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Metabolism of Glycyrrhizin by Human Intestinal Flora. II.[1,2] Isolation and Characterization of Human Intestinal Bacteria Capable of Metabolizing Glycyrrhizin and Related Compounds

Masao Hattori,[a] Tatsuya Sakamoto,[a] Takeyoshi Yamagishi,[b] Kenichi Sakamoto,[b] Kenichi Konishi,[b] Kyoichi Kobashi[c] and Tsuneo Namba*,[a]

Research Institute for Wakan-Yaku (Oriental Medicines),[a] Department of Bacteriology and Immunology, Faculty of Medicine[b] and Department of Biochemistry, Faculty of Pharmaceutical Sciences,[c] Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

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In a survey of intestinal bacteria capable of metabolizing glycyrrhizin (GL), *Ruminococcus* sp. PO1-3 and *Clostridium innocuum* ES24-06 were isolated from human feces. The former strain had the ability to hydrolyze GL to glycyrrhetic acid (GA) and to reduce 3-dehydroglycyrrhetic acid (DGA) to GA while the latter strain had the ability to reduce DGA to 3-*epi*-glycyrrhetic acid (EGA). A mixture of the two strains could not only reduce DGA to both GA and EGA, but also epimerize GA to EGA and *vice versa*, possibly through a 3-dehydro intermediate.

Keywords——glycyrrhizin; glycyrrhetic acid; 3-*epi*-glycyrrhetic acid; 3-dehydroglycyrrhetic acid; metabolism; intestinal bacteria; *Ruminococcus* sp.; *Clostridium innocuum*

In traditional Chinese medicine, most crude drugs are orally administered as decoctions. Active components of the crude drugs are therefore inevitably brought into contact with bacterial flora, mostly composed of anaerobes, in the alimentary tract. Some are transformed by the intestinal bacteria before absorption from the gastrointestinal tract and others after excretion in the bile followed by enterohepatic circulation. Metabolic studies on the components of these drugs by human intestinal flora are of a great importance to an understanding of the mechanisms of their clinical effects.[3-6]

In our previous paper,[1] we reported the metabolism of glycyrrhizin (GL), an active principle of liquorice, *Glycyrrhiza glabra* L. (Legumninosae), by human intestinal flora. GL was shown to be hydrolyzed to the aglycone, 18β-glycyrrhetic acid (GA), which was then transformed to 3-*epi*-18β-glycyrrhetic acid (EGA) *via* a metabolic intermediate, 3-dehydro-18β-glycyrrhetic acid (DGA).

The present paper reports a continuation of our studies on the metabolism of GL by human intestinal flora, based on a survey of intestinal bacteria capable of metabolizing GL and its derivatives.

Materials and Methods

Instruments——Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured with an FX-90Q spectrometer with tetramethylsilane as an internal standard. Ultraviolet (UV) spectra were measured with a Shimadzu UV-210A digital double beam spectrophotometer and infrared (IR) spectra with a Hitachi 260-01 infrared spectrometer. Mass spectra (MS) were measured with a JEOL JMS-DX 300 mass spectrometer at an ionization voltage of 75 eV. All melting points were determined on a Yanagimoto micro melting point apparatus and are

uncorrected. Metabolites on thin layer chromatograms were quantitatively analyzed with a Shimadzu CS-910 dual wavelength thin layer chromatoscanner (TLC-scanner). Gas chromatography (GC) was carried out by using a Shimadzu GC-6A with a flame ionization detector.

Chemicals——GL (monoammonium) and GA were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and purified as described in a previous paper.[1] 18α-Glycyrrhetic acid (18α-GA) was given by Maruzen Seiyaku Co. (Onomichi) and Dr. M. Kanaoka (Toyama Medical and Pharmaceutical University). EGA, DGA and 3-dehydro-18α-glycyrrhetic acid (18α-DGA) were prepared according to the method reported previously.[1] The following components were used for preparing selective culture media: palomomycin sulfate (Kyowa Hakko Kogyo Co., Ltd., Tokyo), neomycin sulfate (Nippon Kayaku Co., Ltd., Tokyo), colimycin (Kaken Chemical Co., Ltd., Tokyo), streptomycin sulfate (C. F. Boehringer and Soehne GmbH, Mannheim), nalidixic acid (Daiichi Seiyaku Co., Ltd., Tokyo), novobiocin (Boehringer Mannheim GmbH), achromycin (Nippon Lederle Co., Ltd., Tokyo), lab-lemco powder (Oxoid Ltd.), proteose peptone No. 3 (Difco, Detroit, Mi) trypticase (BBL, Cockeysville, Md), phytone (BBL) and yeast extract (Difco).

Culture Media——The following media were purchased for cultivation and isolation of intestinal bacteria responsible for the metabolism of glycyrrhizin and related compounds: GAM broth (Nissui Seiyaku Co., Tokyo), GAM agar (Nissui), *Bacteroides* selective agar (Nissui), modified FM agar (*Fusobacterium* selective medium, Nissui), modified LBS agar (*Lactobacillus* selective medium, BBL). BS agar (*Bifidobacterium* selective medium), PO agar (propionate oleandmycin agar), PNC agar (propionate novobiocin colimycin agar), PMS agar (*Peptococcaceae, Megasphaera* selective medium), modified VS agar (*Veillonella* selective medium), modified ES agar (*Eubacterium* selective medium), PS agar (*Peptostreptococcaceae* selective medium), modified ES (*Eubacterium* selective medium), PYG-broth, milk coagulation test medium, gelatin-hydrolysis test medium, indole-nitrate medium and sugar-hydrolysis test medium were prepared according to the reported methods[7,8] except for the use of digested human blood instead of horse blood.

Anaerobes——Human intestinal bacterial strains were provided by Prof. T. Mitsuoka (University of Tokyo).

Thin Layer Chromatography (TLC)——TLC was performed on silica gel plates (Merck, Silica gel 60 F_{254}, layer thickness 0.25 mm) in the following solvent systems: A, AcOH–*n*-BuOH–1,2-dichloroethanol–H_2O (4 : 1 : 4 : 1); B, MeOH–conc. NH_4OH (4 : 1); C, AcOH–*n*-BuOH (10 : 1); D, *n*-BuOH–conc. NH_4OH (5 : 1); E, $CHCl_3$–MeOH (10 : 1); F, $CHCl_3$–petroleum ether–AcOH (5 : 5 : 1). Metabolites were detected on TLC plates under UV light or by spraying with 10% H_2SO_4 and then heating for 10 min over 100 °C. Triterpene alcohols were detected by spraying with 5% $SbCl_5$ (v/v) in $CHCl_3$ and heating for 10 min. The metabolites were quantitatively analyzed with a TLC scanner (λ_s = 250 nm, λ_r = 400 nm) by using calibration lines obtained with authentic samples.

Assay for Ability to Reduce DGA——A human intestinal bacterium or a bacterial mixture was anaerobically cultured for 48 h at 37 °C in GAM broth (20 ml) containing DGA (1 mmol). A portion (10 ml) of the culture was acidified to pH 1 with HCl and extracted with ethyl acetate (EtOAc, 10 ml × 2) after adding NaCl (2 g). The EtOAc solution was concentrated to a volume of 1 ml and an aliquot (12 μl) of it was spotted on a TLC plate, which was then developed with solvent system F. Metabolites GA and EGA were quantitatively analyzed by TLC-densitometry.

Isolation of Intestinal Bacteria Capable of Reducing DGA——A suspension of human feces (1.55 g in 9 ml of a dilution medium[7]) was diluted with the same medium to prepare a series of 10 fold dilutions. An aliquot (50 μl) of each dilution was inoculated into various selective media in 50 ml flasks. The flasks were tightly stoppered with hard rubber and incubated at 37 °C for 48 h. During these procedures, anaerobic conditions were always maintained by flushing with oxygen-free CO_2 gas and replacing all air in the dilution medium and flasks with the CO_2 gas. A portion of the culture was then tested for activity to reduce DGA. If it had the activity, the rest was diluted with the dilution medium and applied to selective media by stabbing and streaking on the plates. The plates were then anaerobically incubated at 37 °C for 40 h. Well-separated colonies obtained from relatively high dilutions were assayed for the reducing activity. Strains capable of reducing DGA were isolated by repeating these procedures.

Determination of Fatty Acids——Each bacterial strain (precultured at 37 °C for 48 h) was inoculated into PYG broth (10 ml) and cultured at 37 °C for 7 d. The cultured broth was then acidified with 50% H_2SO_4 (v/v) and the composition of fatty acids was determined as follows. For volatile fatty acids: a 2 ml aliquot of the medium was extracted with ether (1 ml), and the extract was dried over $MgSO_4$. An aliquot (2 μl) of the extract was injected into a GC column (3 mm i.d. × 2 m) of Chromosorb W (4W, 80—100 mesh) coated with Reopolex 400 (10%). GC was carried out under the following conditions: N_2 gas, 1.0 kg/cm^2; H_2 gas, 1.0 kg/cm^2; air, 2.0 kg/cm^2; detection block and injection temperature, 150 °C; column temperature, 130 °C. For non-volatile fatty acids: MeOH (2 ml) and 50% H_2SO_4 (0.4 ml) were added to the cultured medium (1 ml), which was heated at 55 °C for 30 min. After addition of H_2O (1 ml), the mixture was extracted with $CHCl_3$ (0.5 ml). The $CHCl_3$ solution was analyzed for non-volatile fatty acids by GC in the similar fashion as described above.

Isolation of 3-*epi*-18α-Glycyrrhetic Acid (18α-EGA)——Precultured *Clostridium innocuum* ES24-06 was inoculated into GAM broth (200 ml) and cultured for 5 h at 37 °C. 18α-DGA (94 mg) in EtOH (20 ml) was added to the culture medium, which was further incubated for 10 h at 37 °C. Then 1 N HCl (1000 ml) and NaCl (150 g) were added, and the medium was extracted three times with EtOAc (1.5 l). The EtOAc solution was concentrated to a volume of *ca.* 1000 ml, washed with a saturated NaCl solution (500 ml), dried over Na_2SO_4, and then evaporated to dryness *in*

vacuo below 40 °C. The residue was dissolved in a small volume of $CHCl_3$ and the solution was applied to a column of silica gel (36 × 2 cm). The column was washed with $CHCl_3$ (700 ml) and eluted with $CHCl_3$–MeOH (100 : 1). Fractions I—IV (100 ml each) were pooled, evaporated to dryness *in vacuo*, and washed with H_2O–EtOH. The precipitate (57.4 mg) was purified by preparative thin layer chromatography (Merck, Kieselgel 60 F_{254} S, 2 mm layer thickness) using solvent system F and the product was crystallized from *n*-PrOH–petroleum ether to give colorless prisms (5.1 mg). mp > 300 °C, *Anal.* Calcd for $C_{30}H_{46}O_4$: C, 76.55; H, 9.85. Found: C, 76.54; H, 9.63. ^{1}H-NMR (DMSO-d_6) δ: 0.65, 0.77, 0.84, 1.04, 1.13, 1.16, 1.35 (each 3H, each s, C–C$\underline{H}_3$), 2.8 (1H, br s, C$\underline{H}$–OH), 5.33 (1H, s, C=C$\underline{H}$). UV λ_{max}^{EtOH}: 244 nm. MS *m/z*: 470 (M^+, 2%), 452 (4%), 437 (5%), 303 (100%), 262 (18%), 257 (15%), 175 (15%), 135 (60%). IR ν_{max}^{KBr}: 3480 (OH), 1709 (COOH), 1650 (conjugated C=O), 1615 (conjugated C=C) cm^{-1}. This compound differs in IR and NMR spectra as well as in *Rf* values in various solvent systems from its isomers, 18α-GA, GA and EGA.[1] Based on the half width (*W*/2) of the multiplet or broad singlet attributable to C_3-$\underline{H}$ in the ^{1}H-NMR spectrum (*W*/2 = 8 Hz, *cf.* *W*/2 = 24 Hz for 18α-GA; see reference 1) and a spectroscopic comparison of the oxidized compound with an authentic sample of 18α-DGA, it was determined to be 18α-EGA (3α-hydroxy-11-oxo-18α-olean-12-en-30-oic acid).

Results

Survey of Intestinal Bacterial Strains Capable of Metabolizing GL and Related Compounds

Various stock strains obtained from human feces were screened for metabolizing ability by incubating them anaerobically for 40 h in the presence of GL or DGA, a metabolic intermediate, as reported previously.[1] Among the bacterial strains examined (Table I), *Peptostreptococcus intermedius* could hydrolyze GL to GA in a yield of 31% and *Clostridium perfringens* could hydrolyzed GL slightly. On the other hand, no bacteria could transform DGA to either GA or EGA or both, even by repeated cultivation in the presence of the substrate.

Isolation and Characterization of Human Intestinal Bacteria Capable of Reducing DGA

A 10^4—10^5 fold diluted bacterial suspension obtained from human feces was inoculated into a variety of selective media and cultured for 48 h at 37 °C. The cultures were then assayed for ability to reduce DGA to GA and EGA as well as ability to hydrolyze GL to GA. As listed in Table II, the bacteria grown in the selective media such as *Bacteroides* agar, modified FM agar, modified ES agar, PNC agar and PO agar showed appreciable activity.

From these bacterial mixrures, hundreds of colonies were isolated and each was assayed for transforming ability. Strains PO1-3 and ES24-06 isolated from bacteria grown in the PO selective agar and the modified ES selective agar, respectively, showed appreciable ability to transform DGA to either GA or EGA. These strains were characterized by morphological

Table I. Ability of Stock Strains from Human Feces to Metabolize Glycyrrhizin and Related Compounds

Bacterial strains	Hydrolysis of GL	Reduction of DGA
Peptostreptococcus intermedius EBF 77/25	+ (31%)	−
Bacteroids fragilis ss. *thetaotus*	−	−
Bifidobacterium adolescentis	−	−
Bifidobacterium bifidum a E319	−	−
Lactobacillus xylosus ATCC 155775	−	−
Lactobacillus brevis II-46	−	−
Lactobacillus plantarum ATCC 14917	−	−
Clostridium butyricum	−	−
Clostridium innocuum ATCC 14501		−
Clostridium perfringens To-23	±	−
Eubacterium contortum		−
Streptococcus faecalis II-136	−	−
Proteus mirabilis S_2	−	−

TABLE II. Activity of Human Intestinal Bacteria Grown on Selective Culture Media for the Reduction of DGA

Medium	10^4-Fold dilution[a]		10^5-Fold dilution	
	Metabolites		Metabolites	
	GA	EGA	GA	EGA
Bacteroides agar	++	−	+	−
Modified FM agar	+	+	+	−
Modified ES agar	+	++[b]	+	−
PNC agar	++	+[b]	−	−
PO agar	++	+[b]	+	−
BS agar	−	−	−	−
PMS agar	+	−	−	−
PS agar	−	−	−	−
Modified LBS agar	−	−	−	−
Modified VS agar	+	+	+	−

a) Fresh feces were diluted 10^4-fold with a dilution medium.
b) The substrate disappeared completely.

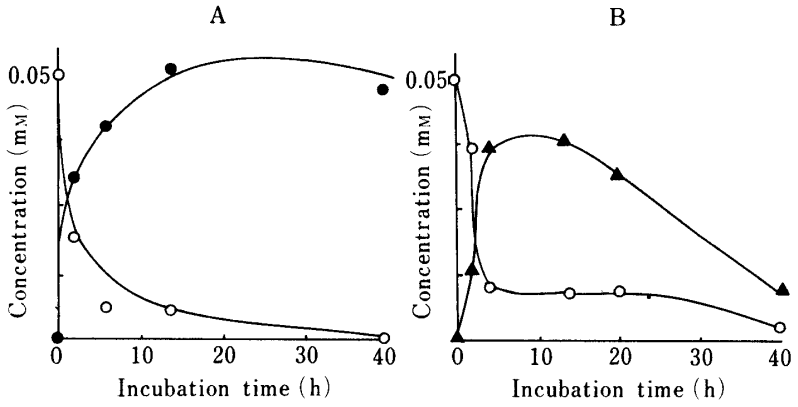

Fig. 1. Time Courses of Metabolism of DGA by *Ruminococcus* sp. PO1-3 (A) and *Clostridium innocuum* ES24-06 (B)
○, DGA; ●, GA; ▲, EGA.

observation and various specific tests, Gram stains, starch hydrolysis, gelation, esculin hydrolysis, milk coagulation, sugar utilization, nitrite reduction, and volatile or non-volatile fatty acid formation. The former strain was identical with the genus *Ruminococcus* (strictly anaerobic gram positive cocci, appeared in chains, required fermentable carbohydrate for growth, and did not produce butyric acid from glucose). The strains showed almost the same characteristics as *R. gnavus* but the species was not defined (Table III). The latter strain was identical with *Clostridium innocuum* (strictly anaerobic gram positive rods, formed round terminal spores, similar in morphology, in gas chromatogram results and in characteristics to *C. innocuum* ATCC 14501 as listed in Table III). However, the authentic strain showed no ability to reduce DGA.

Time Courses of Reduction of DGA by Isolated Human Intestinal Bacteria

Figure 1 shows the time courses of reduction of DGA by *Ruminococcus* sp. PO1-3 and *C. innocuum* ES24-06. The strain PO1-3 completely reduced DGA to GA during incubation for 40 h under anaerobic conditions, while the strain ES24-06 reduced it to EGA only. The amount of the latter metabolite, however, was maximal at 4—18 h, then decreased upon further

TABLE III. Characteristics of Isolates from Fecal Samples

Test	Isolate		*C. innocuum* ATCC	*Ruminococcus* species cited from literature[a]							
	PO1-3	ES24-06	14501	*R. albus*	*R. bromii*	*R. flavefaciens*	*R. callidus*	*R. torques*	*R. gnavus*	*R. lactis*	*R. obeum*
Acid from											
adonitol	−[b]	−	−	−	−	·	·	·	·		·
amygdalin	−	−	−	v	−	−	v	−	−, +	−	−, +
arabinose	+	−	−	−	−	−, (+)	−	−	+	−	+
cellobiose	−	+	+	+, −	−	+, −	+	−	−	−	+
erythritol	−	−	−	−	−	−	·	·	·	·	·
esculin	(+)	+	+	−	−	−	−, (+)	−, (+)	(+)	−	−
fructose	+	+	+	−, (+)	+, −	−, (+)	−, (+)	+	+	+, (+)	+
galactose	+	−	−	−	+, −	−	·	·	·	·	·
glucose	+	+	+	+, −	(+), +	−, (+)	+, −	+	+	+	+
glycerol	−	−	−	·	−	−	·	·	·	·	·
glycogen	−	−	−	−	+, −	−	·	·	−	−	−
inositol	−	−	−	−	−	−	·	·	−	−	−
inulin	+	+	+	−	−	−	·	·	·	·	·
lactose	−	−	−	+, (+)	−	+, −	+, −	+	−, +	+	+
maltose	+	−	−	−	+, (+)	−, (+)	+	(+), −	+	v	+
mannitol	−	+	+	−	−	−	−	−	−	+, (+)	−, +
mannose	+	+	+	+, −	−, (+)	−	−, (+)	(+), −	−	−, +	+
melezitose	−	−	−	−	−	−	−, +	−	·	·	·
melibiose	+	−	−	−	−	−	+, −	−	(+), +	−	+
raffinose	+	−	−	−	−	−	+	−	+	−	+
rhamnose	+	−	−	−	−	−	·	·	+	−	+
ribose	+	+	+	−	−	−	−	−	+	−, +	+
salicin	+	+	+	−, (+)	−	−, (+)	v	v, −	+	−	−, +
sorbitol	−	−	−	−	−	−	−	−	−	−	+, −
starch	(+)	−	−	−	+, −	−	−, (+)	−	−, +	−	−, (+)
sucrose	+	+	+	+, −	−	−	+	−	+, −	−, (+)	+
trehalose	−	+	+	−	−	−	v	−	−	−	−, +
xylose	+	−	−	−	−	−	−, (+)	−	+	−	+
Milk coagulation	−	−	−	−	−	+, −	+, −	+, −	+, −	+	+
Gelatin liquefaction	−	−	−	−, (+)	−	−	·	·	·	·	·
Indole production	−	−	−	−	−	−	·	·	·	·	·
Nitrate reduction	−	−	−	−	−	−	·	·	·	·	·

a) Cited from "Anaerobe Laboratory Manual," 2nd ed., Virginia Polytechnic Institute and State University, Blacksburg, 1973, *Int. J. Syst. Bacteriol.*, **24**, 260 (1974), and *Int. J. Syst. Bacteriol.*, **26**, 238 (1976).

b) Symbols: −, negative reaction; v, variable reaction; (+), weak reaction; +, positive reaction; ·, not reported. Where two reactions are given, the first was the more usual and the second was observed less frequently.

Table IV. Metabolites of Glycyrrhizin and Related Compounds by *Ruminococcus* sp. PO1-3 (A) and *Clostridium innocuum* ES24-06 (B)

Substrate	Incubation time (h)	*Ruminococcus* sp. PO1-3					*Clostridium innocuum* ES24-06				
		Metabolic mixture (%)				Total recovery	Metabolic mixture (%)				Total recovery
		GL	3α-OH	3-Keto	3β-OH		GL	3α-OH	3-Keto	3β-OH	
GL	12	(67)	N.D.	N.D.	24	91	(90)	N.D.	N.D.	N.D.	90
	40	(52)	N.D.	N.D.	34	86	(93)	N.D.	N.D.	N.D.	93
GA	12	—	N.D.	N.D.	(72)	72	—	N.D.	N.D.	(79)	79
	40	—	N.D.	N.D.	(86)	86	—	N.D.	N.D.	(91)	91
DGA	12	—	N.D.	(16)	70	86	—	57	N.D.	N.D.	57
	40	—	N.D.	Faint	72	72	—	36	N.D.	N.D.	36
EGA	12	—	(19)	Faint	N.D.	19	—	(36)	Faint	N.D.	36
	40	—	(19)	Faint	N.D.	19	—	(36)	N.D.	N.D.	36
18α-DGA	40	—	N.D.	(17)	43	60	—	67	N.D.	N.D.	67

3α-OH, 3α-hydroxyl derivative; 3-Keto, 3-dehydro derivative; 3β-OH, 3β-hydroxyl derivative; N.D., not detected. Values in parentheses represent recovery % of the substrate.

Table V. Metabolites of Glycyrrhizin and Related Compounds by a Mixture of *Ruminococcus* sp. PO1-3 and *Clostridium innocuum* ES24-06

Substrate	Incubation time (h)	Metabolic mixture (%)				Total recovery
		GL	3α-OH	3-keto	3β-OH	
GL	12	(101)	N.D.	N.D.	N.D.	101
	40	N.D.	N.D.	N.D.	48	48
GA	12	—	7	N.D.	(77)	84
	40	—	12	10	(83)	105
DGA	12	—	29	(13)	48	90
	40	—	12	(12)	57	81
EGA	12	—	(33)	N.D.	34	67
	40	—	(31)	N.D.	34	65
18α-DGA	40	—	33	(15)	45	93

See Table IV for abbreviations.

prolonged incubation. After 40 h, only 20—25% of metabolites (calculated from added substrate) was recovered, suggesting that EGA was further transformed to other metabolite(s) which could not be detected on the TLC plate under UV light.

Transformation of GL and Related Compounds by Freshly Isolated Bacterial Strains

The isolated strain *Ruminococcus* sp. PO1-3 hydrolyzed GL to GA in a yield of 34% after incubation for 40 h, and reduced either DGA or its 18α-isomer (18α-DGA) to the corresponding 3β-hydroxy derivative (72 or 43%, respectively), but had no ability to epimerize GA to EGA or *vice versa* (Table IV). On the other hand, the strain *C. innocuum* ES24-06 neither hydrolyzed GL to GA nor epimerized GA to EGA or *vice versa*, but reduced the 3-dehydro

derivatives (DGA and 18α-DGA) to 3α-hydroxyl derivatives (36 and 67%, respectively), under the same conditions.

In contrast to the results observed in the individual strains, a mixture of the above two strains had appreciable ability to epimerize GA to EGA and *vice versa* as well as to reduce 18α- and 18β-DGAs to the corresponding mixtures of 3α- and 3β-hydroxyl derivatives (Table V).

Discussion

The results obtained in an earlier study[1] on the metabolism of GL showed that GL was transformed into three metabolites (GA, EGA and DGA) by human intestinal flora. Based on *in vitro* investigations of the metabolic time courses, isomerization of GA to EGA and *vice versa* and reduction of DGA to GA and EGA, the reactions seem to include hydrolysis of GL to GA and glucuronic acid and reversible transformation of GA to EGA *via* a metabolic intermediate (DGA) by the intestinal flora (Fig. 2).

In order to identify human intestinal bacteria responsible for the above metabolic processes, various stock strains from human feces were examined for ability to hydrolyze GL to GA and glucuronic acid and for ability to reduce DGA to GA and/or EGA. However, only *Peptostreptococcus intermedius* and *Clostridium perfringens* showed hydrolytic ability and the others showed no activity in either reaction. Thus, various attempts were made to isolate single strains responsible for these reactions from fresh human feces. *Ruminococcus* sp. PO1-3 and *Clostridium innocuum* ES24-06 were isolated as active bacterial species. The former strain showed ability to hydrolyze GL to GA and reduce DGA to GA and the latter strain showed ability to reduce DGA to EGA. In the case of steroids, the reduction of keto groups of estrone and 3-dehydro bile acids by fecal microorganisms has been reported by Stimmel[9] and Gustafsson *et al.*[10] In the case of triterpenes, however, this is the first report to show that 3-dehydro derivatives of GA as well as of its 18α-isomer (18α-GA) are stereospecifically reduced to the 3α- or 3β-hydroxyl derivative by the action of different species of human intestinal bacteria. Though an attempt to isolate single strains capable of reducing DGA to both GA and EGA was unsuccessful, a mixture of the above two strains could reduce it to both compounds. Furthermore, this mixture could isomerize GA to EGA and *vice versa*, possibly

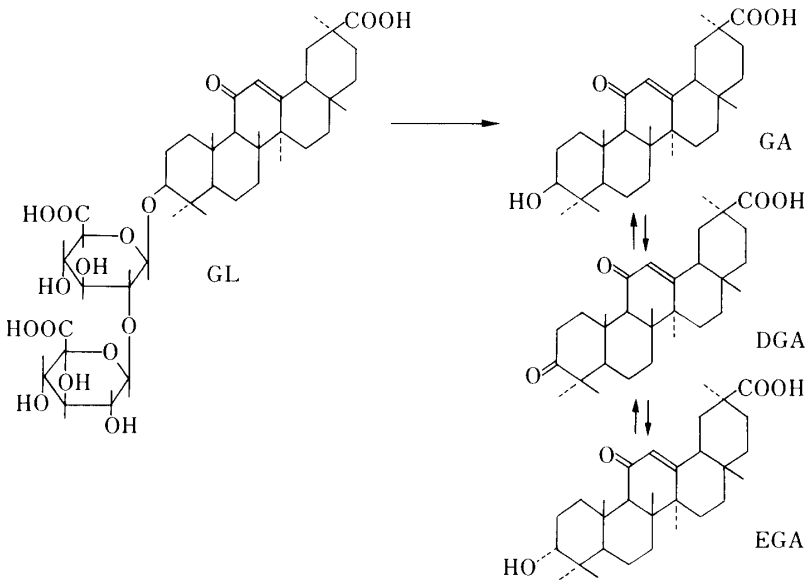

Fig. 2. Metabolic Process of Glycyrrhizin by Human Intestinal Flora

via DGA, suggesting that both strains are responsible for the metabolism of GL in the intestinal tract. It is of interest that GL could not be converted to the three metabolites mentioned above by the action of a single species of bacteria but could be converted by the co-operative action of bacteria responsible for different steps of the metabolic pathways. So far we have examined the metabolic activities of various dilutions of human feces, and we have found that a fairly large number of bacteria showed β-glucuronidase activity to hydrolyze GL to GA, while a moderate number of the bacteria had activity to reduce DGA to either GA or EGA.

Recently Nakano *et al.*[11] reported that in the serum of normal human subjects, the GA level showed two maxima at 1—4 h and 10—24 h after oral administration of GL, as determined by enzyme immunoassay. Though they did not mention any other metabolites in the blood, their observation seems to suggest the enterohepatic circulation of GA, which would enable GA to be transformed to DGA and EGA by the action of intestinal bacreria, and enable it to be re-absorbed together with other metabolites into the body fluid.

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