Yonago Acta medica 2004;47:63–69

Dehydrogenation of Conjugated Cholic Acid by *Escherichia coli*

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7α-Dehydrogenation of taurocholic acid and glycocholic acid by *Escherichia coli (E. coli)* was examined in aerobic and anaerobic culture conditions. Bile acids in the culture medium of *E. coli* were extracted, separated into free, glycine-conjugate and taurine-conjugate fractions by piperidinohydroxypropyl dextran gel column chromatography, hydrolyzed in alkali and analyzed by gas-liquid chromatography. Both conjugated cholic acids were dehydrogenated to the corresponding 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid without deconjugation and no deconjugation of both conjugated cholic acid was detected in aerobic cultures. But there was little transformation in anaerobic cultures. These data suggest that conjugated cholic acids are taken up by *E. coli* in an aerobic culture as conjugate forms, dehydrogenated without deconjugation and excreted from the cell as conjugate forms.

Key words: dehydrogenation; *Escherichia coli*; glycocholic acid; taurocholic acid

Primary bile acids synthesized from cholesterol in the liver are conjugated mainly with taurine or glycine, and excreted with bile into the duodenum. Intestinal microorganisms are well known to transform sterols and bile acids in human and animal gut (Macdonald et al., 1983; Groh et al., 1993; Hylemon and Harder, 1999). In the gut, these primary conjugated bile acids undergo various transformations by intestinal microorganisms. The major reactions are deconjugation, oxidation of hydroxyl groups (dehydrogenation), reduction of oxo groups, and dehydroxylation.

Escherichia coli (*E. coli*) has no deconjugation activity (Drasar and Hill, 1966; Midtvedt and Norman, 1967; Imamura et al., 1979; Chikai et al., 1987; Kayahara et al., 1994; Uchida et al., 1999), but has dehydrogenation activity that forms 7-oxo-bile acids (Macdonald et al., 1973; Heslewood and Haslewood, 1976; Imamura et al., 1979). In a previous paper, we reported that *E. coli* dehydrogenates the 7α-hydroxyl group of both unconjugated cholic acid and taurocholic acid (TCA) (Ogura et al., 2003). In addition, we found that TCA is dehydrogenated by *E. coli* to taurine-conjugated

Abbreviations: CA, cholic acid; GCA, glycocholic acid; GLC, capillary gas-liquid chromatography; G-βα, glycine-conjugated 7β,12α-dihydroxy-5β-cholanoic acid; 7α-HSDH, 7α-hydroxysteroid dehydrogenase; Me-DMES, methyl ester dimethylethylsilyl ether; PHP GEL, piperidinohydroxypropyl dextran gel; TCA, taurocholic acid; T-αβ, taurine-conjugated 7α,12β-dihydroxy-5β-cholanoic acid; T-3α12α7=O, taurine-conjugated 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid; 3α12α7=O, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid; ββ, 7β,12β-dihydroxy-5β-cholanoic acid

Table 1. Relative retention time of bile acid derivatives

	Derivative	Retention time (min)	Relative retention time		
Internal standard	7β,12β-Dihydroxy-5β-cholanoic acid Me-DMES	12.61	1.08	**1.00**	1.28
	7β,12α-Dihydroxy-5β-cholanoic acid Me-DMES	11.62	**1.00**	0.92	1.18
	7α,12β-Dihydroxy-5β-cholanoic acid Me-DMES	9.82	0.85	0.78	**1.00**
Substrate and product	Cholic acid Me-DMES		1.59	1.46	1.88
	3α,12α-Dihydroxy-7-oxo-5β-cholanoic acid Me-DMES		1.72	1.58	2.03
	Unknown		1.90	1.76	2.25

Me-DMES, methyl ester dimethylethylsilyl ether.

3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (T-3α 12α7=O) without deconjugation.

In the present experiments, we examined in detail the dehydrogenation of glycine- and taurine-conjugated cholic acids by *E. coli.*

Material and Methods

Chemicals

Glycocholic acid (GCA), TCA and cholic acid (CA) were purchased from Sigma Chemicals Co., St. Louis, MO. 3α,12α-Dihydroxy-7-oxo-5β-cholanoic acid (3α12α7=O) was prepared by the oxidation of CA with *N*-bromosuccinimide (Fieser and Rajagopalan, 1949). 7β,12β-Dihydroxy-5β-cholanoic acid (ββ), glycine-conjugated 7β,12α-dihydroxy-5β-cholanoic acid (G-βα) and taurine-conjugated 7α,12β-dihydroxy-5β-cholanoic acid (T-αβ) were synthesized as described previously (Arimoto et al., 1982; Yamaga et al., 1987) and used as internal standards for analysis of bile acids by capillary gas-liquid chromatography (GLC). Piperidino hydroxypropyl dextran gel (PHP GEL) was purchased from Shimadzu, Kyoto, Japan and dimethylethylsilylimidazole from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. The other reagents and solvents, of analytical grade, were obtained from Wako Pure Chemical Industries, Osaka, Japan. If not otherwise stated, the solvents were distilled once before use.

Bacteriological procedures

E. coli K-12 was obtained from the American Type Culture Collection (Manassas, VA). Brain-heart infusion (BHI; Becton Dickinson, Sparks, MD) was used for all cultures. *E. coli* was precultured aerobically in BHI medium at 37°C for 1 day. Aliquots ($3 \times 10^7/10$ μL) of *E. coli* were added to 2 mL of BHI medium containing 1 mM bile acids, and the bacteria were cultured at 37°C for 4 days. Aerobic culture was performed by incubation in a thermostat, and anaerobic culture was performed by the gas-pack method (BBL GasPak Pouch System; Becton Dickinson).

Analytical methods

Prior to analysis of bile acids, ββ, G-βα and T-αβ as internal standards were added to 100 μL of the culture medium. The analytical sample with the three internal standards was treated with 8 volumes of ethanol at 80°C for 10 min, and filtered. The filtrate was evaporated to dryness under a stream of nitrogen and the residue was subjected to PHP GEL column chromatography to separate bile acids into nonamidate (free), glycine-conjugate and taurine-conjugate fractions (Yamaga et al., 1987). Glycine- and taurine-conjugate fractions were hydrolyzed individually in alkaline solution (Yamaga et al., 1997). The hydrolysates and nonamidate fraction were acidified with dilute hydrochloric acid, and the bile acids were extracted with ethyl ether. The extracted bile acids were converted into

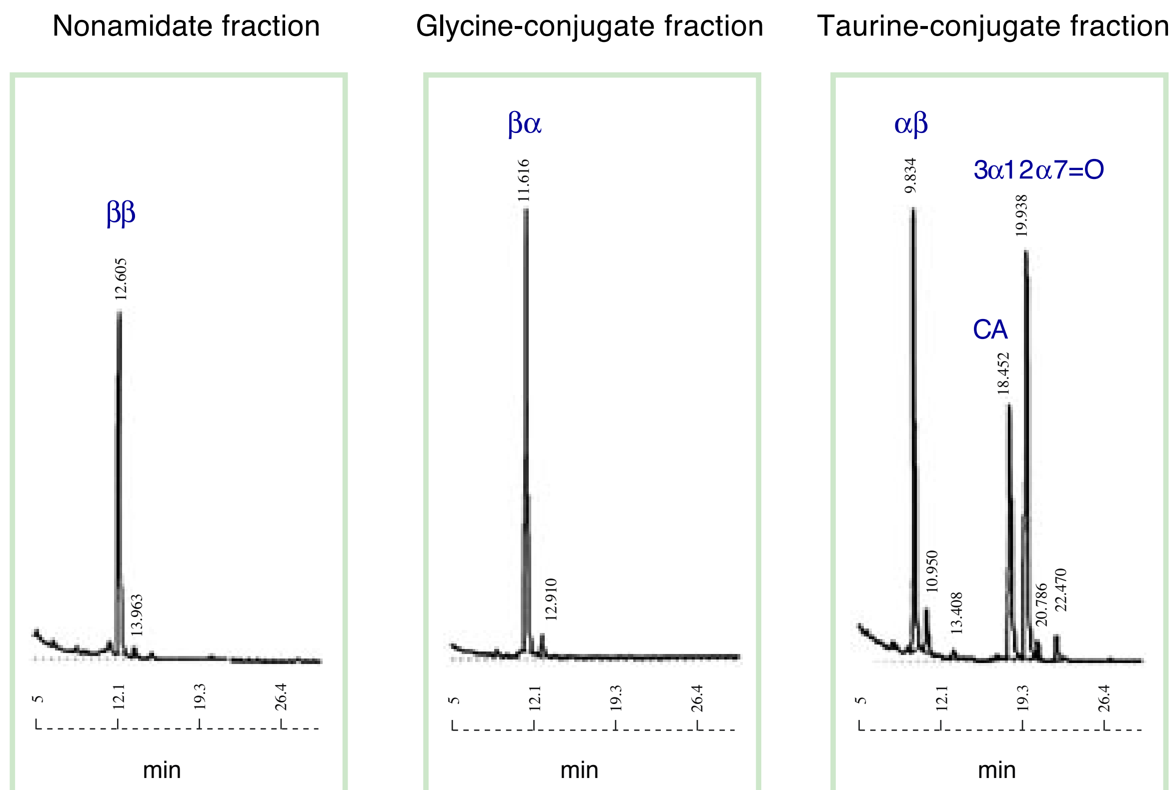

Fig. 1. Gas chromatograms of bile acids in nonamidate, glycine- and taurine-conjugate fractions after a piperidinohydroxypropyl dextran gel (PHP GEL) column of bile acids. The medium cultured with taurocholic acid (TCA) for 4 days added three internal standard compounds, 7β,12β-dihydroxy-5β-cholanoic acid (ββ), glycine-conjugated 7β,12α-dihydroxy-5β-cholanoic acid (G-βα) and taurine-conjugated 7α,12β-dihydroxy-5β-cholanoic acid (T-αβ). The sample was fractionated by PHP GEL column. Each fraction was hydrolyzed, and the bile acid extracts were converted into Me-DMES derivatives. See Table 1 for gas chromatographic condition. CA, cholic acid; 3α12α7=O, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid.

methyl ester dimethylethylsilyl ether (Me-DMES) derivatives as described previously (Yamaga et al., 1987; Yamaga et al., 1996). Briefly, bile acids were methylated with diazomethane, and then left in 70 μL of dimethylethylsilylimidazole in an air-tight vessel at room temperature overnight.

Capillary gas liquid chromatography

An aliquot of the bile acid derivatives dissolved in *n*-hexane was injected into a gas-liquid chromatograph (model GC-14A; Shimadzu) equipped with a flame ionization detector, a solventless injector and a computerized data system (model C-R4A; Shimadzu). A Hicap CBP-1 capillary column (25 m × 0.25 mm i.d.; Shimadzu) was used. The column temperature was maintained at 285°C and helium was used as the carrier gas.

Relative retention times of bile acid derivatives in the present GLC analysis are given in Table 1.

Results

Figure 1 shows gas chromatograms of the bile acids in the nonamidate, glycine- and taurine-conjugate fractions obtained by PHP GEL column chromatography of the medium after culture with TCA for 4 days. Peaks corresponding to CA, 3α12α7=O and αβ (internal standard) were detected in the taurine-conjugate fraction. A peak corresponding

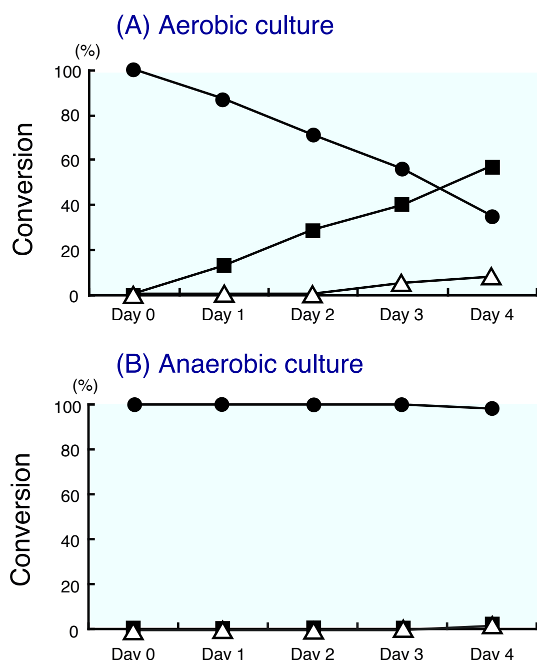

Fig. 2. Conversion of taurocholic acid (TCA) to taurine-conjugated $3\alpha,12\alpha$-dihydroxy-7-oxo-5β-cholanoic acid (T-$3\alpha12\alpha7$=O) by *Escherichia coli*. Changes in percentage composition of individual bile acids were followed during the course of (**A**) aerobic and (**B**) anaerobic incubation for 4 days. ●, TCA; ■, T-$3\alpha12\alpha7$=O; △, Unknown.

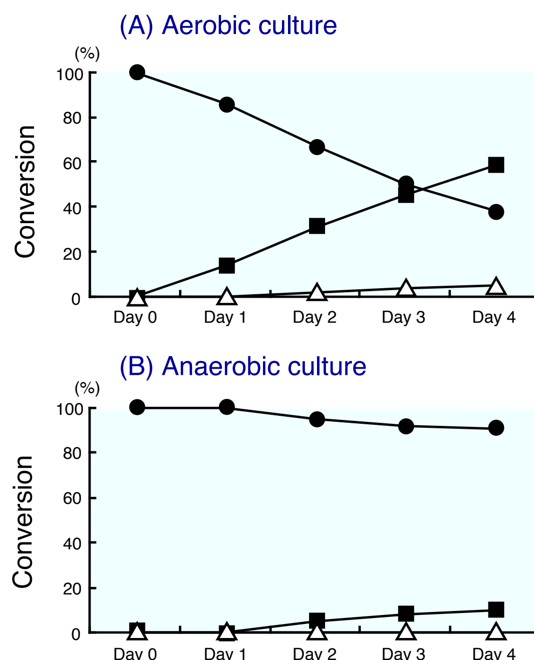

Fig. 3. Conversion of glycocholic acid (GCA) to glycine-conjugated $3\alpha,12\alpha$-dihydroxy-7-oxo-5β-cholanoic acid (G-$3\alpha12\alpha7$=O) by *Escherichia coli*. Changes in percentage composition of individual bile acids were followed during the course of (**A**) aerobic and (**B**) anaerobic incubation for 4 days. ●, GCA; ■, G-$3\alpha12\alpha7$=O; △, Unknown.

to $\beta\beta$ was detected only in the nonamidate fraction, and a peak corresponding to $\beta\alpha$ only in the glycine-conjugate fraction. No more than two internal standard peaks were detected in each fraction. These gas chromatograms demonstrate that the bile acids in the analytical samples were separated completely by PHP GEL column chromatography. This was also confirmed in the experiments with GCA and free CA.

Figure 2 shows the time course of conversion of TCA by *E. coli*. In aerobic culture, TCA was dehydrogenated to T-$3\alpha12\alpha7$=O, and the conversion proceeded almost linearly at a rate of about 15% a day (or 0.15 μmol/day under our experimental conditions). Small amounts of unidentified compounds were formed. However, there was little dehydrogenation of TCA in anaerobic culture.

Figure 3 shows the conversion of GCA by *E. coli*. GCA was dehydrogenated to the corresponding glycine-conjugated 7-oxo-bile acid. The conversion rate was almost the same as that of TCA in aerobic culture, but GCA was slightly more susceptible to the reaction than TCA, especially in anaerobic culture.

Figure 4 shows the conversion of free CA by *E. coli*. In this case, the reaction occurred much more quickly than with TCA or GCA, with 80% conversion after 1 day in aerobic culture and about 50% conversion after 2 days in anaerobic culture.

Medium cultured for 4 days without CA was filtered through a Millipore filter (MILLEX-GV0.22) and the filtrate was incubated aerobically for 1 to 4 days with 1 mM GCA without addition of cofactors. No oxidation was detected at any time of incubation (data not shown), suggesting that no enzyme reaction took place in the medium.
These results suggest that conjugated bile acids are taken up by *E. coli* as conjugate forms, dehydroge-

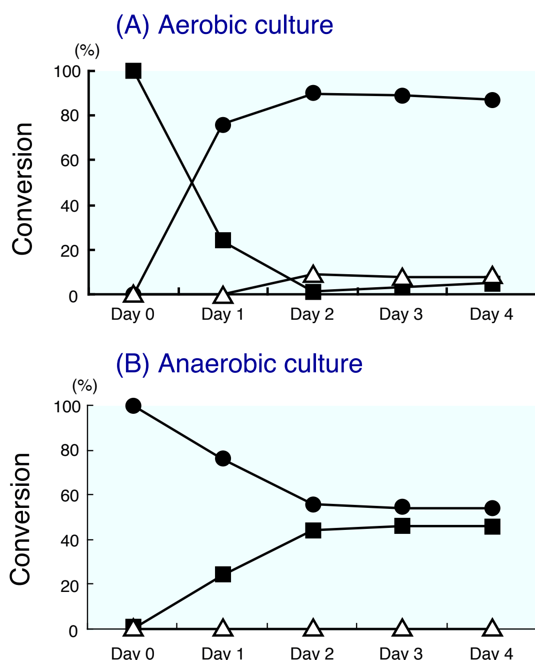

Fig. 4. Conversion of cholic acid (CA) to 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (3α12α7=O) by *Escherichia coli*. Changes in percentage composition of individual bile acids were followed during the course of (**A**) aerobic and (**B**) anaerobic incubation for 4 days. ●, CA; ■, 3α12α7=O; △, Unknown.

nated as conjugate forms and excreted from the cell as conjugate forms.

Discussion

The present experiments demonstrated that *E. coli* dehydrogenates taurine- or glycine-conjugated CA to the corresponding 3α12α7=O without deconjugation. These reactions occurred only under aerobic conditions, and the conversion rates were lower than for free CA. TCA and GCA were about 60% dehydrogenated after 4 days (Fig. 1), whereas free CA was about 80% dehydrogenated after 1 day and almost completely dehydrogenate after 4 days (Ogura et al., 2003).

Haslewood and Haslewood (1976) showed that 7α-hydroxysteroid dehydrogenase prepared from *E. coli* oxidizes both conjugated and free 7α-hydroxyl bile acids at the same rate in an in vitro system. The difference in rates between the present report and the previous report presumably represents differences in substrate penetration into living cells.

It is well known that *E. coli* possesses no hydrolase activity that will hydrolyze conjugated bile acids (Drasar and Hill, 1966; Midtvedt and Norman, 1967; Imamura et al., 1979; Chikai et al., 1987; Kayahara et al., 1994; Uchida et al., 1999). Accordingly, in the present experiments, no deconjugation of TCA and GCA was detected. *E. coli* possesses 7α-hydroxysteroid dehydrogenase (7α-HSDH) (Aries et al., 1969; Macdonald et al., 1973; Prabha et al., 1989) as a non-inducible intracellular enzyme that requires NAD^+ as a cofactor (Macdonald et al., 1973; Prabha et al., 1989). Disruption of *E. coli* during culture might release 7α-HSDH into the medium, but the present experiments ruled out the possibility that released enzyme activity catalyzes the conversion, since the filtrate prepared from medium cultured for 4 days was completely ineffective in 7α-dehydrogenation of GCA.

If conjugated bile acids are taken up by *E. coli*, the corresponding conjugated 7-oxo-bile acids are formed and excreted into the culture medium. However, it remains unclear how conjugated bile acids are take up by *E. coli*. Bile acids may cross the cell membrane by ionic or nonionic diffusion, as demonstrated with intestinal bile acid absorption (Dietschy, 1968). Nonionized bile acids may penetrate the membrane easily, but conjugated bile acids are unlikely to pass through lipid bilayers because their pKa values are low (taurine-conjugated, < 1.5; glycine-conjugated, 3.5–5.2) and they are ionized at the pH of the present culture medium. The pH in our experiments increased to about 9 in aerobic cultures, whereas it decreased to around 6.5 in anaerobic cultures (Ogura et al., 2003). However, the rate of dehydrogenation of conjugated bile acids, presumably reflecting the rate of influx of substrate, was greatest in aerobic culture and least in anaerobic culture.

These observations suggest that *E. coli* possesses an influx mechanism for conjugated bile acids. Mallonee and Hylemon (1996) reported that a bile acid transporter in *Eubacterium* sp. strain

VPI 12708 showed a much higher activity for free bile acids than for glycocholic acid or 7-oxocholic acid. Conversely, Thanassi et al. (1997) demonstrated that *E. coli* possesses a mechanism to actively pump out TCA. T-3α12α7=O was not examined in that report, but it is presumed that this oxo bile acid is excreted by the same mechanism. It is not yet known whether this efflux mechanism is also responsible for influx of bile acids, but if this efflux mechanism is a rate-limiting step, influx of conjugated bile acids will be restricted under anaerobic conditions.

As shown in the present paper, conjugated bile acids are dehydrogenated by *E. coli* without deconjugation. On the other hand, 7α-dehydroxylation of bile acids by intestinal bacteria is considered to take place after deconjugation (Aries and Hill, 1970), and 7α-dehydroxylating bacteria show much higher activities when cultured with bacteria possessing deconjugation activity than when cultured alone (Hirano and Masuda, 1982; Narushima et al., 2002); nevertheless, this dehydroxylation reaction has also been suggested to occur without deconjugation in vivo (Hepner et al., 1972).

Acknowledgments: The authors thank Professor Hisao Ito, Division of Organ Pathology, Department of Microbiology and Pathology, School of Medicine, Tottori University Faculty of Medicine, and Professor Yoshito Irizawa, Division of Legal Medicine, Department of Social Medicine, School of Medicine, Tottori University Faculty of Medicine, for reviewing this manuscript.

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Received September 2, 2004; accepted September 30, 2004

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