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Metabolism of Sennosides by Intestinal Flora[1)]

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Through investigation of the metabolism of sennosides by intestinal contents and feces of the rat, the main metabolic pathways were elucidated and found to be quite different from those previously proposed on the basis of the metabolites produced by individual strains isolated from the human intestine. Sennoside A was hydrolyzed in a stepwise fashion to produce sennidin A *via* sennidin A-8-monoglucoside by β-glucosidase present in the intestinal contents and feces, and sennoside B to sennidin B *via* sennidin B-8-monoglucosides in a similar way. The resulting metabolites, sennidins A and B, were interconvertible under the experimental conditions used, and were further reduced to give rheinanthrone, a purgative active principle of *Rhei Rhizoma* and senna, possibly by reductase bound to cell membranes of intestinal bacteria.

Keywords——sennosides; metabolism; purgative effect; *Rhei Rhizoma*; sennidin; sennidin-8-monoglucoside; rheinanthrone

Introduction

In our previous investigation[2)] on the ability of anaerobes isolated from the human intestine to metabolize sennosides, the species of bacteria were classified into four types: Type I bacteria (*Clostridium sphenoides*, *Clostridium butyricum* and *Bifidobacterium adolescentis*) reductively cleave the C(10)–C(10′) bond of sennoside A to produce 8-glucosylrheinanthrone, which was then converted to sennidin possibly *via* rheinanthrone by β-glucosidase, followed by oxidation. Type II bacteria (*Eubacterium rectale*, *Eubacterium limosum*, *Eubacterium lentum* and *Peptostreptococcus intermedius*) reduce sennoside A to 8-glucosylrheinanthrone, which is re-oxidized to give a mixture of sennosides A and B. Type III bacteria (*Clostridium perfringens* and *Lactobacillus brevis*) produce unknown glucosides. Type IV bacteria, including all species of *Bacteroides* and *Enterobacteria* and several other species of *Bifidobacterium*, have no metabolic activity. On the basis of these findings, the metabolic process of sennosides was deduced, and the role of Type I bacteria in the intestinal flora in manifesting the purgative effect was discussed in connection with individual differences in the medicinal effects of *Rhei Rhizoma*, well known in traditional Chinese medicines.

Since various intestinal bacteria forming a definite flora *in vivo* are considered to be co-operative and/or antagonistic in the gastrointestinal tract, the metabolic pathways of sennosides by intestinal flora could be different from those by individual bacterial strains. Therefore, we investigated the metabolism of the compounds using fresh intestinal contents or feces of rats and humans.

Materials and Methods

Chemicals——Sennosides A and B were isolated and purified from a crude sennoside powder (Lot. DE8741TD, Nippon Funmatsu Yakuhin and Co. LTD.) according to the method reported previously.[2)] Sennidins A and B, rhein, rheinanthrone and 8-glucosylrhein were prepared by the methods of Stoll *et al.*[3,4)]

Preparation of a Suspension of Rat Feces and Its Supernatant Fluid——Fresh feces (20 g) of Wistar rats (female, 180—220 g body weight) were suspended in 100 mM phosphate buffer (200 ml, pH 7.3) containing

0.05% cysteine, which had previously been bubbled through with carbon dioxide to eliminate air. The supernatant fluid was prepared by centrifuging the suspension at 10000 rpm for 10 min.

Incubation of Sennosides with Rat Feces and Quantitative Analysis of Their Metabolites——To 5 ml of a suspension of rat feces was added 500 μl of sennoside A or sennoside B solution (1 mg/ml, dissolved in 100 mM phosphate buffer, pH 7.3). After replacing air in the test tube with carbon dioxide, the mixture was incubated at 37°C for the indicated periods of time. The tube was then immediately cooled and centrifuged at 10000 rpm for 10 min. Next, 2% ethylenediaminetetraacetic acid (EDTA) (0.5 ml), 0.5 N HCl (0.5 ml) and *n*-BuOH (2 ml) were added to 2 ml of the upper layer. After vigorous shaking, the mixture was centrifuged at 3000 rpm for 10 min to separate it into two layers. Five μl of the upper layer was applied on a silica gel thin layer plate (Merck Silica gel 60 F_{254}, layer thickness 0.25 mm). The plate was then developed with a solvent system A, *n*-PrOH: AcOEt: H_2O (4: 4: 3, v/v) containing a few drops of AcOH. The spots on chromatogram were detected under ultraviolet (UV) light and analyzed quantitatively by using a Shimadzu CS-910 chromatoscanner (Shimadzu Seisakusho Ltd., Kyoto) as described in our previous paper.[2]

Isolation of Sennidin A-8-Monoglucoside——Sennoside A (1 g) was incubated with a suspension (2.5 l) of rat feces at 37°C for 30—60 min, followed by centrifugation at 10000 rpm for 20 min. The supernatant fluid was acidified with hydrochloric acid to pH 3, and extracted with *n*-BuOH (2.5 l). The extract was washed with water, neutralized with triethylamine, and concentrated *in vacuo* below 37°C to a small volume (1—2 ml), which was then applied to a column (3×41 cm) of Sephadex LH 20 (Pharmacia Fine Chemicals) and eluted with 70% methanol. Fractions of 2 ml/tube were collected, and the absorbance at 370 nm was monitored. Fractions as shown in Fig. 1 were pooled and evaporated to dryness *in vacuo*. Peak I contained substances mostly derived from feces. Peak II and Peak IV were identified as sennoside A and sennidin A, respectively, by comparing *Rf* values on TLC and absorption spectra with those of authentic samples. Peak III was further purified by repeated Sephadex LH 20 column chromatography to give 2 mg of a chromatographically homogeneous compound (compound **I**): $Rf=0.43$ on TLC developed with solvent system A, UV-VS: λ_{max}, pH 1: 218, 270, 300 (shoulder), 387 nm; pH 7: 220, 270, 365 nm; pH 13: 225, 297 (shoulder), 422 nm.

Acid Hydrolysis of Sennidin A-8-Monoglucoside (Compound I)——Sennidin A-8-monoglucoside (16.3 OD units at 365 nm, $\varepsilon_{365}=1.5\times10^4$) was suspended in 1 ml of 2.4 N H_2SO_4. The suspension was heated at 95°C for 2 h. The precipitates were filtered off, then the filtrate was neutralized with 8 N NaOH and analyzed enzymatically for glucose according to the method of Miwa *et al.*[5]

Incubation of Sennidin A-8-Monoglucoside with Supernatant Fluid of Rat Feces——Sennidin A-8-monoglucoside (0.3 mg/ml) was incubated with supernatant fluid (2.0 ml) of rat feces at 37°C for 2 h, then 2% EDTA (0.5 ml) and 0.5 N HCl (0.5 ml) were added at the indicated time of incubation, followed by extraction of the whole mixture with *n*-BuOH (2 ml) as described above. An aliquot of the extract was applied to a silica gel thin layer plate, which was developed with solvent system A. The spots on the chromatogram were analyzed densitometrically.

Effect of Various Factors on the Isomerization of Sennidins——The effects of bivalent cations, pH, incubation temperature and cysteine on the isomerization of sennidin A were examined under the following conditions.

Ca^{2+} and Mg^{2+}: Mixtures (2 ml) containing sennidin A (0.25 mg/ml), 1—100 mM $CaCl_2$ or $MgCl_2$ and 100 mM Tris-HCl, pH 7.3 were incubated at 37°C for 24 h.

pH: Solutions of sennidin A (final conc. 0.25 mg/ml) dissolved in various buffers (pH 4—11) were incubated at 37°C for 5 h or 20 h.

Incubation Temperature: Solutions (1 ml) of sennidin A (0.25 mg) dissolved in 100 mM phosphate buffer (1 ml, pH 7.3) were incubated at various temperatures for 3 h.

Cysteine: Mixtures (2 ml) containing sennidin A (0.5 mg/ml), 0—100 mM cysteine and 100 mM phosphate buffer (pH 7.3) were incubated at 37°C for 18 h.

After 2% EDTA and 0.5 N HCl had been added, the mixtures were extracted with *n*-BuOH as described above. An aliquot of the extract was applied to a silica gel thin layer plate, which was developed with solvent system A and analyzed densitometrically.

Conversion of Sennidin A to Rheinanthrone and Quantitative Analysis of Rheinanthrone——A solution of sennidin A (3 mg) in 100 mM phosphate buffer (0.6 ml) was added to a suspension (2.4 ml) of rat feces. The mixture was incubated at 37°C, and stopped at the indicated time by adding 1% *p*-nitroso-*N*,*N*′-dimethylaniline reagent (1 ml). After being kept at room temperature for 10 min, the mixture was centrifuged at 10000 rpm for 20 min. Chloroform (1 ml) was added to the supernatant fluid (2 ml), and the mixture was vigorously shaken, and then centrifuged at 3000 rpm for 10 min. The chloroform layer was carefully removed, and filtered immediately. An aliquot (30 μl) of the filtrate was applied to a polyamide thin layer plate, which was developed with solvent system B, pyridine: ammonia–water (100: 3, v/v). Rheinanthrone was quantitatively analyzed by thin layer chromatography (TLC)–densitometry as the azometin derivative formed by treatment with *p*-nitroso-*N*,*N*′-dimethylaniline. The calibration curve, the observed density on TLC *versus* the concentration of rheinanthrone, was a linear function in the range of 0.3—4.5 μg.

Cultivation of Human Intestinal Bacteria in the Presence of Sennidin A——A 0.3 ml aliquot of sennidin A solution (5 mg/ml, in 100 mM phosphate buffer, pH 7.3) which had previously been passed through a mem-

brane filter (0.45 μm, EP 030, Schleicher and Schüll, Dassel, West Germany) was added to sterile GAM (2.6 ml, Nissui Product). The culture medium was then inoculated with a precultured bacterial suspension (0.1 ml) of isolated strains. The cultivation was carried out at 37°C in an anaerobic jar, in which air had been replaced with CO_2 in the presence of activated steel wool. After 24 h cultivation, 1% *p*-nitroso-*N*,*N'*-dimethylaniline reagent (1 ml) was added and then rheinanthrone was analyzed quantitatively by TLC–densitometry as described above.

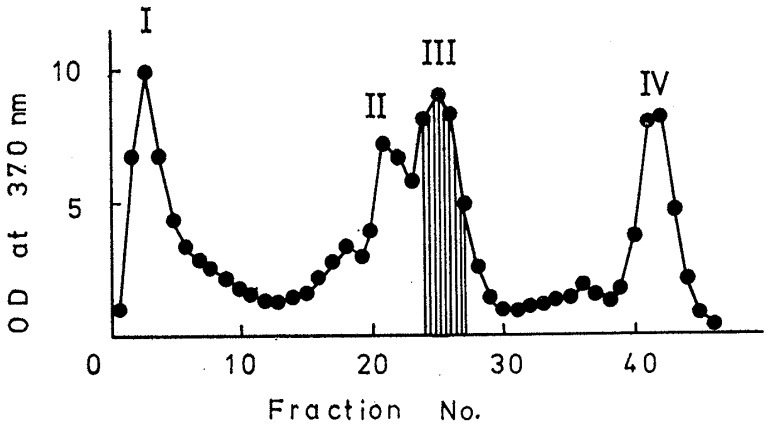

Fig. 1. Elution Profile of the Metabolites on Sephadex LH 20 Chromatography

Results

Sennoside A-Metabolizing Activity in Segments of the Gastrointestinal Tract

Sennoside A was incubated in anaerobic conditions with fresh feces and contents of various segments of the gastrointestinal tract of rats. The reaction mixtures were then extracted with *n*-BuOH and the products were analyzed by thin layer chromatography. Sennoside A was not converted at all when incubated with the contents of the stomach and duodenum, and jejunum, but it was metabolized rapidly when incubated with the contents of the ileum and cecum, and colon, as well as with feces (Fig. 2). Since feces had the strongest metabolic activity for sennoside A and were easier to collect, the following experiments were carried out using rat or human feces.

Metabolites of Sennoside A with Rat Feces

When sennoside A was incubated under anaerobic conditions with rat or human feces, similar patterns of metabolites were detected on TLC. Fig. 3 shows the time-dependent production of the metabolites as measured by densitometry after thin layer chromatographic separation. As sennoside A decreased in amount, sennidin A increased progressively, accompanied with a delayed increase in the amount of sennidin B, an optical isomer of sennidin A. On the other hand, a new substance (*Rf* 0.43,[6] compound I) was also detected during the incubation.

Isolation and Identification of Compound I

In order to isolate compound I, the mixture of metabolites was repeatedly chromatographed on Sephadex LH 20 (Fig. 1). Compound I thus obtained had λ_{max} at 220, 270 and 365 nm (at pH 7), and an intermediate *Rf* value between those of sennosides and sennidins. Acid hydrolysis of compound I gave sennidin A and glucose, which were quantitatively analyzed by UV-VS absorption and enzymic methods, respectively. The molar ratio of the aglycone to glucose was found to be 1.0 to 1.1. In the same procedure, sennoside A gave a ratio of 1.0 to 1.9. Compound I was therefore proved to be sennidin A-8-monoglucoside produced by enzymic partial hydrolysis of sennoside A.

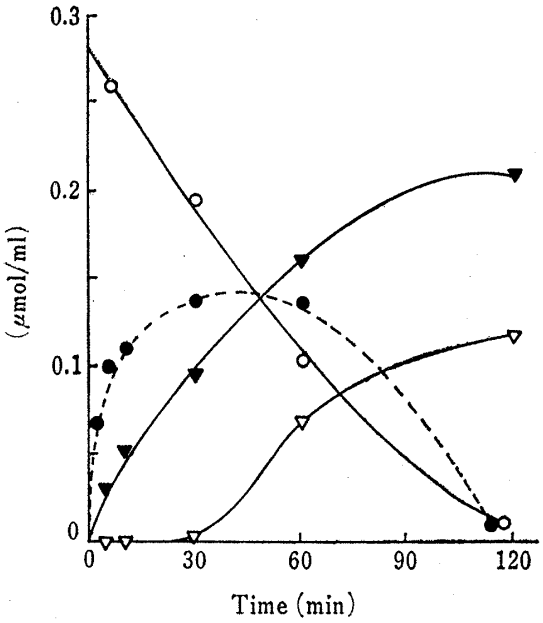

Fig. 2. Metabolism of Sennoside A by a Suspension of Rat Feces

Procedures and conditions were as described in "Materials and Methods." —○—○—, sennoside A; —●—●—, sennidin A-8-monoglucoside, compound I; —▼—▼—, sennidin A; —▽—▽—, sennidin B.

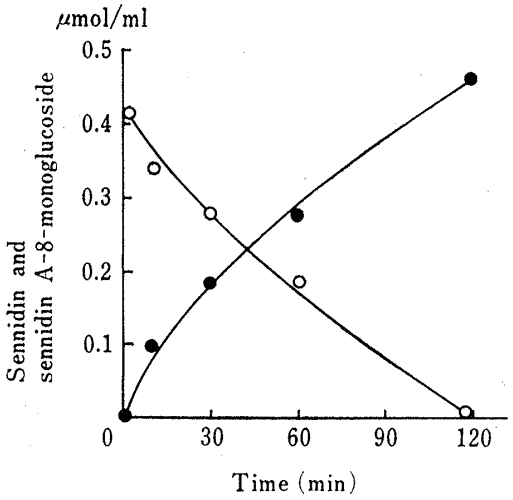

Fig. 4. Hydrolysis of Sennidin A-8-Monoglucoside by Supernatant Fluid of Rat Feces

—○—○—, sennidin A-8-monoglucoside; —●—●—, sennidins A+B.

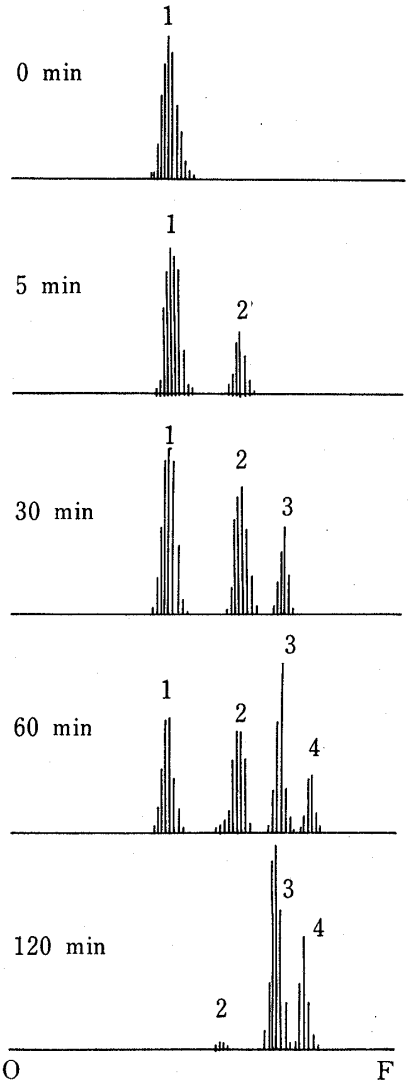

Fig. 3. Densitometric Profiles of the Metabolites of Sennoside A

The metabolites were applied to a silica gel thin layer plate, and developed with solvent system A. The chromatogram was then analyzed by densitometry, using a Shimadzu CS-910 TLC scanner (λ_s=370 nm, λ_r=785 nm). 1, sennoside A; 2, sennidin A-8-monoglucoside; 3, sennidin A; 4, sennidin B; O, origin; F, front.

Incubation of Sennidin A-8-Monoglucoside with Supernatant Fluid of Rat Feces

When sennidin A-8-monoglucoside was incubated with supernatant fluid of rat feces, the compound was completely converted to sennidin A together with small amounts of sennidin B and rhein, indicating that the monoglucoside was an intermediate in the metabolism of sennoside A to sennidins (Fig. 4).

Metabolites of Sennoside B with Rat Feces

Sennoside B, an optical isomer of sennoside A, has a strong purgative activity, as does sennoside A.[7] When sennoside B was incubated with rat feces, the amount of the compound

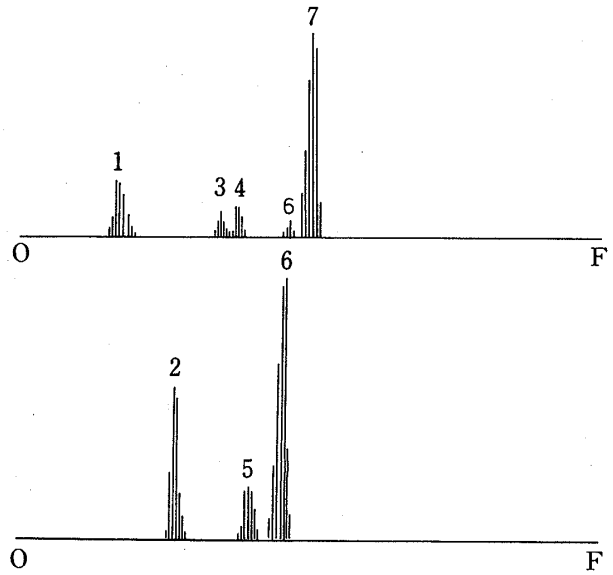

Fig. 5. Densitometric Profile of the Metabolites of Sennoside B in Comparison with that of Sennoside A

Upper: sennoside B. Lower: sennoside A.
1, sennoside B; 2, sennoside A; 3, sennidin B-8-monoglucoside; 4, sennidin B-8′-monoglucoside; 5, sennidin A-8-monoglucoside; 6, sennidin A; 7, sennidin B; O, origin; F, front.

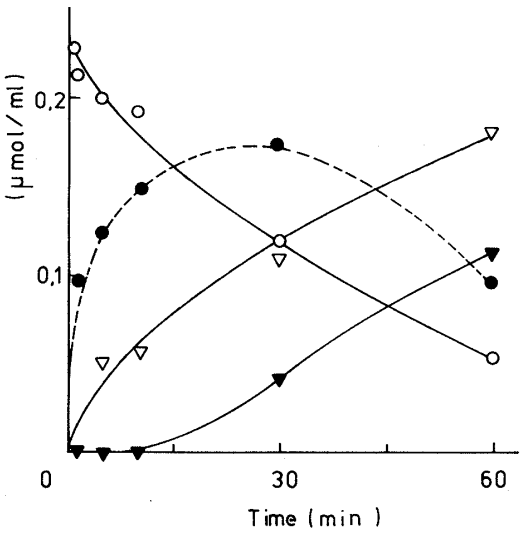

Fig. 6. Metabolism of Sennoside B by a Suspension of Rat Feces

—○—○—, sennoside B; —●—●—, sennidin B-8-monoglucosides; —▽—▽—, sennidin B; —▼—▼—, sennidin A.

decreased progressively with time. In contrast with the case of sennoside A, two spots[8] (*Rf* 0.40 and 0.42, equivalent in density) corresponding to the monoglucosides were detected on TLC during the incubation (Fig. 5). The total amount of the monoglucosides increased for the first 30 min and then decreased gradually (Fig. 6). They were thus metabolic intermediates, like sennidin A-8-monoglucoside in the case of sennoside A metabolism, and were converted to sennidin B.

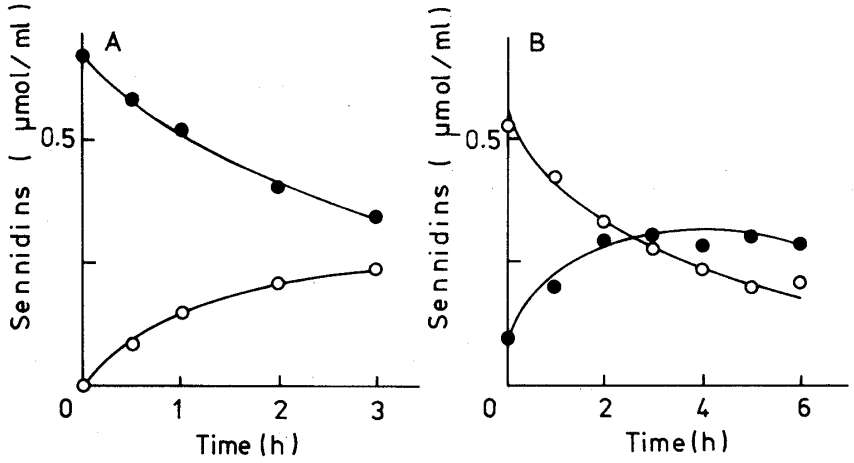

Fig. 7. Isomerization of Sennidins

A: Isomerization of sennidin A to sennidin B. B: Isomerization of sennidin B to sennidin A.
—●—●—, sennidin A; —○—○—, sennidin B.

Isomerization of Sennidin A to Sennidin B and *Vice Versa*

Fig. 7 shows the isomerization of sennidin A to sennidin B by anaerobic incubation with rat feces or supernatant fluid. As sennidin A decreased in amount gradually with time, sen-

nidin B simultaneously increased in amount. The recovery of total sennidins tended to decrease slowly during 3—6 h, and decreased significantly upon more prolonged incubation, possibly due to the conversion of sennidins to rhein and other unidentified compounds. In fact, rhein was always detected qualitatively on TLC during the incubation.

In the same manner, sennidin B was also converted to sennidin A, showing that mutual isomerization between sennidins A and B occurs.

Effects of Bivalent Metal Ions, pH ,Temperature of Incubation and Cysteine on the Isomerization of Sennidins

The isomerization between sennidins A and B proceeded upon incubation not only with the suspension of rat feces and its supernatant fluid, but also with the heat-treated suspension (autoclaved at 120°C for 15 min). The supernatant fluid, however, lost activity appreciably on dialysis against 10 mM EDTA and water, suggesting that low molecular weight substances take part in the isomerization. Various factors which might affect the isomerization reaction were examined as described in "Materials and Methods." Bivalent cations such as Ca^{2+} and Mg^{2+}, which were reported to be present in feces and capable of forming chelates with anthraquinone, did not influence the isomerization reaction at all at final concentrations of 1—100 mM. On the other hand, increase of the pH or temperature of incubation or the addition of cysteine led to the isomerization of sennidins under rather drastic conditions, *i.e.*, above pH 10, at 50—80°C, or with 5—60 mM cysteine. However, with increase of pH, temperature, or the concentration of cysteine, the total recovery of sennidins decreased abruptly. The reaction conditions under which sennidin A was incubated with the suspension of rat feces or its supernatant fluid, *i.e.*, pH 7.3, 37°C and 0.05 mM cysteine, did not affect the isomerization reaction appreciably.

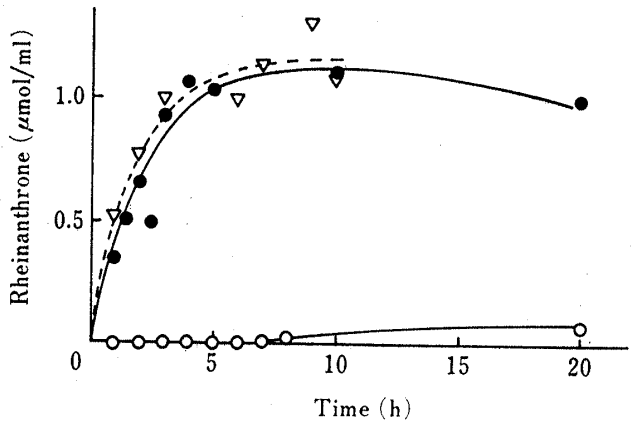

Fig. 8. Conversion of Sennidin A to Rheinanthrone

Rheinanthrone produced was treated with *p*-nitroso-*N*,*N*′-dimethylaniline, and analyzed quantitatively by TLC-densitometry, using a Shimadzu CS-910 TLC scanner (λ_s=650 nm, λ_r=785 nm).
—●—●—, the suspension of rat feces; —○—○—, the supernatant fluid; --▽--▽--, the dialyzed suspension.

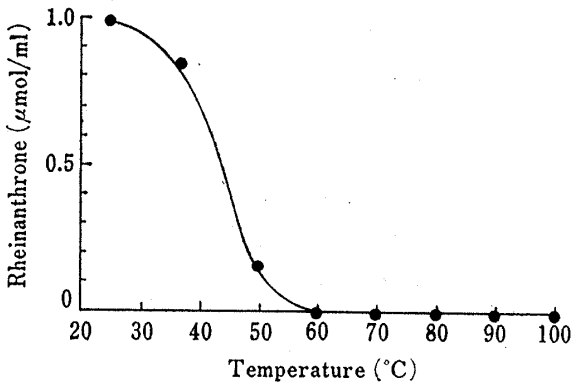

Fig. 9. Effect of Preincubation on the Conversion of Sennidin A to Rheinanthrone

The rat feces suspension was pre-heated at the indicated temperature for 20 min, then incubated with sennidin A at 37°C for 4 h.

Conversion of Sennidins to Rheinanthrone

Sennidin A or B was incubated with the suspension of rat feces under anaerobic conditions and rheinanthrone produced was analyzed by polyamide TLC and densitometry after treatment with a *p*-nitroso-*N*,*N*′-dimethylaniline reagent,[9-12] followed by extraction with chloroform. The amount of rheinanthrone, a reduced product, increased progressively over 3—4 h, and then reached a plateau after 5 h (Fig. 8). In contrast with the isomerization of sennidins, the supernatant fluid of rat feces showed only slight activity for the reduction. The conversion of sennidins to rheinanthrone proceeded similarly with either dialyzed or sonicated feces (data

Table I. Ability of Human Intestinal Bacteria to metabolize Sennidin A to Rheinanthrone

Bacterial species	Rhein-anthrone	Bacterial species	Rhein-anthrone
Clostridium sphenoides	++	*Clostridium butyricum*	±
Clostridium perfringens	++	*Eubacterium rectale*	±
Bifidobacterium adolescentis	++	*Bacteroides fragilis ss thetaiotus*	±
Eubacterium limosum	++	*Bacteroides fragilis ss vulgatus*	±
Peptostreptococcus intermedius	++	*Bifidobacterium longum*	±
Lactobacillus xylosus	++	*Streptococcus faecalis*	±
Eubacterium lentum	+	*Peptostreptococcus anaerobius*	±
Proteus mirabilis	+	*Veillonella parvula ss parvula*	±
Lactobacillus brevis	+	*Veillonella alcalescens*	±
Lactobacillus acidophilus	+	*Lactobacillus leichmannii*	±
Lactobacillus fermenti	+	*Lactobacillus plantarum*	±

Rheinanthrone produced by the cultivation of bacteria in GAM containing sennidin A was analyzed quantitatively by TLC-densitometry after being reacted with *p*-nitroso-*N*,*N*'-dimethylaniline (λ_r=650 nm, λ_s=785 nm). ++, large amount; +, small amount; ±, detectable.

not shown) but decreased after heat treatment of the feces suspension above 50°C for 20 min (Fig. 9), suggesting that the reduction reaction is catalyzed by enzymes present in rat feces.

Conversion of Sennidins to Rheinanthrone by Human Intestinal Bacteria

Through the cultivation of chracterized strains isolated from the human intestine in GAM containing sennidin A, the ability of the bacteria to convert sennidin A to rheinanthrone was investigated and the results are listed in Table I. *Clostridium sphenoides*, *Clostridium perfringens*, *Bifidobacterium adolescentis*, *Eubacterium limosum*, *Peptostreptococcus intermedius* and *Lactobacillus xylosus* produced rheinanthrone to a marked extent from sennidin A, and *Eubacterium lentum*, *Proteus mirabilis*, *Lactobacillus brevis*, *Lactobacillus acidophilus* and *Lactobacillus fermenti* produced it to some extent. The other bacteria did not show any appreciable activity.

Discussion

In studies involving the incubation of sennosides with the gastrointestinal contents or feces of rat, no appreciable metabolic activity was observed in the contents of the stomach and duodenum, and jejunum, but strong activity was observed in the contents of the ileum and cecum, and colon, as well as in the feces. This agrees with the results of Lemli *et al.*[13] who did not find any metabolite of sennosides in the tied-off stomach and jejunum or in the rinsed colon of rats, but did find metabolites in the tied-off cecum where sennosides were injected. Sennoside A was first hydrolyzed to sennidin A-8-monoglucoside and glucose by β-glucosidase derived from bacteria[14] in the lower parts of the intestine and in the feces. The monoglucoside was then further hydrolyzed to sennidin A and glucose by the enzyme. Similarly, sennoside B was converted to sennidin B *via* two isomeric monoglucosides. Sennidins A and B were interconvertible. The isomerization reaction proceeded on incubation with a suspension of rat feces or its supernatant fluid, but the latter lost the activity upon dialysis against 10 mM EDTA and water, suggesting that low molecular weight substances take part in the reaction. Though the isomerization reaction proceeded at higher pH (above pH 9.5), higher temperature (above 45°C) and in the presence of higher concentrations of reducing agents (above 5 mM cysteine) possibly through enolization,[15] radical formation[16] or reductive cleavage at the C(10)–C(10') bond of the molecule, it is unlikely that these factors are responsible for the reaction under the conditions used because no isomerization reaction occurred under the mild conditions without the addition of the suspension of rat feces or its supernatant fluid. As will be described later, the isomerization could proceed to some extent

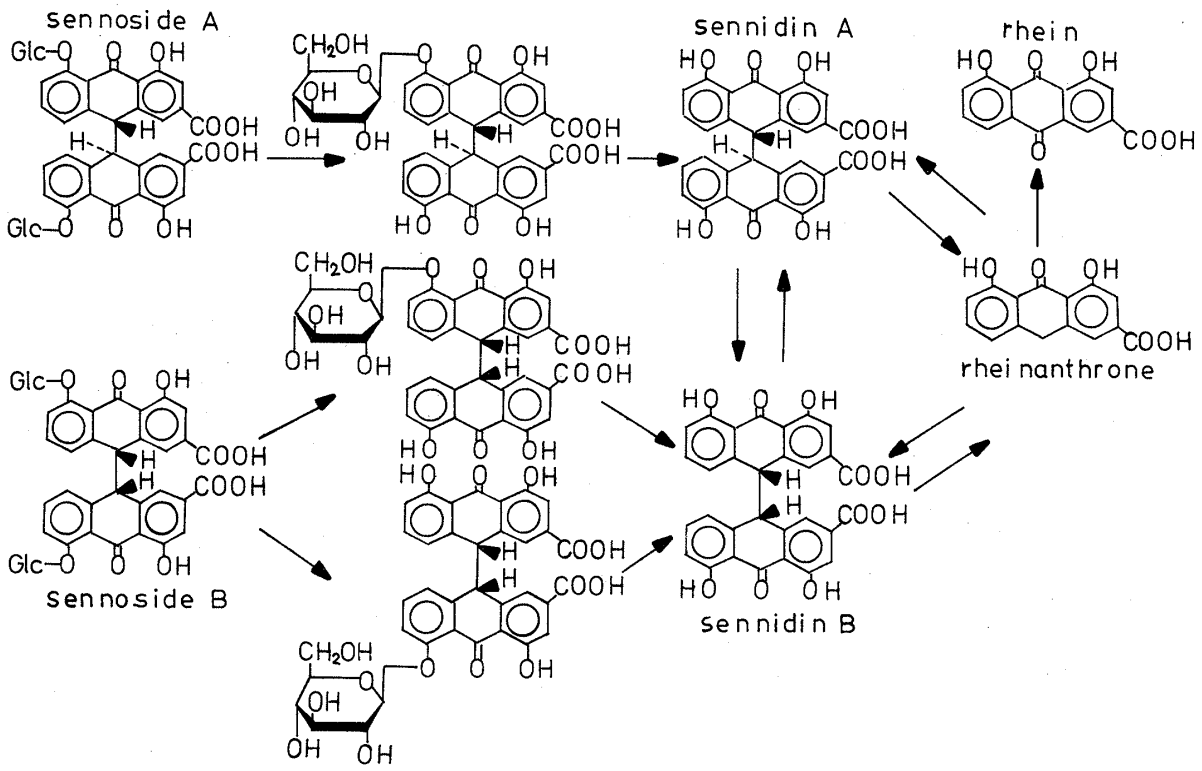

Chart 1. Metabolic Pathways of Sennosides by Intestinal Bacteria

via rheinanthrone due to the enzymic action of intestinal bacteria and subsequent oxidation. However, the isomerization reaction still proceeded in the heat-treated feces suspension or its supernatant fluid, in which the conversion of sennidins to rheinanthrone was negligible, suggesting that a different mechanism takes part in the reaction. Sennidins were then further converted to rheinanthrone, which has a strong purgative effect on direct application to the colon[17,18] and is easily oxidized to rhein and sennidins. The reaction was catalyzed by the action of intestinal bacteria presented in rat feces. The dialyzed or sonicated suspension still had activity for the conversion of sennidins to rheinanthrone, but the supernatant fluid of feces suspension and the heated suspension had no appreciable activity. This suggests that the reaction could be catalyzed by membrane-bound enzymes. Cultivation of intestinal bacterial strains isolated from human feces in media containing sennidin A showed that several species of bacteria markedly stimulated the conversion of sennidin A to rheinanthrone. These bacteria may produce a reductase and play a role in the formation of the purgative principle.

In summary, Chart 1 shows the metabolic processes occurring upon incubation of sennosides and sennidins with rat feces. The metabolic process due to intestinal bacteria in the contents of the gastrointestinal tract and feces of the rat differ from that deduced from the metabolites of individual bacterial strains derived from the human intestine.[2]

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References and Notes

1) A part of this study was presented at the annual meeting of the Japanese Society of Pharmacognosy (Tokyo, 1980).
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5) I. Miwa, J. Okuda, K. Maeda, and G. Okuda, *Clin. Chim. Acta*, **37**, 538 (1972).
6) Under the same conditions, sennoside A, sennoside B, sennidin A and sennidin B had *Rf* values of 0.28, 0.12, 0.50 and 0.55, respectively, on TLC.
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8) Under the same conditions, sennidin A-8-monoglucoside showed an *Rf* value of 0.43 on TLC. A mixture of sennidin A-8-monoglucoside, sennidin B-8-monoglucoside and sennidin B-8′-monoglucoside gave three distinguishable spots on TLC.
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