ON THE MECHANISM OF CHOLIC ACID 7α-DEHYDROXYLATION BY A *CLOSTRIDIUM BIFERMENTANS* CELL-FREE EXTRACT

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Received 21 January 1977

1. Introduction

Samuelsson [1,2] could establish that the 7α-dehydroxylation of cholic acid occurs in vivo in mammal intestine through the formation of 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid. No evidence of a similar in vitro occurrence as far as we know has been revealed. We have demonstrated previously, both with mixed cultures of intestinal microorganisms and with *Clostridium bifermentans* in a pure culture, that the reduction of cholic acid into deoxycholic acid, involving no loss of the H-atom in the 7-position, was associated with an oxido–reductive equilibrium with the corresponding 7-oxo-derivative [3,4].

This note describes the continuation of our investigations on the mechanism of conversion of cholic acid into deoxycholic acid, performed in vitro by a cell-free extract of *Clo. bifermentans* isolated from human faeces [4]. For this purpose the Δ^6- and Δ^7-cholenic acids were tested as possible intermediates. Following investigations carried out using 3α, 12α-5β-[24-^{14}C]chol-6-en-24-oic and 3α, 12α-dihydroxy-5β-[24-^{14}C]chol-7-en-24-oic acids as well as tests of isotope trapping, Δ^6-cholenic acid is to be considered the intermediate in the conversion.

2. Materials and methods

Clostridium bifermentans cp. SD 10 was cultivated in Todd Hewitt Broth (pH 7.2). The cultures were incubated at 37°C in a Vuotomatic Bicasa TN 50 under N_2 and CO_2 (9:1).

The preparation of the cell-free extract was performed as described in the previous paper [5]. The reaction mixture for the enzymatic assay contained: 1 ml enzymatic extract (40 mg protein/ml), 1.5 ml of 0.02 M phosphate buffer (pH 7.0) and 0.5 ml of the sodium salt of the organic acid. After incubation at 37°C under N_2, the evaluation of the enzymatic activity, extraction procedures, thin-layer chromatography and separation of the products, both in the form of methylesters and free acids, were performed as previously described [3].

For isotope trapping experiments 3α,12α-dihydroxy-5β-chol-6-en-24-oic (10 mg) and [24-^{14}C]cholic (0.21 mg, 119 μCi/mg) acids were added, as sodium salts, to 20 ml of the above stated reaction mixture, its components still being present in a same proportion. After incubation at 37°C under N_2 the transformation mixture was extracted and the residue of evaporation of the organic phase was chromatographed on Silica Gel DC Merck plates. Thin-layer chromatography enabled us to separate deoxy-7-oxo-cholic acid and cholic acid from the mixture of 3α,12β-dihydroxy-5β-chol-6-en-24-oic acid and deoxycholic acid. After addition of cold deoxycholic acid, the components of the said mixture were further separated, by thin-layer chromatography on Silica Gel DC Merck plates treated with $AgNO_3$: acetic acid/benzene/ethyl acetate (9:21:69, v/v/v) were used as eluents. The isolated products were crystallised up to a constant radioactivity and counted on a Tricarb Packard Mod. 3320 liquid scintillator (PPO 6.5 g, dimethyl POPOP 0.13 g, naphthalene 104 g, toluene 500 ml, dioxane 500 ml). [24-^{14}C]cholic acid sodium salt was supplied by the Radiochemical Centre, Amersham, Buckinghamshire (England).

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3α,12α-Dihydroxy-5β-chol-6-en-24-oic acid was synthetised starting from deoxy-7-oxo-cholic acid [6] and 3α, 12α-dihydroxy-5β-chol-7-enic acid was obtained from methyl 3α-acetoxy-7α, 12α-dihydroxy-5β-cholanate by subsequent dehydration and hydrolysis [7,8].

3. Results

The activity of the cell-free extract was initially assessed on 3α,12α-dihydroxy-5β-[24-^{14}C]chol-6-en-24-oic acid, added as sodium salt, so as to obtain a 0.03% final concentration. After 1 h of incubation, under N_2 and CO_2 (9:1) at 37°C, the conversion into deoxycholic acid proved to be 34.5%, confirming our previous results obtained operating with unlabelled cholic acid [5].

An experiment of isotope trapping was then carried out with the cell-free extract in the presence of a small amount of [24-^{14}C]cholic acid and a large excess of unlabelled 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid. The labelling pattern shown in table 1 was obtained after an incubation of 30 min, 60 min and 180 min respectively. The finding of the labelled 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid confirms the precedent results.

The incorporation into deoxycholic acid was only 0.1% after incubation of 3α,12α-dihydroxy-5β-[24-^{14}C]chol-7-en-24-oic acid in the experimental conditions used for the corresponding Δ^6-derivative.

Although the observed incorporation was low, experiments of isotope trapping were effected; however the resulting labelling of the 3α,12α-dihydroxy-5β-chol-7-en-24-oic acid was pratically negligible.

4. Conclusions

The survey of the overall data enables to conclude that 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid is to be considered as the actual intermediate in the conversion of cholic into deoxycholic acid, in the case of *Clo. bifermentans*, therefore confirming our previous assumption [5]. Moreover the velocity of the hydrogen diequatorial trans-addition proved to be much higher than the cumulation velocity of Δ^6-cholenic acid.

The relevant loss of radioactivity, encountered in the isotope trapping test, is also partly attributable to an incorporation into other conversion products that, on the other hand, had also been previously observed [5].

The results obtained, within the sensibility limits of the analytical methods, using 3α,12α-dihydroxy-5β-chol-7-en-24-oic acid as a possible intermediate, can be deemed definitely negative.

It can be therefore stated that, also in vitro, the

Table 1
Isotope trapping experiments – labelled products resulting from the transformation of [24-^{14}C]cholic acid by the *Clostridium bifermentans* cell-free extract

	30 min		60 min		180 min	
Cholic acid	(dpm)	(%)	(dpm)	(%)	(dpm)	(%)
	19 188 801	38.38	12 238 328	24.48	5 301 411	11.48
7-Ketodeoxy cholic acid	12 749 612	25.49	9 102 318	18.24	4 122 019	9.16
Deoxycholic acid	1 971 704	3.94	2 294 766	4.58	8 600 088	18.84
3α,12α-Dihydroxy Δ^6 cholenic acid	43 912	0.09	61 182	0.12	388 600	0.82
Recovered total radioactivity	33 954 029	67.90	23 696 594	47.42	18 412 118	40.30

process of dehydration of the hydroxyl-group in the 7-position of cholic acid, involves a diaxial trans-elimination involving the hydrogen in the 6 position. Therefore, this evidence agrees with the observations in vivo by Samuelsson [1,2].

Acknowledgements

This work was supported by CNR (Consiglio Nazionale delle Ricerche, Italy). We wish to thank Mr F. Bruno for his technical assistance.

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