# Formation of ursodeoxycholic acid from chenodeoxycholic acid in the human colon: studies of the role of 7-ketolithocholic acid as an intermediate

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**Abstract** The formation of ursodeoxycholic acid from chenodeoxycholic acid and the role of 7-ketolithocholic acid as an intermediate in this biotransformation were studied in vitro in fecal incubations as well as in vivo in the human colon. [24-$^{14}$C]-Labeled 7-ketolithocholic and chenodeoxycholic acids were studied at various concentrations, and the biotransformation products were analyzed by thin-layer chromatography, gas–liquid chromatography, and mass spectrometry. There was rapid colonic conversion of 7-ketolithocholic acid to ursodeoxycholic acid and, to a lesser extent, to chenodeoxycholic acid. The reduction of 7-ketolithocholic to ursodeoxycholic acid proceeded significantly faster anaerobically and at acid pH than under aerobic and alkaline conditions. When chenodeoxycholic acid was incubated in vitro or instilled into the colon, various amounts of 7-ketolithocholic and ursodeoxycholic acids were formed. The formation of 7-ketolithocholic acid was favored by alkaline conditions. Isotope dilution studies, in which trace amounts of labeled 7-ketolithocholic acid were incubated with unlabeled chenodeoxycholic acid, indicate 7-ketolithocholic acid to be the major intermediate in the intestinal bacterial conversion of chenodeoxycholic to ursodeoxycholic acid.—**Fromm, H., R. P. Sarva, and F. Bazzoli.** Formation of ursodeoxycholic acid from chenodeoxycholic acid in the human colon: studies of the role of 7-ketolithocholic acid as an intermediate. *J. Lipid Res.* 1983. **24:** 841–853.

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Chenodeoxycholic acid (CDC) and its 7$\beta$-epimer, ursodeoxycholic acid (UDC), show promise of being useful in the treatment of cholesterol gallstones (1–12). CDC, which is synthesized in the liver from cholesterol, is a major bile acid in man (13). In contrast, UDC, which is thought to be derived from CDC, is usually found only in small concentrations in human bile (14). One has assumed that UDC originates in the intestine, since it is absent in bile fistula bile (14). Previously it has been shown in our laboratory that UDC can be formed in the liver from 7-ketolithocholic acid (KLC), a putative intermediate in the conversion reaction from CDC to UDC (15). Thus, one mode of UDC formation could involve intestinal bacterial oxidation of CDC to KLC, which, in turn is absorbed and reduced in the liver to UDC. Another possibility could be that the entire biotransformation takes place in the colon without participation of the liver. This has been suggested by in vitro experiments of Federowski et al. (16), in which UDC formation was shown to occur during fecal incubation of CDC. However, in their studies, in which they incubated [7$\beta$-$^{3}$H]- as well as [24-$^{14}$C]-labeled CDC, these authors did not identify KLC or any other intermediate (16), Fedorowski et al. (16) therefore concluded that KLC was not involved in the interconversion of CDC and UDC. Instead, they postulated the occurrence of an unsaturated intermediate such as $\Delta$6 or $\Delta$7-lithocholenic acid, since $^{3}$H label appeared in UDC after fecal incubation of [7$\beta$-$^{3}$H]-labeled CDC. Although this observation provides strong evidence for the existence of a pathway that involves an unsaturated intermediate, it does not exclude the possibility that varying portions of UDC are also formed via KLC. Several findings by other investigators are in support of KLC being an important, though perhaps not the exclusive, intermediate in the biotransformation of CDC to UDC. First, Midtvedt and Norman (17) have shown that several bacterial species, which commonly inhabit the human colon, are capable of oxidizing CDC to KLC. Secondly, KLC can

Abbreviations: CDC, chenodeoxycholic acid; UDC, ursodeoxycholic acid; KLC, 7-ketolithocholic acid; LC, lithocholic acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; C, cholic acid; DC, deoxycholic acid.

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be found in conditions of bacterial overgrowth in the upper small bowel[2] and, physiologically, in the colon (18–20). The aims of the present study, therefore, were to examine, in vitro and in vivo *1*) the colon as the likely site of the formation of UDC from CDC and *2*) the role of KLC in this biotransformation reaction.

## MATERIALS AND METHODS

### Materials

Nonradioactive CDC was supplied in the form of crystalline powder, from Tokyo Tanabe Co., Ltd. (Tokyo, Japan). The purity of this material was assessed by gas–liquid chromatography (GLC). CDC was more than 99% pure, containing less than 0.1% lithocholic acid (LC). [24-$^{14}$C]CDC (sp act 55 $\mu$Ci/mmol) was purchased from New England Nuclear Corp. (Cambridge, MA). It was more than 98% pure by thin-layer chromatography (TLC). Nonradioactive KLC was prepared by the oxidation of CDC with buffered potassium chromate or with N-bromosuccinimide, as described by Fieser and Rajagopalan (21). The melting point was 201–203°C. KLC was more than 98% chemically pure by GLC. Confirmation of the structure was obtained by nuclear magnetic resonance (NMR) at the Hormel Institute, Austin, MN, on a Varian CFT-20 spectrometer, operating in the pulsed Fourier transform mode at 79.54 MHz. This material was labeled with $^{14}$C at the 24 position by halodecarboxylation followed by reaction with [$^{14}$C]cyanide (22). The specific activity was 5.250 mCi/mmol. The synthesized [24-$^{14}$C]KLC was purified by preparative TLC. The final purity was more than 99%.

### In vitro aerobic and anaerobic incubation studies of labeled KLC and CDC

Immediately after evacuation, fresh stool specimens were obtained from four male and one female healthy volunteers, as well as from three female and two male patients with asymptomatic gallstones. Stool samples were homogenized with normal saline (approximately 1:1 v/v). [24-$^{14}$C]CDC and [24-$^{14}$C]KLC, respectively, were incubated simultaneously in different vials with the fresh stool homogenates at a temperature of 37°C under aerobic and anaerobic conditions in a Dubnoff incushaker (Lab-Line Instruments, Melrose Park, IL). The individual incubation reactions were terminated in the different vials after 0, 0.5, 1, 4 and 12 hr, respectively. The 0.5- and 1-hr incubation experiments were carried out in duplicate. The maintenance of the aerobic and anaerobic conditions, respectively, was monitored with a disposable anaerobic indicator (Gas-Pak, Becton, Dickinson and Co., Cockeysville, MD) suspended in the vials above the incubation media. In control experiments, this indicator was found to be reliable in discriminating between aerobic and anaerobic conditions. It turned blue if the caps of the incubation vials were loosened for the purpose of exposing the media to atmospheric oxygen (aerobic conditions), but showed no color change if the samples were kept under a nitrogen stream before the vials were tightly capped (anaerobic conditions). KLC and CDC were incubated at concentrations of 0.40 ± 0.10 mM and 0.46 ± 0.06 mM (mean ± SEM), respectively. These concentration figures encompass both the endogeneous and the exogenously added unlabeled KLC and CDC, respectively. The endogenous fecal bile acid concentrations were as follows: CDC, 0.19 ± 0.06 mM; UDC, 0.12 ± 0.03 mM; LC, 1.18 ± 0.26 mM; DC, 1.89 ± 0.45 mM; and C, 0.04 ± 0.03 mM. No measurable quantity of endogeneous KLC was identified in any of the fecal samples. For these incubation experiments, stock solutions of [24-$^{14}$C]CDC and [24-$^{14}$C]KLC, respectively, were prepared. The respective isotope was dissolved in 0.1 M sodium pyrophosphate buffer at a concentration appropriate for the incubation experiment to be performed.

### Aerobic incubations

Solutions of [$^{14}$C]KLC and [$^{14}$C]CDC, respectively, (50–500 $\mu$l) were pipetted into sterile vials and mixed with 7 ml of sterile normal saline. This solution was then mixed with 1 g of fresh stool homogenate. Normal saline was used in order to dilute the samples and assure good mixing of the labeled bile acids with the fecal material. The vials were capped and the incubation media were then mixed in a test-tube stirrer (Vortex Genie, Scientific Industries, Springfield, MA). Subsequently, the caps were loosened to expose the media to atmospheric oxygen. In order to study the influence of pH on the biotransformation reactions, the incubations were carried out at a pH ranging from 5.1 to 9.2. The stool pH was adjusted with 1 N NaOH. Two types of incubation experiments were then performed. In one the stool pH was not readjusted during the incubation period. In these experiments the pH dropped by 0.95 ± 0.17 within 4 hr. In the second type of study the pH was kept constant throughout the incubation. The pH was monitored using a gel-filled combination pH electrode (Orion Research, Cambridge, MA, Model 91-05), which was placed into one of the incubation vials. If the pH changed, readjustment to the original value was effected by addition of 1 N NaOH. The same amount of NaOH necessary to keep the pH constant in the pH-monitored medium was added to the other incubation vials. The final pH in the different vials, which was recorded in all incubation studies, showed only minor vari-

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ations (mean difference from mean pH value was 0.10 ± 0.01). The incubations were terminated at the different time intervals by addition of concentrated HCl.

### Anaerobic incubations

Homogenized fresh stool was kept under a nitrogen stream. $^{14}$C-Labeled KLC and CDC, respectively, were pipetted into sterile vials and mixed with 7 ml of prereduced anaerobically sterilized chopped meat-glucose medium (Scott Laboratories, Fiskeville, RI). This medium was then mixed with 1 g of fresh stool homogenate. These preparations of the incubation medium were carried out under a stream of nitrogen. Before capping, the vials were flushed with nitrogen. Termination of the incubations at the different time intervals was effected by addition of 2.5 ml of 33% KOH and 10 ml of absolute ethanol (16). No attempt was made in these anaerobic studies to keep the pH constant. The pH values at the beginning of the incubations ranged from 5.9–7.9.

### In vitro isotope dilution studies

In order to study the role of KLC as an intermediate in the biotransformation of CDC to UDC, six series of isotope dilution studies were performed. Trace amounts of [24-$^{14}$C]KLC and unlabeled CDC were first mixed together and then mixed with fresh stool homogenate, as described above. The total concentration of CDC at the beginning of the incubations was 0.25 ± 0.10 mM. The endogenous fecal bile acid concentrations in the fecal incubates were as follows: CDC, 0.11 ± 0.10 mM; UDC, 0.08 ± 0.04 mM; LC, 0.55 ± 0.21 mM; DC, 0.60 ± 0.19 mM; and C, 0.03 ± 0.03 mM. One of the four subjects from whom the fecal samples for the isotope dilution studies were obtained, a healthy volunteer, showed unusually high endogenous concentrations of CDC and UDC. The latter represented 43% and 18%, respectively, of the total fecal bile acids. In the other three subjects, CDC constituted always less than 1% and UDC less than 7% of the fecal bile acids. Aerobic incubations were carried out for 0, 0.5, 1, 3, 5, 7, 9, 11, and 24 hr with fecal samples of two female gallstone patients at native pH values of 6.25 and 7.14, respectively. In four other experiments, fecal samples from two healthy male volunteers were incubated at a native pH of 5.40 and 6.25, respectively, and at pH values that were adjusted to 7.12 and 7.16.

The specific activities of CDC, KLC, UDC, and LC were derived from the GLC measurements of the mass and the TLC determinations of the proportional radioactivity of the respective bile acids (vide infra). The precursor-product relationships were evaluated using specific activity time curves of these compounds (23). In the calculations of the specific activities, the bile acids that were endogenously present in the fecal incubates were considered. The calculation of the specific activities of CDC was based on the mass of both the endogeneous and exogenous CDC, since both can be expected to participate in a comparable fashion in the biotransformation reactions. For the computation of the specific activities of KLC, UDC, and LC, the endogenous components were subtracted from the respective total measurements. This treatment of the data was thought to provide the best approximation for the specific activities of CDC, KLC, and UDC. However, the calculated specific activities of LC are probably slightly lower than the true values, since they also reflect the formation of LC from endogeneous UDC.

### In vivo studies

Two female and two male subjects (Tables 1 and 2) were admitted to our in-hospital Cooperative Care facility. Routine laboratory studies, including liver function tests, were normal in these subjects. An oro-colonic tube with a bag containing 0.7 cm$^3$ of mercury at its tip was passed (24). In three of the four subjects, a single-lumen tube (Tygon R-3603, ID 1.5 mm, OD 3.0 mm (Norton Plastics and Synthetics Division, Akron, OH) and, in the fourth, a double-lumen tube (ID 1.5 mm × 2, OD 4.5 mm) was used (**Table 1**). The tip of the tube was placed into the colon. A second oro-intestinal tube was passed with the tip in the second portion of

TABLE 1. In vivo biotransformation of $^{14}$C-labeled 7-ketolithocholic acid during steady-state perfusion[a] of ascending colon with a double-lumen tube

| Subject, Sex, Age | Concentrations of Infused KLC (mM) | pH of Collected Sample | Radioactive Metabolite (% on TLC) | | | | Bile Acid Composition by GLC (mM) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | KLC | CDC | UDC | LC | KLC | CDC | UDC | LC | C | DC |
| M.L., F, 50 yr | 0 (Control) | 6.4 | | | | | | 0.015 | 0.008 | 0.318 | | 0.061 |
| | 0.5 | 4.8 | 3 | 16 | 56 | 25 | | 0.003 | 0.128 | 0.189 | | 0.205 |
| | 1.0 | 4.8 | 3 | 14 | 61 | 22 | | 0.066 | 0.258 | 0.311 | | 0.372 |
| | 1.5 | 4.8 | 2 | 17 | 66 | 14 | | 0.173 | 0.515 | 0.337 | | 0.332 |

[a] Eight ml per minute steady-state infusion of sodium KLC, 0.5, 1.0, and 1.5 mM, respectively; glucose, 150 mM and NaCl, 30 mM. During the infusion of each of the three KLC solutions, two 15-min collections were obtained following a 15-min equilibration period. The samples were collected in the ascending colon 15 cm distal to the infusion site.

the duodenum. The position of both tubes was confirmed by an abdominal roentgenogram. The colonic pH values ranged from 6.2 to 6.8. At 8:00 AM of the day of the biotransformation study, 10 $\mu$Ci (1 mmol) of $^{14}$C-labeled sodium CDC and KLC, respectively, (10 mM solution in normal saline) were instilled through the orocolonic tube into the colon. In one of the four subjects, the colonic instillation of the 10 mM solution of [$^{14}$C]KLC was preceded by a steady-state perfusion study of three different concentrations of this compound (Table 1). The subjects were fasting for 10 hr prior to the experiment. Following the colonic instillation of the labeled precursor, the tube was flushed with normal saline. Colonic aspirations were performed hourly. When material was obtained from the colon, it was either freshly analyzed or immediately frozen at −20°C for later analysis. Gallbladder contraction was effected by i.v. injection of 0.02 $\mu$g/kg of Kinevac®, and approximately 10-ml samples of bile were obtained through the duodenal tube. Individual stool collections were made over a period of 48 hr (Table 2) and either analyzed freshly after each bowel movement or frozen immediately at −20°C for later analysis.

### TLC analysis of chemical forms of radioactivity

The bile acids in the in vitro and in vivo fecal samples were extracted by a previously established method (24). As described in detail elsewhere, the bile acids in the in vitro fecal incubates were extracted with Amberlite-XAD-7 resin (Polysciences, Inc., Washington, PA), purified by percolation through a Florisil column, and esterified using ethereal diazomethane (24). The recovery (following these extraction and purification steps) of the total radioactivity incubated as [$^{14}$C]CDC and [$^{14}$C]KLC, respectively, was 90 ± 1.0%. The in vivo fecal samples (stool and colonic aspirates) were first subjected to alkaline hydrolysis and then processed identically to the in vitro incubates (24). The biliary bile acids were analyzed as previously described (15, 25, 26). The chemical form of the radioactivity of the extracted bile acid methyl esters was determined by TLC, using chloroform–acetone–methanol 75:23:2 (v/v) as a solvent system (15). The distribution of the radioactivity on the TLC plates was determined by zonal scraping of the silica gel. Complete zonal scraping was carried out on all TLC plates. The radioactive bile acid metabolites were identified by relating the distribution of the radioactivity to that of pure reference standards (15). The recovery of the radioactivity from the TLC plates was 98 ± 1.2%.

### GLC and mass spectrometry (MS) analyses of bile acid composition

The in vivo fecal samples and in vitro fecal incubates were also analyzed by GLC for unlabeled metabolites of CDC and KLC. In addition, in vitro fecal incubates were analyzed by MS. For GLC determinations, nordeoxycholic acid was used as internal standard for bile acid quantification. After the described methylation step, the bile acids were acetylated (15, 24–26). The methyl ester acetates were dissolved in dimethyl formamide and analyzed by GLC using a flame ionization detector (Gas Chromatograph, Model 421, Packard Instrument, Downers Grove, IL): 1.8-meter U-columns, 2 mm ID, packed with 3% AN-600 on gas-CHROM Q 100–120 mesh (Supelco, Inc., Supelco Park, Bellefonte, PA), (15, 24–26). In the in vitro studies, correction was made for the endogenously present bile acids. The mass of the bile acids present in the stool samples before addition of the respective precursor was subtracted from that measured afterwards at the different times of incubation.

For preparation of the samples for MS, the individual bands representing CDC, KLC, and UDC were scraped off the TLC plates. The bile acids were eluted from the silica gel with methanol. Following preparation of the bile acid methyl ester acetates, GLC/MS analysis was performed by Dr. Erwin H. Mosbach, Director, Lipid Research Laboratory, Beth Israel Medical Center, New York, on a Hewlett-Packard Model 5992B GLC/MS.

### Statistical analysis

The paired comparison *t*-test was used for the statistical evaluation of the effect of different conditions (pH, aerobic versus anaerobic) on in vitro bile acid biotransformation. The correlation between fecal pH and in vitro KLC formation from CDC was statistically analyzed by least squares regression.

## RESULTS

### Colonic biotransformation of KLC

*In vitro fecal incubation of labeled KLC.* Various proportions of $^{14}$C-labeled KLC were reduced to both UDC and CDC in the aerobic as well as in the anaerobic fecal milieu (Figs. 1–3). In most incubations more UDC than CDC was formed. The time curves of the reaction under aerobic conditions at a native pH ranging from 5.1 to 6.5 are shown in **Fig. 1.** After 4 hr of incubation, only about 35% of the radioactivity was still present in KLC. After 12 hr, this figure had decreased to approximately 13%. The peak of UDC formation, which averaged about 35% of the original KLC radioactivity, occurred usually at 4 hr. As the radioactivity of KLC declined, there was progressive accumulation of LC (Fig. 1). The reduction of KLC to UDC proceeded significantly faster at acid than at alkaline pH levels (**Fig. 2**). The rate and pattern of biotransformation of KLC were similar in experiments in which the pH of the incubation medium

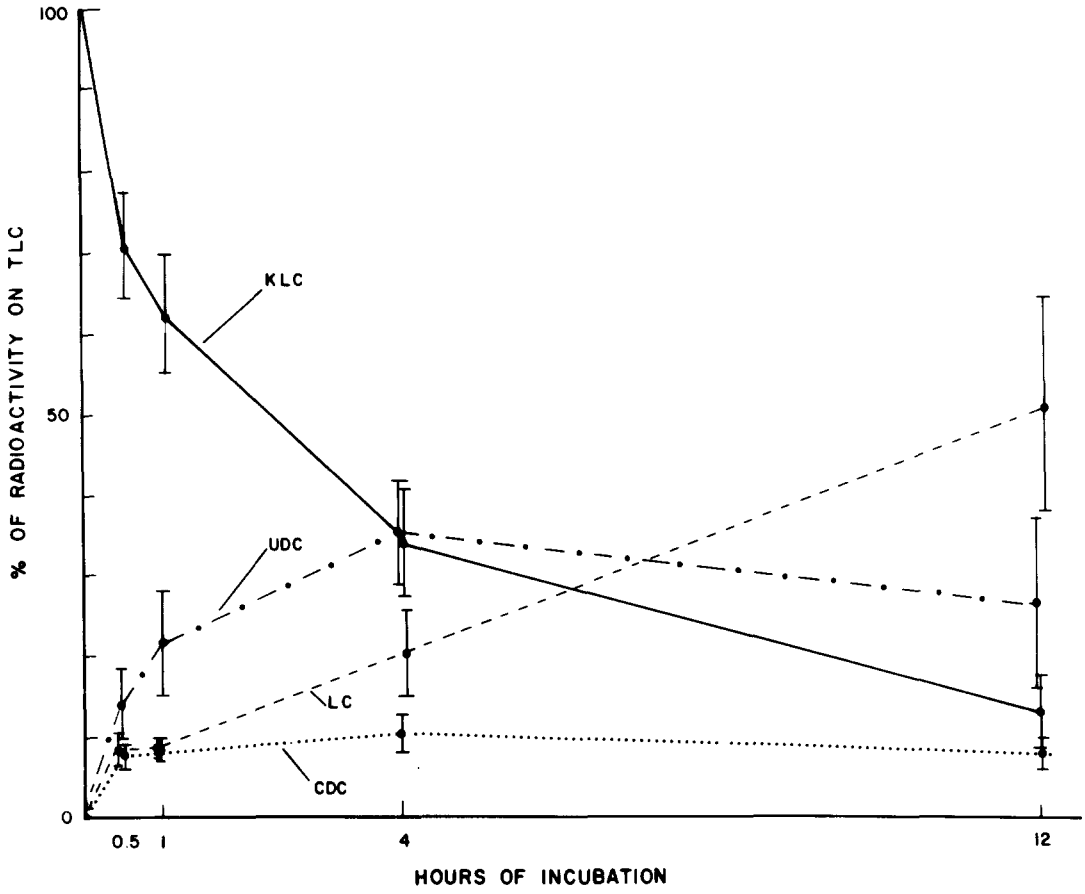


**Fig. 1.** Aerobic in vitro fecal biotransformation of $^{14}$C-labeled KLC at native pH ranging from 5.1 to 6.5 in three control and one gallstone subjects (mean ± SEM). A total of 11 incubation studies of KLC were carried out at a concentration of 0.40 ± 0.10 mM.

was only initially adjusted to higher levels and in those in which the higher pH was maintained throughout the incubations. Anaerobic conditions were significantly more conducive than aerobic conditions to the reduction of KLC to UDC, both at lower and higher fecal pH values (**Fig. 3**).

The identity of KLC, UDC, and CDC bands separated by TLC was confirmed by GLC/MS. No attempt was made to identify the structure of compounds that eluted from the TLC plates with KLC, CDC, and UDC, but they constituted less than 10% of the respective total bile acid. These compounds possibly represented 3$\beta$-hydroxy epimers (27, 28).

*In vivo infusion of labeled KLC into colon.* The biotransformation of KLC shown in vivo after infusion into the colon of two human subjects (Table 1 and **Table 2**) was comparable to that found in the in vitro incubation experiments (Figs. 1–3). The proportion of KLC converted in vivo in the colon to UDC within 1 hr ranged from about 56% to 77%. This rate of UDC formation in vivo (Tables 1 and 2) resembled the rate observed in vitro under anaerobic conditions more closely than that under aerobic conditions (Fig. 3). The results of the studies obtained by TLC analysis of the radioactive precursor and metabolites were congruent with those of the GLC analyses of the corresponding unlabeled compounds.

### Colonic biotransformation of CDC

*In vitro fecal incubation of labeled CDC.* In the anaerobic fecal incubation experiments, about 90% of CDC was metabolized to LC within 12 hr (**Fig. 4**). In addition, various proportions of CDC were transformed to KLC and UDC (Fig. 4). The correlation between percent of radioactivity as KLC on TLC and fecal pH was significant for the aerobic incubation studies ($n = 23$, $r = 0.6079$, $P < 0.01$) as well as the aerobic and anaerobic incubations combined ($n = 33$, $r = 0.5980$, $P < 0.001$). However, the correlation between fecal pH and KLC formation was not significant if only the data of the ten anaerobic incubation series were used for the calculation. The results of the TLC and GLC analyses were, again, confirmed by MS.

*In vitro incubation of unlabeled CDC with trace amounts of labeled KLC (isotope dilution studies).* As shown in **Fig. 5,** there was rapid biotransformation of significant

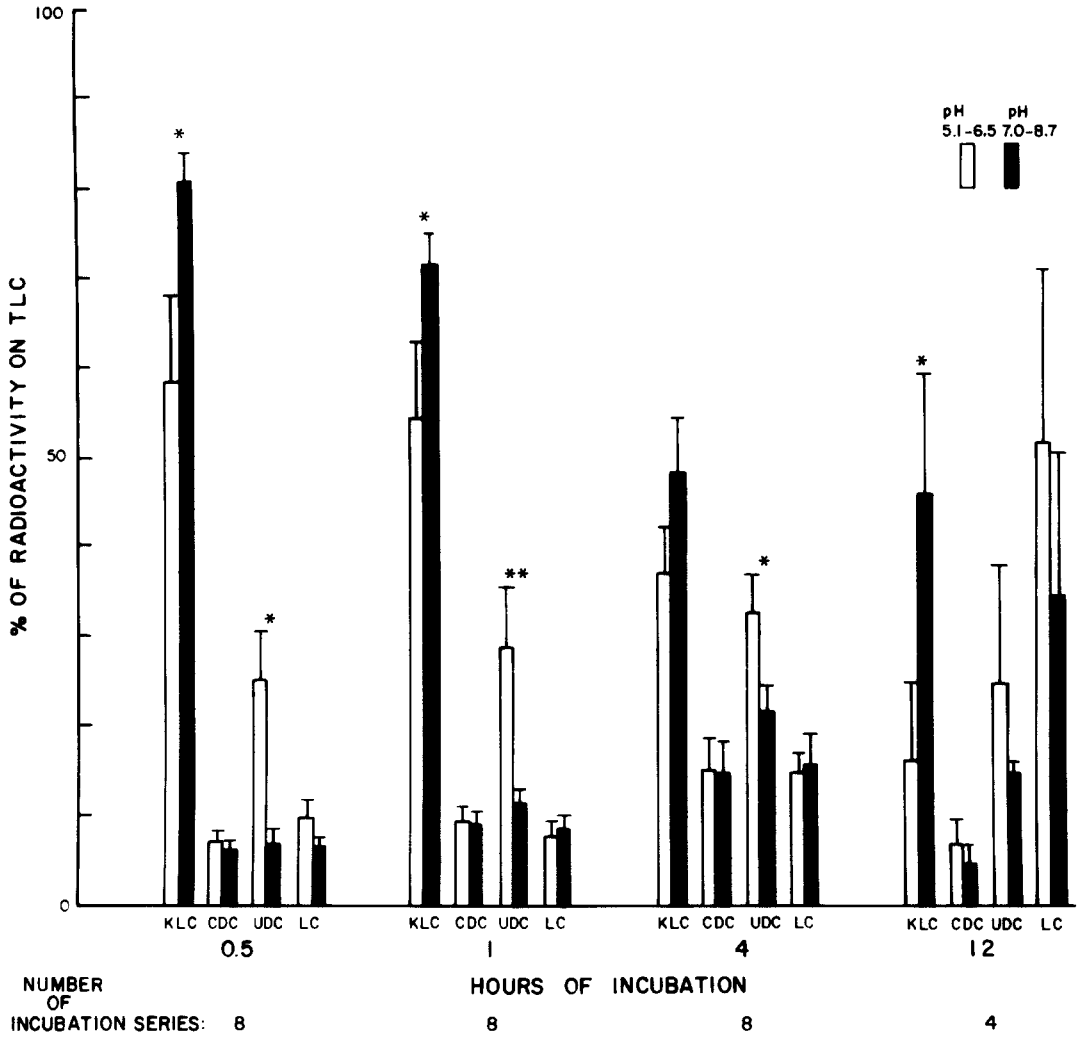


**Fig. 2.** Effect of alkalinization on in vitro aerobic fecal biotransformation of $^{14}$C-labeled KLC in ten comparative incubation series of samples from one control and one gallstone subject (mean ± SEM). The KLC concentration at the beginning of the incubations was 0.41 ± 0.12 mM. *, Significant at $P < 0.05$; **, significant at $P < 0.02$.

amounts of unlabeled CDC to KLC, UDC, and LC in the six series of isotope dilution studies using incubations of fecal samples from four subjects. The biotransformation of the $^{14}$C-labeled KLC, which was incubated in trace amounts in these studies, was similar to that shown in other experiments in Fig. 1. After 1 hr, 36 ± 3.6% of the radioactivity incubated as KLC appeared in UDC, 16 ± 2.5% in CDC, 24 ± 2.8% in LC, and 24 ± 3.8% in the original compound. The corresponding percentages of radioactivity after 5 hr of incubation were 26 ± 3.5 in UDC, 28 ± 3.5 in CDC, 26 ± 2.9 in LC, and 21 ± 3.0 in KLC. The time curves of the specific activities of CDC, KLC, UDC, and LC in these studies are shown in **Fig. 6.** A rapid decline in the specific activity of KLC was accompanied by a rise in that of UDC, CDC, and LC. In every study, both at acid and alkaline fecal pH levels, the KLC intersected with the UDC and/or LC curves.

*In vivo infusion of labeled CDC into the colon.* Formation of KLC and UDC from CDC was also observed in vivo in the two subjects in whom [$^{14}$C]CDC was infused into the transverse colon (Table 2). It is of interest that one of the two subjects (W.D., who had severe bile acid malabsorption due to a distal ileal resection) was found, prior to the infusion of CDC, to have not only this bile acid but also KLC and UDC in considerable concentrations in the stool. The formation of KLC and UDC in the colon was then further documented by fecal analysis of the radioactive metabolities after colonic infusion of $^{14}$C-labeled CDC. In addition, after CDC was instilled into the colon, there was a marked rise in the colonic concentration of unlabeled KLC and UDC. In both subjects, in the patient with ileal resection as well as in the one with asymptomatic gallstones, 21% and 17%, respectively, of [$^{14}$C]CDC were converted to [$^{14}$C]UDC in 2 hr. The corresponding figures for fecal KLC for-

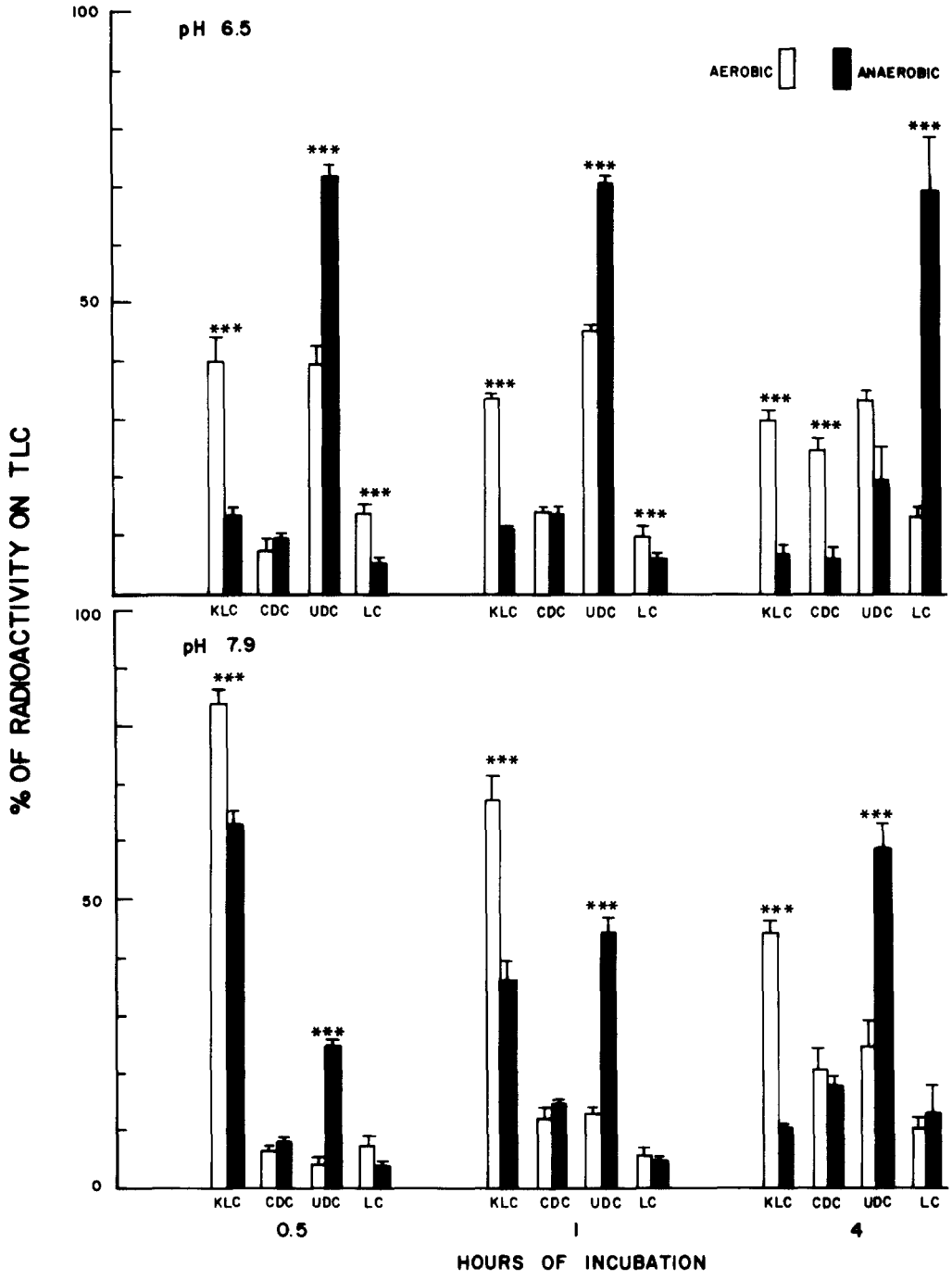


**Fig. 3.** In vitro comparison of aerobic and anaerobic biotransformation of trace amounts of $^{14}$C-labeled KLC at a pH of 6.5 and 7.9, respectively, in a fecal sample of a gallstone subject. At both pH levels, aerobic and anaerobic incubation series were carried out at CDC concentrations of 0.17, 0.33, 0.49 and 0.65 mM, respectively. *, Significant at $P < 0.05$; ***, significant at $P < 0.01$.

mation during the same time interval were 7% and 2%, respectively. (Table 2).

### Biliary bile acids following colonic infusion of KLC and CDC, respectively

At 1.5 hr after colonic infusion of [$^{14}$C]KLC in subject M.L., 53% of the radioactivity appearing in duodenal bile was found in CDC, 24% in UDC, 7% in LC, and 16% unchanged in KLC (Table 2). The corresponding figures for the chemical form of the radioactivity in bile, 1 hr after colonic infusion of [$^{14}$C]CDC in subject W.D., were 77% for CDC, 6% for UDC, 12% for LC, and 5% for KLC (Table 2). A similar distribution of the radioactivity in biliary bile acids was found in the third subject, from whom duodenal bile was obtained after co-

TABLE 2. In vivo biotransformation of $^{14}$C-labeled 7-ketolithocholic and chenodeoxycholic acid, respectively, following intracolonic infusion[a]

| Subject, Sex, Age; Site of Infusion | Bile Acid Infused | Origin of Sample | Hour of Collection after Colonic Infusion | pH of Collected Sample | Radioactive Metabolite (% on TLC) | | | | Bile Acid Composition by GLC (Fecal bile acids in mM, duodenal bile acids in %) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | KLC | CDC | UDC | LC | KLC | CDC | UDC | LC | C | DC | Other |
| M.L., F, 50 yr;[b] Ascending colon | KLC | Stool | 0 (Control) | 6.8 | —[d] | — | — | — | — | 0.097 | 0.043 | 0.531 | 0.001 | 0.995 | — |
| | | | 24 | 5.9 | — | 10 | 29 | 61 | — | 0.120 | 0.360 | 0.763 | — | 0.766 | — |
| | | Duodenal bile | 0 (Control) | N.A. | — | — | — | — | — | 36 | 8 | 2 | 20 | 34 | — |
| | | | 1.5 | N.A.[e] | 16 | 53 | 24 | 7 | 1 | 44 | 12 | 3 | 17 | 23 | — |
| | | | 2.0 | N.A. | 18 | 48 | 24 | 9 | 2 | 42 | 12 | 5 | 16 | 24 | — |
| H.N., F, 35 yr; Transverse colon | KLC | Transverse colon | 1.0 | 6.2 | 17 | 4 | 76 | 2 | — | — | 0.893 | 0.238 | — | 0.174 | — |
| | | Stool | 46 | 8.3 | 2 | 12 | 11 | 75 | — | 0.064 | 0.114 | 0.511 | — | 0.376 | — |
| W.D., M, 33 yr;[c] Transverse colon | CDC | Stool | 0 (Control) | 6.7 | — | — | — | — | 0.030 | 1.715 | 0.315 | 0.022 | 1.582 | 0.010 | — |
| | | | 2.0 | 7.4 | 7 | 59 | 21 | 13 | 0.031 | 1.270 | 0.352 | 0.005 | 0.908 | — | — |
| | | | 15 | 7.2 | 13 | 37 | 39 | 10 | 0.064 | 1.584 | 1.372 | 0.018 | 1.418 | 0.003 | 0.605 |
| | | Duodenal bile | 0 (Control) | N.A. | — | — | — | — | — | 62 | 10 | 1 | 26 | — | — |
| | | | 1.0 | N.A. | 5 | 77 | 6 | 12 | 2 | 69 | 11 | 2 | 15 | — | — |
| C.C., M, 67 yr; Transverse colon | CDC | Transverse colon | 0 (Control) | 6.5 | — | — | — | — | — | 0.154 | — | 0.072 | — | 0.131 | — |
| | | | 1.0 | 7.3 | — | 68 | 10 | 23 | — | 0.380 | — | 1.104 | — | 0.501 | — |
| | | | 2.0 | 7.0 | 2 | 56 | 17 | 25 | — | 0.911 | 0.202 | 1.319 | — | 0.582 | — |
| | | | 3.0 | 7.0 | 8 | 42 | 13 | 37 | N.A. | N.A. | N.A. | N.A. | N.A. | N.A. | N.A. |
| | | | 24 | 6.5 | 12 | 11 | 7 | 70 | N.A. | N.A. | N.A. | N.A. | N.A. | N.A. | N.A. |
| | | Stool | 0.3 | 7.7 | 4 | 80 | 5 | 10 | — | 0.628 | 0.398 | 2.434 | — | 1.153 | 0.250 |
| | | | 6.5 | 8.0 | — | 5 | 3 | 92 | — | 0.041 | 0.186 | 1.148 | — | 0.462 | 0.298 |
| | | | 23 | 7.8 | — | 3 | 2 | 95 | — | 0.066 | 0.276 | 1.546 | — | 0.625 | 0.077 |
| | | Duodenal bile | 0 (Control) | N.A. | — | — | — | — | — | 35 | 5 | 5 | 15 | 40 | — |
| | | | 24 | N.A. | 2 | 77 | 8 | 13 | — | 42 | 4 | 3 | 20 | 32 | 1 |

[a] A bolus of 10 μCi of 1 mmol of $^{14}$C-labeled sodium KLC and CDC, respectively (respective bile acid, 10 mM; glucose, 200 mM; NaCl, 30 mM) was infused at a rate of 15–20 ml per min into the colon.

[b] In this subject, the bolus infusion was performed after completion of steady-state perfusion study (Table 1).

[c] Patient with status post 60 cm of distal ileal resection for Crohn's disease and documented bile acid malabsorption. The other three subjects were healthy with the exception of their having asymptomatic gallstones.

[d] —, No measurable radioactivity or quantity.

[e] N.A., Not analyzed.

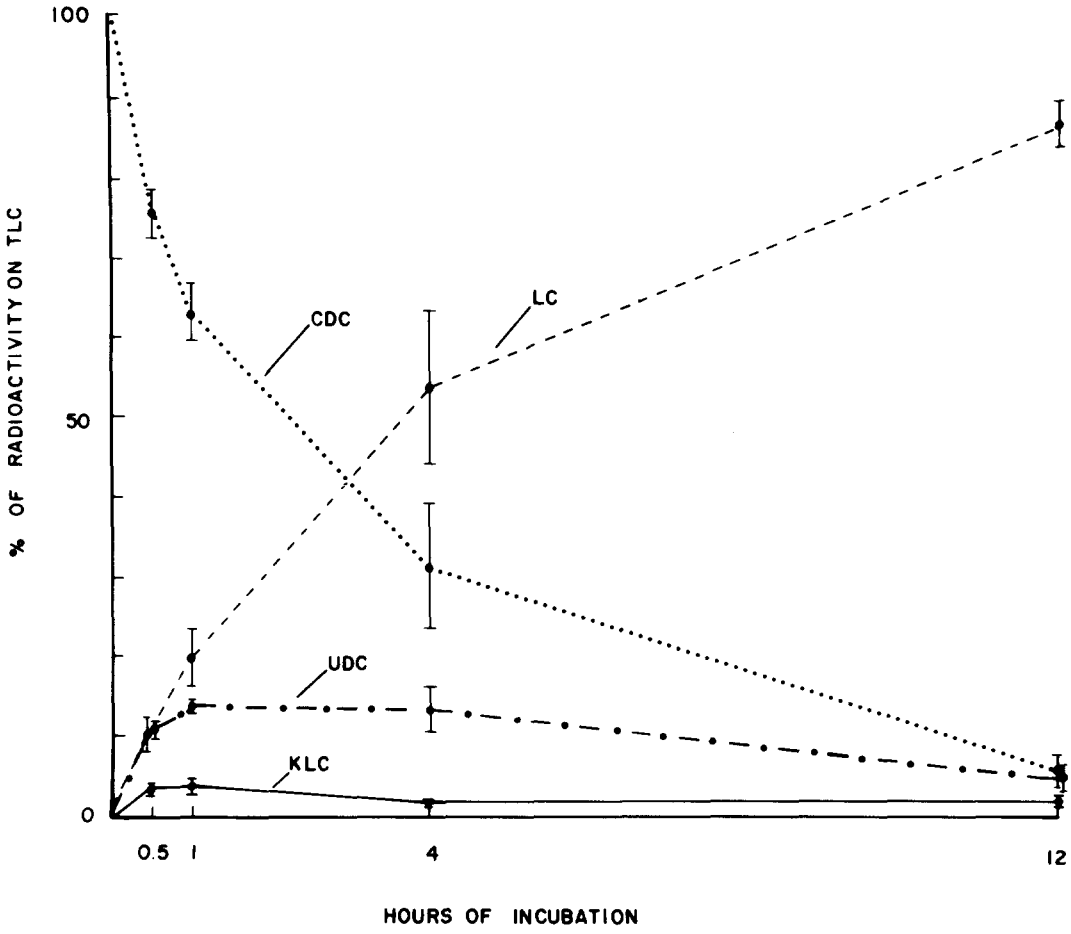


**Fig. 4.** In vitro anaerobic fecal biotransformation of $^{14}$C-labeled CDC in eight subjects, in whom a total of ten incubation series were carried out. The CDC concentration at the beginning of the incubation was 0.46 ± 0.06 mM.

lonic instillation of a labeled bile acid. In this subject, C.C. (Table 2), duodenal bile was obtained 24 hr after instillation of [$^{14}$C]CDC into the colon. Seventy-seven percent of the $^{14}$C-label in bile appeared in CDC, 8% in UDC, 13% in LC, and 2% in KLC.

## DISCUSSION

Previously it has been shown in our laboratory that KLC, a putative intermediate in the conversion of CDC to UDC, is absorbed in the small intestine and reduced in the liver to CDC and, to a lesser degree, to UDC (15). In the present study, this biotransformation reaction was, for the first time, also found to take place in vivo in the human colon. KLC was rapidly biotransformed both in in vitro fecal incubations and in vivo after colonic instillations. It is of note that, in contrast to the reaction in the liver (15), more KLC was transformed to UDC than to CDC in the colon. These findings are in agreement with in vitro anaerobic fecal incubation studies by Higashi, Setogushi, and Kazuki (29). One reason for the appearance of more UDC than CDC during the colonic biotransformation of KLC could be that intestinal bacterial enzymes preferentially catalyze a 7$\beta$-reduction of this compound. Another reason could be that CDC is degraded more rapidly than UDC to LC. Previous studies in our laboratory indicate the former possibility to be the more likely one, since we showed that the 7-dehydroxylations of CDC and UDC to LC are, in most cases, very similar (30).

There are few sources of information in the literature that relate to our observations regarding the effects of aerobic conditions and changes in fecal pH on the biotransformation of KLC. Both factors significantly influenced the reaction; more UDC was formed at an acid pH (pH 5.1 to 6.5) and in an anaerobic milieu than under alkaline (pH 7.0 to 8.7) and aerobic conditions. On the other hand, the formation of KLC from CDC was favored by an alkaline fecal pH. These findings are consistent with studies by Macdonald and Roach (31), which showed the pH optimum to be higher for the 7$\alpha$-hydroxysteroid dehydrogenase than for the 7$\beta$-hydroxysteroid dehydrogenase.

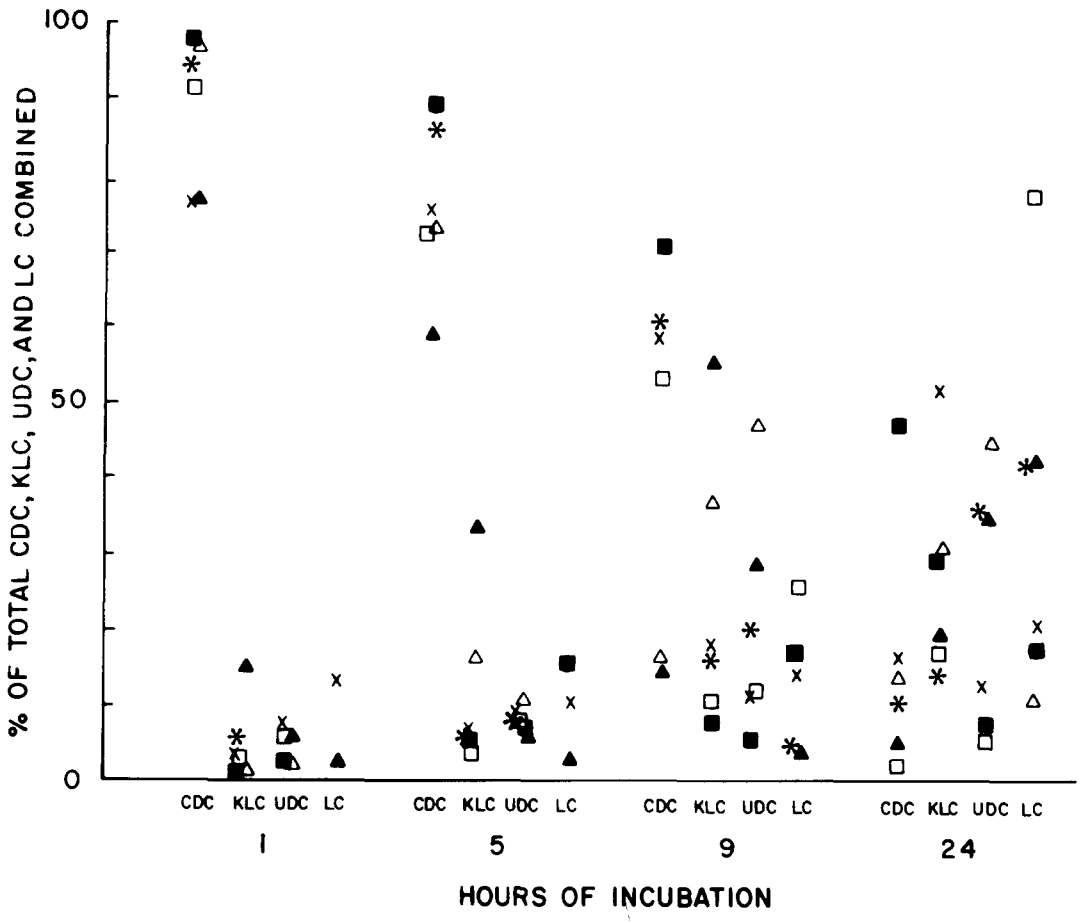


**Fig. 5.** Time curves of the fecal biotransformation of unlabeled CDC to KLC, UDC, and LC in the isotope dilution studies, which were performed in six incubation series on fecal samples of two male control subjects and two female asymptomatic gallstone patients. The fecal CDC concentrations at the beginning of the incubations ranged from 0.13 to 0.55 mM. The studies in the two control subjects were carried out at both acid (open squares and triangles) and alkaline (filled squares and triangles) pH levels.

Similar to Salen et al. (32), we had previously observed significant increases of biliary UDC in gallstone patients treated with CDC (25). In several of the patients, the UDC content exceeded 25% of the total bile acid pool. This suggested intestinal bacterial transformation of CDC as the major source of UDC formation. The liver is only involved in the formation of that portion of biliary UDC that is produced by hepatic reduction of KLC. This portion can only be small, since the majority of KLC is converted in the liver to CDC and less than 10% to UDC (15). The present study also represents, to our knowledge, the first in vivo demonstration of the biotransformation of CDC to UDC and its effect on biliary bile acid composition. The biotransformation of CDC, after infusion of this compound into the colon, differed considerably between the two subjects studied (Table 2). Consistent with our previous observations in patients with diarrhea due to ileal resection and bile acid malabsorption (24), the fecal bile acids in subject W.D. contained a relatively small proportion of lithocholic acid. It is of interest that, in spite of this apparent depression of 7-dehydroxylation, other bacterially catalyzed reactions, such as the dehydrogenation of the $\alpha$-OH substituent at C-7 and the stereospecific reduction of the newly formed oxo moiety to a 7$\beta$-OH group, were active, as evidenced by the presence of substantial amounts of KLC and UDC in feces. Therefore, marked increases in the fecal CDC concentrations, as they were present in this patient to a level of 1.72 mM, do not appear to suppress bacterial 7$\alpha$- or 7$\beta$-hydroxysteroid dehydrogenase activity. These in vivo observations are consistent with our in vitro incubation studies, in which changes in the fecal CDC concentration from 0.32 to 0.93 mM did not result in any noticeable depression of KLC or UDC formation. Also, the deconjugation reaction was apparently normal since the fecal bile acids were present in the deconjugated form. The second subject studied, who except for having asymptomatic gallstones was healthy, showed both a normal rate of LC formation and the appearance

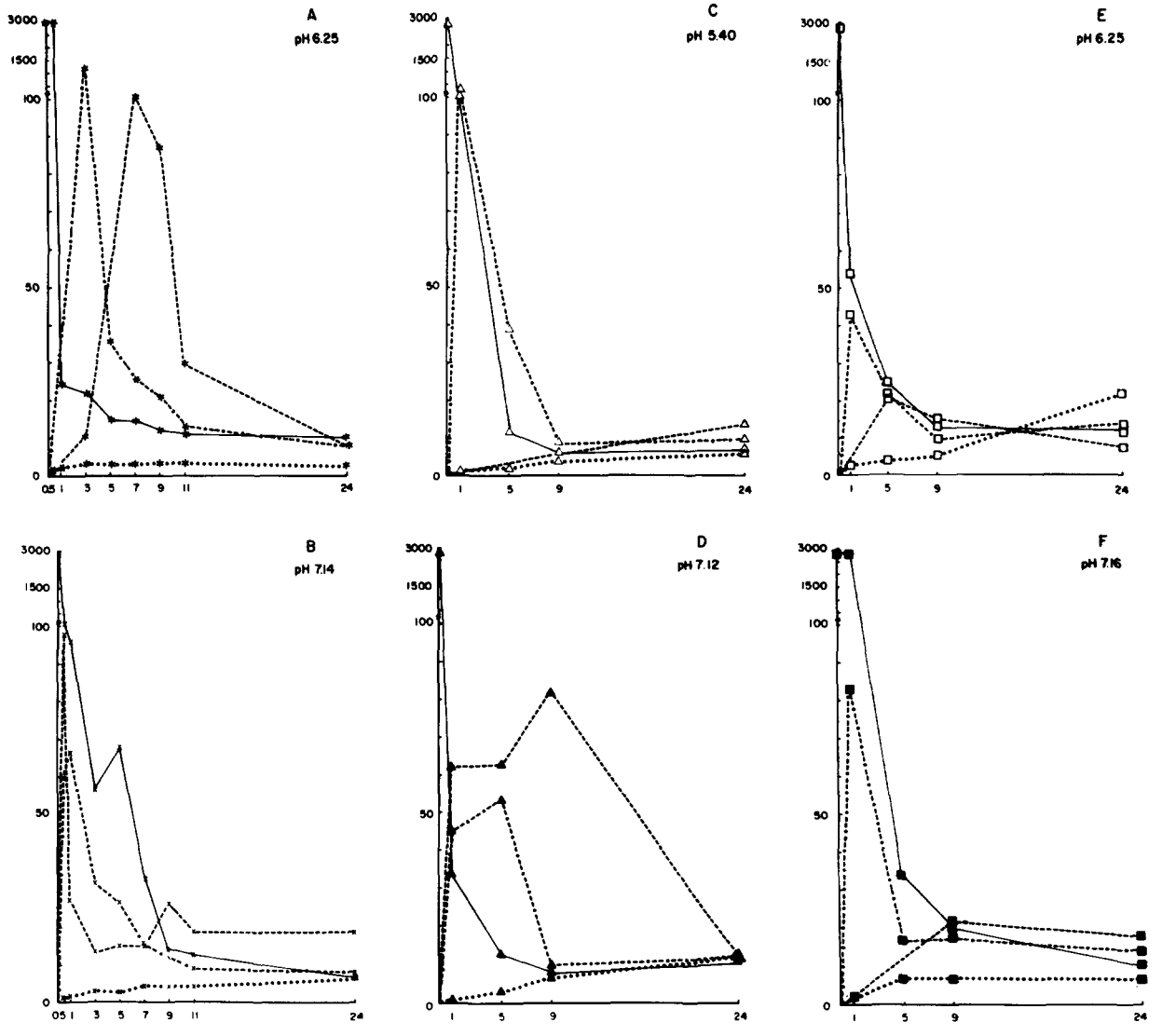


**Fig. 6.** Time curves of specific activities of $^{14}$C-labeled KLC, UDC, CDC, and LC after in vitro fecal incubation of trace amounts of $^{14}$C-labeled KLC with unlabeled CDC (isotope dilution studies). Each set of curves represents the results of one incubation series. A total of six incubation series was carried out on fecal samples of four subjects (see also legend of Fig. 5 for incubation conditions). The biotransformation rate and pattern of the unlabeled CDC for each study is shown in Fig. 5. The solid line represents the [$^{14}$C]KLC, the dotted line the [$^{14}$C]CDC, the dashed line the [$^{14}$C]LC, and the dotted-dashed line the [$^{14}$C]UDC specific activity. The specific activity (DPM $\times$ mg$^{-1}$ $\times$ 10$^4$) is depicted on the y-axes and hours of incubation on the x-axes.

of sizable amounts of UDC. However, no KLC was identified in the fecal samples, thus indicating a very rapid reduction of KLC to UDC as well as CDC and/or the involvement of an intermediate other than KLC.

The composition of radioactively labeled bile acids in duodenal bile after colonic infusion of [$^{14}$C]KLC and [$^{14}$C]CDC, respectively, is consistent with previous studies in our laboratory (15). The radioactive bile acids in bile represent the product of bacterial biotransformation, colonic absorption, hepatic biotransformation and biliary excretion of the respective labeled bile acid instilled into the colon. After colonic infusion of [$^{14}$C]KLC, the percentage of the label appearing in CDC was higher in bile than in the colon (Table 2), since a major portion of KLC that is absorbed is converted in the liver predominantly to CDC (15). The proportion of the $^{14}$C-label found in biliary CDC was also, as expected, very high when [$^{14}$C]CDC was infused into the colon. In this instance, the percentage of the biliary radioactivity found in CDC is determined by the rate of both the colonic absorption and the intracolonic biotransformation of [$^{14}$C]CDC (CDC is not altered at the steroid nucleus during its passage through the liver (15)).

Our in vivo observations are in agreement with our previous studies of the hepatic metabolism of KLC, CDC, and UDC (15), as well as with the in vitro data

of our own study and those of most other investigators who have studied the bioconversion of CDC in fecal incubation systems or intestinal bacterial cultures (16, 17, 26, 27, 33). However, neither our GLC nor TLC systems allowed a separation of 3$\beta$-hydroxy epimers from the corresponding normally occurring 3$\alpha$-hydroxy bile acids. 3$\beta$-Hydroxy epimers, such as isolithocholic acid, have been isolated in feces by other investigators (27, 28). According to Hirano, Masuda, and Oda (27), isolithocholic acid may constitute up to 15% of the fecal metabolites of CDC and UDC. Our nearly complete recovery of the total radioactivity on the TLC plates in the LC, KLC, CDC, and UDC bands is consistent with the observation on GLC systems that the corresponding 3$\alpha$- and 3$\beta$-hydroxy epimers elute closely to each other (27). In other words, isolithocholic acid and other 3$\beta$-hydroxy epimers, if they occur, would be measured together with the respective 3$\alpha$-hydroxy compounds. The latter does, however, not affect the main purpose of our studies, namely the evaluation of the role of KLC as an intermediate in the conversion of CDC to UDC.

The ability of several anaerobic as well as aerobic intestinal bacterial species to oxidize CDC to KLC was first reported by Midtvedt and Norman (17). More than a decade later, Fedorowski et al. (16) showed that the entire biotransformation of CDC to UDC can take place in the mixed bacterial milieu of anaerobically incubated stool samples. Although these authors did not identify any KLC during the formation of UDC, we and other authors (26, 27, 33) showed this putative intermediate to be formed in the course of this reaction. However, in agreement with Fedorowski et al. (16), we initially suspected that KLC is neither the only, nor necessarily the most important, intermediate in the conversion of CDC to UDC (26). This suspicion grew out of in vitro and in vivo biotransformation studies of labeled as well as unlabeled CDC, in which the amount of KLC formed was observed to be considerably smaller than that of UDC. However, in the interpretation of this finding, one has to consider the complex interaction of the multiple enzymatic reactions that are involved in the intestinal bacterial metabolism of CDC. The reversible reactions CDC $\rightleftharpoons$ KLC $\rightleftharpoons$ UDC are not only influenced by the comparative availability and reactivity of specific dehydrogenases and reductases in the colon, but also by the velocity of the irreversible bacterial dehydroxylation of CDC and UDC to LC. The isotope dilution experiments carried out in this study, in which trace amounts of [$^{14}$C]KLC were incubated with unlabeled CDC, show a precursor-product relationship between CDC, KLC, UDC, and LC. In every experiment, the specific activity curve of [$^{14}$C]KLC intersected with that of [$^{14}$C]UDC and/or [$^{14}$C]LC. An intersection of the [$^{14}$C]KLC and [$^{14}$C]UDC specific activity curves can be expected to occur if the formation of KLC proceeds at a rate similar to that of its transformation to UDC and LC. In contrast, the specific activity curve of [$^{14}$C]KLC intersects only with that of [$^{14}$C]LC if the velocity of the KLC $\rightarrow$ UDC $\rightarrow$ LC is higher than the CDC $\rightarrow$ KLC reaction. It is noteworthy that the precursor-product relationship between CDC, KLC, and UDC was evident at acid as well as at alkaline fecal pH levels. These findings are consistent with KLC being the major intermediate in the intestinal bacterial conversion of CDC to UDC, regardless of whether fecal pH is in the acid or alkaline range.

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