Figure 2 shows the reduction of externally added cytochrome c in the presence or absence of xanthomegnin. Addition of NADH produced an increase in the absorbance at 550 nm suggesting that the externally added cytochrome c was reduced by NADH-cytochrome c reductase of mitochondrial outer membranes (curve b). In the presence of xanthomegnin, cytochrome c was reduced at a higher rate than that in the control experiment (curve a). This suggests that the redox response of xanthomegnin increased the reduction rate of cytochrome c.

The reduction of cytochrome c by reduced xanthomegnin has already been demonstrated by us using ascorbate-loaded liposomes[18]. These data suggest that xanthomegnin, reduced by NADH-cytochrome c reductase present in the outer membranes was oxidized by cytochrome c. Cytochrome c, localized on the cytoplasmic-side of the mitochondrial inner membranes, may be reduced by xanthomegnin in a similar fashion as described above. This may give rise to a xanthomegnin-bypass as presented in figure 3.

As demonstrated in our previous communication[19], xanthomegnin was also reduced by mitochondria respiring succinate or L-glutamate as a substrate. But the reduction was initiated when the dissolved oxygen of reaction mixture was completely consumed. The reduction was completely inhibited by antimycin A or cyanide. This evidence indicates that xanthomegnin can form a bypass only in the presence of NADH. Thus xanthomegnin may oxidize cytoplasmic NADH by means of mitochondrial electron transport system.

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The inability of nuclear dehydrogenating clostridia to oxidize bile salt hydroxyl groups[1]

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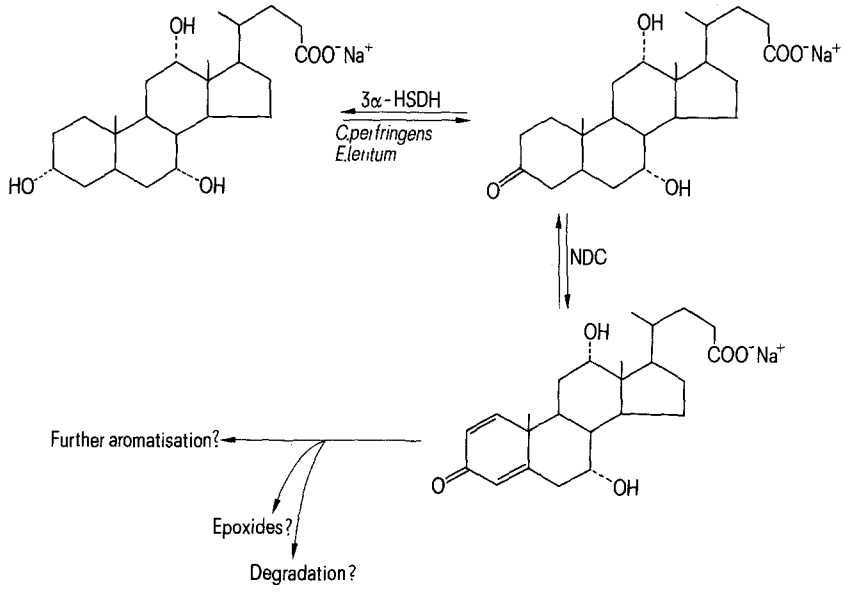
Summary. In a survey of the intracellular bile salt oxidoreductase activity in fecal bacteria, 16 strains of nuclear dehydrogenating clostridia and 2 strains of non-nuclear dehydrogenating *C. paraputrificum* were demonstrated unable to oxidize cholate at any of the 3 OH groups. Since nuclear dehydrogenation at the Δ-1 and Δ-4 position requires a 3-oxo precursor steroid, it appears that these organisms require the presence of a 3α-hydroxysteroid dehydrogenating organism for nuclear dehydrogenation.

In the intestinal flora, the primary bacteria capable of introducing double bonds into the steroid skeleton are the lecethinase negative clostridia: predominantly *C. paraputrificum, C. indolis,* and *C. tertium*[2]. These organisms contain nuclear dehydrogenases (NDH) active at the Δ-1 and Δ-4 positions of the A ring of 3 oxo-bile salts and 3 oxo-steroids[3,4]. High fecal populations of nuclear dehydrogenating clostridia (NDC) and high fecal concentrations of bile salts have been associated with bowel cancer patients and may be thereby implicated in the aetiology of colon cancer. Putative aromatisation of bile salts and steroids by gut bacteria has been a proposed mechanism of carcinogen formation in the human bowel.

The requirement of NDC for 3 oxo-bile salts or 3 oxo-steroids for the introduction of unsaturations into the A ring has prompted a search in these organisms for intracellular 3α-hydroxysteroid dehydrogenase (HSDH) activity which could then provide substrates for the NDH.

Methods and materials. Nuclear dehydrogenating and non-nuclear dehydrogenating clostridia were isolated from clinical sources[5] and prescreened for the presence of NDH as demonstrated by the formation of Δ1 and Δ4 unsaturations into 5β-androstan-3, 17 dione and 3 oxo-5β-cholanoate as described before[3]. These organisms were maintained in cooked meat medium at 4 °C and grown in 10 ml Brain Heart Infusion medium containing 0.1% sodium thioglycolate and 1.0×10^{-3} M sodium cholate for 48 h.

Cultures were centrifuged at 6000 × g for 20 min at 4 °C in an International B-20 centrifuge. The pellet was resuspended in 1.5 ml of 0.1 M sodium phosphate containing 10^{-3} M



Degradation of primary bile salt by 3α-HSDH containing organisms and NDC, a proposed in vivo pathway.

EDTA and the bacteria were ruptured in a French Pressure Cell. The cell-free preparation was centrifuged at 6000 × g for 20 min at 4 °C and the supernatant fluid was decanted from the pellet.

The cell-free preparation was assayed for 3α, 7α, and 12α-HSDH activity using as substrates: chenodeoxycholate, deoxycholate, taurolithocholate, and 3α-hydroxy-5α-androstan-17-one and cofactors: NAD and NADP (8 substrate-cofactors combinations). The formation of reduced nucleotide was followed at 25 °C at 340 mm using a Beckman spectrophotometer with a 25 cm Beckman recorder. Each assay cuvette contains 17×10^{-3} M NAD(P)+, 0.17 M glycine/NaOH buffer (pH 9.5), 10^{-3} M dihydroxy-substrate or 10^{-3} M monohydroxy-substrate and 100 µl of freshly prepared cell-free preparation. The spent bacterial medium containing undegraded cholate and putative metabolites was adjusted to pH 3.5 and extracted with an equal volume of diethyl ether. The extract was blown to dryness (N_2), reconstituted in 1/10 volume methanol/water, 4/1 (v/v) and plated (10 µl) onto thin layer silica plates. Samples were chromatographed in either chloroform/methanol/acetic acid 40/4/2 (v/v/v) or in benzene/dioxane/acetic acid 70/20/2 (v/v/v). Plates were sprayed with either anisaldehyde[6] or p-hydroxybenzaldehyde[7], heated with heat gun and inspected in day light and under the UV lamp.

Results and discussion. None of the above described clostridia contained any demonstrable 3α, 7α, or 12α-hydroxysteroid dehydrogenase activities in the presence of cell-free preparations and NAD or NADP. None of the above organisms significantly metabolized sodium cholate on inspection of TLC plates although trace amounts of methyl cholate were evident in many of the organisms. A notable absence of 7α, 12α-dihydroxy-3 oxo-5β-cholanoate was evident in all strains. No products visible under the UV lamp were detectable. This was expected in the absence of a suitable hydrogen acceptor such as menadione.

The presence of NDH yet apparent absence of HSDH in this group of organisms suggest a dependence of NDC on the presence of 3α-HSDH containing organisms in the gut (e.g. *C.perfringens*[8] or *E.lentum*[9]). The presence of NAD-dependent 3α-HSDH active against sodium taurolithocholate or 3α-hydroxy-5α-androstan-17-one appears present in the vast majority of mixed fecal cultures in one study[10]. Moreover, *C.perfringens* (most strains of which contain 3α-HSDH) is a commonly carried organism[11].

The absence of HSDH in 16 NDC does not, however, preclude the possibility that other strains of NDC do exist which also contain 3α-HSDH. It does, however, allow the authors to propose a bile salt degradative pathway involving 2 or more organisms (figure). The fate of unsaturated bile salts in the human intestine remains unknown. Aromatized bile salts have not been, to the authors' knowledge, found in either stool or bile of the human, although aromatized products of cholesterol metabolism by the gut flora have been demonstrated in guinea-pig urine[12]. The reversion to saturated primary bile salts in a steady-state fashion is more likely and is in keeping with the instability of 2,2',4,4' tritiated bile salts in the presence of human colonic flora[13]. The possibility of potentially carcinogenic epoxide formation[14] in the human gut, although yet undemonstrated, remains an alternative which could explain the apparent association of NDC with bowel cancer[5].

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