdot 10^{-4}$ M DC, C) grown in a 50-ml cylinder with $2 \cdot 10^{-4}$ M DC. Symbols as in Fig. 1.

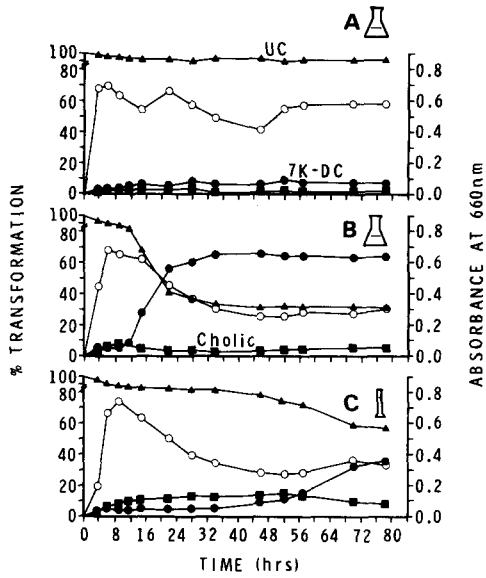

Fig. 5. Time course curve for the degradation of $5 \cdot 10^{-5}$ M [24-^{14}C]UC by a 50-ml culture of *C. absonum* A) grown in a flask without DC, B) grown in a flask in the presence of $2 \cdot 10^{-4}$ M DC, C) grown in a cylinder in the presence of $2 \cdot 10^{-4}$ M DC. Symbols as in Fig. 1.

grew similarly to those in flasks (squares), but higher absorbance values between 12 and 48 hr in the former suggest that the cells in the cylinder may have survived in greater numbers. This observation was confirmed by the viable count data which showed that at 24 hr there were about 3.2×10^8 organisms/ml in the flask culture (no bile acid added) compared to 3.9×10^8 organisms/ml in a cylinder culture. At 72 hr there were 3.8×10^4 and 2.2×10^7 organisms/ml, respectively. Differences in the bile acid degradation patterns between flask cultures and cylinder cultures cannot, however, be explained on the basis of viable cell differences since much of the metabolic activity occurs in the first 12 hr when there are no measurable differences in absorbance values at 660 nm.

When the pH value was studied with respect to time of culture, the pH initially dropped (regardless of whether the culture was in a flask or cylinder or whether it contained bile acid or not) and then recovered (Fig.

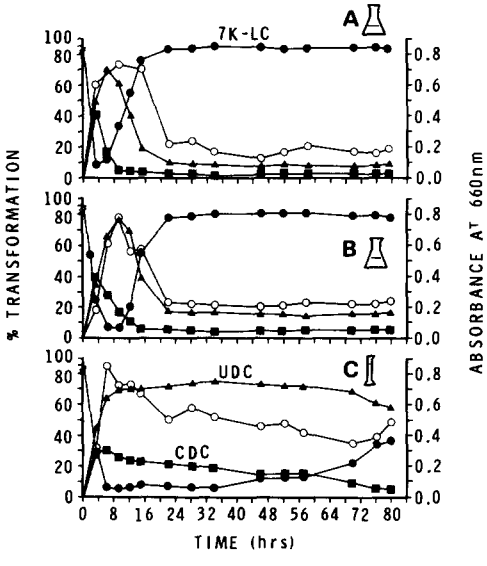

Fig. 4. Time course curve for the degradation of $2 \cdot 10^{-4}$ M [24-^{14}C]7K-LC by a 50-ml culture of *C. absonum*. Sequence A–C as in Fig. 3; symbols as in Fig. 2.

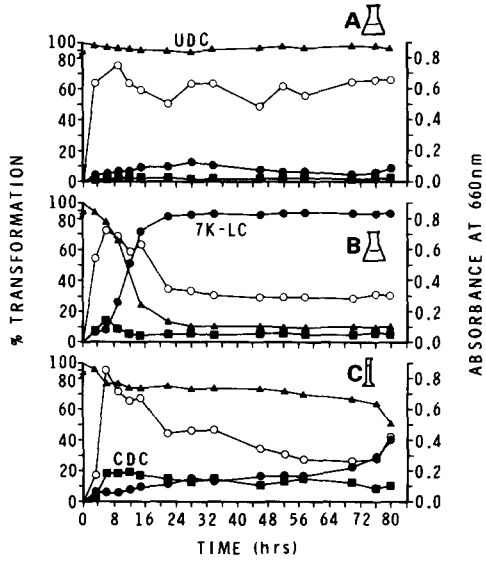

Fig. 6. Time course curve for the degradation of $5 \cdot 10^{-4}$ M [24-^{14}C]UDC by a 50-ml culture of *C. absonum*. Sequence A–C identical to Fig. 5; symbols as in Fig. 2.

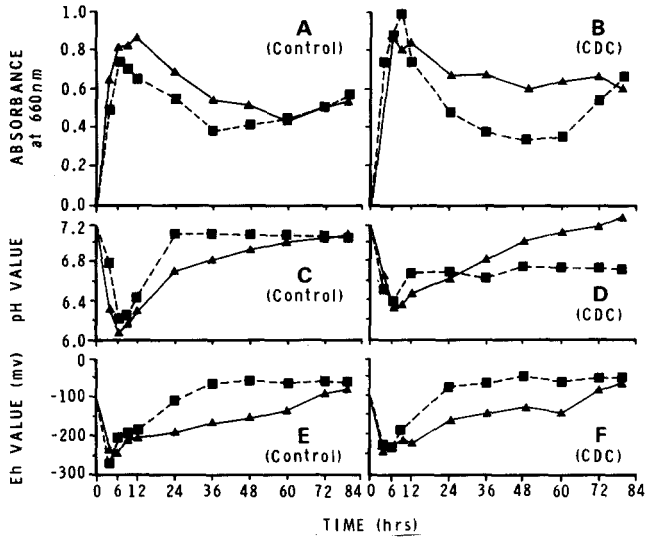

Fig. 7. Time course curves of *C. absonum* cultures showing the effect of time on A) absorbance values at 660 nm without CDC in the medium, B) absorbance values at 660 nm with CDC in the medium, C) pH values without CDC present, D) pH values with CDC present, E) Eh values without CDC present, and F) Eh values with CDC present. Symbols: ■ —— ■, flask cultures; ▲ —— ▲, cylinder cultures.

7c and 7d). The rise back to neutrality was slow when cylinders were used and not much affected by the addition of bile acid. But the rise back to neutrality was relatively rapid in a flask and never recovered to neutrality when CDC was added. Similar results were found when other bile acids were tested (results not shown) including 7K-LC ($2 \cdot 10^{-4}$ M), a mixture of cholic acid and DC (each $2 \cdot 10^{-4}$ M), and a mixture of 7K-DC and DC (each $2 \cdot 10^{-4}$ M). However, when $5 \cdot 10^{-5}$ M UC was added, the pH curves were similar to those of the control system (no bile acid) (Fig. 7c). When the Redox potential was studied (Fig. 7e and 7f), the Eh value was shown to drop from about −100 mv (achieved by autoclaving medium) to around −250 mv by the growth of the organism (all systems). The primary difference between the flask and cylinder cultures was that the cylinder was effective in retarding the rise in Eh with time as the culture aged.

Fig. 8 shows 7-HSDH activities measured against *a*) cholic acid (pH 10.5), *b*) UDC (pH 9.5), *c*) CDC (pH 10.5) (all with NADP), and *d*) cholic acid (pH 9.5) with NAD. The inducer bile acid at $4 \cdot 10^{-4}$ M is represented on the abscissa. As can be seen here, both CDC and DC are effective in inducing all enzyme systems; 7K-LC and cholic acid work at an intermediate level, while 7K-DC and the urso-bile acids are ineffective. Note that the activity against UDC (7β-HSDH) is totally absent in the control and requires an inducer, while a finite amount of 7α-HSDH is present without an inducer.

DISCUSSION

This work, in effect, represents an extension of a previous study (14) and further demonstrates that the epimerization of bile acids by *C. absonum* can occur via an oxidation-reduction mechanism which is mediated by 7-oxo bile acids. The reaction scheme: primary (7α-OH) bile acid ⇄ 7-keto bile acid ⇄ urso (7β-OH) bile acid appears to be partially reversible under appropriate conditions. Two features of the *C. absonum* culture appear to be most important in determining the outcome of the microbial degradation of the starting bile acid: *a*) the ability of the initial bile acid to induce the bile acid metabolic system of the culture or the presence of an added inducer (i.e., DC), and *b*) the shape of the fermentation vessel.

a) As established earlier, DC has the unusual property of inducing the 7α- and 7β-HSDH systems (15), yet itself is not catabolized. Thus DC provides a useful tool in probing the metabolism of bile acids such as 7K-DC and urso bile acids which (in contrast to CDC, cholic,

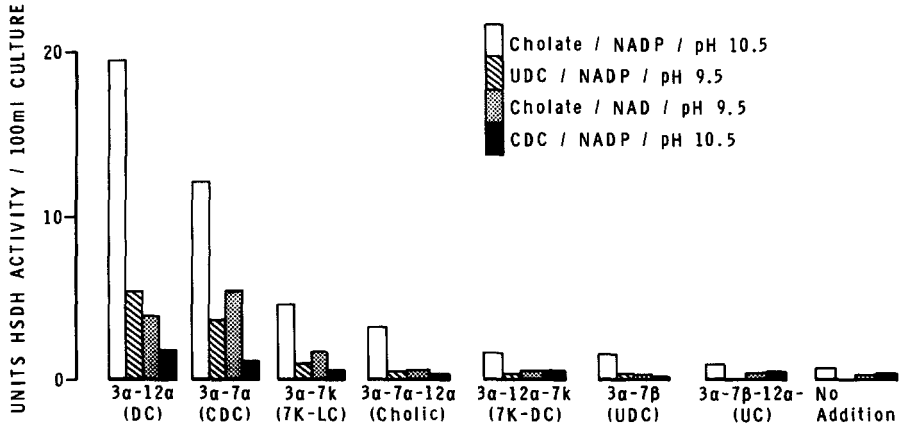

Fig. 8. Levels of HSDH activity induced by the addition of various bile acids to the culture as designated.

and 7K-LC) would otherwise not react or react very poorly since they are substrates, but non-inducers (or at best, poor inducers) of the 7α- and 7β-HSDH systems (14).

b) In an earlier study (14) it was unclear to us why the oxo-intermediate should accumulate and the viable cell count should drop rapidly in the aging culture. The current study suggests that the cells are able to generate and maintain a low Eh value in their culture for only the first 6–9 hr (probably by CO_2 evolution (14)) after which they are at the mercy of the rate of oxygen diffusion into the system, and the production of 7-oxo bile acid may be one manifestation of oxygen poisoning which, in a flask, causes the abrupt death of most of the organisms. A cylinder restricts oxygen diffusion and delays the death of the organisms and the formation of 7-oxo bile acid. We have also shown that layering 0.5 cm of olive oil on a culture in a flask or growing the organisms in a flask in a Gas Pak System is equally effective in preventing formation of 7-oxo bile acids.[2] We proposed earlier (14) that an increasing intracellular ratio of NADP/NADPH due to other metabolic activities just prior to the death phase of the cells might give rise to greater amounts of 7-oxo bile acid. Our current data are still consistent with this hypothesis. The reason why formation of 7K-LC in the CDC cultures in flasks is more prominent than formation of 7K-DC in the cholic system is not yet clear, but may relate to rate differences in the metabolism of the two primary bile acids (compare Fig. 1b with Fig. 2b).

Additionally, there appears to be an "anaerobic" equilibrium (Eh value < -100 mV) and an "aerobic" (Eh value > -100 mV) equilibrium. When a difunctional bile acid (CDC, 7K-LC, or UDC) was substrate and sufficient inducer was present, there is at equilibrium about 75% UDC, 20% CDC, and 5% 7K-LC (compare Figs. 2d, 4c, and 6c) if the culture stays sufficiently anaerobic. As the culture becomes more aerobic (Eh value rising above -100 mV or so), the equilibrium shifts to give about 87% 7K-LC, 8% UDC, and 5% CDC (compare Figs. 2b, 4b, and 6b). (The primary difference between the flask and cylinder experiments was that, in the former, the culture became aerobic faster.)

A similar relationship may exist for the trifunctional bile acids, except that the reaction is markedly slower even in the presence of DC inducer. (This may simply relate to differences in enzyme kinetics, e.g., K_m value for *C. absonum* 7α-HSDH is considerably higher for cholic than CDC (15).) In fact, in some cases, the reaction may not actually reach "anaerobic" equilibrium before the aerobic phase commences. It is, however, particularly difficult to drive the reaction when UC is the starting substrate (Fig. 5a–c).

Lastly, there was a correlation between the relative ability of a given bile acid to induce 7α- and 7β-HSDH (Fig. 8) and the ability of *C. absonum* to transform the bile acid to other products (Figs. 1–6). Urso bile acids and 7K-DC were very poor inducers and they required the presence of DC; cholic and 7K-LC were intermediate inducers and their transformation by *C. absonum* was marginally enhanced by the presence of DC in the culture. CDC was a good inducer and its metabolism could be only very slightly enhanced by DC addition. The difficulty in forcing the reverse reaction (particularly with UC) even in the presence of DC may be because urso bile acids behave as repressors. (In an earlier communication (15), we demonstrated that UDC does indeed behave as a repressor.)

The relationship between primary, 7-oxo, and urso bile acids has also been studied using mixed fecal flora and difunctional bile acids (9, 11). However, neither Hayase and coworkers (13) nor the authors of this report[2] have succeeded in isolating *C. absonum* from human feces, thus suggesting that one or more of the other species of fecal bacteria may be responsible for the in vivo formation of UDC (17). The recent report by Edenharder and Knaflic (12) suggests that the organisms normally responsible for 7-epimerization in the human intestine may be lecithinase-negative/lipase-negative *Clostridia* (12). Epimerization by these organisms is also evidently mediated by a 7-oxo derivative and, similarly to *C. absonum*, UDC is not metabolized (no inducer added). Further comparisons between *C. absonum* and the human fecal clostridial isolates must await further studies.

The ability of *C. absonum* to epimerize bile acids in the presence of mixed fecal cultures is currently under study.

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