Utilization of Orally Administered D-[¹⁴C]Mannitol via Fermentation by Intestinal Microbes in Rats

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Summary To investigate the available energy of orally administered [¹⁴C]mannitol via intestinal microbes, [¹⁴C]mannitol (222 kBq, 105 mg) or [¹⁴C]glucose (222 kBq, 105 mg) was administered to conventional rats and antibiotics-treated rats whose intestinal microbes were depleted by drinking water containing antibiotics, respectively. The exhausted CO₂, feces and urine were then separately collected at 2, 4, 6, 8, 10, 12 and 24 h after administration of the test solution. In the conventional rats, 45% of administered radioactivity was recovered as ¹⁴CO₂ in the administration of [¹⁴C]mannitol, while 57% of administered radioactivity was recovered as ¹⁴CO₂ following the administration of [¹⁴C]glucose for 24 h. The time sequence for the ¹⁴CO₂ excretion from [¹⁴C]mannitol was delayed as compared to [¹⁴C]glucose by about 4–6 h (*p* < 0.05). However, when [¹⁴C]mannitol was orally administered to antibiotics-treated rats, only 3% of administered radioactivity was excreted as ¹⁴CO₂ for 24 h. The total radioactivity of the gastrointestinal contents and feces for 24 h after administration was over 70%, much higher than those of the conventional rats (*p* < 0.05). When a half dose (222 kBq, 52.5 mg) of [¹⁴C]mannitol was administered to conventional rats, the recovery as ¹⁴CO₂ for 24 h (%) was significantly higher than that of a regular dose of [¹⁴C]mannitol (105 mg). When cold mannitol (105 mg) was orally administered to the antibiotics-treated rats, about 9% of intact mannitol was excreted in feces within 48 h after administration. However, no intact mannitol was detected in the conventional rats. These results demonstrate that more than 95% of mannitol administered orally is utilized via fermentation by intestinal microbes.

Key Words mannitol, antibiotics, intestinal microbes, fermentation, rats

D-Mannitol (mannitol) is a hexitol (C₆H₁₄O₆; molecular weight, 182.17) that distributes widely in a variety of plants, seaweeds and fungi (1). Mannitol has been used as a sugar substitute in processed foods, as a diuretic medicine by parenteral administration (2–4) and in an intestinal permeability test to assess intestinal mucosal barrier function (5–8).

Previous studies have demonstrated that about half of administered mannitol is converted to CO₂ (9, 10); in spite of being orally administered, hardly any mannitol is absorbed (11, 12). In addition, the metabolic pathway of the production of ¹⁴CO₂ had not been clarified until the present study. Dwivedi has speculated that mannitol which is not absorbed from the small intestine might be fermented by intestinal microbes, and that the short-chain fatty acids (SCFAs) produced might be utilized by the host (1). Recently, we clarified that the organic acids in cecal contents were increased by feeding of a mannitol-containing diet to conventional rats (13). Nishiyama et al. also reported similar results (14). However, it has not been clarified how intestinal

microbes convert orally administered mannitol into CO₂.

In previous reports, it was demonstrated that 77–97% of the administered radioactivity was excreted into the urine within 24 h, and only 2–3% of the radioactivity was oxidized to ¹⁴CO₂ in the intraperitoneal injection of [¹⁴C]mannitol in rats (11). These results suggest that mannitol absorbed from the small intestine is not metabolized to produce energy. On the other hand, when 240 mg of [¹⁴C]mannitol was orally given to non-fasted conventional rats, 56% of the administered radioactivity was recovered in ¹⁴CO₂ within 24 h (11). In addition, 40% of the radioactivity was emitted as ¹⁴CO₂ within 24 h in the oral administration of 500 mg of [¹⁴C]mannitol per kg of body weight in fasted conventional rats (9). Although these findings demonstrate that ingested mannitol was metabolized to ¹⁴CO₂, they did not demonstrate the mechanism or the metabolic pathway of ¹⁴CO₂ production. Furthermore, the oral administration of mannitol to fasted rhesus monkeys (15) and the feeding of mannitol-containing diets to rats (16) and dogs (17) led to a small increase of liver glycogen (15–17). These results indicate that mannitol is not utilized as an energy source by the absorption in

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the small intestine. However, the fermentation and utilization of mannitol via intestinal microbes remains ambiguous.

Sugar alcohols such as maltitol or sorbitol, which are not digested or absorbed in the small intestine, are fermented by intestinal microbes and converted to SCFAs, CO₂, H₂ and CH₄. Most of the SCFAs produced are absorbed from the lower intestine and utilized as energy by the host (18–21). We previously clarified that the cecal contents of rats fed a mannitol-containing diet showed a remarkable increase of organic acids (13). This suggests that orally ingested mannitol may be metabolized via fermentation by intestinal microbes. In addition, the decrease of pH value in cecal content and increments of cecal and colon weight were observed in mannitol-fed rats (14, 22). However, there was no evidence in terms of the degree of fermentation and utilization of mannitol via intestinal microbes in *in vivo* experiments.

Oku and Tokunaga et al. clarified that fructooligosaccharide, which is a nondigestible sugar substitute, was metabolized into CO₂ via fermentation by intestinal microbes in experiments in which the [¹⁴C]fructooligosaccharide was orally administered to conventional, antibiotics-treated and germ-free rats and the ¹⁴CO₂ excretion was time-dependently observed (23, 24). Rats given antibiotics-containing drinking water were used as the intestinal microbes-depleted rats. Although the conversion of [¹⁴C]fructooligosaccharide to ¹⁴CO₂ was negligible in antibiotics-treated rats and germ-free rats, about 54.6% of the total radioactivity administered was exhaled as ¹⁴CO₂ within 24 h in conventional rats after the oral administration of [¹⁴C]fructooligosaccharide. These results demonstrate that fructooligosaccharide, which is not digested in the small intestine, is metabolized via fermentation by intestinal microbes (23, 24). In the present study, we investigated the utilization of mannitol via fermentation by intestinal microbes and the degree of fermentation using the same methods as were used with fructooligosaccharide.

The purpose of this study is to directly confirm that mannitol is metabolized to CO₂ via fermentation by intestinal microbes. The degree of fermentation was estimated by the administration of [¹⁴C]mannitol to conventional and antibiotics-treated rats. Although mannitol is clinically used via oral administration as a mucosal permeability marker in the small intestine, the fermentability and available energy of mannitol via intestinal microbes remains unclear. It is essential to estimate the degree of fermentation by orally administered mannitol and to evaluate energy efficiency for safe uses. Furthermore it could contribute to develop the utilization of mannitol. Clarifying the metabolism of orally administered mannitol can contribute to new applications for human health and the treatment of diseases.

MATERIALS AND METHODS

1. Materials. D-Mannitol (Marinecrystal®, purity >99%), which was kindly provided by Mitsubishi Shoji

Table 1. The dose of test solutions orally administered to rats in [¹⁴C]mannitol tracer experiment.

Treatment of rat	Conventional		Antibiotics
	[¹⁴ C]Glucose	[¹⁴ C]Mannitol	[¹⁴ C]Mannitol
Glucose (mg)	105.0	—	—
Mannitol (mg)	—	105.0	52.5
			105.0

Each test material was dissolved in 1.5 mL of sterile water with 222 kBq of respective radioactive compounds. Dose level of 105 mg of mannitol was the maximum permissive dose which did not cause osmotic diarrhea in rats.

Foodtech Co., Ltd. (Tokyo, Japan), mannitol, D-[¹⁴C] (specific activity 1.85 GBq/mmol, American Radiolabeled Chemicals Inc., USA), and glucose, D-[¹⁴C(U)] (specific activity 74 MBq/mmol, American Radiolabeled Chemicals Inc.) were used.

2. Animals and diets. Twenty male Wistar rats (8 wk old, 150 g, CLEA Japan, Inc., Tokyo, Japan) were housed in air-conditioned cages (room temperature, 23±1°C; humidity, 50±10%) and maintained on a 12 h light-dark cycle (light, 08:00–20:00; dark, 20:00–08:00). They were raised on a standard solid diet (MF®, Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum* for 3 d to adapt before being used in the administration experiment and then fed a AIN 93-M cellulose-free diet (cellulose was replaced with β -cornstarch). For depletion of intestinal microbes, 5 rats were given the sterilized water containing 50 units/mL of benzylpenicillin potassium (23, 24) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2.0 mg/mL of neomycin sulfate (25, 26) (Wako) and 0.5 mg/mL of cefoperazone sodium salt (27, 28) (Wako) *ad libitum* for 10 d before the oral administration of [¹⁴C]mannitol experiment. The remaining 15 rats were given distilled water *ad libitum*.

Twelve male Wistar rats (8 wk old, 150 g, CLEA Japan) were fed a standard solid diet *ad libitum* for 3 d and randomly assigned to 2 groups. Six rats were given sterilized water containing the same dose of antibiotics as mentioned above, and an AIN 93-M cellulose-free diet fed *ad libitum* for 10 d before the oral administration of mannitol experiment. Six rats were given the distilled water without antibiotics and the same diet *ad libitum*.

3. Preparation of test solutions. Table 1 shows the amount of [¹⁴C]mannitol and its radioactivity in the test solutions of oral administration. Based on our preliminary experiments, 105 mg of mannitol was used as the maximum permissive dose which did not cause transitory osmotic diarrhea in rats, and was the appropriate dose level to measure the radioactivity of feces, urine, tissues and organs using a scintillation counter, as well as to analyze the intact mannitol in feces and urine by HPLC.

To clarify the utilizable capacity of [¹⁴C]mannitol via intestinal microbes, 222 kBq of [¹⁴C]mannitol was dissolved in 1.5 mL of sterilized water containing 52.5 mg

Fig. 1. Diagram of Metabolica apparatus. To clarify the metabolism of $[^{14}\text{C}]$ mannitol via intestinal microbes, 5 conventional rats were orally administered 222 kBq $[^{14}\text{C}]$ mannitol and 105 mg of mannitol/1.5 mL; 222 kBq of $[^{14}\text{C}]$ mannitol and 52.5 mg of mannitol/1.5 mL; and 222 kBq of $[^{14}\text{C}]$ glucose and 105 mg of glucose/1.5 mL. Five antibiotics-treated rats were administered 222 kBq of $[^{14}\text{C}]$ mannitol and 105 mg of mannitol/1.5 mL. Immediately after the administration by a stomach sonde, each rat was transferred to the Metabolica apparatus and observed for 24 h. The expired $^{14}\text{CO}_2$ was trapped with 500 mL of monoethanolamine, and sampling (2 mL) was carried out at 2, 4, 6, 8, 10, 12 and 24 h after the administration. Feces and urine were collected at the same time. KOH and soda lime removed CO_2 in air, and H_2SO_4 and silica gel removed H_2O .

or 105 mg of mannitol. As the control, 222 kBq of $[^{14}\text{C}]$ glucose was dissolved in 1.5 mL of sterilized water containing 105 mg of glucose.

4. *Oral administration of $[^{14}\text{C}]$ mannitol to measure the $[^{14}\text{C}]$ distribution into carbon dioxide, gastrointestinal contents, feces, urine, tissues and organs in conventional and antibiotics-treated rats.* To measure the radioactivity from orally administered $[^{14}\text{C}]$ mannitol into $^{14}\text{CO}_2$, the contents of the gastrointestinal tracts, feces, urine, tissues and organs, the rats were fasted for 15 h before the administration of the test solutions and supplied drinking water only. Five conventional rats were each administered the test solution containing 222 kBq (105 mg) of $[^{14}\text{C}]$ mannitol or $[^{14}\text{C}]$ glucose using a stomach sonde. In order to estimate the metabolism of mannitol via fermentation by intestinal microbes, 5 antibiotics-treated rats were administered 222 kBq (105 mg) of $[^{14}\text{C}]$ mannitol. Furthermore, to estimate the dose response of $^{14}\text{CO}_2$ exhalation, the utilizable capacity of $[^{14}\text{C}]$ mannitol via intestinal microbes and the incorporation of radioactivity into organs and tissues, 222 kBq of $[^{14}\text{C}]$ mannitol (52.5 mg) was administered to 5 conventional rats.

5. *Collection and preparation of expired CO_2 , feces, urine, contents of gastrointestinal tract, organs and tissues, and determination of the radioactivity.* Immediately after $[^{14}\text{C}]$ mannitol or $[^{14}\text{C}]$ glucose was administered to each rat, the rat was transferred to a glass metabolic apparatus (Fig. 1) (Metabolica CO-2, Sugiyamagen Co. Ltd., Tokyo, Japan) with an interior temperature maintained at $23 \pm 1^\circ\text{C}$, and $^{14}\text{CO}_2$, urine and feces were collected

for 24 h. The rats transferred to the glass metabolic apparatus were fasted for a further 6 h after the administration of the test solution and given only drinking water. The expired $^{14}\text{CO}_2$ was trapped with 500 mL of monoethanolamine (Wako) and 2 mL of monoethanolamine was removed to measure the radioactivity at 2, 4, 6, 8, 10, 12 and 24 h after the administration. After the exhausted $^{14}\text{CO}_2$ was trapped (absorbed) into monoethanolamine for 12 h after administration, it was changed to a new glass container with 500 mL of monoethanolamine, and $^{14}\text{CO}_2$ collection was continued for a further 12 h.

Feces and urine were separately collected in bottles fixed to the metabolic apparatus, and these bottles were changed for new bottles at 2, 4, 6, 8, 10, 12 and 24 h after administration. The rats were killed at 24 h after administration of radioactive material, and the blood, contents of stomach and small intestine, cecum and colon, liver, kidneys, gastrocnemius muscle of the right leg, perirenal and epididymal adipose tissues and small intestinal mucosa were collected to measure the radioactivity incorporated. Serum was obtained by centrifugation at $2,500 \times g$ for 15 min at 20°C (High Speed Refrigerated Centrifuge 6900, Kubota Co., Tokyo, Japan).

Five hundred microliters of monoethanolamine containing $^{14}\text{CO}_2$ was taken to a counting vial, and the radioactivity was determined with a liquid scintillation counter (LSC-6000, Aloca Co., Ltd., Tokyo, Japan) after the addition of 5 mL of scintillation cocktail (Hionic-Fluor, Perkin Elmer Japan, Kanagawa, Japan) and 1 mL

of Cellosolve (Wako). The tissues, organs, contents of stomach, small intestine, cecum and colon, and feces were homogenized using a polytron homogenizer (Kinematica Co., Ltd., Swiss) in 9 volumes of 0.9% NaCl solution. Three hundred microliters of homogenates or serum were taken to a counting vial and solubilized with 0.5 mL of solubilizer (Soluen 350, Perkin Elmer Japan) at 50°C for 12 h. The feces and contents of the cecum and colon were decolorized by the addition of 0.3 mL of 30% of hydrogen peroxide (Wako). Five milliliters of scintillation cocktail (Pico-fluor 40, Perkin Elmer Japan) was added to the solubilized homogenates and urine in the counting vial, and the radioactivity was determined with a scintillation counter. The counting efficiency was approximately 90%.

6. *Measurement of intact mannitol in feces and urine of conventional and antibiotics-treated rats using HPLC.* After 15 h fasting, 6 conventional and 6 antibiotics-treated rats (B.W. 250 g) were orally administered 105 mg of mannitol dissolved in 1.5 mL of sterilized water using a stomach sonde. Immediately after administration, each rat was transferred to a stainless steel metabolic apparatus, and the feces and urine were separately collected at 6, 12, 24, 36 and 48 h after administration.

The feces collected were weighed and homogenized in 9 volumes of 0.9% NaCl solution using a polytron homogenizer (Kinematica Co., Ltd.), and the homogenate was heated at 100°C for 7 min to inactivate the intestinal microbes and enzymes. After the homogenate was centrifuged by a high-speed refrigerated micro centrifuge (MX-160, Tomy Seiko Co., Ltd., Tokyo, Japan) at $8,500 \times g$ for 15 min at 20°C, the supernatant was filtered with a membrane filter (0.22 μm , Millex-GV, Millipore Co., USA) and used for HPLC analysis to measure the mannitol concentration. The total volume of the urine collected was measured and the urine was then heated for 5 min at 100°C and used for HPLC analysis after filtration. The samples prepared were stored at -20°C until HPLC analysis.

7. *Conditions of HPLC for mannitol analysis.* The amount of intact mannitol in the feces and urine were analyzed by an HPLC system (SCL-10A, Shimadzu Corp., Kyoto, Japan) with a refractive index detector (RID-10A, Shimadzu Corp.), using an Asahipak NH₂P50-4E column (4.6 mm I.D. $\times$ 250 mm L., Showa Denko Co., Ltd., Tokyo, Japan). The analysis was performed at a constant temperature of 40°C. The sample was eluted with CH₃CN : H₂O = 75 : 25 at a flow rate of 0.6 mL/min and the injection volume of the sample was 10 μL .

8. *Calculation and statistical analysis.* The percentage of radioactivity recovered in the ¹⁴CO₂, tissues, organs and contents of the gastrointestinal tracts versus oral administered radioactivity were calculated.

Data were calculated as means and standard deviations (SD). After the normality test, the data were analyzed by Student's *t*-test or one-way ANOVA and Dunnett's post hoc test was used to evaluate significant differences among groups. A *p*-value of less than 0.05

Fig. 2. Comparison of the time course of expired ¹⁴CO₂ between conventional rats and antibiotics rats for 24 h after oral administration of [¹⁴C]mannitol. Values were mean $\pm$ SD (*n* = 5). —●—, [¹⁴C]glucose (conventional); —■—, [¹⁴C]mannitol (conventional); —◆—, [¹⁴C]mannitol (antibiotics-treated). [¹⁴C]Mannitol or [¹⁴C]glucose was orally administered to conventional or antibiotics-treated rats. a–n: Significant differences were evaluated by the ANOVA and values with the same superscripts were significantly different at *p* < 0.05 by Dunnett's post hoc test.

was considered significant. The statistical analysis was performed using SPSS version 16.0 for Windows, Japan (SPSS Inc., Tokyo, Japan) (29).

9. *Ethics.* The study protocol was approved by the respective committee on Animal Experiments of the University of Nagasaki, Siebold. The rat experiment was performed according to the guidelines on the care and use of laboratory animals of the University of Nagasaki, Siebold and the standards relating to the care and management of experimental animals (Notification No. 88, April 28, 2006 from the Prime Minister's office).

RESULTS

1. Conversion of [¹⁴C]mannitol into ¹⁴CO₂ and radioactivity of [¹⁴C]mannitol remaining in gastrointestinal contents in conventional rats

In Fig. 2, when [¹⁴C]mannitol solution (222 kBq, 105 mg) was administered to a conventional rat, 44.8% of the administered radioactivity was excreted as ¹⁴CO₂ for 24 h. In contrast, 56.5% of the administered radioactivity was excreted as ¹⁴CO₂ for 24 h after administration of [¹⁴C]glucose (222 kBq, 105 mg). The radioactivity excreted as ¹⁴CO₂ within 24 h was significantly lower in [¹⁴C]mannitol-administered rats than in [¹⁴C]glucose administration (*p* < 0.05). In addition, the elimination of ¹⁴CO₂ at each time point for 24 h after administration was significantly less in the [¹⁴C]mannitol-administered rats than in [¹⁴C]glucose-administered rats (*p* < 0.05).

Figure 3 shows that ¹⁴CO₂ excretion in the [¹⁴C]mannitol-administered rats started at around 2 h after administration. The peak time of the ¹⁴CO₂ excretion

Fig. 3. Comparison of the time course of expired $^{14}\text{CO}_2$ between administration of [^{14}C]glucose and [^{14}C]mannitol in conventional rats. Values were mean $\pm$ SD ($n=5$). —●—, [^{14}C]glucose (conventional); —■—, [^{14}C]mannitol (conventional). [^{14}C]Mannitol was orally administered to conventional or antibiotics-treated rats. a–e: Significant differences were evaluated by Student's t -test and values with the same superscripts were significantly different at $p<0.05$.

from [^{14}C]mannitol was delayed as compared to [^{14}C]glucose by about 4–6 h.

Figure 4 shows the percentage of radioactivity versus administered amounts in the gastrointestinal contents and feces. When [^{14}C]mannitol solution (222 kBq, 105 mg) was administered to a conventional rat, the percentage of radioactivity excreted into feces within 24 h was 6.1%, and excretion by the administration of [^{14}C]glucose was 5.4% of radioactivity. The percentages were not significantly different. However, the percentage of radioactivity of the contents of the gastrointestinal tracts in antibiotics-treated rats was significantly higher in conventional rats when [^{14}C]mannitol was administered ($p<0.05$). The percentage of radioactivity excreted to the urine within 24 h was 6.3% for [^{14}C]glucose and 6.1% for [^{14}C]mannitol (Table 2).

2. Comparison of conversion from [^{14}C]mannitol into $^{14}\text{CO}_2$ and remaining radioactivity in gastrointestinal contents between conventional rats and antibiotics-treated rats

In the antibiotics-treated rats, the radioactivity expired as $^{14}\text{CO}_2$ was only 3.3% in 24 h (Fig. 2) and the conversion of [^{14}C]mannitol into $^{14}\text{CO}_2$ was remarkably disturbed by the depletion of intestinal microbes with antibiotics treatment. In contrast, 44.8% of the radioactivity of [^{14}C]mannitol administered orally was expired as $^{14}\text{CO}_2$ in conventional rats in 24 h (Fig. 4). Furthermore, the elimination of $^{14}\text{CO}_2$ from [^{14}C]mannitol at each time point was significantly lower in the antibiotics-treated rats than in the conventional rats ($p<0.05$). The radioactivity recovered in the contents of gastrointestinal tracts is shown in Fig. 4. They were calculated on three sections as follows: stomach, and small intestine; cecum, colon and rectum; feces. They were more than 7-times higher in the antibiotics-treated rats than in the conventional rats administered [^{14}C]mannitol ($p<0.05$) (Fig. 4).

Fig. 4. Radioactivity in gastrointestinal contents and feces at 24 h after oral administration of [^{14}C]mannitol in conventional rats and antibiotics-treated rats. Values were mean $\pm$ SD ($n=5$). □, Contents of stomach and small intestine; ▒, contents of cecum and colon; ■, contents of rectum and feces. [^{14}C]Mannitol was orally administered to 5 conventional rats and 5 antibiotics-treated rats, and [^{14}C]glucose was administered to 5 conventional rats. a, b: Significant differences were evaluated by one-way ANOVA and values of contents of stomach, small intestine, cecum colon and rectum and feces with the same superscripts were significantly different at $p<0.05$ by Dunnett's post hoc test.

3. Incorporation of radioactivity into tissues and organs after oral administration of [^{14}C]mannitol to conventional and antibiotics-treated rats

The radioactivities incorporated into the liver, kidneys, and perirenal and epididymal adipose tissues were significantly higher in conventional rats administered [^{14}C]mannitol than in conventional rats administered [^{14}C]glucose (Table 2) ($p<0.05$). However, the radioactivities incorporated into the organs and tissues of antibiotics-treated rats were significantly less than those in the conventional rats administered [^{14}C]mannitol (Table 2) ($p<0.05$).

4. Comparison of the time course of expired $^{14}\text{CO}_2$ in conventional rats for 24 h after oral administration of 52.5 and 105 mg [^{14}C]mannitol

To investigate the utilizable capacity of [^{14}C]mannitol via intestinal microbes, [^{14}C]mannitol solutions (222 kBq, 105 mg) and (222 kBq, 52.5 mg) were prepared and administered to conventional rats. Figure 5 shows that the conversion of [^{14}C]mannitol to $^{14}\text{CO}_2$ and incorporation of radioactivity into organs and tissues were compared between 52.5 mg and 105 mg of the administration.

The cumulative conversion percentages of [^{14}C]mannitol to $^{14}\text{CO}_2$ by the administration of 105 mg and 52.5 mg of [^{14}C]mannitol were not significantly different until 2 and 4 h after administration. However the percentage was significantly higher in the 52.5 mg of [^{14}C]mannitol than in the 105 mg of [^{14}C]mannitol administration at each time point of 6, 8, 10, 12 and 24 h after administration ($p<0.05$). The excretion of $^{14}\text{CO}_2$ for 24 h after the administration of [^{14}C]mannitol was 54.8% of 52.5 mg of [^{14}C]mannitol and 44.8% of

Table 2. Incorporation percentages of radioactivity into tissues and organs at 24 h after oral administration of [^{14}C]mannitol.

Treatment	Conventional		Antibiotics
	[^{14}C]Glucose	[^{14}C]Mannitol	[^{14}C]Mannitol
Tissue distribution (% of dose/g wet weight)			
Liver	0.19 $\pm$ 0.04 ^a	0.39 $\pm$ 0.07 ^{a,b}	0.03 $\pm$ 0.01 ^b
Gastrocnemius muscle	0.10 $\pm$ 0.02	0.08 $\pm$ 0.01 ^c	0.01 $\pm$ 0.00 ^c
Perirenal and epididymal adipose tissue	0.10 $\pm$ 0.01 ^d	0.31 $\pm$ 0.01 ^{d,e}	0.01 $\pm$ 0.00 ^e
Kidneys	0.13 $\pm$ 0.01 ^f	0.16 $\pm$ 0.02 ^{f,g}	0.03 $\pm$ 0.01 ^g
Small intestinal mucosa	0.53 $\pm$ 0.05	0.65 $\pm$ 0.23	0.53 $\pm$ 0.16
Serum	0.01 $\pm$ 0.01	0.01 $\pm$ 0.00 ^h	0.00 $\pm$ 0.00 ^h
Urinary elimination (% of dose)	6.30 $\pm$ 0.71	6.10 $\pm$ 1.17 ⁱ	1.10 $\pm$ 0.46 ⁱ

Values were means $\pm$ SD ($n=5$).

[^{14}C]Mannitol or [^{14}C]Glucose (222 kBq, 105 mg) were orally administered to conventional or antibiotics-treated rats.

^{a-i}Significant differences were evaluated by one-way ANOVA and values with the same superscripts were significantly different at $p<0.05$ by Dunnett's post hoc test.

Fig. 5. Comparison of the time course of expired $^{14}\text{CO}_2$ in conventional rats for 24 h after oral administration of 52.5 and 105 mg [^{14}C]mannitol. Values were mean $\pm$ SD ($n=5$). —●—, [^{14}C]glucose (conventional); —■—, [^{14}C]mannitol 105 mg (conventional); —▲—, [^{14}C]mannitol 52.5 mg (conventional). [^{14}C]Mannitol solutions containing 105 mg or 52.5 mg of mannitol was orally administered to conventional rats, respectively. a–e: Significant differences were evaluated by ANOVA and values with the same superscripts were significantly different at $p<0.05$ by Dunnett's post hoc test.

105 mg of [^{14}C]mannitol, respectively. The radioactivities incorporated into the liver, kidneys, serum, gastrocnemius muscle, perirenal and epididymal adipose tissues, and small intestinal mucosa were significantly higher in the 105 mg of [^{14}C]mannitol administration than in the 52.5 mg of [^{14}C]mannitol administration ($p<0.05$, data not shown).

These results indicate that when the capacity of fermentation in intestinal microbes is enough to metabolize mannitol, mannitol is quickly fermented, the organic acids are produced, absorbed from the large

Fig. 6. Cumulative excretion of intact mannitol excretion into feces in conventional rats and antibiotics-treated rats. Values were mean $\pm$ SD ($n=6$). ▨, 0–6 h; ▩, 6–12 h; ▤, 12–24 h; ▥, 24–36 h; □, 36–48 h. Six conventional or antibiotics-treated rats were administered 105 mg of mannitol solution orally. Feces were separately collected at 6, 12, 24, 36 and 48 h, and the mannitol contents were analyzed by HPLC. a: Significant differences were evaluated by Student's t -test and values with the same superscripts were significantly different at $p<0.05$.

intestine, and incorporated into the tissues.

5. Excretion of intact mannitol into feces and urine after oral administration of cold mannitol to conventional rats and antibiotics-treated rats

In order to confirm the fecal and urinary excretion of intact mannitol, 105 mg of cold mannitol was orally administered to both conventional and antibiotics-treated rats, and the feces and urine were collected for 48 h after administration. The difference in the radioactivity recovered in feces between conventional and antibiotics-treated rats is shown in Fig. 6. Nine percent of the intact mannitol was excreted to feces within 48 h in the antibiotics-treated rats, while no intact mannitol was detected in the feces of conventional rats after the administration of 105 mg of mannitol. The difference between the conventional rats and antibiotics-treated

rats was significant ($p < 0.05$). These results strongly suggest that orally administered mannitol is decomposed spontaneously by intestinal microbes.

In conventional rats, 1.2% of the administered mannitol was excreted to urine in 48 h, and 1.4% of mannitol was detected in the urine of antibiotics-treated rats. The excretion of mannitol to urine was not significantly different between the conventional and antibiotics-treated rats.

DISCUSSION

When [^{14}C]mannitol (105 mg) was orally administered to conventional rats, about 45% of the radioactivity administered was expired as $^{14}\text{CO}_2$, and the time sequence for $^{14}\text{CO}_2$ excretion was delayed as compared to [^{14}C]glucose by about 4–6 h. Oku and Tokunaga et al. demonstrated that a time lag of about 4 h in $^{14}\text{CO}_2$ excretion occurred between the two groups when [^{14}C]sucrose, which is readily digested, and [^{14}C]fructooligosaccharide, which is not digested by intestinal enzymes, were administered separately to conventional rats (23, 24). Therefore, the time lag of 4–6 h of $^{14}\text{CO}_2$ excretion in [^{14}C]mannitol administration to conventional rats is considered to be the period when [^{14}C]mannitol administered orally arrives at the cecum where intestinal microbes live. [^{14}C]Mannitol which arrived at the cecum might be rapidly metabolized by intestinal microbes and metabolized to SCFAs. Finally, SCFAs were metabolized by the host as available energy, because [^{14}C]SCFAs which were directly injected to the cecum of conventional rats were quickly metabolized to $^{14}\text{CO}_2$ (23, 24). When [^{14}C]mannitol was administered to antibiotics-treated rats, only 3.3% of the radioactivity administered was expired as $^{14}\text{CO}_2$ in 24 h; in contrast, 45% of the radioactivity administered was expired as $^{14}\text{CO}_2$ in conventional rats. Additionally, the radioactivity remaining in the gastrointestinal tracts of antibiotics-treated rats was 7 times higher than that in conventional rats. Orally administered [^{14}C]mannitol was incorporated dose-dependently in the liver, gastrocnemius muscle and perirenal and epididymal adipose tissues in conventional rats, while the incorporation of the radioactivity in antibiotics-treated rats was markedly lower than that in conventional rats ($p < 0.05$). Furthermore, when 105 mg of cold mannitol was administered to conventional rats, intact mannitol was not detected in the feces. These results strongly demonstrate that orally administered mannitol is transferred to the large intestine and is readily fermented by intestinal microbes. It is possible that orally administered mannitol was finally utilized by the host, because organic acids markedly increased in cecal content after mannitol feeding in rats (13). It is considered that orally administered mannitol that reaches to the lower intestine might not be metabolized to SCFAs in antibiotics-treated rats whose intestinal microbes were depleted by antibiotics.

In the present study, 105 mg of [^{14}C]mannitol was administered to fasted conventional rats and about 45% of the radioactivity administered was expired as $^{14}\text{CO}_2$

within 24 h (Fig. 2); in contrast, about 55% of the radioactivity administered was excreted to $^{14}\text{CO}_2$ in 24 h in the administration of 52.5 mg of [^{14}C]mannitol to conventional rats (Fig. 5). The total of $^{14}\text{CO}_2$ excretion for 24 h depends on the dose level in [^{14}C]mannitol administered rats.

However, although the expiration of $^{14}\text{CO}_2$ continued for 24 h in the administration of the larger amount (105 mg) of [^{14}C]mannitol, the expiration of $^{14}\text{CO}_2$ reached a plateau at 10 h after the administration of the smaller amount (52.5 mg) of [^{14}C]mannitol. The [^{14}C]radioactivities incorporated into the organs and tissues were higher in the administration of 105 mg (500 mg/kg B.W.) of [^{14}C]mannitol than in that of 52.5 mg (250 mg/kg B.W.) of [^{14}C]mannitol (data not shown). We have investigated the dose which did not cause osmotic diarrhea in rats and the maximum permissive dose was 500 mg/kg B.W. in rats (data not shown). We considered this to be the maximum amount of orally administered mannitol which intestinal microbes can completely metabolize to SCFAs, carbon dioxide, hydrogen, methane and others via fermentation in rats (30–33). When 52.5 mg of mannitol is administered, it is rapidly fermented by intestinal microbes and quickly utilized as energy by the host.

We previously clarified that rats fed a mannitol-containing diet showed a remarkable increase in the SCFAs of the cecal contents (13). In addition, Nishiyama et al. reported a similar increase of organic acids of the cecal contents in mannitol-fed rats (14). Furthermore, hardly any $^{14}\text{CO}_2$ was excreted following the administration of [^{14}C]mannitol to antibiotics-treated rats (Fig. 2). Similar results were obtained in the experiments using [^{14}C]fructooligosaccharide (23, 24). When [^{14}C]fructooligosaccharide (74 kBq, 4 mg) was orally administered to conventional rats, about 55% of the radioactivity was expired as $^{14}\text{CO}_2$. However, when [^{14}C]fructooligosaccharide was administered to antibiotics-treated rats or germfree rats, the excretion of $^{14}\text{CO}_2$ was negligible (23, 24). The results obtained in the present study strongly demonstrate that orally ingested mannitol, which is hardly absorbed from the small intestine, is finally metabolized to CO_2 via intestinal microbes and utilized as an energy source in the host (9, 10).

When [^{14}C]glucose (222 kBq, 105 mg) was administered to conventional rats, 56.5% of the radioactivity administered was excreted as $^{14}\text{CO}_2$ and 5.4% of the radioactivity was excreted into feces within 24 h. Furthermore, 1.8% of radioactivity remained in the contents of the gastrointestinal tracts, and 6.3% of radioactivity was excreted to the urine in 24 h. Glucose administered orally is quickly absorbed from the small intestine and readily metabolized by the body. Therefore, the radioactivity excreted to urine appears to be derived from the metabolites of glucose absorbed from the small intestine, and the radioactivity of the contents of the gastrointestinal tracts is derived from the metabolites of glucose which are absorbed and excreted to the small intestine with the digestive fluid. On the other hand, when [^{14}C]mannitol was orally administered to

conventional rats, 6.1% of radioactivity was excreted to the urine, 6.1% was excreted into the feces within 24 h, and 6.4% of radioactivity remained in the contents of the gastrointestinal tracts. The radioactivity excreted to the urine (6.1%) might be derived from the metabolites of SCFAs produced from [^{14}C]mannitol by fermentation, because the excretion of [^{14}C] radioactivity was decreased by the antibiotics treatment (1.1%). The radioactivities of feces and contents of the gastrointestinal tract may be the result of SCFAs produced from mannitol being absorbed from the large intestine and further metabolized by some organs, and these metabolites being secreted with digestive fluid. In fact, intact mannitol was not detected in the feces of conventional rats. Almost all of the orally administered mannitol is transferred to the lower intestine, and is completely fermented by intestinal microbes. A small amount might be excreted as the components of the microbes. It was thus estimated that more than 95% of mannitol administered orally was fermented by intestinal microbes.

The fermentation of nondigestible and/or nonabsorbable sugar substitutes by intestinal microbes produces SCFAs, CO_2 , CH_4 and H_2 (19–21). The SCFAs are absorbed from the large intestine and further metabolized by the host to produce energy (19–21). This energy value is considered to be less than 50% of the energy coefficient of sucrose (4 kcal/g). Then the available energy is 2 kcal/g when nondigestible/nonabsorbable sugar substitutes are completely fermented by intestinal microbes. Therefore, the available energy of mannitol is considered to be 2 kcal/g, because it is mostly fermented by intestinal microbes.

In conclusion, it was directly demonstrated in the administration of [^{14}C]mannitol to the conventional rats and antibiotics-treated rats that mannitol administered orally was mainly metabolized and utilized via fermentation by intestinal microbes, and the utilizable percentage was estimated to be more than 95% in conventional rats. As in rats, mannitol in humans appears to be utilized via intestinal microbes. In addition, the energy coefficient of mannitol was estimated to be 2 kcal/g in the present study. Mannitol is used in many applications, such as medical and pharmaceutical products and the food industry. The evidence provided by the present study on the utilization of mannitol could contribute to the development of additional health-related uses of mannitol.

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