Gastroenterologia Japonica
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Vol. 11, No. 3
Printed in Japan

—Original Article—

DECONJUGATION OF BILE ACIDS BY HUMAN INTESTINAL BACTERIA

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Summary

The purpose of this report is to present the deconjugation of bile acids by numbers of strains of bacteria in the small intestine and feces. The small intestinal juice was aseptically aspirated by a double lumen tube with a rubber cover on the tip devised by us ("Fukushima Type 1"). Bile acids were analyzed with thin layer chromatography. The results: 1) Among aerobic bacteria, species of which all of the strains split conjugated bile acids was enterococcus, and most of the strains split were Staphylococcus (S.) epidermidis and Lactobacillus (L.) bifidus. Species of which none of the strains split were Escherichia (E.) coli, E. communior, E. freundii, L. plantarum, L. acidophilus, L. buchneri, L. cellobiosus, L. bulgaricus, S. aureus, Aerobacter aerogenes, Pseudomonas aeruginosa, candida, proteus, serratia, and almost none of the species split was Intermediate coliform bacilli. 2) Among anaerobic bacteria, species of which all of the strains split were Bacteroides (B.) vulgatus, B. thetaiotaomicron, B. uniformis, Corynebacterium (C.) granulosum, C. avidum, Peptostreptococcus (Peptostrept.) putridus, Eubacterium (Eubact.) lentum, Peptococcus (Pept.) grigoroffii, Pept. anaerobius, Veillonella (V.) orbiculus, and most of the strains split were Coryne. diphtheroides, Eubact. parvum, Peptostrept. intermedius. Species of which none of the strains split were Coryne. parvum, Peptostrept. micros, V. alcalescens, V. parvula, Catenabacterium (Catena.) catenaforme, and Catena. filamentosum. 3) All or none, or almost all or none, of the strains of each species tested split conjugated bile acids, and it seems probable that the presence or absence of this ability would be a proper character of each species.

Key Words: *human intestinal bacteria, bile acids metabolism, deconjugation of bile acids.*

Introduction

The blind loop syndrome and Crohn's disease[1,2] are often associated with steatorrhea. According to a current hypothesis[3], the steatorrhea is caused by excessive proliferation of bacteria in the lumen of the small bowel.

Conjugated bile acids synthesized from cholesterol at the liver are secreted into the duodenal lumen. In the above mentioned diseases, they were then converted into free bile acids by bacteria in the lumen of the upper small bowel and consequently the concentration of conjugated bile acids is reduced to the levels below the critical micellar concentration. Since anaerobes are so active in deconjugating bile acids, it is presumed that the anaerobes in the small bowel may play an important role in the pathogenesis of the steatorrhea[4,5,6].

On the other hand, in patients with hepatic diseases, especially in hepatic cirrhosis, bacterial proliferation was found by us in the lumen of their small bowels. It is pronounced

by the overgrowth of aerobic gram negative bacilli and anaerobes[7,8]. But, the causal relation between the hepatic disease and the enterohepatic circulation of bile acids under the influence of bacterial overgrowth had not been elucidated.

The several literatures on deconjugation of bile acids by various bacteria have been reported since the evidence of bacterial splitting was first presented by Norman and Grubb (1955)[9] who isolated bacteria from rat faeces capable of hydrolyzing conjugated bile acids. But many reports are rather fragmental and we can not get a general view of bacterial deconjugation.

The purpose of this report is to present the deconjugation of bile acids by numbers of strains of bacteria found in the small intestine.

Material and Methods

Materials: The majority of strains tested were isolated from the small intestinal juice obtained from patients with hepatic diseases admitted to the Yokohama City University hospital. Some of the bacteria were isolated from the feces and intestinal juices of patients without hepatic diseases. Anaerobes and aerobic gram-negative bacilli except lactobacillus and enterococcus were identified according to Bergey's Manual of determinative bacteriology in 1957[10] and Schaub's diagnostic bacteriology[11] respectively. Enterococcus was identified by SF medium "Eiken" and lactobacillus was identified according to Modern Media[12] and Bergey's Manual of determinative bacteriology in 1957[10]. The small intestinal juice was aseptically aspirated by a double lumen tube with a rubber cover on the tip devised by us ("Fukushima Type 1")[8]. This tube was the best suited for aspirating the fluid aseptically from the small intestine. The rubber cover was blasted off by instillation with the saline just before aspiration at the desired site in the small intestine. The kinds of bacteria tested were strains of Escherichia (Esch.) coli, Esch. communior, Esch. freundii, lactobacillus (Lact.), Staphylococcus (Staph.) epidermidis, Staph. aureus, Intermediate coliform bacilli, Aerobacter aerogenes, Pseudomonas aeruginosa, candida, enterococcus, proteus, serratia, bacteroides (Bact.), corynebacterium (Coryne.), peptostreptococcus (Peptostrept.), eubacterium (Eubact.), peptococcus (Pept.), veillonella (V.), catenabacterium (Catena.).

Methods: A loopful of organisms from a plate culture was inoculated into 10 ml of broth containing 0.5 ml of sterilized ox gall (**Fig. 1**). All organisms except strictly anaerobic bacteria were grown in glucose broth containing 0.5 ml of sterilized ox gall. The anaerobic bacteria were grown in Thioglycollate liquid medium containing 0.5 ml of sterilized ox gall. Cultures were incubated at 37°C for 48 hours. The broth was acidified to pH 1 with hydrochloric acid and extracted three times with double volume of butanol. The butanol phases were washed free of hydrochloric acid and evaporated to dryness. The residues were dissolved in 2 ml of methanol

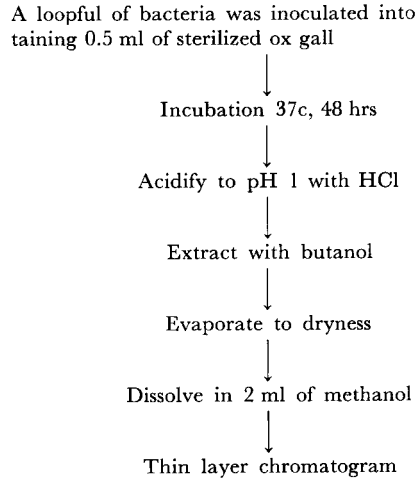

Fig. 1. Preparation.

Table 1. Rf's of Free and Conjugated Bile Acids

	Rf Values	
	Solvent I	Solvent II
Free bile acids		
Cholic	0.16	0.75
Hyodeoxycholic	0.39	0.80
Chenodeoxycholic	0.53	0.85
Deoxycholic	0.59	0.88
Lithocholic	0.82	0.91
Conjugated bile acids		
Taurocholic	0	0.07
Taurochenodeoxycholic	0	0.16
Taurodeoxycholic	0	0.16
Glycocholic	0	0.48
Glycochenodeoxycholic	0.08	0.68
Glycodeoxycholic	0.08	0.70

Composition of Solvent I	
acetic acid	5
carbon tetrachloride	20
di-isopropyl ether	30
iso-amyl acetate	40
n-propanol	10
benzene	10
Composition of Solvent II	
propionic acid	15
iso-amyl acetate	20
water	5
n-propanol	10

Hofmann, A.F.: J. Lip. Res., 3, 127, 1962. (14)

and the aliquots (0.004 ml) were taken for thin layer chromatographic analysis.

Commercial cholic acid and deoxycholic acid were used as reference substances. Ox gall was used instead of conjugated bile acids. Bile acid of ox gall is composed of six conjugated bile acids, namely, glycocholic acid (GC), glycochenodeoxycholic acid (GCD), glycodeoxycholic acid (GD), taurocholic acid (TC), taurochenodeoxycholic acid (TCD), taurodeoxycholic acid (TD). And ox gall contains almost no free bile acid[13]. Bile acids were analyzed with thin layer chromatography. Thin layer chromatographic equipment made by Yamato Chemistry co. was used. A slurry of 30 g of Kiesel-gel G in 58 ml of distilled water was spread 0.25 mm thick on glass plates measuring 20 by 20 cm. The chromatoplates were activated in an oven at 110–120°C for 1–3 hr before use. The samples to be analyzed were dissolved in methanol (**Fig. 1**) and applied to the film through a sharpened micropipette. The glass plates were developed with two kinds of solvent, Solvent I, consisting of acetic acid, carbon tetrachloride, di-isopropyl ether, iso-amyl acetate, n-propanol and benzen in proportion of 5: 20: 30: 40: 10: 10, for 1.5 hours and Solvent II, consisting of propionic acid, iso-amyl acetate, water and n-propanol in proportion of 15: 20: 5: 10, for 3.5 hours (**Table 1**). All runs were performed by the ascending technique at room temperature (18–20°C). When the solvent front was 17–18 cm (Solvent II) or 15–16 cm (Solvent I) from the starting line, the plates were removed and dried in an oven at 150°C. The plates were sprayed with 10% solution of phosphomolybdic acid in alcohol and heated for 5 min. in an oven at 120°C until the characteristic blue spots appeared[14,15,16,17,18,19].

Results

The representative chromatograms after use of Solvent II and Solvent I are shown in **Fig. 2** and **Fig. 3** respectively. The average Rf values of bile acids in these two solvent systems reported by Hoffmann[14] are listed in **Table 1**. The figures written at the bottom of these plates (**Fig. 2** and **Fig. 3**) represent the following substances: 1, commercial cholic acid (reference substance); 2, commercial deoxycholic acid (reference substance); 3 and 4, ox gall incubated with Bact. vulgatus from small intestinal juice; 5, ox gall with Bact. vulgatus isolated from faeces; 6, ox gall with Bact. thetaiotaomicron isolated from faeces; 7, ox gall with Bact. thetaiotaomicron

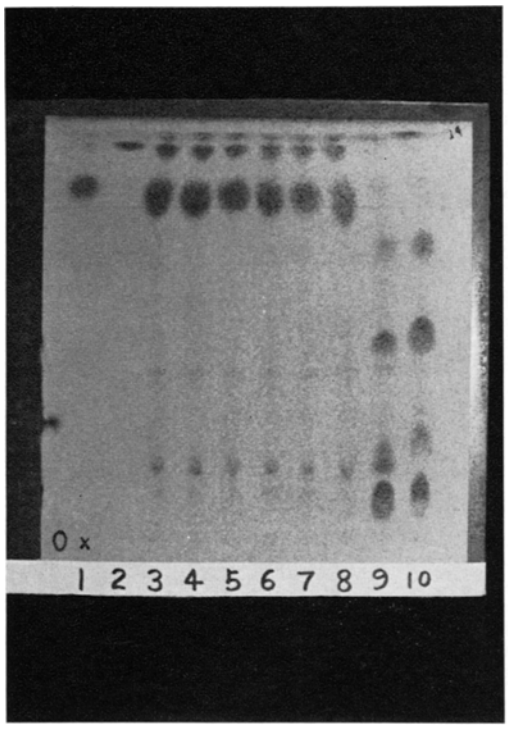

Fig. 2. Thin layer chromatogram developed with Solvent II.
The 4 blue spots of conjugated bilie acids which are detected in the position 9 and 10 are not detected in the position 3–8, and the 2 blue spots of free bile acids are detected in the upper end of the position 3–8.

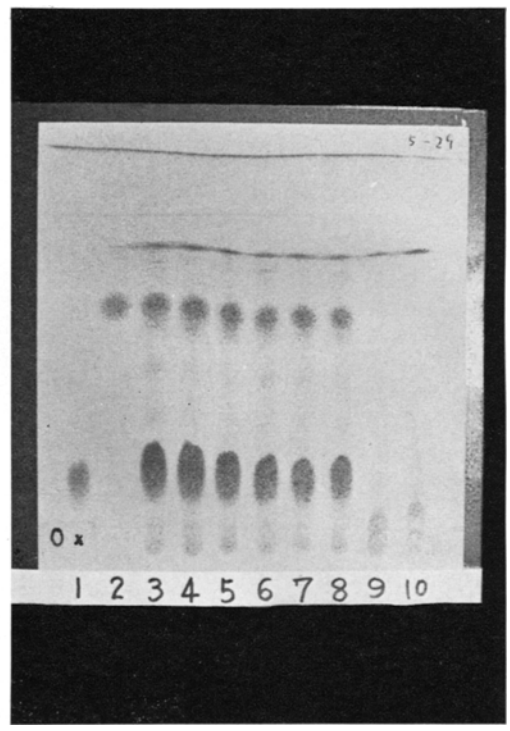

Fig. 3. Thin layer chromatogram developed with Solvent I.
The spots of free bile acids are clearly showed in the position 3–8, but none of the spots are showed in the position 9 and 10.

isolated from small intestinal juice; 8, ox gall with Bact.uniformis isolated from small intestinal juice; 9, broth with ox gall (control) and 10, ox gall only.

The spots in **Fig. 2** are identified as follows. When more than two spots are present in a sample, they are enumerated in their respective order from the starting line. The weak spots appearing in some mixtures are due to impurities in some of the samples and other metabolites. O = origin. Position 1: cholic acid (3α,7α, 12α-Trihydroxycholic acid), 2: deoxycholic acid (3α,12α-Dihydroxycholic acid), 3–8: cholic acid, deoxycholic acid, 9 and 10: taurocholic acid, taurochenodeoxycholic acid and taurodeoxycholic acid, glycocholic acid, glycochenodeoxycholic acid and glycodeoxycholic acid. The spots of conjugated bile acids which are detected in the position 9 and 10 are not detected in the position 3–8, whereas, the spots of free bile acids are able to detected in the upper end of the position 3–8.

The spots in **Fig. 3** are identified as follows. When more than two spots are present in a sample, they are enumerated in their respective order from the starting line. The weak spots appearing in some mixtures are due to impurities in some of the samples and other metabolites. O = origin. Position 1: cholic acid, 2: deoxycholic acid, 3–8: cholic acid, chenodeoxycholic acid (3α,7α-Dihydroxycholic acid) and deoxycholic acid, 9 and 10: con-

Table 2. Bile acids deconjugation by aerobic bacteria

Species		Incubation period (hr)	No. of strains tested	No. of strains capable of deconjugation	%
Escherichia	coli	48	43	0	0
	communior	48	5	0	0
	freundii	48	5	0	0
Lactobacillus	bifidus	48	13	9	69.2
	plantarum	48	2	0	0
	acidophilus	48	2	0	0
	buchneri	48	2	0	0
	cellobiosus	48	1	0	0
	bulgaricus	48	1	0	0
Staphylococcus	epidermidis	48	50	44	88.0
	aureus	48	55	0	0
Intermediate coliform bacilli		48	23	2	8.7
Aerobacter aerogenes		48	38	0	0
Pseudomonas aeruginosa		48	35	0	0
candida		48	26	0	0
enterococcus		48	40	40	100.
proteus		48	8	0	0
serratia		48	1	0	0

Table 3. Bile acids deconjugation by anaerobic bacteria

Species		Incubation period (hr)	No. of strains tested	No. of strains capable of deconjugation	%
Bacteroides	vulgatus	48	15	15	100
	thetaiotaomicron	48	6	6	100
	uniformis	48	1	1	100
Corynebacterium	granulosum	48	25	25	100
	avidum	48	2	2	100
	diphtheroides	48	5	3	60
	parvum	48	1	0	0
Peptostreptococcus	intermedius	48	14	13	92.9
	putridus	48	1	1	100
	micros	48	1	0	0
Eubacterium	parvum	48	6	5	83.3
	lentum	48	1	1	100
	limosum	48	1	1	100
Peptococcus	grigoroffii	48	5	5	100
	anaerobius	48	1	1	100
Veillonella	alcalescens	48	6	0	0
	parvula	48	2	0	0
	orbiculus	48	1	1	100
Catenabacterium	catenaforme	48	1	0	0
	filamentosum	48	1	0	0

jugated bile acid. The spots of free bile acids, cholic acid, chenodeoxycholic acid and deoxycholic acid, are clearly detected in the position 3–8, but none of the spots of free bile acids are detected in the position 9 and 10. The Rf values vary somewhat with each run, and in **Fig. 3**, the Rf values of cholic acid, chenodeoxycholic acid, and deoxycholic acid at the

position 3–8 are 0.24, 0.58, and 0.69 respectively.

The results of splitting of conjugated bile acids by 446 strains of bacteria tested are summarized in **Table 2** and **Table 3**. Among aerobic bacteria (**Table 2**), none of the strains of Esch.coli (43 strains), Esch. communior (5 strains), Esch.freundii (5 strains), Lact. plantarum (2 strains), Lact. acidophilus (2 strains), Lact. buchneri (2 strains), Lact. cellobiosus (1 strain), Lact. bulgaricus (1 strain), Staph. aureus (55 strains), Aerobacter aerogenes (38 strains), Pseudomonas aeruginosa (35 strains), candida (26 strains), proteus (8 strains), and serratia (1 strain) could hydrolyze the conjugated bile acids. And 2 of 23 strains (8.7%) of Intermediate coliform bacilli split them. Whereas, 44 of 50 strains (88%) of Staph. epidermidis, 9 of 13 strains (69.2%) of Lact. bifidus and 40 of 40 strains (100%) of enterococcus could split them. Among anaerobic bacteria (**Table 3**), all of the strains tested of Bact. vulgatus (15 strains), Bact. thetaiotaomicron (6 strains), Bact. uniformis (1 strain), Coryne. granulosum (25 strains), Coryne. avidum (2 strains), Peptostrept. putridus (1 strain), Eubact. lentum (1 strain), Eubact. limosum (1 strain), Pept. grigoroffii (5 strains), Pept. anaerobius (1 strain), V. orbiculus (1 strain) could split the conjugated bile acids. And then 3 of 5 strains (60%) of Coryne. diphtheroides, 13 of 14 strains (92.9%) of Peptostrept. intermedius, and 5 of 6 strains (83.3%) of Eubact. parvum split them. On the other hand, the 1 strain tested of Coryne. parvum, Peptostrept. micros, Catena. catenaforme, and Catena. filamentosum could not hydrolyze them, and then none of the strains of V. alcalescens (6 strains) and V. parvula (2 strains) could also hydrolyze them.

Discussion

In recent years, several reviews of bile acids metabolism have appeared, since the techniques of bile acid analysis, thin layer and gas liquid chromatography, had taken rapid strides. As far as we know, the metabolism of conjugated bile acids during their passage through the intestinal tract is almost exclusively brought about by the action of bacteria indigenous to the intestine. Among the several structural alterations, the most widely studied appears to the hydrolytic cleavage of the peptide bond of conjugated bile acids by bacterial enzymes[20]. In 1955, Norman and Grubb[9] suggested the bacterial deconjugation of bile acids. According to their report, Cl. perfringens, Cl. septicum, Cl. multifermentans, Cl. sphenoides, Cl. tertium, Cl. fallax, and enterococcus were found to be capable of splitting glycocholic acid and taurocholic acid. Drasar and Hill (1966)[21] demonstrated that among 97 strains isolated from saliva, jejunum, and faeces, 15 of 25 Bacteroides spp. were found to be capable of splitting conjugated bile acids, but enterococcus (18 strains), Esch. coli (32 strains), Pseudomonas spp. (20 strains), St. salius (2 strains) could not deconjugate bile acids. Then Hill and Drasar (1968, 1969)[22][23] have demonstrated that 10 of 47 Staph. aureus, 55 of 109 St. fecalis, 119 of 237 Bacteroides sp., 15 of 16 clostridium, 34 of 89 Bifidobacterium sp., and 14 of 17 Veillonella sp. were found to be capable of splitting conjugated bile acids. Midtvedt and Norman (1967, 1968)[24,25] have demonstrated that enterococcus, streptococcus, Lactobacillus sp., Eubacterium sp., Catenabacterium sp., Ramibacterium sp., Lactobacillus bifidus, Butyribacterium rettgeri, Clostridium sp., and B. necrophorus commonly deconjugate bile acids. In 1969, evidence of a bacterial splitting was presented by Shimada, Bricknell, and Finegold[26], who isolated bacteria from human faeces capable of hydrolyzing conjugated bile acids. In

their report, following bacteria had the ability of deconjugating bile acids, namely, Bact. fragilis, Sphaerophorus necrophorus, Bact. melaninogenicus, Bifid. adolescentis, Bifid. longum, Bifid. breve, Bifid. bifidum, Bifid. liberorum, Bifid. parvulorum, Catena. catenaforme, Cl. perfringens, Cl. paraputrificum, Streptococcus fecalis, Strep. lactis, Lact. buchneri. In Japan, Sasaki[27] has reported that streptococcus, lactobacillus, bacteroides, and clostridium have been found to deconjugate bile acids in vitro, which retained their ability in germ free intestines. But Esch. coli, proteus, staphylococcus, shigella, and El Tor vibrio had not the ability. He indicated that the bacteria which were found in the upper small intestine, had the ability of deconjugating bile acids.

As mentioned above, some differences have been noted among the results of these authors. Our result clearly shows that, all or none, or almost all or none, of the strains of each species tested split conjugated bile acids. And it is presumed that the ability of splitting conjugated bile acids might be a proper character of each bacterial species.

No report has been made on in vivo study on deconjugation of bile acids by overgrowth of intestinal flora on liver cirrhosis. In 1957, Martini[28] has described that nearly all of 12 cirrhotic patients tested had increased concentrations of colonic bacteria (coliform organism and Strep. faecalis) in the jejunum. Tarao (1969)[7] and Saito (1972)[8] have descrived that aerobic bacteria, especially gram-negative bacilli, and anaerobic bacteria were more frequently found in the small intestinal juice of hepatic diseased patients, especially cirrhotic patients, than non-hepatic diseased patients. In order to study that conjugated bile acids are really split in more amount in the small bowel under these abnormal conditions resulting from bacterial overgrowth, we gave glycine-1-^{14}C cholate (glycocholic acid-26-^{14}C) to cirrhotic patients orally and measured $^{14}CO_2$ specific activity of expired air by breath-analysis technic on one hand, and we isolated small intestinal flora and tested their deconjugation ability in vitro on the other hand in the same patients. The results revealed that abnormally increased specific activity and overgrowth of bacteria with the deconjugation ability were found concomitantly in some of the patients with liver cirrhosis and, in the other patients, either of them was not found[29,30]. Thus, this study in vitro coincides well with our in vivo study.

Conclusions

1) Deconjugation of bile acids by bacteria obtained from small intestine and faeces has been studied in vitro.

2) All or none, or almost all or none, of the strains of each species tested split conjugated bile acids, and it seems probable that the presence or absence of this ability might be a proper character of each species.

Acknowledgment

We wish to express thanks to prof. Taro Kazuno for his advice on experimental method, and to Drs. Shigeki Odagiri, Kazuo Tarao, Yoichi Saito, and Ryuichiro Yamazaki for help.

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Editor's note:

The Bergey's Manual of Determinative Bacteriology, 7th Ed., Williams and Wilkins, cited in Reference No. 10, was revised in 1974.

Received December 1, 1975
Accepted April 19, 1976
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