BIOTRANSFORMATION OF BILE ACIDS BY CLOSTRIDIA

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SUMMARY. The metabolism of bile acids by nuclear dehydrogenating clostridia (NDC) was studied. NDC were able to desaturate the A-ring of 5 β -cholan-3-oxo-24-oic acid, 12 α -hydroxy-5 β -cholan-3-oxo-24-oic acid, 7 α -hydroxy-5 β -cholan-3-oxo-24-oic acid, 6 α -hydroxy-5 β -cholan-3-oxo-24-oic acid, 7 α , 12 α -dihydroxy-5 β -cholan-3-oxo-24-oic acid, 3,12-dioxo-5 β -cholan-24-oic acid but not 3,6-dioxo-5 β -cholan-24-oic acid, 3,7-dioxo-5 β -cholan-24-oic acid and 3,7,12-trioxo-5 β -cholan-24-oic acid. In each case the sole product possessed a 4-ene-3-one structure. Desaturation of bile acids was more efficient than that of androstanes. NDC are, therefore, capable of introducing double bonds into the nucleus of bile acids as well as that of androstanes. The physiological significance of such reactions in relation to large bowel cancer has yet to be elucidated.

INTRODUCTION

Certain clostridia, indigenous to the human intestine are capable of oxidising the A-ring of steroids to give a 4-ene-3-one configuration (Aries *et al.*, 1971; Goddard *et al.*, 1975). Such organisms are termed nuclear dehydrogenating clostridia (NDC). The ability to perform nuclear dehydrogenation (NDH) is present in > 90% of lecithinase-negative clostridia but in only *c.* 3% of lecithinase-positive clostridia (Goddard *et al.*, 1975). The presence in the bowel of clostridia able to perform NDH forms an integral part of the hypothesis put forward by Aries *et al.* (1969) that implicates unsaturated bile acids in colon carcinogenesis. Persons suffering from colon cancer have a carriage rate of NDC 40% higher than control populations (Hill *et al.*, 1971).

Previous reports (Aries *et al.*, 1971; Goddard *et al.*, 1975) have been concerned with the desaturation of androstanes because they are more water-soluble and thus easier to study than bile acids. However, NDC do not oxidise the 3 α -hydroxyl group of bile acids (Macdonald and Hill, 1978) because a C₃ oxo-group is required to induce NDH enzymes. There is, however, abundant evidence that 3-oxo-bile acids are produced *in vivo* (Eneroth *et al.*, 1966) and *in vitro* (Macdonald *et al.*, 1979) by lecithinase-positive clostridia and other genera common in the human intestinal tract.

The present study was undertaken to ascertain whether clostridia indigenous to the human intestine can metabolize bile acids in anaerobic conditions.

MATERIALS AND METHODS

Steroids. 5 β -Androstan-3,17-dione, 17 β -hydroxy-5 β -androstan-3-one, androst-4-ene-3,17-dione, 17 β -hydroxyandrost-4-ene-3-one and androsta-1,4-diene-3,17-dione were obtained from Koch-Light Laboratories Ltd (Colnbrook, Bucks). Cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid), chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid), deoxycholic acid (3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid), hyodeoxycholic acid (3 α ,6 α -dihydroxy-5 β -cholan-24-oic acid) and lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid) were obtained from Steraloids Ltd, Worthing, Sussex.

The 3-oxo-derivatives of bile acids were prepared by selective oxidation of the 3 α -hydroxyl group with silver carbonate on celite (Tserng, 1978). The identity of the products was confirmed by spectroscopy and gas-chromatography-mass spectrometry by comparison with authentic standards (Owen, 1980).

Bacterial strains. The organisms selected for study comprised nine NDH-positive and two NDH-negative strains. The NDH-positive strains were *Clostridium paraputrificum* strains 16, 17, 7, 1172, 10, 1090 and 510; *C. tertium* strain 667 and *C. butyricum* strain 493. The NDH-negative strains were *C. perfringens* strain 11/11 and *C. paraputrificum* strain 1152. The strains were isolated from human faeces by the methods of Drasar and Crowther (1971) and identified by the methods of Holdeman and Moore (1975).

Growth conditions. An exponential-phase cooked-meat culture (500 μ l) was added to 4.0 ml of freshly steamed Todd-Hewitt Broth containing steroid 50 μ g/ml as inducer. The cultures were incubated in an atmosphere of H₂ 90% CO₂ 10% for 24 h at 37°C. To this culture was added 500 μ l of an ethanolic solution containing steroid as substrate and menadione (as artificial electron acceptor) to give a final concentration of steroid 0.01% and menadione 0.35%. The cultures were re-incubated for 18 h and the products were extracted twice with equal volumes of ethyl acetate. The extracts were dried over anhydrous MgSO₄ and solvent was removed *in vacuo* on a Buchi rotary evaporator.

Analysis and identification of bile-acid products. Culture extracts were analysed by thin-layer chromatography (TLC) on Polygram Sil/UV₂₅₄, 0.25-mm plastic backed plates (Camlab, Cambridge) with the developing solvents, methanol-dichloromethane (1:19 v:v), benzene-dioxan-water (100:10:1 v:v:v) and 2,2,4-trimethylpentane-ethyl acetate-acetic acid (45:45:10 v:v:v). Unsaturated bile acids were located under ultraviolet light at 254 nm; the TLC plates were then sprayed with anisaldehyde reagent (Kritchevsky *et al.*, 1963) and heated for 10 min to locate all steroids. TLC made possible the presumptive identification of products by comparing colour characteristics and mobilities with authentic standards.

Bile-acid products were analysed quantitatively as their methyl esters by gas-liquid chromatography (GLC) on a Pye 204 gas chromatograph fitted with a 2 M $\times$ 4 mm, 3% OV-1 100-120 mesh supelcoport glass column at 260°. Before GLC, bile acids and their bacterial products were methylated with diazomethane in ether by the method of De Boer and Backer (1954).

Infra red spectra were obtained on KBr discs with a Unicam SP 1200 spectrophotometer. Ultra-violet spectra were measured in methanolic solutions in silica cells (path length 1 cm) with a SP 1800 recording spectrophotometer.

GC-mass spectra were obtained with a Dupont 21-491 series mass spectrometer equipped with a 21-0948 data system and coupled to a Varian aerograph 2700 gas chromatograph.

RESULTS

The results show that NDC were able to desaturate, at C₄-C₅, a range of bile acids carrying on oxo group at C₃ (table). Of the nine NDH-positive strains tested each was able to desaturate 5 β -cholan-3-oxo-24-oic acid to chol-4-ene-3-one-24-oic acid, 12 α -hydroxy-5 β -cholan-3-oxo-24-oic acid to 12 α -hydroxychol-4-ene-3-one-24-oic acid, 7 α -hydroxy-5 β -cholan-3-oxo-24-oic acid to 7 α -hydroxychol-4-ene-3-one-24-oic acid and 6 α -hydroxy-5 β -cholan-3-oxo-24-oic acid to 6 α -hydroxychol-4-ene-3-one-24-

TABLE
The percentage conversion of 3-oxo-bile acids to 4-en-3-one derivatives by nuclear dehydrogenating clostridia

Substrate	Product	Percentage of strains performing the reaction	Mean percentage conversion
5 β -Cholan-3-oxo-24-oic acid	Chol-4-ene-3-one-24-oic acid	100	53
12 α -Hydroxy-5 β -cholan-3-oxo-24-oic acid (I)	12 α -Hydroxychol-4-ene-3-one-24-oic acid (II)	100	76

7 α -Hydroxy-5 β -cholan-3-oxo-24-oic acid	7 α -Hydroxychol-4-ene-3-one-24-oic acid	100	91
6 α -Hydroxy-5 β -cholan-3-oxo-24-oic acid	6 α -Hydroxychol-4-ene-3-one-24-oic acid	100	84
7 α ,12 α -Dihydroxy-5 β -cholan-3-oxo-24-oic acid (III)	7 α ,12 α -Dihydroxychol-4-ene-3-one-24-oic acid (IV)	67	75

3,12-Dioxo-5 β -cholan-24-oic acid	3,12-Dioxochol-4-ene-24-oic acid	78	49
3,6-Dioxo-5 β -cholan-24-oic acid	None	...	...
3,7-Dioxo-5 β -cholan-24-oic acid	None	...	...
3,7,12-Trioxo-5 β -cholan-24-oic acid	None	...	...
5 β -Androstan-3,17-dione	Androst-4-ene-3,17-dione	100	44
17 β -Hydroxyandrostan-3-one	17 β -Hydroxyandrost-4-ene-3-one	100	47

oic acid. Two other bile-acid substrates were metabolised by NDC. However, only 67% of the NDC were capable of desaturating 7 α -12 α -dihydroxy-5 β -cholan-3-oxo-24-oic acid to 7 α -12 α -dihydroxychol-4-ene-3-one-24-oic acid and 78% of desaturating 3,12-dioxo-5 β -cholan-24-oic acid to 3,12-dioxochol-4-ene-24-oic acid.

The 3-oxo bile acids that also possessed a 6-oxo group (3,6-dioxo-5 β -cholan-24-oic acid) or a 7-oxo group (3,7-dioxo-5 β -cholan-24-oic acid and 3,7,12-trioxo-5 β -cholan-24-oic acid) were not desaturated by NDC.

It is of interest that NDC gave a higher percentage conversion (mean 25%) of 3-oxo bile acids than of androstanes. NDH-negative strains did not desaturate 3-oxo bile acids or androstanes.

DISCUSSION

The results show that NDC, mainly strains of *C. paraputrificum*, oxidise the A-ring of 3-oxo bile acids efficiently to yield products with a 4-ene-3-one structure. Oxidation of 3-oxo bile acids is more efficient than that of androstanes and the presence of single hydroxyl groups at C₆, C₇ and C₁₂ appears to enhance enzyme induction and activity. Oxo-groups at C₆ and C₇ completely inhibit the NDH reaction, presumably because of the proximity of such groups to the enzyme binding site. Experiments to test this conclusion showed that, even after enzyme induction, oxidation was completely inhibited. The presence of a 12-oxo group on a 3-oxo bile acid inhibited oxidation by 22% of the clostridia, whilst the presence of hydroxy groups at C₇ and C₁₂ inhibited the NDH reaction in 33% of the clostridia. These results were consistent in six experiments and indicate that the ability of NDC to perform the NDH reaction depends, to a certain degree, on the number and type of substituent groups carried by a 3-oxo bile acid. In the described conditions, products of the 4-ene-3-one configuration only were detected without evidence for the presence of 1,4-dienone intermediates. Unsaturated C₂₂ and C₁₉ intermediates were not detected indicating that NDC are unable to degrade the side-chain of bile acids. This was confirmed by quantitative recovery of substrate plus product from the fermentations.

This study conclusively shows that, *in vitro*, NDC can efficiently desaturate the A-ring of the 3-oxo derivatives of the major colonic bile acids—lithocholic acid, deoxycholic acid, chenodeoxycholic acid and cholic acid. There is no evidence to suggest that these reactions take place in the large bowel because the unsaturated bile acids described in this report have not been detected in faeces. There may be several reasons for this. The amounts of artificial electron acceptor used in this and other studies (Aries *et al.*, 1971; Goddard *et al.*, 1975) are probably unphysiological and represent a much higher concentration than the available electron source in colonic contents. Moreover, the techniques employed for faecal bile-acid analysis may degrade unsaturated bile acids, and unsaturated intermediates, if produced *in vivo* by NDC, may be transient because of immediate reduction by other colonic bacteria.

Several studies (Fernandez and Hill, 1978; Fernandez *et al.*, 1985) have been conducted in an attempt to evaluate the concentration of faecal electron acceptors which are mainly plant and bacterial quinones. Absolute measurement of quinones has proved technically difficult because the quinones are unstable. It is, therefore, premature to conclude that their colonic concentration is too low to support the NDH reaction *in vivo*.

Recently, several reports have highlighted the advances made in faecal bile-acid analyses (Setchell *et al.*, 1983; Owen *et al.*, 1984). The techniques now employed involve mild extraction steps and fractionation of faecal steroids by diethylaminohydroxypropyl (DEAP-LH-20) sephadex column chromatography and it is known that unsaturated bile acids survive such processing (Owen, unpublished). Unsaturated bile acids have not been detected in faeces probably because their appearance is only transient. It has recently been shown (Owen and Bilton, in press) that, when unsaturated 4-ene-3-one, 1,4-diene-3-one and 4,6-diene-3-one bile acids are co-incubated with mixed faecal bacteria *in vitro*, they become fully reduced within 72 h. Metabolism to the fully reduced products is, however, substantially inhibited in the presence of menadione. Evidence for the transient appearance of unsaturated bile acids in faeces has been supplied by Kallner (1967) who demonstrated that intestinal bacteria are responsible for the inversion of 5β -bile acids to 5α -bile acids (allo-bile acids) via 4-ene-3-one intermediates. It is, therefore, possible that NDC may be an important group of organisms responsible for the small amounts of allo-bile acids detected in faeces.

It has recently been demonstrated that allo-bile acids have co-mutagenic activity (Wilpart *et al.*, 1983) and it is possible that the positive correlation observed between high NDC carriage and colon cancer (Aries *et al.*, 1969; Hill *et al.*, 1971) is due more to allo-bile acid than to unsaturated bile-acid formation. This view is favoured by recent evidence that unsaturated bile acids are not potent mutagens (McKillop *et al.*, 1983); however, their role as co-mutagens has yet to be evaluated.

Studies are in progress to elucidate the effect of carbohydrates, fibre and other intestinal bacteria on the metabolism of 3-oxo-bile acids by *Clostridium* species.

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