

Effect of bile acid oxazoline derivatives on microorganisms participating in 7 α -hydroxyl epimerization of primary bile acids

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Abstract We tested bile acid oxazoline derivatives of chenodeoxycholic (CDC-OX), 7-ketolithocholic (7-KLC-OX), ursodeoxycholic (UDC-OX), and deoxycholic (DC-OX) as inhibitors of the 7-epimerization of the primary bile acids cholic acid (CA) and CDC in cultures of four species of bacteria and the human fecal flora. The organisms tested elaborate a 7 α - and/or 7 β -hydroxysteroid dehydrogenase (HSDH); they were *Escherichia coli* (7 α -HSDH), *Bacteroides fragilis* (7 α -HSDH), *Clostridium absonum* (7 α - and 7 β -HSDH) and *Eubacterium aerofaciens* (7 β -HSDH). None of the oxazolines affected 7 α -OH oxidation of CA or CDC by *E. coli* or the growth of the organism. All the oxazolines (except UDC-OX) inhibited the growth of *B. fragilis* and its 7 α -HSDH. In contrast, only DC-OX blocked 7 α -OH epimerization of CA by *C. absonum*. Surprisingly, the other three oxazolines enhanced 7 α -OH epimerization of CA, but not that of CDC, which was inhibited (CDC-OX > 7-KLC-OX \gg UDC-OX). Enzymic data suggest that CDC-OX in the presence of CA can induce a greater level of both 7 α - and 7 β -HSDH than CA or CDC-OX alone, CDC-OX being more toxic in the presence of CDC. Formation of urso-bile acid from 7-keto substrates by *E. aerofaciens* is totally blocked by the oxazolines (except UDC-OX). Similarly, suppression of urso-bile acid formation from primary bile acids by the human fecal flora was evident with DC-OX > 7-KLC-OX > CDC-OX \gg UDC-OX, the last being ineffective. The inhibitory activity of the oxazolines on the 7-dehydroxylation of primary bile acids by human fecal flora followed the same order.—**Macdonald, I. A., J. D. Sutherland, B. I. Cohen, and E. H. Mosbach.** Effect of bile acid oxazoline derivatives on microorganisms participating in 7 α -hydroxyl epimerization of primary bile acids. *J. Lipid Res.* 1983. **24**: 1550–1559.

Supplementary key words 7-hydroxysteroid dehydrogenase • chenodeoxycholic acid • cholic acid • human fecal flora

A number of intestinal microorganisms, including *Escherichia coli* (1, 2), *Bacteroides fragilis* (3, 4), *Eubacterium aerofaciens* (5, 6), *Peptostreptococcus productus* (6, 7), *Clostridium absonum* (8, 9), and certain lecithinase-lipase-negative *Clostridia* (10) can participate in the epimerization of primary (7 α -hydroxyl) bile acids to urso (7 β -hydroxyl) bile acids. In fact, ursodeoxycholic acid (UDC) formed from chenodeoxycholic acid (CDC) by microbial action

in the intestine (or by a combination of microbial and hepatic action) is the fourth most prevalent bile acid in human bile (11). Recent studies show that oral administration of UDC to cholesterol gallstone patients successfully dissolves gallstones (12, 13). In vivo, UDC is formed from CDC largely via the intermediate 7-KLC by microbial 7 α -hydroxysteroid dehydrogenases (7 α -HSDH). Subsequently, the reduction of 7-KLC giving UDC occurs by the action of 7 β -HSDH-containing organisms. This process may take place a) by the action of two different organisms containing 7 α - and 7 β -HSDH respectively (e.g., *B. fragilis* and *E. aerofaciens* (5)) or b) by the action of a single organism containing both enzymes (e.g., *C. absonum* or lecithinase-lipase-negative *Clostridia* (10)) or c) via oxidation by 7 α -HSDH-elaborating intestinal organisms (e.g., *E. coli*) and reduction of the keto-intermediate to UDC by the liver (14–16). There is evidence that all three mechanisms may be operating in humans. Ursodeoxycholic acid appears to be less toxic and more hydrophilic than CDC, thus its formation may be beneficial (17).

In contrast, 7-dehydroxylation of primary bile acids yields products which are less hydrophilic and more toxic than the primary bile acids. Organisms elaborating 7-dehydroxylase include *C. leptum* (18, 19) and a *Eubacterium* sp. (20, 21). This is carried out by a single species in pure

Abbreviations: Systematic names of bile acids and oxazoline derivatives are as follows: CA, cholic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid; CDC, chenodeoxycholic acid, 3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid; DC, deoxycholic acid, 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid; UDC, ursodeoxycholic acid, 3 α ,7 β -dihydroxy-5 β -cholan-24-oic acid; LC, lithocholic acid, 3 α -hydroxy-5 β -cholan-24-oic acid; 7-KLC, 7-ketolithocholic acid, 3 α -hydroxy-7-oxo-5 β -cholan-24-oic acid; 7-KDC, 7-ketodeoxycholic acid, 3 α ,12 α -dihydroxy-7-oxo-5 β -cholan-24-oic acid; 12-KCDC, 12-ketochenodeoxycholic acid, 3 α ,7 α -dihydroxy-12-oxo-5 β -cholan-24-oic acid; UC, ursocholic acid, 3 α ,7 β ,12 α -trihydroxy-5 β -cholan-24-oic acid; ICDC, isochenodeoxycholic acid, 3 β ,7 α -dihydroxy-5 β -cholan-24-oic acid. Oxazoline derivatives of bile acids were abbreviated with "OX" after the bile acid abbreviation (e.g., CDC-OX, UDC-OX). Thus CDC-OX is 2-(3 α ,7 α -dihydroxy-24-nor-5 β -cholanyl)-4,4-dimethyl-2-oxazoline.

culture and proceeds via a $\Delta 6,7$ intermediate (21, 22). Recent studies show that, in addition to primary bile acids, UDC may also be 7-dehydroxylated by *Eubacterium* sp. (23) and by the human fecal flora (24, 25), but not by *C. leptum* (18, 19). The resulting secondary bile acids, DC and LC, collectively represent the third most prevalent bile acids in human bile and are the predominant fecal bile acids (15).

Unfortunately, LC is a potent liver toxin in animal models (26, 27) and is co-mutagenic in the Ames assay (28, 29); moreover, formation of LC from CDC or UDC administered to gallstone patients may represent both a drain on the pharmacologically active agent and a source of toxicity during therapy. Additionally, high fecal concentrations of dihydroxy-bile acids (particularly DC) can cause diarrhea at high fecal pH values (30). In man, the main mechanism of detoxification of LC is sulfation by the liver before biliary secretion (15). However, an elevated serum glyco-LC-sulfate level, induced by CDC administration in gallstone patients, appears to be associated with a high serum transaminase level (31).

Thus mechanisms of preventing bacterial 7-dehydroxylation may be of practical significance. Recently, Ayengar and coworkers (32) synthesized a series of bile acid oxazoline derivatives and showed that oral administration of CDC-oxazoline (CDC-OX) in low amounts effectively blocks 7-dehydroxylation in rats (33). The structure of CDC-oxazoline is shown in Fig. 1. Hylemon, Fricke, and Mosbach (34) have shown that CDC-OX inhibits the growth of dehydroxylating organisms and other bacteria by causing the cells to lyse spontaneously. The effect of bile acid oxazolines on 7-epimerization has not yet been investigated. In this study we report the effect of bile acid oxazolines on four organisms that can participate in 7-epimerization, and on human mixed fecal flora.

METHODS AND MATERIALS

Cultures

Model cultures used in this study were: *i*) *E. coli*, strain #23 (35) (ATCC #29532); *ii*) *B. fragilis*, strain #18 (3); *iii*) *C. absoum*, strain VPI 6905 (ATCC #27555), and *iv*) *E. aerofaciens*, strain ATCC #25986. Strains were maintained in cooked meat cultures prior to inoculation into Brain Heart Infusion (BHI) starter cultures for transformation tests.

Bile acids and oxazolines

Cholic acid (CA) was from J. T. Baker Chemicals, Phillipsburg, NJ; deoxycholic acid (DC) was from Calbiochem-Behring, San Diego, CA; chenodeoxycholic (CDC) and ursodeoxycholic (UDC) acids were from Sigma Chemicals,

Oxazoline Derivative of Chenodeoxycholic Acid

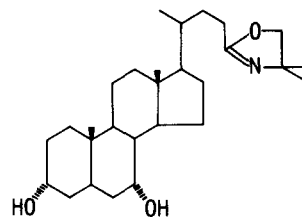


Fig. 1. Chemical structure of the oxazoline derivative of chenodeoxycholic acid.

St. Louis, MO. 7-Ketodeoxycholic acid (7-KDC) and 7-ketolithocholic acid (7-KLC) were from Steraloids, Wiltschire, NH. The bile acid oxazolines (CDC-OX, UDC-OX, 7-KLC-OX, and DC-OX) were synthesized according to the method of Ayengar et al (32). All the above bile acids gave one spot on TLC using chloroform-methanol-acetic acid 40:4:2 (v/v/v) and similarly the oxazoline derivatives gave one spot using chloroform-methanol 40:2 (v/v).

Labeled $[24-^{14}\text{C}]$ CA and CDC were products of New England Nuclear, Lachine, Quebec. Labeled 7-keto bile acids $[24-^{14}\text{C}]$ 7-KDC and $[24-^{14}\text{C}]$ 7-KLC were made by growing 10-ml cultures of *E. coli* for 24 hr in the presence of $[24-^{14}\text{C}]$ CA or $[24-^{14}\text{C}]$ CDC and purifying the respective 7-keto-intermediate by TLC. In all cases, over 99% of the label was associated with the spot by TLC corresponding to the radiolabeled bile acid in question. The final specific activity of each of the four radiolabeled bile acids used was approximately $1.0 \times 10^{-3} \mu\text{Ci}/\mu\text{mol}$.

Effect of bile acid oxazolines on the growth of model organisms and the transformation of bile acid substrates

Nine-ml cultures of freshly boiled BHI broth containing 0.2 mM CDC (plus 0.01 μCi of ^{14}C -labeled CDC) or 0.2 mM CA (plus 0.01 μCi of ^{14}C -labeled CA) were inoculated with a 10% overnight starter culture (10% volume) of the desired organism and grown at 37°C. A final concentration of 0.10 mM CDC-OX, 7-KLC-OX, UDC-OX, or DC-OX (all unlabeled) was added from stock solutions in methanol. All cultures were grown in air except *E. aerofaciens* which was grown in a Gas Pak system. Cultures were sampled by the removal of 3-ml aliquots at 6 hr, 24 hr, and 48 hr. Samples were acidified to pH 3, extracted, and subjected to TLC analysis as described before (8, 9, 36). Bile acid products were separated on TLC by chloroform-methanol-acetic acid 40:4:2 (v/v/v) for CA and its degradation products and chloroform-methanol-acetic acid 40:2:1 (v/v/v) for CDC and degradation products.

Effect of bile acid oxazolines on the transformation of primary bile acids by the human fecal flora

These experiments were performed as described above, except that starter cultures consisted of 10 ml of freshly boiled BHI inoculated with approximately 1 g of fresh human feces.

Inhibition of crude microbial 7 α - and 7 β -HSDH preparations in vitro by CDC-OX and UDC-OX

Lyophilized preparations of *i*) *E. coli* 7 α -HSDH (35), *ii*) *B. fragilis* 7 α -HSDH (3), *iii*) *C. absonum* 7 α - and 7 β -HSDH (26, 37), and *iv*) *E. aerofaciens* 7 β -HSDH (5) were made as described in earlier communications. Approximately 10–20 mg of lyophilized preparation (for the first three organisms) (or 60 mg of *E. aerofaciens* 7 β -HSDH) were dissolved in 1.0 ml of twice-distilled water, and 50 μ l of enzyme solution was used for each assay. In all systems we used CA as a model substrate for 7 α -HSDH and UDC for 7 β -HSDH. Assay systems consisted of: 1.0 mM NAD, 2.0 mM CA in 0.30 M glycine–NaOH buffer, pH 9.5 (for *E. coli* and *B. fragilis* 7 α -HSDH); 1.0 mM NADP, 2.0 mM CA in 0.30 M glycine–NaOH, pH 10.5 (for *C. absonum* 7 α -HSDH); 1.0 mM NADP, 2.0 mM UDC in 0.30 M glycine–NaOH, pH 9.5–10 (for *C. absonum* and *E. aerofaciens* 7 β -HSDH, respectively). The enzymes were tested in the presence (and absence) of 0.050 mM and 0.10 mM CDC-OX and UDC-OX. The total volume of the reaction mixture was 1.0 ml. All reactions were initiated by enzyme addition and were run at 25°C. Formation of NAD(P)H was monitored in a Beckman DBG T spectrophotometer/recorder at 340 nm.

Effect of bile acid oxazolines on the induction of *C. absonum* 7 α - and 7 β -HSDH by CDC and CA

Experiments were performed as described earlier (37, 38). We grew 100-ml cultures of *C. absonum* in graduated cylinders (9) in the presence of *a*) no bile acid, *b*) 0.05 mM CDC-OX, 7-KLC-OX, or UDC-OX, *c*) 0.2 mM CA, *d*) 0.2 mM CA plus the above oxazolines at 0.05 mM, *e*) 0.2 mM CDC, and *f*) 0.2 mM CDC plus 0.05 mM CDC-OX for comparison. The bacteria were harvested at 6 hr and cell preparations were made and assayed for 7 α - and 7 β -HSDH as described before (37, 38).

Extraction and thin-layer chromatography of the bile acid oxazolines in pure and mixed fecal cultures

Five-ml cultures of the above described organisms and mixed fecal cultures were incubated in the presence of 0.10 mM CDC-OX, 7-KLC-OX, UDC-OX, and DC-OX (20 combinations) for 48 hr and extracted twice *without prior acidification* (to avoid artifactual degradation of oxazoline) by two volumes of ether. Combined ether extracts

were allowed to evaporate, 50 μ l of methanol–water 4:1 (v/v) was added, and all of the reconstituted extract was spotted on a 20 \times 20 cm \times 250 μ m TLC plate which was then developed in chloroform–methanol 40:2 (v/v). Plates were sprayed with Komarowsky's reagent and examined under visible and UV light (36).

Protein estimations

Protein was estimated according to Bradford (39).

RESULTS

Inhibition of bile acid transformation by oxazolines

As shown in **Table 1**, none of the oxazolines were inhibitory to *E. coli*; however, CDC-OX, 7-KLC-OX, and DC-OX were inhibitory by varying degrees to whole cell cultures of *B. fragilis*, *C. absonum*, and *E. aerofaciens*, while UDC-OX was usually less inhibitory to these species. Thus the bile acid transformations by these three organisms that participate in the reaction: primary bile acid \rightleftharpoons 7-keto bile acid \rightleftharpoons urso bile acid, were generally inhibited in proportion to the inhibition of bacterial growth. The order of decreasing potency was generally DC-OX \approx CDC-OX \approx 7-KLC-OX $>$ UDC-OX. Although UDC-OX suppressed the growth of these three organisms to some extent, it apparently did not suppress bile acid transformation at 0.10 mM. The most surprising feature of the study was that CDC-OX, 7-KLC-OX, and UDC-OX, when added to *C. absonum* cultures containing CA, actually enhanced the transformation of CA to UC (while DC-OX was so inhibitory that transformation was almost totally abolished). Additionally, 7-KLC-OX effectively blocked the transformation of 7-KLC to UDC, but not of 7-KDC to UC in *E. aerofaciens* (Table 1).

When the same series of oxazoline derivatives was added to cultures inoculated with human fecal flora (one human volunteer studied) (**Fig. 2**), it can be seen that the oxazolines were partially inhibitory to epimerization of CDC to UDC with DC-OX $>$ 7-KLC-OX $>$ CDC-OX \gg UDC-OX. Inhibition of the dehydroxylation of CA and CDC to DC and LC, respectively, followed a similar pattern (Fig. 2). A minor band tentatively identified as isochenodeoxycholic acid (3 β ,7 α ; ICDC) (40), occurred immediately above the UDC band. The amount of label in this band was not depressed by oxazoline addition to the culture (Fig. 2). However, enhancement of formation of 7-KDC (or 12-KDC) and 7-KLC can occur in the presence of oxazoline presumably because of the suppression of competing reactions (i.e., 7-epimerization and 7-dehydroxylation). Unfortunately the TLC system (see Methods) does not separate 12-KCDC from 7-KDC (both derived from CA) or LC from 3-oxo-5 β -cholan-24-oic

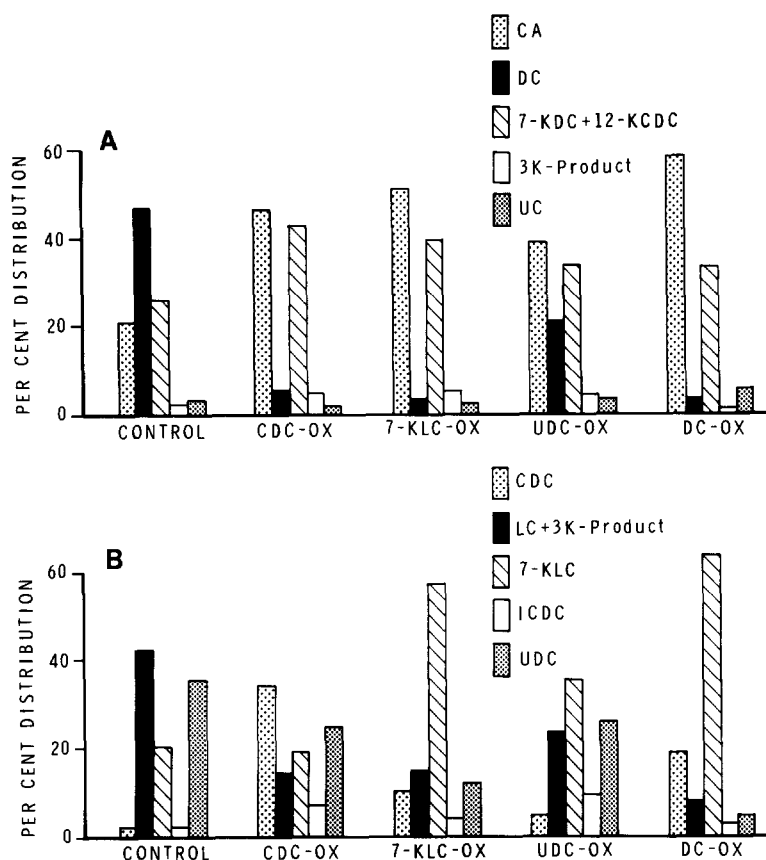


Fig. 2. Effect of various bile acid oxazolines (0.10 M) on the transformation of primary bile acids (0.20 M) by the human fecal flora: (A) CA transformation; (B) CDC transformation. Products as indicated in the figure (growth time, 24 hr).

acid (both derived from CDC) (Fig. 2A and B). None of the oxazolines were inhibitory to bacterial growth in the human fecal cultures. In CA-containing cultures, we were able to detect only little conversion to UC; this process appears to be inhibited by the oxazolines analogous to UDC formation from CDC.

When a series of concentrations of CDC-OX was studied for each of the four organisms and in mixed fecal cultures, a somewhat greater inhibition of transformation at the 7 position could be seen by increasing the CDC-OX concentration from 0.05 mM to 0.10 mM with little or no increase in inhibition at 0.20 mM in most cases (Fig. 3 and Fig. 4). Concentrations of CDC-OX less than 0.05 mM were ineffective. Thus comparisons of the abilities of the four oxazolines to inhibit transformation of primary bile acids (Table 1) were made at the minimal effective CDC-OX concentration of 0.10 mM. Interestingly, some inhibition of 7-KDC formation from CA in *E. coli* cultures by 0.20 mM CDC-OX was evident even though growth of this organism was not affected (Fig. 3A). Consistent with Table 1, the transformation of CA to UC by *C. absonum* was enhanced by CDC-OX even at a concentration of 0.01 mM (Fig. 3C). This enhancement was evident in spite of some inhibition of growth of this

organism at high CDC-OX concentrations. A similar phenomenon did not occur when CDC was introduced into *C. absonum* cultures (compare Fig. 4C with Fig. 3C).

In mixed fecal cultures, formation of UC from CA (which amounted to less than 15% even in the absence of oxazoline) was inhibited by CDC-OX, but required 0.10 mM CDC-OX for total inhibition (Fig. 3E). A similar observation was made for UDC formation from CDC by human fecal flora, but this reaction was only partially inhibited (Figs. 2B and 4E). Formation of 7-keto bile acids by mixed fecal cultures was not suppressed by the presence of bile acid oxazolines, but was enhanced to varying degrees (Figs. 2, 3E, and 4E).

Chemical degradation of bile acid oxazolines in culture

Thin-layer chromatography revealed that the oxazolines tended to degrade in the pure cultures, as well as in mixed fecal cultures, giving primarily the parent bile acid and several other bands visible after spraying with Komarowsky's reagent. Similar results were obtained by incubation of oxazoline in sterile medium which had been adjusted to pH 5.5, but not for unadjusted medium at

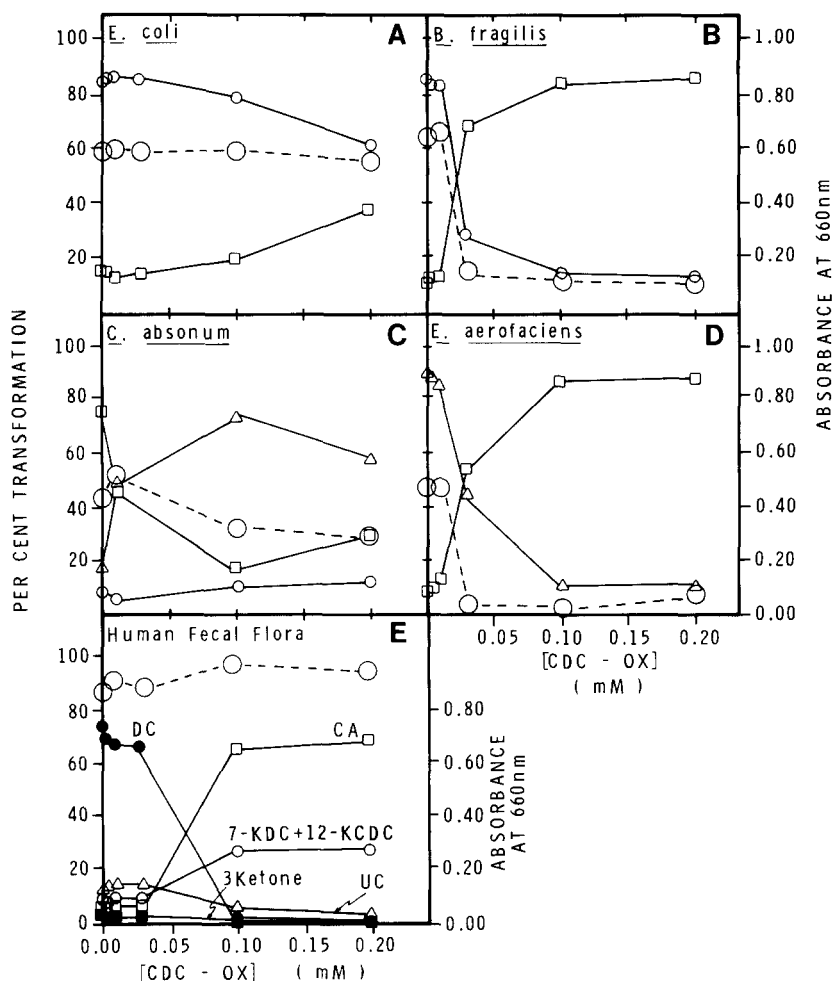


Fig. 3. Effect of CDC-OX concentration on the transformation of (A) CA ($3\alpha,7\alpha,12\alpha$ -) to 7-KDC ($3\alpha,12\alpha,7$ -keto-) by *E. coli*; (B) CA to 7-KDC by *B. fragilis*; (C) CA to 7-KDC and UC ($3\alpha,7\beta,12\alpha$ -) by *C. absonum*; (D) 7-KDC to UC by *E. aerofaciens*; and (E) CA to various products by the human fecal flora (growth time, 24 hr). Symbols: CA, (\square — \square); 7-KDC, (\circ — \circ); UC, (Δ — Δ); DC, ($3\alpha,12\alpha$ -) (\bullet — \bullet); 3-keto derivative, (\blacksquare — \blacksquare) and absorbance at 660 nm (\circ — \circ).

pH 7.4, suggesting this degradation is a non-enzymic, pH-dependent process.

Inhibition of 7α - and 7β -hydroxysteroid dehydrogenase preparations in vitro by bile acid oxazolines

As seen in **Fig. 5**, some degree of inhibition of 7α - and 7β -HSDH preparations by CDC-OX and UDC-OX was evident, with the former invariably being the more potent inhibitor. The greatest degree of inhibition was seen with *C. absonum* 7α -HSDH and CA as substrate. Further investigation revealed that the type of inhibition was noncompetitive (results not shown). In contrast, no inhibition of *E. coli* or *B. fragilis* 7α -HSDH could be obtained with underivatized DC, UDC, or 7-KLC; similarly, no inhibition of *E. aerofaciens* 7β -HSDH by CDC, DC, or 7-KLC occurred (up to 10^{-3} M was tested in each case).

Induction of 7α - and 7β -hydroxysteroid dehydrogenases in *C. absonum* by CA, CDC, and CDC-OX

The 7α - and 7β -HSDH cell preparation made from *C. absonum* grown in the presence of 0.2 mM CDC showed a tenfold increase in specific activity as compared to CA-induced culture preparations, in which the activity was similar to the uninduced culture. However, the addition of both 0.2 mM CA and 0.02 mM CDC-OX to the culture resulted in about a fivefold enhancement in the specific activities of 7α - and 7β -HSDH, while 0.02 mM CDC-OX alone was ineffective. No further increase in specific activities of the HSDH's could, however, be observed by a similar addition of CDC and CDC-OX to the culture. In fact, CDC-OX, and to a greater degree CDC and CDC-OX together, were toxic to the culture, while the toxicity

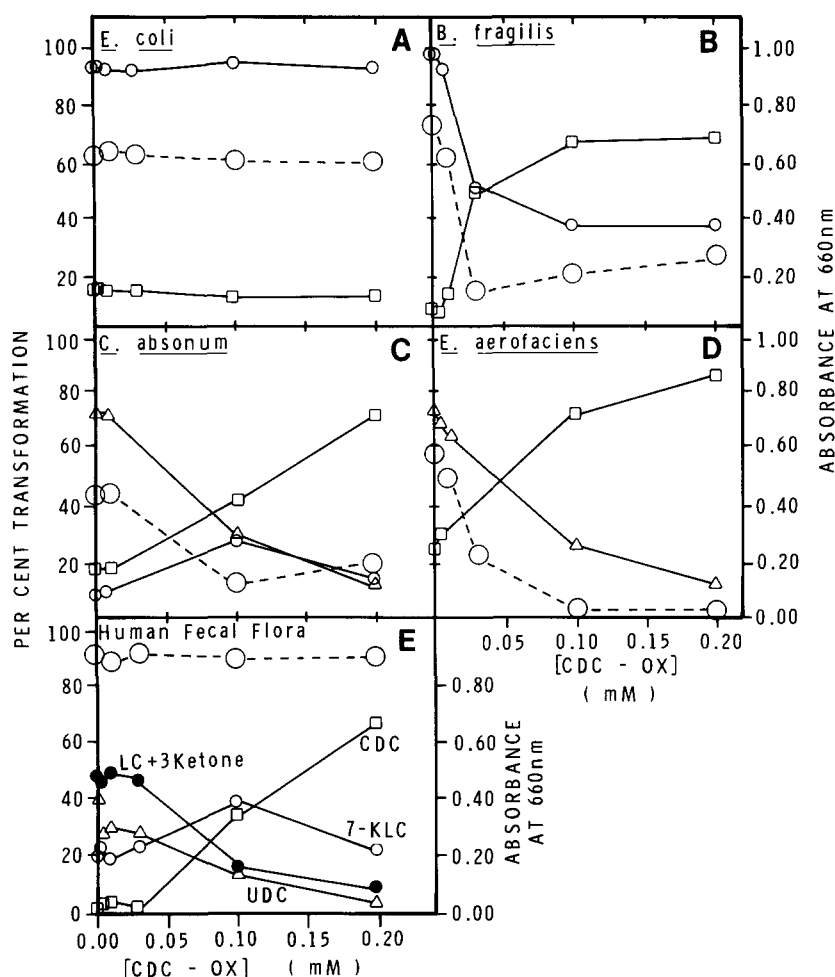


Fig. 4. Effect of CDC-OX concentration on the transformation of (A) CDC (3 α ,7 α -) to 7-KLC (3 α ,7-keto-) by *E. coli*; (B) CDC to 7-KLC by *B. fragilis*; (C) CDC to 7-KLC and UDC (3 α ,7 β -) by *C. absonum*; (D) 7-KLC to UDC by *E. aerofaciens*; and (E) CDC to various products by the human fecal flora (growth time, 24 hr). Symbols: CDC, (□ — □); 7-KLC, (○ — ○); UDC, (Δ — Δ); mixture of LC (3 α -) and 3-keto derivative, (● — ●); and absorbance at 660 nm (○ — ○).

could be relieved by the addition of CDC-OX and CA together (**Fig. 6**).

A similar series of results could be obtained by substituting 7-KLC-OX or UDC-OX for CDC-OX (results not shown). (However, DC-OX at 0.02 mM was so inhibitory that it was not possible to perform the experiment even in the presence of CA).

DISCUSSION

The bile acid oxazolines appear to interfere with the epimerization of primary bile acids at the 7-position by inhibiting the growth of a number of organisms that can participate in the reaction (with the notable exception of *E. coli*). No similar effect can be observed by the addition of the same concentration of parent bile acid.¹ Trans-

formation data at 6 hr and 48 hr confirm and extend the data presented in Table 1 and Figs. 3 and 4.² Thus it is not surprising that formation of 7-KDC and 7-KLC from CA and CDC by the human fecal flora is not inhibited, but enhanced. The enhancement of formation of 7-KDC and 7-KLC appears to be largely a result of suppression or elimination of competing reactions within the ecosystem. Moreover, it appears that the *in vitro* inhibition of various preparations of 7 α - and 7 β -HSDH (**Fig. 5**) is secondary to the inhibitory effects of the oxazolines on the growth of different cultures. This may largely explain the results observed *in vivo* (33). For example, the specific activity of *C. absonum* 7 β -HSDH greatly exceeds that of *E. aerofaciens* 7 β -HSDH in cell preparations identically prepared (**Fig. 5**). The same is true, even when *C. absonum* 7 β -HSDH is assayed in the presence of 0.10 mM CDC-OX and compared to that of uninhibited *E.*

¹ Macdonald, I. A. Unpublished observations.

² Macdonald, I. A., and J. D. Sutherland. Unpublished data.

TABLE 1. Effect of bile acid oxazolines on formation of 7-keto and 7 β -hydroxyl compounds from precursor bile acids (0.2 mM) by four model organisms (growth time = 24 hr)^a

Oxazoline at 0.1 mM	Bile Acid Product (or Starting Material) and Growth ^b	<i>E. coli</i>		<i>B. fragilis</i>		<i>C. absonum</i>		<i>E. aerofaciens</i>	
		+CDC	+CA ^c	+CDC	+CA	+CDC	+CA	+7-KLC	+7-KDC
None	Primary bile acid	10 ^d	11	8	9	12	73	0	0
	7-Keto bile acid	90 ^e	89	92	91	16	9	18	12
	7 β -Hydroxy bile acid	0 ^f	0	0	0	72	18	82	86
	Growth	+++++	+++++	++++	++++	++++	++++	++++	++++
CDC-OX	Primary bile acid	9	20	64	85	47	18	0	0
	7-Keto bile acid	91	80	36	15	18	4	96	78
	7 β -Hydroxy bile acid	0	0	0	0	35	78	4	22
	Growth	+++++	+++++	+	+	+	++	+	+
7-KLC-OX	Primary bile acid	10	12	57	88	14	16	0	0
	7-Keto bile acid	90	88	43	12	20	8	92	6
	7 β -Hydroxy-bile acid	0	0	0	0	66	76	8	94
	Growth	+++++	+++++	+	+	++	+++	+	+
UDC-OX	Primary bile acid	10	12	14	20	17	16	0	0
	7-Keto bile acid	90	88	86	80	10	8	10	5
	7 β -Hydroxy-bile acid	0	0	0	0	73	78	90	95
	Growth	+++++	+++++	++	++	+++	+++	++	++
DC-OX	Primary bile acid	8	21	58	78	78	70	0	0
	7-Keto-bile acid	92	79	42	22	10	15	94	92
	7 β -Hydroxy-bile acid	0	0	0	0	12	15	6	8
	Growth	+++++	+++++	+	+	+	+	+	+

^a Figures given in percent bioconversion on analysis of radiolabeled products on TLC after 24 hr growth of the culture.

^b Growth on the basis of Klett units and graded from + to +++++.

^c Starting material either CDC, CA, 7-KLC, or 7-KDC as designated.

^d Percent of primary bile acid remaining at end of 24 hr.

^e Percent of corresponding 7-keto bile acid at end of incubation.

^f Percent of corresponding 7 β -hydroxyl bile acid at end of incubation.

Abbreviations: CDC, 3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid; CA, 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid; 7-KLC, 3 α -hydroxy-7-oxo-5 β -cholan-24-oic acid; 7-KDC, 3 α ,12 α -dihydroxy-7-oxo-5 β -cholan-24-oic acid; UDC, 3 α ,7 β -dihydroxy-5 β -cholan-24-oic acid; DC, 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid. The suffix "-OX" designates the oxazoline derivative.

aerofaciens 7 β -HSDH (Fig. 5). Yet *E. aerofaciens* in the presence of a 7 α -HSDH-elaborating organism is more efficient in the transformation of CDC to UDC than *C. absonum* (5, 9).

Our results are consistent with those of Hylemon, Fricke, and Mosbach (34) who showed that a number of species in the human fecal flora are sensitive to bile acid oxazolines, while others are resistant; these derivatives act against a spectrum of organisms rather like antibiotics. Their mechanism of action is not yet well understood, although they cause the susceptible cells to lyse. It also appears that the sensitivity of the cells to oxazolines is not necessarily a function of the presence of bile acid-metabolizing enzymes in the sensitive organisms (34), although all of the anaerobic organisms participating in 7-epimerization in the human fecal flora thus far studied appear to be oxazoline-sensitive.

The case of *C. absonum* appears to be unique. Both Nakamura et al. (41) and ourselves (40) have failed to isolate *C. absonum* from human feces. The *C. absonum* data in Table 1 and Figs. 3C and 4C, when compared to data on bile acid metabolism in the fecal flora (Figs.

2, 3E, and 4E), are consistent with the proposal that *C. absonum* is not a normal resident of the human intestine, contrary to our original proposal (8).

The induction of 7 α - and 7 β -HSDH in *C. absonum* (37, 38) is a necessary feature for efficient transformation of bile acids by the organism. Enzyme induction in *C. absonum* cultures appears to result from an appropriate level of toxicity of the inducer to the cell. This also results in the synthesis of a number of polypeptides in addition to 7 α - and 7 β -HSDH (42). Thus CA, although a substrate, is a poor inducer; while DC is a good inducer, but not a substrate (37, 38). The addition of CDC-OX, 7-KLC-OX, or UDC-OX to CA-containing cultures results in levels of toxicity which are not totally inhibitory to growth, but which induce the 7 α - and 7 β -HSDH activity, thereby enhancing bile acid transformation. Thus "co-induction" in these instances (Fig. 6) explains enhanced formations of UC from CA (Table 1 and Fig. 3C). On the other hand, the mixtures of CDC and bile acid oxazolines (with the exception of UDC-OX) that we tested were so toxic to the cultures that both growth and transformation were markedly depressed. We propose that only in *C. absonum*

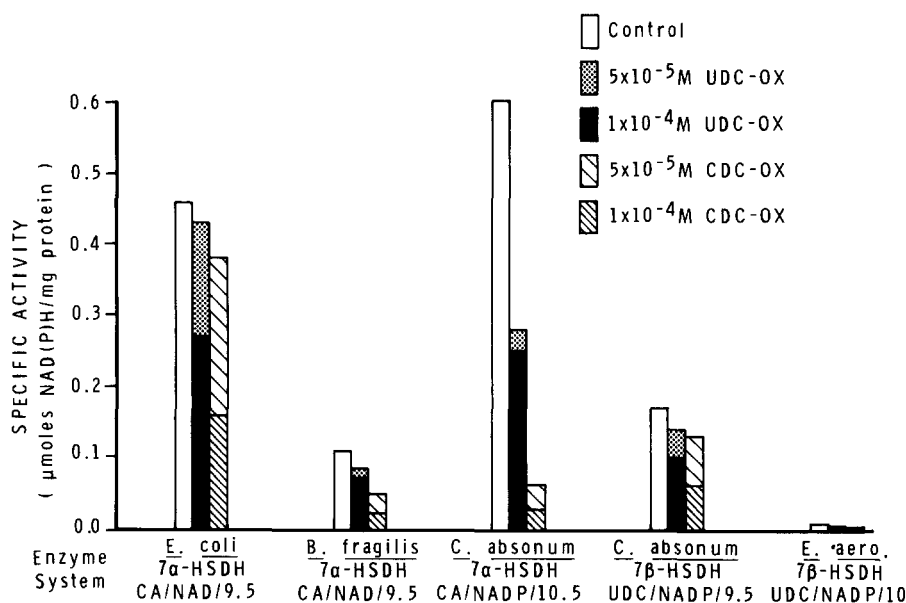


Fig. 5. Inhibition of 7 α - and 7 β -HSDH by CDC-OX and UDC-OX as measured in vitro in cell preparations. Enzymes and organisms are as designated.

is induction of 7 α - and 7 β -HSDH a factor in the ability of the organisms to transform bile acids and this mechanism does not appear to be operating in any of the other organisms studied so far or in mixed fecal cultures.

In all of the above cultures and in the human fecal flora, UDC-OX appears to be the least effective inhibitor of 7-epimerization and 7-dehydroxylation. It is already

established that UDC is more hydrophilic (and therefore less toxic) than CDC, 7-KLC, or DC (17); thus, it should not be surprising that an analogous relationship may exist with the oxazoline derivatives. On the other hand, DC-OX appears to be the most toxic oxazoline and, with the exception of *E. coli*, is inhibitory to all the pure cultures tested in this study. Lower concentrations of this deriv-

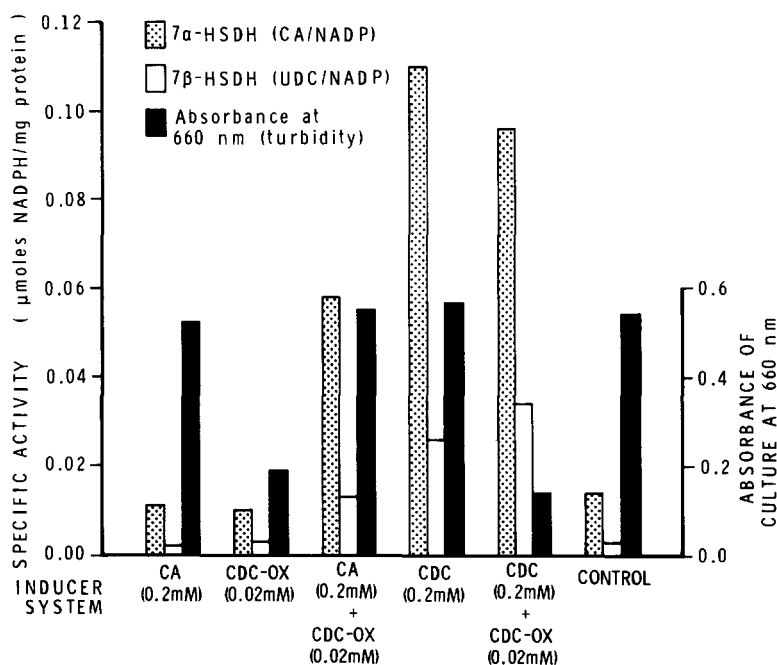


Fig. 6. Induction of *Clostridium absonum* by CA, CDC, and CDC-OX and combinations of primary bile acids and CDC-OX. Specific activity of 7 α - and 7 β -HSDH and absorbance at 660 nm of the culture as indicated.

ative remain to be tested. In contrast to the results with the above microbial populations, it appears that the bile acid oxazolines are nontoxic to human liver cells in culture³ and are nontoxic to rats when orally administered (33).

Although no specific microbial transformations of bile acid oxazolines have been noted, our results suggest that these derivatives degrade in the presence of hydrogen ions, giving parent bile acids as well as other products yet to be identified. The degradation appears to be relatively slow at pH values higher than 6.0, most of the oxazoline being intact in the log phase of the culture. At pH values below 6.0, the rate of degradation is marked; the use of acetic acid-containing solvent systems or acidification of spent medium prior to extraction results in the complete artifactual degradation of bile acid oxazolines on the TLC plate² and therefore must be avoided. The slow degradation of oxazolines in cultures suggests that a similar degradation of these derivatives may also occur in the enterohepatic circulation of animals and man, which remains to be demonstrated.

The administration of bile acid oxazolines in combination with CDC or UDC for the dissolution of cholesterol gallstones should inhibit both 7-dehydroxylation and 7-epimerization of the bile acid administered. However, inhibition of 7-epimerization appears to be only partial, while inhibition of 7-dehydroxylation is somewhat more thorough.

We conclude that 7-epimerization inhibition by the oxazolines in pure cultures and in the human fecal flora is closely related to growth inhibition of the active species. Inhibition of active enzymes in vitro, although often evident, is a secondary effect. ■■

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