A study on the mechanism of the epimerization at C-3 of chenodeoxycholic acid by *Clostridium perfringens*

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The mechanism of 3-hydroxy epimerization of chenodeoxycholic acid by *Clostridium perfringens* was investigated in $3\alpha,7\alpha$ -dihydroxy-[2,2,4,4- $^2\text{H}_4$]-, $3\alpha,7\alpha$ -dihydroxy-[3β - ^2H]- and $3\beta,7\alpha$ -dihydroxy-[3α - ^2H]- 5β -cholanoic acid transformations. Our findings rule out a dehydration–rehydration pathway and agree with a redox mechanism involving 3-oxochenodeoxycholic acid as intermediate.

3β -Hydroxy bile acids have been detected in human and animal faeces (Carey, 1973), and the origination of these compounds from the 3α -hydroxy epimers was shown in mixed and pure cultures of intestinal micro-organisms (Drasar & Hill, 1974; Hirano & Masuda, 1981; Hirano *et al.*, 1981a).

More recently, ursodeoxycholic acid ($3\alpha,7\beta$ -dihydroxy- 5β -cholanoic acid, UDCA), the 7-epimer of chenodeoxycholic acid ($3\alpha,7\alpha$ -dihydroxy- 5β -cholanoic acid, CDCA), has also been shown to derive from CDCA, and, because of their current use as efficient cholesterol-gallstone-dissolving agents (Iser *et al.*, 1975; Maton *et al.*, 1977; Stiehl *et al.*, 1978), there is new interest in their interconversion *in vivo* and *in vitro* (Fedorowski *et al.*, 1979; Hirano *et al.*, 1981a; Macdonald *et al.*, 1981; Edenharder & Knaflitz, 1981). In spite of this strong interest, the 7-epimerization mechanism of bile acids has not been definitely established (Fedorowski *et al.*, 1979; Sarva *et al.*, 1980; Edenharder & Knaflitz, 1981; Sutherland & Macdonald, 1982), and experimental evidence for the 3-epimerization mechanism is limited (Hayaishi *et al.*, 1955; Hirano *et al.*, 1981b). Two different pathways can be considered for this transformation: (1) oxidation–reduction; (2) dehydration–rehydration (Scheme 1). A third pathway of ‘direct epimerization’ (Salen *et al.*, 1974; Edenharder & Knaflitz, 1981) seems less likely, because no examples in enzymic reactions have yet been reported of alcohol epimerization involving activa-

tion of hydroxy group, with its leaving with $\text{S}_{\text{N}}2$ substitution by a hydroxy group from water.

The present paper is concerned with a study of 3-hydroxy epimerization of deuterated chenodeoxycholic acids ($3\alpha,7\alpha$ -dihydroxy-[2,2,4,4- $^2\text{H}_4$] 5β -cholanoic acid, $3\alpha,7\alpha$ -dihydroxy-[3β - ^2H] 5β -cholanoic acid and $3\beta,7\alpha$ -dihydroxy-[3α - ^2H] 5β -cholanoic acid) with a strain of *Clostridium perfringens* isolated from human faeces, in order to obtain some information about this transformation mechanism.

Materials and methods

Micro-organism

The micro-organism, from freshly voided faeces of a healthy man, was isolated by growth on sulphite/polymyxin/milk/agar plates (Mevisen-Verhage *et al.*, 1982). The strain was identified by using the Api 20 A System (Ayerst Italiana, Latina, Italy). All manipulations of stool specimens and the cultures of isolated micro-organism were carried out in an anaerobic glove cabinet (Forma Scientific, Marietta, OH, U.S.A.) in which the air was replaced by an atmosphere of $\text{N}_2/\text{H}_2/\text{CO}_2$ (17:2:1) sterilized by filtration (FHL P 02500 filter; Millipore Corp., Bedford, MA, U.S.A.). The micro-organism was maintained in cooked-meat broth, grown overnight at 37°C, stored at 4°C and used for no more than 2 weeks as a culture source.

Transformation of bile acids by growing cells

Starter cultures in Todd Hewitt broth (Biolife, Milan, Italy) were inoculated with cooked-meat cultures (10% inoculum) and grown for 24 h at 37°C. Cultures for bile acid transformation were

Abbreviations used: CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid.

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Scheme 1. Alternative pathways for epimerization at C-3 of CDCA

performed by inoculating starter cultures (10%) in pre-reduced Todd Hewitt broth containing 0.02% of the tested bile acid, and incubated for 48 h. The bile acids, as sodium salt solution, were sterilized by membrane filtration (HAWF 1300 filter; Millipore Corp.) and added to autoclaved broth.

Transformation of bile acids by resting cells

Cl. perfringens was grown in Todd Hewitt medium containing CDCA at 0.01%. The cells, harvested after 24 h of incubation, were washed in 0.02 M-potassium phosphate buffer, pH 7.0, by centrifuging three times and resuspended in the same buffer (1 mg dry wt. of cells/ml) to which 0.02% of the bile acid being tested had been added. After 48 h of anaerobic incubation at 37°C, the cell suspension was assayed for bile acid transformation and/or extracted for isolation of transformation products.

Identification of bile acids and isolation of 3α,7α-dihydroxy-5β-cholanoic acid and 3β,7α-dihydroxy-5β-cholanoic acid

The cultures or the cell suspensions were acidified to pH 2.0 with 5 M-HCl and extracted with methylene dichloride/ethyl acetate (1:1, v/v). To identify the transformation products, the extracts were analysed by t.l.c. (0.2 mm DC silica-gel plates; Merck, Darmstadt, West Germany) with chloroform/acetone/acetic acid (28:12:1, by vol.) as solvent system and H₂SO₄ as detector. To isolate CDCA and its 3-epimer, the organic extracts were washed with water and evaporated under vacuum. The two epimers were separated by flash chromatography (Still *et al.*, 1978), by elution

with the same solvent system used for t.l.c. CDCA was crystallized from ethyl acetate/heptane [m.p. 119°C; ¹H n.m.r. δ 3.45 p.p.m. (m, 1 H, 3β-H; *W*₁ = 20 Hz), 3.85 p.p.m. (m, 1 H, 7β-H)] and its 3-epimer from ethyl acetate [m.p. 193°C; ¹H n.m.r. δ 3.85 p.p.m. (m, 1 H, 7β-H), 4.05 p.p.m. (m, 1 H, 3α-H)]. The ratio between the intensity of the signals of hydrogen at C-3 and C-7 was used to evaluate the percentage of label at C-3 in deuterated CDCA and its 3-epimer.

Methyl 7α-hydroxy-3-oxo-5β-cholanoate

Methyl 7α-hydroxy-3-oxo-5β-cholanoate was obtained from methyl chenodeoxycholate by selective Oppenauer oxidation (Jones *et al.*, 1949). Methyl chenodeoxycholate (8.3 g), obtained by methylation of CDCA with diazomethane, was refluxed for 24 h with aluminium *t*-butoxide (10 g) in dry benzene (230 ml) and acetone (75 ml). The reaction was quenched by adding distilled water (50 ml) and acidified to pH 2.0 with 1 M-H₂SO₄. The mixture was then extracted with diethyl ether, and the organic extracts were washed with aq. 10% (w/v) NaHCO₃ solution, then water, and evaporated under vacuum. The residue (8.8 g) was purified by flash chromatography by elution with ethyl acetate/benzene (3:7, v/v). The methyl 7α-hydroxy-3-oxo-5β-cholanoate obtained (4.7 g) was crystallized from ethyl acetate/heptane (m.p. 124–125°C).

7α-Hydroxy-3-oxo-[2,2,4,4-²H₄]5β-cholanoic acid

7α-Hydroxy-3-oxo-[2,2,4,4-²H₄]5β-cholanoic acid was prepared in accordance with the method reported by Reich *et al.* (1969) for steroids. Methyl

7 α -hydroxy-3-oxo-5 β -cholanoate was hydrolysed with methanolic 10% (w/v) KOH solution under refluxing for 2 h. 7 α -Hydroxy-3-oxo-5 β -cholanoic acid (6g) obtained by standard work-up was kept for 14 h at 80°C with $^2\text{H}_2\text{O}$ (45 ml), dry dioxan (27 ml) and sodium methoxide (2g), and then the reaction mixture was acidified to pH 3.0 with aq. 37% (w/v) HCl solution, concentrated under vacuum and extracted with methylene dichloride. The organic extracts were washed with water and evaporated. The residue was purified by flash chromatography by elution with chloroform/methanol (19:1, v/v) and crystallized from ethyl acetate/heptane [5.2g; m.p. 85°C (decomp.)].

3 α ,7 α -Dihydroxy-[2,2,4,4- $^2\text{H}_4$]5 β -cholanoic acid

3 α ,7 α -Dihydroxy-[2,2,4,4- $^2\text{H}_4$]5 β -cholanoic acid was obtained by direct reduction of 7 α -hydroxy-3-oxo-[2,2,4,4- $^2\text{H}_4$]5 β -cholanoic acid with NaBH_4 . 7 α -Hydroxy-3-oxo-[2,2,4,4- $^2\text{H}_4$]5 β -cholanoic acid (2.5g) was dissolved in dry dimethylformamide (60 ml) and $^2\text{H}_2\text{O}$ (20 ml), and then NaBH_4 (500 mg) was added. After 14 h at room temperature the reaction was quenched by adding water (500 ml) and 5M-HCl (10 ml), and the mixture was then extracted with methylene dichloride. The organic extracts were washed with water and evaporated under vacuum. The 3 α ,7 α -dihydroxy-[2,2,4,4- $^2\text{H}_4$]5 β -cholanoic acid was purified by flash chromatography as described above for undeuterated compounds; 1350 mg was obtained. The introduction of four deuterium atoms was assessed by ^2H -n.m.r. spectroscopy of the methyl ester obtained with deuterated diazomethane (from Deutero-Diazald Prep Set; Aldrich, Milwaukee, WI, U.S.A.). In the ^1H -n.m.r. spectrum the sharp signal ($W_1 = 1.6\text{ Hz}$) at 3.45 p.p.m., due to the 3 β -proton with no coupling with neighbouring protons, confirmed the presence of four deuterium atoms at C-2 and C-4.

3 α ,7 α -Dihydroxy-[3 β - ^2H]5 β -cholanoic acid and 3 β ,7 α -dihydroxy-[3 α - ^2H]5 β -cholanoic acid

To a solution of methyl 7 α -hydroxy-3-oxo-5 β -cholanoate (3.1 g) in methanol (50 ml) was added NaB^2H_4 (150 mg). After 24 h at room temperature the reaction mixture was acidified with 2M-HCl and evaporated under vacuum, and the residue was dissolved in methanol and evaporated under vacuum several times to eliminate boric acid. The crude products were then hydrolysed with methanolic 10% (w/v) KOH for 2 h, and the two deuterated epimers were separated by flash chromatography as described above for undeuterated compounds, yielding 3 α ,7 α -dihydroxy-[3 β - ^2H]5 β -cholanoic acid (1400 mg; m.p. 118°C) and 3 β ,7 α -dihydroxy-[3 α - ^2H]5 β -cholanoic acid (295 mg; m.p. 191–192°C).

The absence of the signal at 3.45 and at 4.05 p.p.m. respectively from ^1H -n.m.r. spectra for these two compounds gave evidence for the complete introduction of deuterium at positions 3 β and 3 α .

Spectral analyses

^1H - and ^2H -n.m.r. spectra were recorded on a Varian XL-200 system operating in the Fourier-transform mode at 200.000 and 30.701 MHz respectively. Chemical shifts are in p.p.m. (δ) from tetramethylsilane. ^1H -n.m.r. and ^2H -n.m.r. spectra were recorded for C^2HCl_3 and C^1HCl_3 solutions, respectively.

Chemicals

All reagents were analytical grade. NaB^2H_4 (99 atom% ^2H) and $^2\text{H}_2\text{O}$ (99.95 atom% ^2H) were from Fluka, Buchs, Switzerland.

Results and discussion

One strain, identified as *Cl. perfringens*, was isolated from human faeces. It was able to transform CDCA in 20% yield into a compound with chromatographic, physical and chemical characteristics identical with those of an authentic sample of 3 β -hydroxy-CDCA. *Cl. perfringens* was also able to perform the reverse reaction in 80% yield and to reduce 3-oxo-CDCA to a mixture of 3 α - and 3 β -hydroxy derivatives in 4:1 ratio. 3-Oxo-CDCA was never observed during CDCA epimerization in anaerobic conditions, but was found in aerobic conditions with resting cells as reported for other strains of *Cl. perfringens* (Hirano *et al.*, 1981b). Moreover, when ZnSO_4 (50 mM), which inhibits dehydrogenation of cholic acid (Archer *et al.*, 1982), was added to resting cells, no transformation of CDCA was observed.

The results of all these experiments suggest that 3-oxo-CDCA is the intermediate of the epimerization at C-3; however, the accumulation of a supposed intermediate and the ability to transform it into the final metabolite cannot be considered sufficient evidence for a pathway involving this compound. Further investigations were carried out to study the transformation of deuterated chenodeoxycholic acids.

To assess the occurrence of the dehydration-rehydration pathway, 3 α ,7 α -dihydroxy-[2,2,4,4- $^2\text{H}_4$]5 β -cholanoic acid was administered to a culture of *Cl. perfringens*. The 3 β -hydroxy epimer isolated after the transformation was methylated with deuterated diazomethane to evaluate the percentage of label at C-2 and C-4. The relationship between the intensity of the broad overlapping signals centered at 2 p.p.m. from deuterium atoms at C-2 and C-4 and the intensity of the signal

at 3.60 p.p.m. of the three deuterium atoms at the methyl ester group is evidence of complete retention of deuterium at C-2 and C-4. This retention was confirmed by the sharp signal ($W_1 = 1.6$ Hz) at 4.05 p.p.m. due to the 3α -hydrogen in the ^1H -n.m.r. spectrum with no detectable coupling with neighbouring protons. These results rule out the dehydration-rehydration pathway because, according to this mechanism, loss of one deuterium atom at C-2 or C-4 would occur.

To trace the fate of hydrogen at C-3 during the epimerization reaction, $3\alpha,7\alpha$ -dihydroxy-[3β - ^2H]5 β -cholanoic acid was fed to a growing culture of *Cl. perfringens*. The ^1H -n.m.r. spectrum of the isolated 3β -hydroxy epimer showed the signal at 4.05 p.p.m. (m, 0.2H) due to the 3α -hydrogen with intensity 0.2:1 by comparison with the signal at 3.85 p.p.m. due to the 7β -hydrogen (m, 1H), whereas the ^1H -n.m.r. spectrum of recovered $3\alpha,7\alpha$ -dihydroxy-5 β -cholanoic acid did not show the signal of the 3β -hydrogen at 3.45 p.p.m. These results show the total retention of deuterium at the C-3 of the recovered $3\alpha,7\alpha$ -dihydroxy-[3β - ^2H]5 β -cholanoic acid, but a loss of 20% of the deuterium during the conversion of the latter into its 3β -hydroxy epimer.

Edenharder & Knaflitz (1981), interpreting the results obtained by Fedorowski *et al.* (1979) on the transformation of CDCA by human intestinal bacteria, ruled out an oxidoreductive pathway for the C-7 epimerization on the basis of label retention at C-7, and pointed to another mechanism proceeding via an unsaturated intermediate. Since we have excluded the dehydration-rehydration pathway in our first experiment, our results can be explained in terms of an oxidoreductive mechanism assuming that the reduction of the 3-oxo group to 3β -hydroxy has been performed by an $\text{NAD(P)}^2\text{H}/\text{NAD(P)H}$ mixture in which deuterated NAD(P)H derives from the first oxidative step.

To confirm this interpretation, we carried out experiments on the transformation of CDCA and 3β -hydroxy-CDCA with two different concentrations of resting cells (Table 1). In all the experi-

ments with a low cell concentration the two isolated epimers showed total label retention at C-3. In the transformation of [3β - ^2H]CDCA with a high cell concentration, 3β -hydroxy-CDCA retained 80% of deuterium, whereas untransformed CDCA was completely deuterated. Unexpectedly, in the reverse transformation of 3β -hydroxy-[3α - ^2H]CDCA, completely labelled CDCA was obtained, whereas the recovered untransformed acid contained 80% deuterium.

The complete deuterium retention observed in the experiments with low cell concentration suggests that 3-oxo-CDCA reduction to both epimers is performed only by NAD^2H , which strongly prevails over endogenous NADH because of the high substrate/cell ratio. Conversely, with a low substrate/cell ratio, an appreciable participation of NADH from endogenous metabolism can be supposed, and the consequent partial label at C-3 was observed. A similar behaviour occurs in redox reactions of the malolcoholic fermentation pathway (Maconi *et al.*, 1984).

The reason why label loss was observed only on the 3β -hydroxy-CDCA in both direct and reverse epimerization is not easily explicable on the basis of our results. The influence of a possible occurrence of an NADH compartmentation or of different rates in $\text{CDCA} \rightleftharpoons 3\text{-oxo-CDCA}$ and $3\beta\text{-hydroxy-CDCA} \rightleftharpoons 3\text{-oxo-CDCA}$ reaction can be hypothesized, but evidence for this phenomena requires a different experimental approach.

In conclusion, the inhibition of the epimerization by Zn^{2+} , the 3-oxo-CDCA accumulation in aerobic conditions, its reduction to both epimers, and lastly all the results obtained in the transformations of deuterated compounds are consistent with a redox mechanism of this epimerization reaction.

Our results give evidence that tritium or deuterium retention at a carbon atom bearing a hydroxy group may be in agreement with a ketonic intermediate, as already reported in 4-UDP-glucose isomerization (Glaser, 1972) and in malolcoholic fermentation (Maconi *et al.*, 1984). Thus, also, the tritium retention at C-7 observed by Fedorowski *et al.* (1979) for $\text{CDCA} \rightleftharpoons \text{UDCA}$

Table 1. Percentage of deuterium atoms at C-3 in the transformation products of deuterated chenodeoxycholic acid and 3β -hydroxychenodeoxycholic acid

Transformations were performed by resting cells of *Cl. perfringens* at different cell concentrations. For details see the text.

Substrate	Cell concn. (mg/ml)	Deuteration (%)	
		CDCA	3β -Hydroxy-CDCA
[3β - ^2H]CDCA	0.05	100	100
	1.5	100	80
3β -Hydroxy-[3α - ^2H]CDCA	0.05	100	100
	1.5	100	80

epimerization can be explained in terms of a redox mechanism involving hydrogen at C-7 and nucleotide recycling (Macdonald & Roach, 1981) rather than the less likely hydration of the isolated double bond of chol-6-enoic acid.

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