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Degradation of bile salts by human intestinal bacteria

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It is now well established that some component of the intestinal flora is capable of degrading bile salts, since the rate of turnover of ^{14}C-cholic acid in germ-free rats has been shown to be only one fifth of that in normal rats (Gustafsson, Bergström, Lindstedt, and Norman, 1957). Further, caecal fluid and faecal suspensions degrade cholic acid, producing a variety of products, including deoxycholic acid and a number of keto acids (Norman and Bergman, 1960; Hamilton, 1963; Norman and Shorb, 1962; Portman, Shah, Antonis, and Jorgensen, 1962; Danielsson, Eneroth, Hellström, and Sjövall, 1962; Norman and Widström, 1964).

The bile acids as secreted into the intestine are conjugated with taurine or glycine, but those isolated from the faeces are largely unconjugated (Danielsson, Eneroth, Hellström, Lindstedt, and Sjövall, 1963; Norman and Palmer, 1964). Although the deconjugation has generally been attributed to the bacteria of the intestine, attempts to isolate in pure culture the organisms able to perform this hydrolytic reaction (or any other step in the degradation of bile) have been largely unsuccessful. Clostridia and enterococci have been shown to deconjugate bile (Norman and Grubb, 1955) and *Escherichia coli* and *C. perfringens* can produce a keto acid from cholate (Norman and Bergman, 1960) but these organisms are minor components of the intestinal flora (Drasar and Shiner, 1967, in preparation), and it seems unlikely that they could account for all of the deconjugation and degradation that occurs.

In a preliminary communication (Drasar, Hill, and Shiner (1966) reported that *Bacteroides* spp. were able to deconjugate bile salts (Fig. 1a). In this paper we report an extension of that study. We have now tested a much greater number of strains of bacteria and a wider range of species for their ability to deconjugate bile salts (the first step in bile degradation) and also for their ability to carry out a further step in the degradation, the removal of the 7-hydroxyl group from free cholic acid (Fig. 1b).

MATERIALS AND METHODS

ORGANISMS Pure cultures of intestinal bacteria were isolated and identified in the course of an investigation of the human intestinal flora, in which specimens of saliva, jejunal juice, and faeces were examined with the use of methods specially devised for the isolation of strictly anaerobic bacteria (Drasar, 1967). In addition to the intestinal bacteria, a collection of yeasts from the intestine and cultures of *Pseudomonas aeruginosa* and *Staph. aureus* from various non-intestinal sources were also examined. Nineteen strains of *Strep. faecalis* of known serotype were obtained from the Cross Infection Reference Laboratory, Colindale.

GROWTH CONDITIONS AND MEDIA All organisms except the strictly anaerobic bacteria were grown in glucose broth

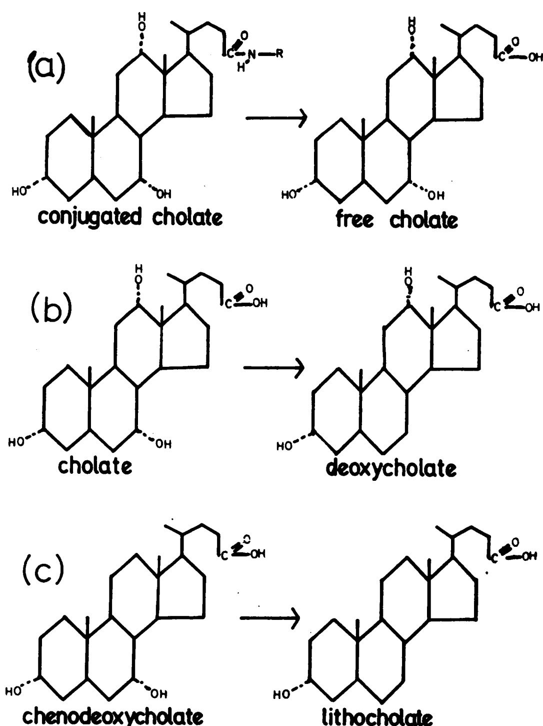

FIG. 1 *Diagrammatic representation of the reaction carried out by taurocholate amidase (reaction a) and cholate-7-dehydroxylase (reaction b) which may also act on chenodeoxycholate (reaction c).*

(Southern Group Laboratory) overnight at 37°C. The anaerobic bacteria were grown in a medium containing 1% tryptone, 1% yeast extract, and 1% soya peptone with added cysteine as described elsewhere (Drasar, 1967).

DETECTION OF BILE DEGRADATION Broth culture, 50 ml, was centrifuged and the cell deposit washed in physiological saline and resuspended in 1-2 ml saline. Of this suspension, 0·5 ml and 0·5 ml of the culture supernatant was each incubated at 37°C overnight with an equal volume of 1% taurocholate (Maybridge Chemical Co., Tintagel). The presence of unconjugated cholate was then detected by thin-layer chromatography (Fig. 2) as described previously (Drasar *et al.*, 1966). Microbial degradation of cholate and chenodeoxycholate was studied in the same way, suitable markers being used on each plate.

ENZYME LOCATION Bacterial cells were disrupted by Mickle disintegration and the wall/membrane fraction isolated by centrifugation. The presence of activity in the supernatant fluid after removal of the cell debris was considered to demonstrate intracellular enzyme. The wall/membrane fraction was washed to remove cytoplasmic material and then tested for activity as above.

*p*H OPTIMUM AND THE EFFECT OF VARIOUS AGENTS The *p*H optimum of the enzyme was assayed quantitatively by formyl titration of the released amino acid. Of the enzyme sample (extracted soluble enzyme, cell suspension, or culture supernatant), 40 ml was adjusted to the appropriate *p*H, then 0·4 g sodium glycocholate was dissolved in the sample. Of the reaction mixture, 10 ml was immediately pipetted into 25 ml of 20% formaldehyde solution; the remainder was incubated at 37°C and samples were taken periodically and treated with formaldehyde in the same way. The samples were titrated to neutrality with 0·01 N NaOH using a direct-reading *p*H meter.

Enzyme inhibition was tested by mixing equal volumes of enzyme sample (cell suspension or culture supernatant) with the test solution. After a short time an equal volume of 1% taurocholate was added and the mixture incubated at 37°C overnight as before.

RESULTS

ABILITY OF VARIOUS ORGANISMS TO DECONJUGATE BILE In our preliminary communication we reported that a high proportion of the bacteroides strains tested were able to deconjugate bile salts, whilst this property was possessed by none of the other organisms tested. A survey of a much larger collection of organisms has now revealed that the ability to deconjugate bile is widespread amongst the strictly anaerobic intestinal organisms, namely, *Bacteroides* spp., *Veillonella* spp., *Clostridium* spp. and *Bifidobacteria* (anaerobic lactobacilli) and is also found in many strains of *Str. faecalis* and some of *Staph. aureus* (Table I). The enzyme responsible

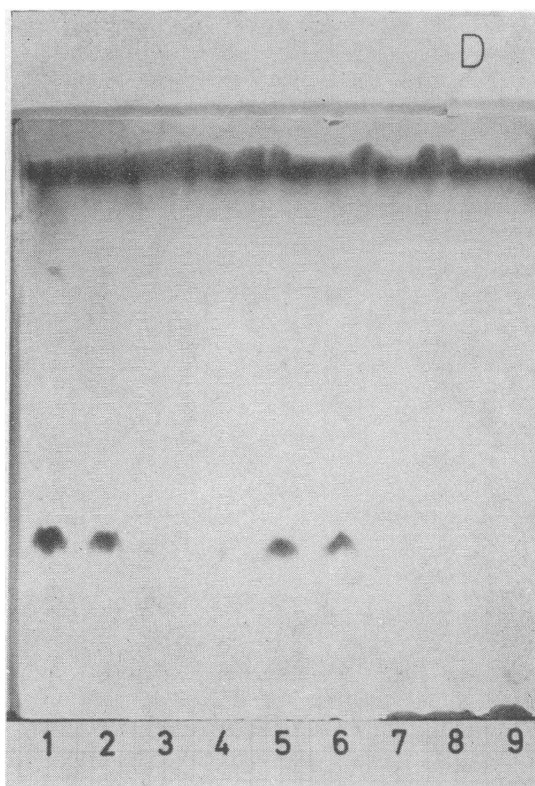

FIG. 2. *TLC plate of various mixtures after development with chloroform: acetic acid: acetone (7:1:2 v/v). Samples 3, 4, 7, 8, and 9 contain undegraded taurocholate. In samples 1, 2, 5, and 6 the taurocholate has been deconjugated yielding cholic acid, and, in samples 1 and 6, the cholate has been 7-dehydroxylated to give deoxycholate.*

(taurocholate amidase) was absent from all the strains tested of *E. coli*, *Ps. aeruginosa*, non-faecal streptococci, aerobic lactobacilli, and the yeasts.

There is a relation between the proportion of strains of bacteroides, veillonella, and bifidobacteria able to split bile and their source in the gastrointestinal tract (Fig. 3). Thus only 5% of oral bacteroides strains split bile conjugates compared with 75% of faecal strains. A number of oral strains of bacteroides, veillonella, and bifidobacteria were subcultured repeatedly on growth medium containing 10% Difco ox bile. Many oral strains of bacteroides and bifidobacteria are sensitive to bile and only one of the bifidobacteria was able to survive this treatment. After a number of subcultures many of the strains were able to deconjugate bile (5/5 bacteroides, 1/1 bifidobacteria and 5/12 veillonella), indicating that exposure to bile of bile-resistant strains of these species favours production of the

TABLE I

DISTRIBUTION OF TAUROCHOLATE AMIDASE AND CHOLATE-7-DEHYDROXYLASE AMONGST VARIOUS BACTERIAL GENERA

Species	*No. of Strains Tested*	*Percentage Showing Taurocholate Amidase*	*Percentage Possessing Cholic Acid-7-Dehydroxylase*
Aerobic bacteria			
Pseudomonas sp.	58	0	0
Facultative anaerobic bacteria			
Esch. coli	75	0	0
Proteus mirabilis	1	0	0
Staph. aureus	47	21	0
Strep. pyogenes	2	0	0
Strep. faecalis	109	50	2
Strep. salivarius	30	0	0
Strep. viridans	12	0	0
Anaerobic bacteria			
Bacteroides sp.	237	48	8
Clostridium sp.	16	94	12
Bifidobacterium sp.	89	38	0
Veillonella sp.	47	30	8
Microaerophilic bacteria			
Lactobacillus sp.	12	0	0
Fungi			
Candida albicans	28	0	0
Candida sp.	6	0	0
Saccharomyces sp.	4	0	0
Torulopsis sp.	7	0	0

bile-deconjugating enzyme. However, none of the faecal strains of *E. coli* and none of the yeasts (all isolated from faeces and therefore long exposed to bile) possessed the enzyme and all attempts to produce deconjugating strains of *E. coli* and *Ps. aeruginosa* by repeated subculture in the presence of bile failed.

Bacteria able to deconjugate taurocholate were also active on glycocholate, taurodeoxycholate, and glycodeoxycholate. The enzyme was not restricted to any phage group of *Staph. aureus* or any serotype of *Strep. faecalis*.

ENZYME LOCATION The location of the enzyme was determined by testing washed cell deposits and culture supernatants for the ability to deconjugate bile. With the bifidobacteria the activity was usually located principally in the culture supernate, *i.e.* the enzyme was extracellular, but with the other species tested the enzyme was mainly cell bound (Table II).

The extracellular enzyme of *Bifidobacterium* sp. strain H[4] could be precipitated with 30% saturated ammonium sulphate. Since the culture medium of this organism is complex, no further purification of the enzyme was attempted.

The location within the cell of the enzyme in *Str. faecalis* strain E41 and bacteroides strain NCTC 9343 was further investigated. The bacteroides enzyme was very labile and all activity was lost overnight from a cell suspension at 0°C unless stored anaerobically or in tightly capped bottles in

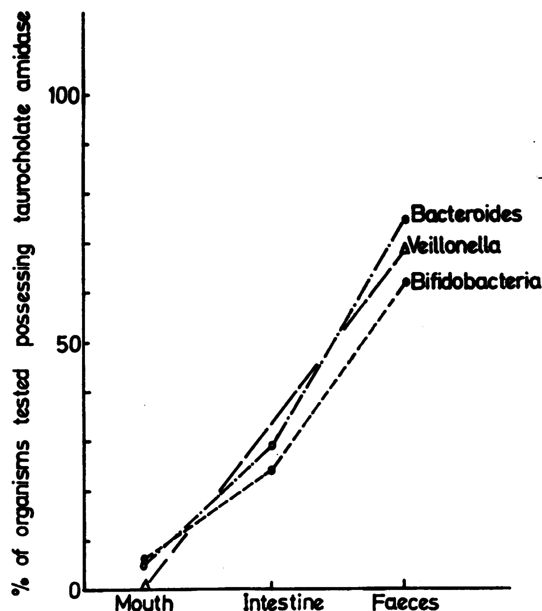

FIG. 3. *Variation in the proportion of strains possessing taurocholate amidase with source.*

the presence of a reducing agent (*e.g.* 1% cysteine). When the cells were disrupted, all activity was lost in less than 20 minutes at 0°C regardless of the precautions taken, so that it way not possible to wash the wall/membrane fraction. Nevertheless, there was sufficient difference in activity between the intracellular fraction and the wall/membrane to indicate a cytoplasmic location for this enzyme. The enzyme of *Str. faecalis* was much more stable. Most of the activity was found in the wall/membrane fraction, but could be eluted readily by repeated washing with 0·9% sodium chloride solution. Soluble enzyme obtained in this was was further purified by ammonium sulphate precipitation followed by chromatography on Sephadex G-200. Three elution peaks were obtained, an initial peak containing the high molecular weight material not

TABLE II

LOCATION OF TAUROCHOLATE AMIDASE IN VARIOUS BACTERIAL GENERA

Species	*No. of Strains Tested*	*Percentage with Cell-bound Enzyme*
Str. faecalis	49	100
Staph. aureus	10	100
Bifidobacterium	29	34
Bacteroides spp.	68	85
Veillonella spp.	14	86
Clostridium spp.	15	87

taken up by the Sephadex particles, a second broad peak (which contained all of the enzyme activity), and a third small peak. Enzyme purified in this way is unstable, but is at present under investigation with a view to further purification and characterization.

*p*H OPTIMUM OF TAUROCHOLATE AMIDASE The *p*H optima of the amidase enzymes derived from *Bifidobacterium* Hl, *Bacteroides* NCTC 9343, and *Str. faecalis* E41 were determined as described previously. Enzyme from 500 ml of culture supernatant of Hl was made 30% saturated with ammonium sulphate and stored at 4°C overnight to ensure complete precipitation. The precipitate was redissolved in 250 ml distilled water and used as the enzyme sample. The *p*H optimum (Fig. 4) was between 6 and 6·5. After three hours' incubation of the reaction mixtures, the *p*H optimum was also estimated qualitatively by comparing the relative intensities of the cholate spots on TLC plates. This qualitative *p*H optimum was also between 6 and 6·5.

Crude soluble enzyme from E41, obtained by extraction of wall/membrane deposits with physiological saline, was used to determine the *p*H optimum of this strain, which was found to be 6·5-7·0 (Fig. 4).

In the case of the bacteroides strain, whole cells had to be used as the enzyme source, since extracted enzyme was so unstable. Resting cells are slowly extracted by bile salts (Hill, 1967), but the extracted protein interfered with the formol titration and in consequence the results obtained for this strain are much less reliable; nevertheless the *p*H optimum obtained by the qualitative method (6·5-7·0) agreed with that obtained by the quantitative method.

EFFECT OF VARIOUS AGENTS ON ENZYME ACTIVITY The effect of various agents on the activity of taurocholate amidase was investigated for a number of representative strains. Enzyme from all strains tested was inhibited by periodate (Table III) and by cupric ions. Whereas enzyme from *Str. faecalis* was destroyed by merthiolate and unaffected by formaldehyde, the reverse was true for enzyme derived from

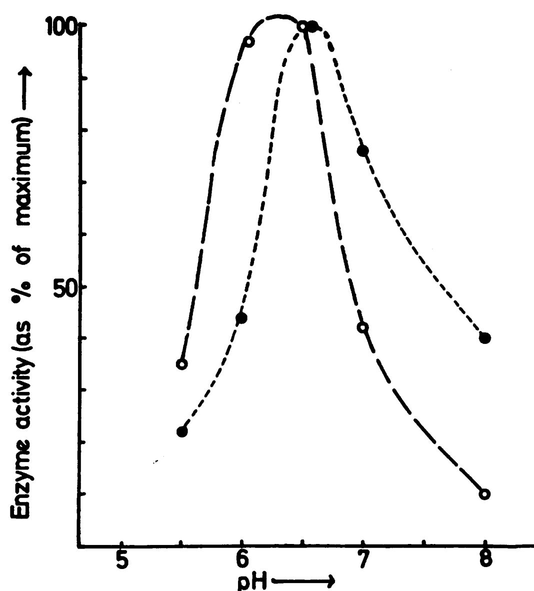

FIG. 4. *Variation in activity of taurocholate amidase with pH.*

○—— ——○ Strain H 1
● - - - - ● Strain E 41

the strictly anaerobic bacteroides and bifidobacterium strains. The enzyme from bacteroides is much more oxygen-sensitive than that from the other species, but is also much more heat resistant; it is also trypsin resistant.

DECOMPOSITION OF CHOLATE BY INTESTINAL ORGANISM In addition to the deconjugating enzyme responsible for reaction A in Fig. 1, we have also investigated the enzyme responsible for reaction B in Fig. 1, cholate-7-dehydroxylase, which removes the 7-OH group from cholic acid yielding deoxycholate. This enzyme, like the deconjugating enzyme, is not restricted to any group of bacteria (Table I) but was not detected in any organism which did not also

TABLE III

THE EFFECT OF VARIOUS AGENTS ON THE ACTIVITY OF TAUROCHOLATE AMIDASE

Species	*Enzyme Activity in the Presence of the Test Reagent*							
	Control	*HCHO*	*Merthiolate*	IO_4	Mg^{++}	Cu^{++}	F^-	N_3^-
Str. faecalis								
E41	+++	+++	+	–	+++	–	+++	+++
E48	+++	+++	–	–	+++	–	+++	+++
Bacteroides								
9343	+++	–	+++	–	+++	–	+++	+++
B22	+++	–	+++	–	NT	NT	NT	–
Bifidobacterium								
H1	+++	–	+++	–	+++	–	–	+++
H2	+++	–	+++	–	+++	–	+++	+++

possess the deconjugating enzyme. No bifidobacteria have been found which possess this dehydroxylase enzyme. Only merthiolate, of the agents tested, had any marked inhibitory effect on the enzyme, which has a *p*H optimum determined qualitatively, of *p*H 6 to 7.

Strains of bacteroides and veillonella isolated from the mouth and unable to attack cholate were repeatedly subcultured on a medium containing bile. Populations were produced that were able to remove the 7-hydroxyl group and to produce other unidentified products from cholate. The 7-dehydroxylase enzyme was present in three of five bacteroides treated in this way but in none of the veillonella strains.

DISCUSSION

Our studies have shown that the ability to deconjugate bile salts is common in strains of *Bacteroides* and *Bifidobacteria*. In some other studies, which are summarized schematically in Table IV, we have shown that these bacteria are by far the most numerous of the intestinal bacteria (Drasar and Shiner, in preparation). Previously the only bacteria known to possess this enzyme were *Str. faecalis* and *Cl. perfringens* (Norman and Grubb, 1955), both of which are minor components of the flora. That some strains of the dominant organisms also possess this ability explains the scarcity of conjugated bile salts in faeces.

There is a relationship between the proportion of strains of *Bacteroides*, *Veillonella*, and *Bifidobacterium* able to split bile and their source in the gastro-intestinal tract. This could be due to selection or to adaptation since repeated subculture of oral organisms in the presence of bile gives rise to populations able to deconjugate bile. This ability may give some selective advantage to the organisms and may be significant in the ecology of the strictly anaerobic bacteria and of *Str. faecalis*.

TABLE IV

THE NUMBERS OF BACTERIA PER GRAM OF SPECIMEN

Species	*Specimen*		
	Saliva	*Jejunal Juice after a Meal*	*Faeces*
Aerobic bacteria			
Enterobacteria	10	0	10^6
Streptococcus faecalis	0	0	10^3
Streptococcus salivarius	10^6	10^3	10^2
Lactobacilli	10^3	10^2	10^4
Anaerobic bacteria			
Bacteroides	10^4	10^3	10^{11}
Bifidobacteria	10^2	10^3	10^{11}
Clostridia	0	0	10^2
Veillonella	10^5	0	10^3

The presence of the bile deconjugating enzyme in *Staph. aureus* is more difficult to explain, but perhaps it has a wider role in steroid metabolism than the deconjugation of bile.

The physiological significance of the bile salt deconjugation reaction and of the 7-dehydroxylation is difficult to estimate. In some disorders, *e.g.* the blind loop syndrome, where the upper small intestine is heavily colonized by bacteria, there is an obvious possibility that the enzyme has clinical significance. This problem is under study and will be discussed elsewhere.

The liver cells synthesize only cholate and chenodeoxycholate; the presence of deoxycholate conjugates in bile has been explained by postulating caecal absorption of the deoxycholate formed in the large intestine. We have shown that, after exposure to bile, many oral strains of bacteroides and veillonella are capable of removing the 7-hydroxyl groups from cholate yielding deoxycholate. Small numbers of bacteria may pass through the intestine during a meal so that thus ingested bacteria could break down cholate to deoxycholate in the small intestine which could then be absorbed in the terminal ileum and recirculated.

Nair, Gordon, and Reback (1967) have described a deconjugating enzyme obtained from *Cl. perfringens*. This enzyme has a *p*H optimum of 5·6 to 5·8 and is inhibited by agents blocking sulphydryl groups and by cupric ions. Norman and Widström (1964) have also found a deconjugating enzyme in cell-free rat caecal fluid, with a *p*H optimum of 6 to 7. Our results would indicate that the enzyme of Norman and Widström was mainly derived from *Bifidobacterium* spp., since it was extracellular, and the *p*H optima were in agreement. None of our enzyme samples had a *p*H optimum as low as that of the *Cl. perfringens* strain of Nair *et al.* Our *Str. faecalis* enzyme was like the *Cl. perfringens* enzyme with respect to inhibition by sulphydryl blocking substances (a property not shared by the enzyme from bacteroides and bifidobacterium) and all of our amidase enzymes were inhibited by cupric ions. The variation in the properties of amidase enzymes from different bacterial sources indicates that much fruitful comparative work in this field remains to be done.

SUMMARY

The ability to deconjugate bile salts is a property widely distributed amongst intestinal bacteria. Some strains originally unable to deconjugate developed the ability after repeated subculture on medium containing bile.

The *p*H optima of enzymes derived from *Bactero-*

ides, *Strep. faecalis* and *Bifidobacterium* were between 6 and 7, but the three enzymes showed differing responses to various inhibitors.

A cholate 7 dehydroxylase has been detected in a variety of intestinal organisms. This enzyme also has a *p*H optimum between 6 and 7. Only merthiolate, of the agents tested, had an inhibitory effect on the enzyme.

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