Studies on Absorption, Metabolism and Physiological Effects of Ethyl α-D-Glucoside in Rat

(ラットにおける Ethyl α-D-glucoside の吸収、代謝および生理効果に関する研究)

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Contents

Abbreviations

α-EG, ethyl α-D-glucoside

β-EG, ethyl β-D-glucoside

α-MG, methyl α-D-glucoside

SGLT, sodium-dependent glucose transporter

GLUT, facilitative glucose transporter

HPLC, high-performance liquid chromatography

IC_{50}, concentration required for 50% inhibition of the reaction

Introduction

Sake, Japanese traditional alcoholic beverage, is brewed from rice. The koji mold (*Aspergillus oryzae*) produces enzymes that convert rice starch to sugar, and Sake yeast (*Saccharomyces cerevisiae*) metabolizes sugar to ethanol (Kondo 1984). The produced enzymes in koji mold showed the transglucosidation reaction. On fermentation process of Sake, ethyl α-D-glucoside (α-EG, Fig. A) was formed by the transglucosidation reaction of glucose moiety in α-1,4-glucan to ethanol by fungal transglucosidase (Oka & Sato 1976). α-EG has been known not only as the 4th major component (1~ 7 mg/mL) next to water, ethanol and glucose, but also as a peculiar component in Sake (Hayakawa *et al.* 2000, Imanari & Tamura 1971, Oka & Sato 1976, Oka *et al.* 1976).

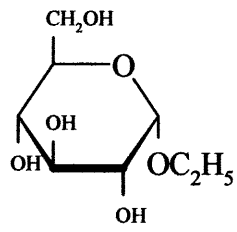

Fig. A Structure of ethyl α-D-glucoside

Imanari & Tamura (1971) and Teague *et al.* (2004) showed that α-EG was detected in the urine of the man who took Sake or rice wine. The history of "α-EG ingestion" was appeared by analysis of urinary α-EG. On the other hand, Sake is used in cooking as flavor enhancer. Therefore exposure by α-EG was quite likely as a result of the consumption of essentially non-alcohol containing food. Thus urinary α-EG was not necessarily derived by drinking of Sake.

In general, 90~ 95% of ethanol is eliminated by oxidation mainly in the liver and 0.5% of ethanol elimination was bio-transformed to ethyl glucuronide *via* conjugation with activated glucuronic acid. Ethyl glucuronide was detected in urine and blood (Jaakonmaki *et al.* 1967), and was detectable in hair over a few months (Yegles *et al.* 2004). Furthermore fatty acid ethyl esters were enzymatically formed in a side route of the ethanol metabolism in

almost all tissues (Auwärter *et al.* 2001, Pragst *et al.* 2001). Fatty acid ethyl esters were detectable in blood up to 24 h after the end of drinking and accumulated in fat tissues. Ethyl glucuronide and fatty acid ethyl esters were two major markers of the non-oxidative ethanol metabolism. On the other hand, despite extensive research into the metabolic fate of ethanol over many years, glucosidation to ethanol as accepter in mammalian body has not been described. Therefore α-EG might not be appeared in urine unless it was ingested.

Some specific properties and functions of α-EG have been known. α-EG showed instantaneous sweet taste like glucose with slow-acting bitter taste. According to Oka *et al.* (1976), threshold value for sweetness and bitter taste of α-EG was assessed 1.2 and 0.063 g/100mL, respectively. Therefore α-EG was considered to bring characteristic taste for Sake. Recently, Kitamura *et al.* (1997) reported the physiological effects of α-EG that transepidermal water loss levels after ultraviolet B irradiation was lowered when it was applied to mouse skin, and formation of cornified envelopes differentiated type keratin in human keratinocytes was accelerated by applying α-EG in the medium. On the other hand, ethyl β-D-glucoside (β-EG) was detected in peel of yuzu (*Citrus hassaku* HORT) (Matsubara *et al.* 1989). According to Matsubara *et al.* (1989), β-EG decreased blood pressure on SHR-SP rat at 1 mg /100 g body weight dose. Furthermore, according to Kitamura *et al.* (1997), β-EG did not show any effects on proliferation of human keratinocytes. Thus it was reported that α- and β-EG showed interesting physiological effects.

Although Japanese have ingested α-EG from Sake since ancient times, its nutritional availability has not been described. Nutritional availabilities in mammalian body of some glucosides, for example vitamin-glucosides and flavonoid-glucosides have been reported (Day *et al.* 2000, Gregory 1998, Trumbo *et al.* 1990). If α-EG was hydrolyzed in animal body, it would provide glucose and ethanol as nutritional sources.

Glucose and its analogues were polar molecule and it does not readily diffuse across

the hydrophobic plasma membrane. Therefore, specific carrier molecules exist to mediate the specific uptake of this sugar. In polarized epithelial cells in the lumen of small intestine and in the proximal tubules of the kidneys, there is an energy-dependent Na^+/glucose co-transporter (sodium-dependent glucose transporter; SGLT). This transport protein utilizes the movement of Na^+ down its electrochemical gradient to drive the complete uptake of glucose in intestine and kidneys. In the intestine, this glucose transporter serves to efficiently absorb the dietary-presented glucose. In kidneys, filtered glucose is reabsorbed back into the blood. In contrast to the highly restricted tissue specificity of the SGLT, all mammalian cells contain one or more members of the facilitative glucose transporter (GLUT) gene family. These GLUTs are characterized by a high degree of stereo-selectivity, providing for the bi-directional transport of glucose, with passive diffusion solely down its concentration gradient (Olson & Pessin 1996). Landau *et al.* (1962) reported that α-EG was extensively concentrated in everted hamster intestine. Ramaswamy *et al.* (1976) demonstrated that hamster intestinal SGLT showed affinity to β-alkyl glucosides, and the affinity of alkyl glucosides for SGLT1 increases with the increase in the length of the alkyl chain. Furthermore, Kipp *et al.* (1996) also demonstrated that hog renal SGLTs showed differences of affinity to alkyl chain length in alkyl glucosides. Thus α-EG was actively transported in small intestine (probably *via* SGLT1), which indicated that α-EG might be effectively absorbed into blood stream.

At present, few research papers dealing with the metabolic status of α-EG ingested have been appeared. In this thesis, the author intended to reveal how α-EG is hydrolyzed, absorbed, excreted and how it affects the rats physiologically.

Chapter 1

Hydrolysis of ethyl α-D-glucoside by rat organs and rat intestinal acetone powder

Disaccharides are hydrolyzed by α-glucosidases localized in the small intestinal mucosa (Dahlqvist 1964, Gutschmidt *et al.* 1979) and released monosaccharides are absorbed through glucose transporters distributed in small intestinal membrane (Harmon & McLeod 2001, Wood & Trayhurn 2003).

Some glucosides taken orally were hydrolyzed by enzymes distributed in small intestine as well as disaccharides. According to Takenaka & Uchiyama (2001), (2*S*)-1-*O*-α-D-glucosylglycerol, which was also contained in Sake, was hydrolyzed by sucrase in sucrase-isomaltase complex in rat intestine. Furthermore, vitamin-glucosides (Gregory 1998, Trumbo *et al.* 1990) and flavonoid-glucosides (Day *et al.* 2000) were hydrolyzed in animal body, and they provide glucose and aglycons (nutrients in these cases). On the other hand, methyl α-D-glucoside (α-MG), non-metabolic glucoside, is usable for analyzing transport activity of SGLT1 (González *et al.* 1998, Kimmich & Randles. 1981, Shinohara *et al.* 1993).

If α-EG was hydrolyzed in rat organs, it provides glucose and ethanol as conventional nutrients. In this Chapter, α-EG hydrolyzing activities by crude enzymes prepared from rat organs and intestinal acetone powder are compared with disaccharides hydrolyzing activities. Furthermore effects of α-EG on disaccharides hydrolysis in small intestine are examined.

Materials and methods

Regents and animals

Reagent grade α-EG (purity> 98%), α-MG, acarbose and Glucose B-Test Wako were purchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). Rat intestinal acetone powder was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Male Wistar ST clean rats were purchased from Japan SLC Inc. (Hamamatsu, Japan), and care and use of the rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals (1980), and animal experiments were approved by ethical committee of Faculty of Agriculture in Gifu University.

Preparation of crude enzyme solution from rat organs

Wistar ST rats, approximately 10~ 12 wk old and approximately weighing 250~ 350 g, were killed by drawing blood under anesthesia, and tissues (small intestine, liver, kidneys, spleen, brain, dorsal muscles and heart) were excised immediately and rinsed briefly with cold physiological saline. Small intestine was cut open and mucosa was scraped off with a slide glass on ice (intestinal mucosa). The residual tissue was served as intestinal residue.

Crude enzyme solutions from rat intestinal mucosa and other tissues were prepared according to the method of Dahlqvist (1964). Same volume (v/w) of 0.2 M sodium phosphate buffer (pH 6.0) was added to each tissue (liver, kidneys, spleen, brain, dorsal muscles, heart, intestinal mucosa and intestinal residue), and then homogenized with HG30 homogenizer (HITACHI High-Technologies Co., Tokyo, Japan). Homogenates were sonicated for 15 min using a Sharp UT-104 Silentsonic (Sharp Manufacturing System Co., Osaka, Japan) in ice bath. To remove large particles, homogenates were centrifuged at 2,000x *g* for 30 min at 4°C using a refrigerated centrifuge RS-20 IV (TOMY SEIKO Co., Ltd., Tokyo, Japan). Each supernatant as crude enzyme solution was collected in a sample tube. Protein concen-

tration in each supernatant was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

α-EG hydrolyzing activity by homogenates from rat organs

Reaction mixture, which was consisted of 200 μL of 500 mM α-EG or α-MG dissolved in 0.2 M sodium phosphate buffer (pH 6.0) and 100 μL of the crude enzyme solution, was incubated for 180 min at 37°C. The reaction was stopped by adding 100 μL of 1 M NaOH. After neutralization with 1 M HCl, reaction mixture was centrifuged at 12,000x *g* using high-speed micro refrigerated centrifuge MR-150 (TOMY SEIKO Co., Ltd.). The amount of glucose in supernatant was determined with Glucose B-Test Wako. Reaction was performed in triplicate. Hydrolyzing activity of α-EG was expressed as the μmol of glucose liberated per mg protein per hour. Furthermore, maltose hydrolyzing activity was also measured for crude enzyme solutions from small intestinal mucosa and kidneys. Hydrolyzing activity of maltose was expressed as half the amount (μmol) of glucose liberated per mg protein per hour, since 1 mol maltose is hydrolyzed to liberate 2 mol of glucose.

Preparation of crude enzyme solution from rat intestinal acetone powder

Crude enzyme solution was prepared from rat intestinal acetone powder as described by Takenaka & Uchiyama (2001). Two grams of the acetone powder was suspended with 10 volume (w/v) of 0.2 M sodium phosphate buffer (pH 6.0) and then homogenized with HG30 homogenizer. Homogenate was sonicated for 15 min using a Sharp UT-104 Silentsonic in ice bath. To remove large particles, homogenate was centrifuged at 3,000x *g* for 30 min at 4°C using a refrigerated centrifuge RS-20IV. Supernatant as crude enzyme solution was collected in a sample tube, and kept on ice until use. Protein concentration in each supernatant was determined by the method of Lowry *et al.* (1951) as described above.

Measurement of hydrolyzing activity for α-EG, α-MG and disaccharides

The crude enzyme solution was diluted with 0.2M sodium phosphate buffer (pH6.0)

appropriately. Substrate solution (α-EG, α-MG, maltose, sucrose and lactose) was diluted at various concentrations with the same buffer. After preincubation of 200 μL of each sugar solution for 5 min, reaction was started by adding 100 μL of crude enzyme solution. Incubation was performed at 37°C for 180 min for α-EG and α-MG or 30 min for disaccharides. To stop the reaction, 1 M NaOH was added and then neutralized with 1 M HCl. Reaction was performed in triplicate. After centrifugation at 12,000x *g* for 10 min with centrifuge MR-150, supernatant was used for glucose determination by Glucose B-Test Wako.

Hydrolyzing activity for maltose was expressed as half the amount (μmol) of glucose liberated per mg protein per hour, since 1 mol maltose is hydrolyzed to liberate 2 mol of glucose.

Assessment of IC_{50} for disaccharides hydrolysis by α-EG and α-glucosidase inhibitors

To start hydrolyzing reaction, 100 μL of the crude enzyme solution was added to 200 μL of 50 mM disaccharide (maltose, sucrose and lactose) solution containing various concentration of α-EG, arabinose or acarbose. Hydrolyzing reaction and glucose analysis were performed by the method described above.

Assessment of inhibition for disaccharides hydrolysis by α-EG

To start reaction, 100 μL of the crude enzyme solution was added to 200 μL of various concentration of maltose or sucrose solution containing various concentrations of α-EG. Hydrolyzing reaction and glucose analysis were performed by the method described above.

Results

Hydrolyzing activities for α-EG were detected in crude enzyme solutions prepared from small intestinal mucosa and kidneys, which showed 0.064 and 0.029 μmol/mg protein/h, respectively (data not shown). No other organ showed any detectable activity for α-EG hydrolysis in this experimental conditions. On the other hand, hydrolyzing activities for maltose in small intestinal mucosa and kidneys were 10.58 and 6.53 μmol/mg protein/h, respectively (data not shown). Any detectable activity for α-MG hydrolysis was not shown in all enzyme preparations (data not shown).

Saturation curve of α-EG hydrolysis by crude enzyme prepared from rat kidneys was shown in Fig. 1-1. Lineweaver-Burk plot for α-EG hydrolysis by crude enzyme preparation from rat kidneys was indicated in Fig. 1-2. *K*m and *V*max values for α-EG hydrolysis were 63.1 mM and 0.023 μmol/mg protein/h, respectively. On the other hand, *K*m and *V*max values for maltose hydrolysis were 1.8 mM and 7.62 μmol/mg protein/h, respectively (data not shown).

α-EG was hydrolyzed by crude enzyme solution prepared from rat intestinal acetone powder (Fig. 1-3) and α-MG was not hydrolyzed (data not shown). Lineweaver-Burk plot for α-EG hydrolysis by crude enzyme preparation from rat intestinal acetone powder was indicated in Fig. 1-4. Table 1-1 summarized *K*m and *V*max values for α-EG and disaccharides in this hydrolysis experiments. *V*max values for the hydrolysis of α-EG, maltose, sucrose and lactose were 0.09, 13.1, 4.46 and 0.22 μmol/mg protein/h, respectively. Figures 1-5 and 1-6 shows Lineweaver-Burk plot for maltose and sucrose hydrolysis by rat intestinal crude enzyme with or without α-EG. α-EG showed mixed type inhibition for hydrolysis of maltose and sucrose. The *K*i values of α-EG for hydrolysis of maltose and sucrose were 285 mM and 93 mM, respectively.

Figure 1-7 showed effect of α-EG on hydrolyzing of disaccharides by rat intestinal

crude enzyme solution. Hydrolyzing activities for maltose (a) and sucrose (b) were decreased by adding α-EG to reaction mixture, however, that for lactose (c) was decreased only slightly in the presence of α-EG. Table 1-2 summarized the concentrations required for 50% inhibition of the reaction (IC_{50}). In α-EG, IC_{50} for maltose and sucrose was 408 mM and 101 mM, respectively. In acarbose, IC_{50} for maltose and sucrose was 2.6 μM and 2.5 μM, respectively. In arabinose, IC_{50} for sucrose hydrolysis was 3.4 mM, whereas maximal inhibition rate for maltose was 20.9% at 667 mM.

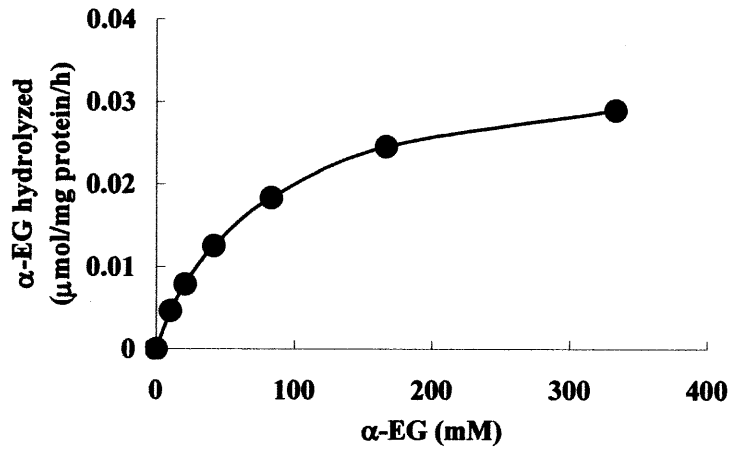

Fig. 1-1 Hydrolysis of α-EG by crude enzyme preparation from rat kidneys

Incubation was performed for 180 min at 37°C. Concentrations of α-EG were set at 0, 10, 21, 42, 83, 167, and 333 mM. Reaction was performed in triplicate.

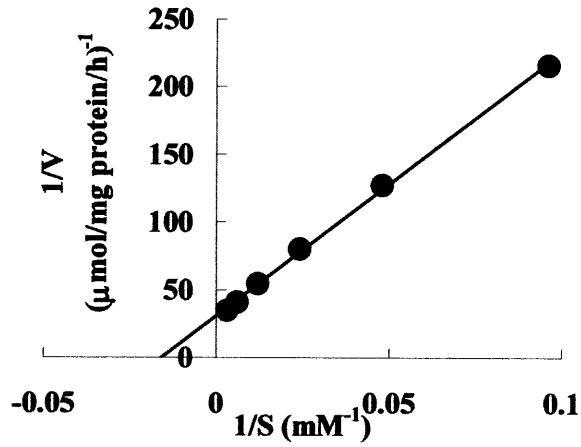

Fig. 1-2 Lineweaver-Burk plot for α-EG hydrolysis by crude enzyme preparation from rat kidneys

For experimental conditions, see legend for Fig. 1-1.

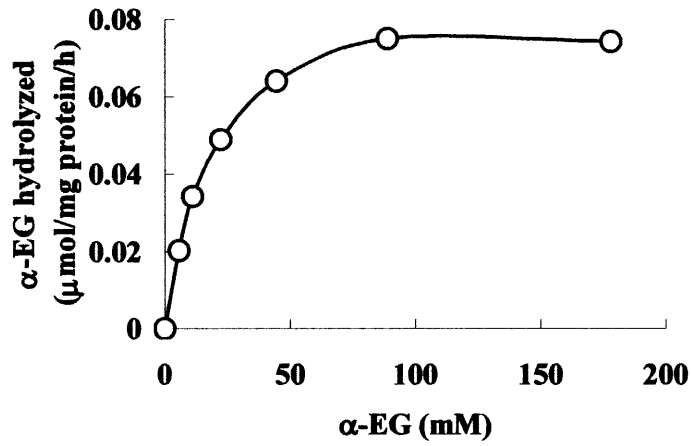

Fig. 1-3 Hydrolysis of α-EG by crude enzyme preparation from rat intestinal acetone powder

Incubation was performed for 180 min at 37°C. Concentrations of α-EG were set at 0, 5, 10, 21, 42, 83, and 167 mM. Reaction was performed in triplicate.

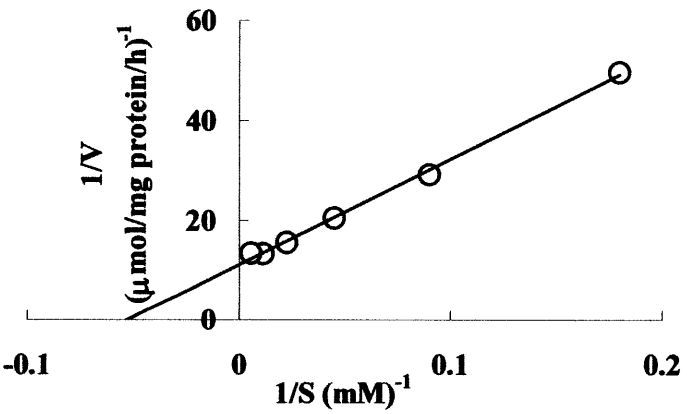

Fig. 1-4 Lineweaver-Burk plot for α-EG hydrolysis by crude enzyme preparation from rat intestinal acetone powder

For experimental conditions, see legend for Fig. 1-3.

Table 1-1 Kinetics constants for the hydrolysis of glucose conjugates by crude enzyme preparation from rat intestinal acetone powder

	*K*m (mM)	*V*max (μmol/mg protein/h)
α-EG	18.7	0.09
Maltose	14.1	13.1
Sucrose	19.9	4.46
Lactose	22.5	0.22

Incubations were performed at 37°C for 180 min for α-EG or 30 min for disaccharides. Reaction was performed in triplicate.

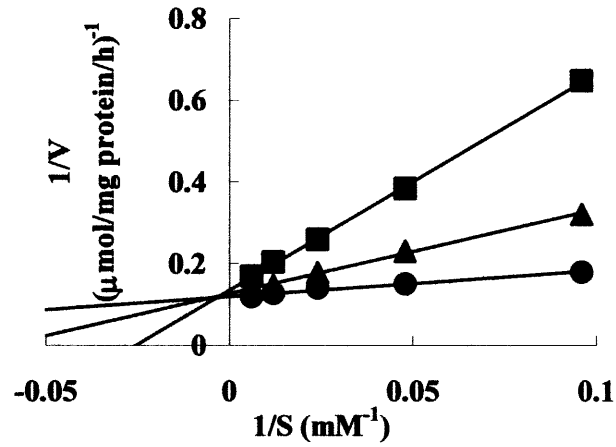

Fig. 1-5 Lineweaver-Burk plot for maltose hydrolysis in the presence and absence of α-EG

Incubations were performed for 30 min at 37°C. Concentration of α-EG was set at 0mM (●), 167 mM (▲) or 667 mM (■). Reaction was performed in triplicate.

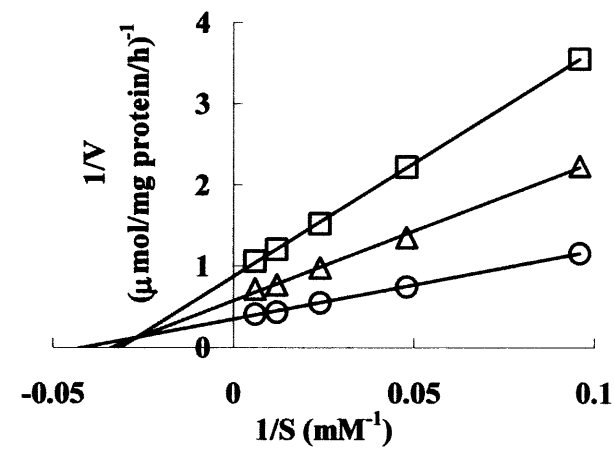

Fig. 1-6 Lineweaver-Burk plot for sucrose hydrolysis in the presence and absence of α-EG

Incubations were performed for 30 min at 37°C. Concentration of α-EG was set at 0mM (○), 83 mM (△) or 167 mM (□). Reaction was performed in triplicate.

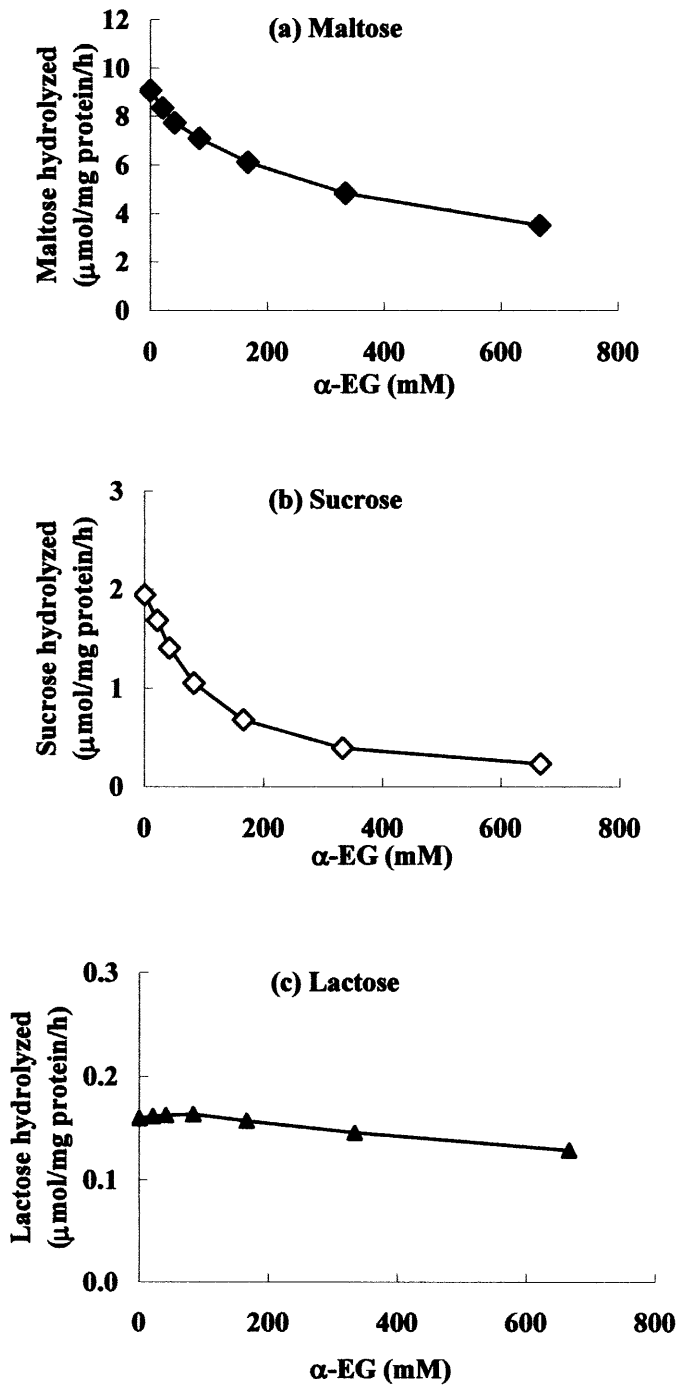

Fig. 1-7 Inhibition of disaccharide hydrolysis by α-EG

Concentrations of α-EG were set at 0, 21, 42, 83, 167, 333 and 667 mM. Incubations were performed for 30 min at 37°C.

Reaction was performed in triplicate.

Table 1-2 IC_{50} values of α-EG, arabinose or acarbose for disaccharide hydrolysis by crude enzyme preparation from rat intestinal acetone powder

	IC_{50} values		
	α-EG	Arabinose	Acarbose
Maltose	408 mM	ND*	2.6 μM
Sucrose	101 mM	3.4 mM	2.5 μM

IC_{50}, Inhibitory concentration required for 50% inhibition (IC_{50}) for disaccharide hydrolysis. Incubation was performed at 37°C for 30 min. Reaction was performed in triplicate.

* IC_{50} was not determined. Inhibition was maximum (20.9%) at 667 mM

Discussion

Landau *et al.* (1962) suggested presence of α-EG hydrolyzing enzymes, glucosidases, in hamster intestine. In this investigation, hydrolyzing activity for α-EG was detected in crude enzyme solutions prepared from small intestinal mucosa and kidneys of Wistar rats. α-EG might be hydrolyzed by α-glucosidases, which were reported to be distributed in small intestinal mucosa (Harmon & McLeod 2001, Sanai *et al.* 1997) and kidneys brush border membrane (Hirsh *et al.* 1997, Kageyama *et al.* 1997, Sato *et al.* 1993). However, hydrolyzing activity for α-EG was much lower than that for maltose (see the Results section in this Chapter).

Rat intestinal acetone powder was used for measurement of hydrolyzing activity in small intestine because it was difficult to obtain enough sample of small intestinal mucosa. α-EG was hydrolyzed by rat intestinal preparation from acetone powder as shown in Fig.1-3. Hydrolyzing activity for α-EG was lower than those for disaccharides (Table 1-1), which showed the possibility that α-EG might be a low caloric food component. Furthermore a small amount of glucose and ethanol liberated from α-EG were consumed safely as conventional nutrients.

Disaccharides, *e.g.* maltose and sucrose are absorbed as monosaccharides after membrane digestion by small intestinal enzymes (Tamai & Tsuji 1996). These enzymes could hydrolyze various glucose conjugates (Day *et al.* 2000, Gregory 1998, Trumbo *et al.* 1990). Pyridoxine 5'- phosphate concentration in plasma was increased following administration of pyridoxine-α-glucosides as well as administration of pyridoxine, which suggested that pyridoxine-α-glucosides were hydrolyzed in rat body (Maeno *et al.* 1997). Riboflavin-5'-α-D-glucoside and ascorbyl-2-α-D-glucoside could exhibit full bioavailability as a source of each vitamin nutrient, because they were hydrolyzed in mammalian body (Gregory 1998). α-EG showed mixed-type inhibition for maltose and sucrose hydrolysis (Figs.

1-5 and 1-6), suggesting that α-EG might be hydrolyzed by maltase and sucrase. On the other hand, α-MG was not hydrolyzed by the intestinal crude enzyme preparation (data not shown). At present, α-MG is considered as a non-metabolic glucoside because it was not hydrolyzed by mammalian enzymes (González *et al.* 1998, Kimmich & Randles 1981, Shinohara *et al.* 1993). Therefore α-EG hydrolyzing enzymes in rat small intestine might recognize alkyl carbon chain length in aglycon.

As shown in Table 1-2, IC_{50} values of α-EG for maltose and sucrose hydrolysis were higher than those of arabinose and acarbose. Arabinose showed uncompetitive inhibition for sucrose hydrolysis (Sanai *et al.* 1997) and thus it was expected for prevention of increasing plasma glucose level after sucrose ingestion. Acarbose strongly inhibited disaccharides hydrolysis by intestinal enzymes, therefore it is used as diabetic medicine (Hirsh *et al.* 1997, Kageyama *et al.* 1997). Weak effect of α-EG as inhibitor for disaccharides hydrolysis suggested that suppression of glucose level increase by α-EG after disaccharides ingestion was less as compared to arabinose and acarbose.

α-glucosidase in mammalian small intestine has been reported to catalyze transglucosidation (Muto *et al.* 1990, Sato *et al.* 1993, Takenaka & Uchiyama 2001, Yamamoto *et al.* 1990). However, despite extensive research into the metabolic fate of ethanol over many years, glucosidation to ethanol as accepter in mammalian body has not been described. Therefore α-EG might not be detected in mammalian body unless it was ingested from Sake or rice wine.

Chapter 2

Absorption of ethyl α-D-glucoside by everted rat intestinal sac

Glucose was polar molecule and it does not readily diffuse across the hydrophobic plasma membrane. Therefore specific carrier molecules exist to mediate the specific uptake of this sugar. In polarized epithelial cells in the lumen of small intestine and in the proximal tubules of kidneys, there is an energy-dependent Na^+/glucose cotransporter (sodium- dependent glucose transporter; SGLT). This transporter protein utilizes the movement of Na^+ down its electrochemical gradient to drive the complete uptake of glucose. It serves to efficiently absorb the dietary-presented glucose in intestine and reabsorbs the filtered glucose back into blood in kidneys. In contrast to highly restricted tissue specificity of SGLTs, all mammalian cells contain one or more members of the facilitative glucose transporter (GLUT) gene family. These facilitative glucose transporters are characterized by a high degree of stereo-selectivity, providing for the bi-directional transport of glucose, with passive diffusion solely down its concentration gradient (Olson & Pessin 1996).

Landau *et al.* (1962) demonstrated α-EG was effectively concentrated in everted hamster intestinal sac. α-EG might be absorbed into blood stream through the small intestinal wall of rats as well as hamster. The purpose of this study is to investigate whether α-EG is absorbed through the rat intestinal wall using everted rat intestinal sac.

Materials and Methods

Reagents and animals

Reagent grade α-EG (purity> 98%) was obtained from Kanebo Cosmetics Ltd. (Tokyo, Japan). Glucose assay kit (Glucose B-Test Wako) and α-glucosidase (EC 3.2.1.20) from *Saccharomyces* sp. were purchased from Wako Pure Chemicals Co. Ltd. Other reagents were purchased from Nacalai Tesque Inc. Male Wistar ST clean rats were purchased from Japan SLC Inc., and care and use of the rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals (1980), and animal experiments were approved by ethical committee of Faculty of Agriculture in Gifu University.

Preparation of everted rat intestinal sacs

Wistar ST rats, approximately 10~ 12 wk old and approximately weighing 250~ 350 g, were killed by drawing blood under anesthesia, and small intestine was excised immediately and rinsed briefly with cold Krebs-Ringer buffer. The preparation of everted rat intestinal sac was performed by the method described by Wilson & Wiseman (1954). The adherent mesenteric tissue and fat were trimmed off from jejunum region. The jejunum was divided into tubes of similar size (4 cm in length), and then everted. Both ends of these tubes were bound tightly with silk string after inclusion of 500 μL Krebs-Ringer buffer.

Sugars absorption by everted rat intestinal sacs in the presence of α-EG

Intestinal sacs were dipped into Krebs-Ringer buffers containing several concentrations of α-EG, which were bubbled with O_2/CO_2 (95: 5, v/v) gas adequately. After incubation for 30 min at 37°C, serosal solution was used for determination of glucose (Glucose B-Test Wako). Total sugar (α-EG plus glucose) was determined by the same kit after hydrolysis by α-glucosidase from *Saccharomyces* sp. α-EG absorbed was calculated by subtracting glucose from total sugar. Furthermore, α-EG absorption assay was performed by replacing

sodium-ion with equimolar amount of potassium-ion in the Krebs-Ringer buffer (Shimizu *et al.* 2000). Absorption activity was expressed as the nmol of sugar absorbed per cm small intestine per minutes.

Time course of α-EG absorption by everted rat intestinal sacs

The preparation of everted rat intestinal sac was performed by the method described above. Intestinal sacs were incubated in each experimental solution containing 20 mM α-EG as indicated in parenthesis; Control (Krebs-Ringer buffer), Na^+-free (Krebs-Ringer buffer replaced sodium-ion with equimolar amount of potassium-ion) and phlorizin (Krebs-Ringer buffer containing 1.55 mM phlorizin). After incubation for 0 (blank), 1, 5, 15, 30 or 45 minutes at 37°C, serosal solution was collected in sample tube. Same volume (w/v) of ice-cold Krebs-Ringer buffer was added to residual intestinal tissue, and then homogenized with HG30 homogenizer. After centrifugation at 12,000x *g* for 10 min at 4°C with centrifuge MR-150, supernatant was collected in sample tube. Serosal solution and supernatant from tissue homogenate were used for determination of sugars. Sugar analysis was followed by the method mentioned above. Absorption of α-EG was expressed as the nmol of α-EG absorbed per 100 mg small intestine.

Statistical analysis

Statistical significance among means was estimated at $P< 0.05$ according to student's *t*-test (Shinjo 2004).

Results

Figure 2-1 shows the results of α-EG absorption by everted rat intestinal sacs at several concentrations. α-EG was detected in all serosal solutions and its absorption in the absence of sodium-ion was significantly lower than that in the presence of sodium-ion at any concentration tested ($P< 0.01$). Although glucose liberated from α-EG was detected in serosal solution, it was extremely low level (less than 1% of absorbed α-EG; Fig. 2-2).

Figures 2-3 and 2-4 shows the time course of α-EG absorption in small intestinal tissue (cells), and in serosal solution of everted rat intestinal sacs, respectively. α-EG absorption in the small intestinal cell and serosal solution were reduced by elimination of sodium-ion from experimental solution, which coincided well with the results as shown in Fig. 2-1. Furthermore α-EG absorption was reduced by adding phlorizin in the mucosal solution.

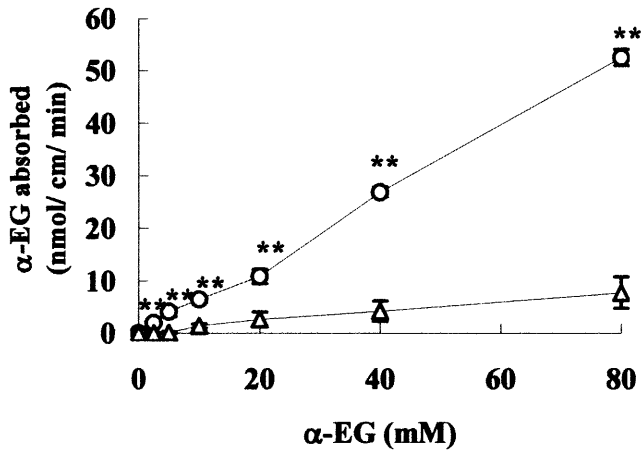

Fig. 2-1 Absorption of α-EG by everted rat intestinal sac

α-EG absorption in rat everted small intestine. Everted rat intestinal sacs were incubated for 30 min in Krebs-Ringer buffer containing sodium-ions (○) or in Krebs-Ringer buffer containing potassium-ion (△). Final concentrations of α-EG in each buffer solution were 2.5, 5, 10, 20, 40, and 80 mM, respectively. α-EG absorption was calculated by subtracting glucose from totally absorbed sugar (glucose plus a-EG). ** Significant difference at each concentration (P<0.01). Each point represents mean ± standard error (n=8).

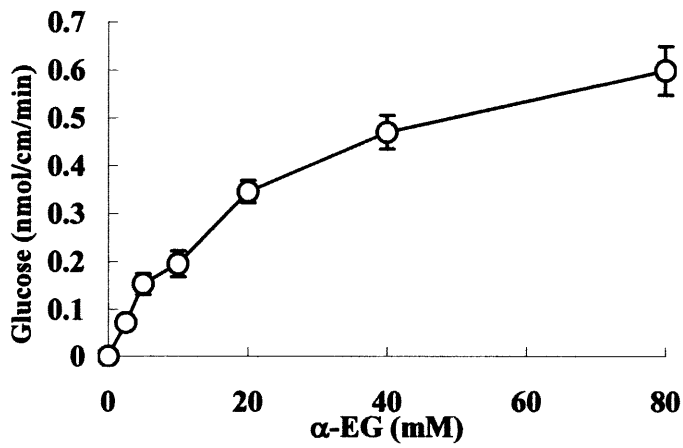

Fig. 2-2 Absorption of glucose liberated from α-EG in rat everted small intestine

Everted sacs were incubated in the Krebs-Ringer buffer containing sodium-ion (○). For calculations, see legend for Fig. 2-1. Each point was mean ± standard error (n= 8).

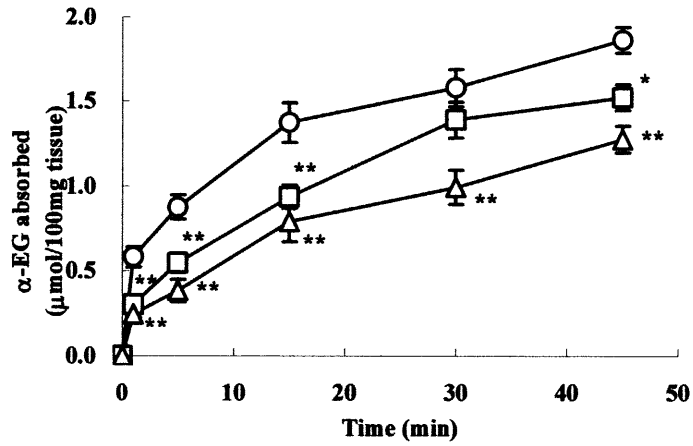

Fig. 2-3 Time course of α-EG absorption into intestinal tissue on everted rat intestinal sacs

α-EG absorption into small intestinal tissue of everted rat intestinal sac was examined. Everted rat intestinal sacs were incubated for 0, 1, 5, 15, 30 or 45 min in the Krebs-Ringer buffer containing sodium-ion (○), in the Krebs-Ringer buffer without sodium-ion (△) in which potassium-ion was substituted by sodium-ion or in the Krebs-Ringer buffer with 1.55 mM phlorizin (□). α-EG absorption was calculated by subtracting glucose from totally absorbed sugar (glucose plus α-EG). Significant difference at each concentration was indicated by asterisk (**; $P< 0.01$ and *; $P< 0.05$). Each point was mean ± standard error (n=10 in Control and n= 6 in Na^+-free and +phlorizin).

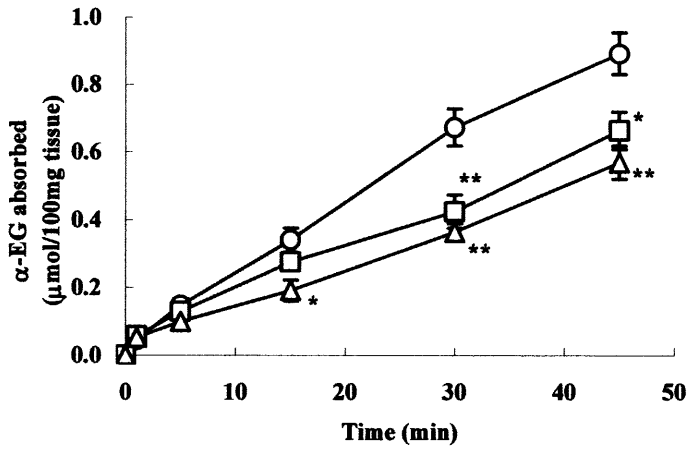

Fig. 2-4 Time course of α-EG absorption into serosal solution on everted rat intestinal sacs

α-EG absorption into serosal solution of everted rat intestinal sac was examined. Everted rat intestinal sacs were incubated for 0, 1, 5, 15, 30 or 45 min in the Krebs-Ringer buffer containing sodium-ion (○), in the Krebs-Ringer buffer without sodium-ion (△) in which potassium-ion was substituted by sodium-ion or in the Krebs-Ringer buffer with 1.55 mM phlorizin (□). α-EG absorption was calculated by subtracting glucose from totally absorbed sugar (glucose plus α-EG). Significant difference at each concentration was indicated by asterisk (**; $P< 0.01$ and *; $P< 0.05$). Each point was mean ± standard error (n=10 in Control and n= 6 in Na^+-free and +phlorizin).

Discussion

Brash border membrane transport in small intestinal mucosa proceeds *via* SGLT1 and GLUT5. Basolateral membrane transport is also mediated by GLUT2, which has substrate specificity similar to but distinct from that of GLUT5. The substrate specificity of SGLT1 evaluated by examining the transport and inhibitory activities of many hexoses in *Xenopus laevis* oocytes injected with SGLT1 cRNA is in the order of D-glucose> α-MG> D-galactose> 3-*O*-methylglucoside>> mannitol and L-glucose (Ikeda *et al.* 1989). Alkyl glucosides were also substrates for SGLTs and are poorly transported or not transported by GLUT transporters (Kimmich & Randles 1981).

Disaccharides are hydrolyzed to monosaccharides by the brush-border α-glucosidases, and then monosaccharides are absorbed into blood stream by SGLT and GLUT transporters distributed in intestinal membrane (Tamai & Tsuji 1996). In Chapter 1, the author revealed that α-EG hydrolyzing activity was detected in rat intestine. As shown in Fig. 2-2, glucose liberated from α-EG was detected in serosal fluid after incubation. These results suggested that glucose liberated from α-EG was absorbed through the small intestinal wall. However, absorbed glucose was less than 1% of absorbed α-EG (Figs. 2-1 and 2-2). Therefore, not all of ingested α-EG was hydrolyzed in small intestine, and α-EG ingested might be absorbed through the small intestinal wall as intact form.

According to Landau *et al.* (1962), α-alkyl glucosides with the smallest aglycone, *i.e.*, methyl, ethyl and isopropyl glucosides were extensively concentrated in everted hamster intestine. Furthermore hamster intestinal SGLT showed affinity to β-alkyl glucosides, and the affinity of alkyl glucosides for SGLT1 increased with the alkyl chain length. Only β-methyl, β-ethyl and β-propyl glucosides are translocated, whereas the longer chain compounds are competitive and non-transported inhibitors (Ramaswamy *et al.* 1976). In this experiment using everted rat intestinal sac, α-EG absorption into small intestinal cells was

reduced by elimination of sodium-ion from the mucosal solution (Fig. 2-3). Similar observation was reported on transport of glucose (Shimizu *et al.* 2000). Furthermore, as shown in Fig. 2-3, α-EG absorption into intestinal cells was reduced under the presence of phlorizin, which is a specific inhibitor of SGLT transporters (Koepsell *et al.* 1990). These data suggested that α-EG was absorbed into small intestinal cells *via* SGLT1 in brush-border membrane. As shown in Fig. 2-4, α-EG was transported into serosal solution, which suggested that α-EG absorbed into intestinal cells was transported to serosal side *via* GLUT2. However α-EG was almost saturated in intestinal cells after 15 min of incubation (Fig. 2-3). The transport activity for α-MG by GLUT was lower than that by SGLT (Kimmich & Randles 1981). α-EG absorbed was accumulated into small intestinal cells (Fig. 2-3), suggested that the transport activity for α-EG by GLUT was lower than that by SGLT as well as α-MG.

Although ability of GLUT5 and other transport systems on α-EG transport in small intestine remained unknown, results in this Chapter showed that α-EG was transported into intestinal cell through SGLT1 distributed in brush-border membrane and then transported to serosal side by GLUT2 distributed in basolateral membrane.

In conclusion, only a part of α-EG ingested was hydrolyzed in small intestine, and most of α-EG was absorbed as intact form through small intestinal wall. Consequently, α-EG might be absorbed into blood stream as intact form.

Chapter 3

Feeding experiment of ethyl α-D-glucoside solution to adult rats

In Chapter 1, experiment *in vitro* demonstrated that hydrolyzing activity for α-EG was detected in crude enzyme solution prepared from rat small intestinal mucosa, however, it was lower than those for disaccharides. Furthermore, α-EG was absorbed through rat small intestinal wall as intact form (Chapter 2). Imanari & Tamura (1971) and Teague *et al.* (2004) reported that α-EG was detected in the urine of the man who took Sake. These results indicated that α-EG was absorbed into blood stream. In this chapter, the author investigated whether α-EG was detected in plasma and urine of Wistar rat after α-EG ingestion.

Materials and Methods

Reagents and animals

α-EG sample solution, which consisted of 9.5% α-EG (0.46 M), 0.91% glucose, 0.39% maltose and 1.6% isomaltose, was obtained from Ozeki Co. Glucose assay (Glucose B-Test Wako) and insulin enzyme immunoassay (EIA insulin assay) kits were purchased from Wako Pure Chemicals Co. Ltd. and Kanto Kagaku Co. (Tokyo, Japan), respectively. Other reagents were purchased from Nacalai Tesque Inc. unless otherwise indicated. Male Wistar ST clean rats (24 wks old, weighing 450~ 550 g) were purchased from Japan SLC Inc., and care and use of the rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals (1980), and animal experiments were approved by ethical committee of Faculty of Agriculture in Gifu University.

HPLC apparatus and analytical conditions

α-EG in plasma and urine was kindly analyzed at Ozeki Co. A chromatographic system

manufactured by Gilson Medical Electonics Inc. (Middletown, WI, USA) equipped with an SZ5532 (sugar) column (Showa Denko K. K., Kawasaki, Japan) and a Sedex model 55 evaporative light scattering detector (SEDERE, Alfortville, Cedex, France) was used. The column was eluted with a linear gradient from 80% CH_3CN and 20% H_2O to 70% CH_3CN and 30% H_2O in 10 min. The elution was continued in the latter solvent until 20 min. Flow rate was set at 1.0 mL/min throughout the analysis.

Conditions of the feeding experiment

Rats were divided into three groups (n= 5) and individually housed in a wire-bottom stainless steel cage in a temperature-controlled room (23±1°C) with a 12-h light/ 12-h dark cycle (light; 6:00~ 18:00). They were given free access to powder MF diet (Oriental Yeast Co., Tokyo, Japan) and each test solution was administered for 36 days as indicated in parenthesis: Control group (tap water, *ad libitum*), α-EG group (0.46 M α-EG sample solution, *ad libitum*), and PF group (0.46 M α-EG sample solution, pair-fed to the Control group). During the feeding period, drink and food intakes were measured every day and 24-hour urine was collected to check its volume once a week.

On day 36 of the feeding period, blood was drawn from an abdominal aorta through a syringe treated with 1% heparin-Na under ether anesthesia. Plasma was obtained after centrifugation at 1,500x *g* for 20 min at 4°C with a centrifuge RS-20IV. Glucose and insulin in plasma were determined with Glucose B-Test Wako and insulin enzyme immunoassay kit, respectively. α-EG in plasma and urine was analyzed by the HPLC, and excretion rate of α-EG in urine was expressed as the ratio of urinary α-EG to ingested α-EG.

Statistical analysis

Statistical significance among means was estimated at $P< 0.05$ according to ANOVA and Duncan's multiple range-test (Shinjo 2004).

Results

Table 3-1 lists total food and water intakes and the levels of insulin, glucose and α-EG in plasma together with other growth parameters. α-EG was detected in plasma of the α-EG and PF groups, but plasma glucose and insulin levels were not affected by α-EG feeding. Total food intake in the α-EG group was significantly less than that of the Control group, and there was no significant difference in the final body weight between the Control and α-EG groups. In contrast, total drink intake in the α-EG group increased significantly as compared to that of the Control or PF group. Final body weight in the PF group was decreased from initial body weight, and it was significantly less than those of other groups. α-EG was detected from urine in the α-EG and PF groups. Excretion rate of α-EG was approximately 0.6~ 0.9 during the experimental period (Fig. 3-1). Urine volume in the α-EG group was significantly larger than those of the other groups during the experimental period (Fig. 3-2).

Table 3-1 Initial body weight, final body weight, total food intake, total drink intake, and levels of glucose, α-EG, and insulin in plasma

	Group		
	Control	α-EG	PF
Initial body weight (g)	505 ± 17	507 ± 16	506 ± 15
Final body weight (g)	521 ± 17 [a]	509 ± 20 [a]	432 ± 15 [b]
Total food intake (g)	591 ± 12 [a]	410 ± 26 [b]	352 ± 21 [b]
Total drink intake (g)	1048 ± 108 [b]	2411 ± 119 [a]	1056 ± 96 [b]
Plasma glucose (mM)	14.6 ± 1.3 [a]	14.7 ± 2.1 [a]	9.8 ± 0.8 [b]
Plasma α-EG (mM)	ND	9.5 ± 0.8 [a]	3.5 ± 0.1 [b]
Plasma insulin (ng/mL)	1.08 ± 0.24 [a]	1.07 ± 0.19 [a]	0.73 ± 0.05 [b]

ND, not detected

Values are means ± standard error for five rats. Means in the same row not sharing the same superscript are significantly different at $P< 0.05$. Test drinks were tap water *ad libitum* (Control), 0.46 M α-EG sample solution (Ozeki Co.) *ad libitum* (α-EG), and 0.46 M α-EG sample solution pair-fed to the Control group (PF).

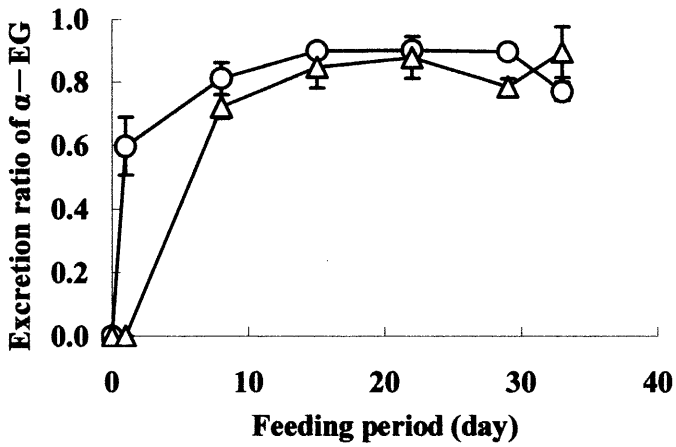

Fig. 3-1 Urinary excretion ratio of α-EG during the feeding period

Urinary excretion ratio of α-EG in the feeding experiment was expressed as the ratio of urinary α-EG to ingested α-EG and plotted. The Control group was given tap water *ad libitum*, the α-EG group (○) was given 0.46 M of α-EG solution (Ozeki Co.) *ad libitum*, and the PF group (△) was given 0.46 M of α-EG solution that was pair-fed to the Control group. α-EG was not detected in the urine from the Control group. Each point represents mean ± standard error (n = 5).

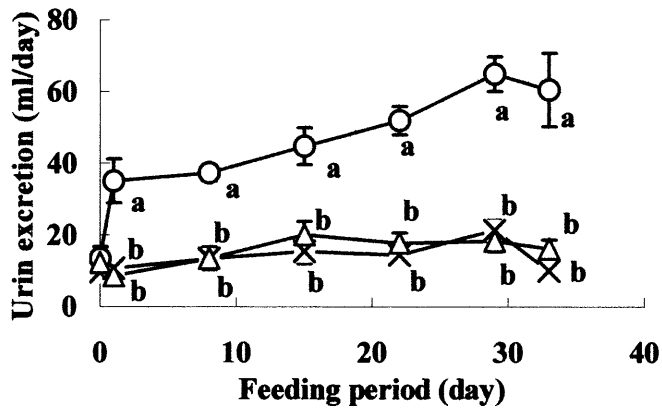

Fig. 3-2 Urine volume during the feeding period

Urine volume during the feeding period was plotted. The Control group was given tap water *ad libitum*, the α-EG group (○) was given 0.46 M of α-EG solution (Ozeki Co.) *ad libitum*, and the PF group (△) was given 0.46 M of α-EG solution that was pair-fed to the Control group. For feeding conditions, see legend for Fig.3-1. Each point was mean ± standard error (n= 5). Means in the same day not sharing the same alphabet are significantly different at $P< 0.05$.

Discussion

As shown in Chapter 2, α-EG absorption was reduced by elimination of sodium-ions from the experimental solution (Figs. 2-1, 2-2 and 2-3), which suggested that α-EG was absorbed through small intestinal wall *via* SGLT1. Thus, in this experiment *in vivo*, intact form of α-EG was detected in plasma of rats fed α-EG (Table 3-1).

All of glucose filtered from plasma by kidneys is normally reabsorbed back into the blood by SGLTs in the proximal tubules (Wright 2001). Kipp *et al.* (1996) reported that renal SGLTs showed affinity to alkyl glucosides and transported short chain alkyl glucosides. As demonstrated by the *in vivo* experiment shown in Fig. 3-1, α-EG absorbed in bloodstream was excreted in urine, and the excretion ratio was approximately 0.6~ 0.9. These data suggested that not all the α-EG filtered in kidneys was reabsorbed by SGLTs in proximal tubules. Imanari & Tamura (1971) found α-EG in the urine of the man who took Sake, which contained trace α-EG (1~ 7 mg/mL). This means that α-EG was excreted into urine even when a minute amount of α-EG was ingested. On the other hand, Bormans *et al.* (2003) reported that α-MG was almost completely reabsorbed from the primary glomerular filtrate, which suggested that α-MG was retained in the body. α-EG absorbed into blood stream would disappear from the body even if α-EG had been reabsorbed by the kidneys because kidneys had α-EG hydrolyzing activity (Chapter 1). In other words, α-EG ingested would not be retained in the body different from α-MG.

Food intake of the α-EG group was less than that of the Control group in spite of no significant difference in final body weight between the Control and α-EG groups (Table 3-1). This result showed that a part of α-EG might liberate glucose and ethanol and were utilized as conventional nutrients. As Shown in Chapter 1, hydrolyzing activities for α-EG were detected in crude enzyme solutions prepared from small intestinal mucosa and kidneys. However, hydrolyzing activity for α-EG was much lower than those for disaccharides,

and insulin secretion was not enhanced by α-EG ingestion in spite of high total sugar concentration (α-EG plus glucose) in plasma of the α-EG group (Table 3-1). α-EG was a low energy food component which would not induce insulin secretion directly.

As shown in Fig. 3-2, there was a significant difference in urine volume between the α-EG group and the other two groups. The author surmised that α-EG filtered in glomerulus might prevent water reabsorption by increasing osmotic pressure at the renal tubule side, and increase urine volume. Thus α-EG could be used as a diuretic similarly to mannitol, which is an osmotic diuretic used in various clinical settings, including brain edema, glaucoma, and acute renal failure (Lameire & Vanholder 2001).

Kitamura *et al.* (1997) reported that α-EG applied to mouse skin has physiologic effect, and α-EG in medium accelerates cell differentiation. In this investigation, an intact form of α-EG was detected in plasma. This surmised the possibility that α-EG might affect skin cell differentiation by ingestion without direct application to skin.

In conclusion, only a part of α-EG ingested was hydrolyzed in small intestine, and most α-EG was absorbed into blood stream intact through small intestinal wall. Absorbed α-EG was hydrolyzed in kidneys to some extent, and excreted into urine. Moreover it was found that α-EG was an insulin-independent, diuretic, and less-nutritive glucoside that was safely applicable to food.

Chapter 4

Effects of ethyl α-D-glucoside feeding on urine excretion and morphologic changes of rat kidneys

In Chapter 3, it was revealed that α-EG was detected in plasma and approximately 60~ 90% of α-EG ingested was recovered from urine of the rats received α-EG solution *ad libitum*. Therefore it was surmised that α-EG was low energy food component. Ingestion of α-EG solution increased urine volume significantly. Several papers reported that ingestion of some materials, *e.g.* erythritol, which were absorbed into blood stream and excreted in urine, caused morphologic changes of renal tissue without any detectable damage (Bär *et al.* 1995, Ogino *et al.* 1994, Til *et al.* 1996). Hence it is interesting and important to investigate how α-EG ingestion affects kidneys. In this Chapter, effects of α-EG ingestion on urine excretion and morphologic change of kidneys are investigated using rats.

Materials and Methods

Reagents and Animals

α-EG solution (34%) was obtained from Ozeki Co. Uric acid assay (Uric acid C-Test Wako) and urea assay (Urea nitrogen B-Test Wako) kits were purchased from Wako Pure Chemicals Co. Ltd. Other reagents were purchased from Nacalai Tesque Inc. unless otherwise indicated. Male Wistar ST clean rats (4 wks old, weighing 80~ 100 g) were purchased from Japan SLC Inc. Care and use of the rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals (1980), and animal experiments were approved by ethical committee of Faculty of Agriculture in Gifu University.

HPLC apparatus and analytical conditions

α-EG in urine was kindly analyzed at Ozeki Co. as indicated in Chapter 3. In brief, the chromatographic system equipped with a sugar column and an evaporative light scattering detector was used. The column was eluted with a linear gradient of CH_3CN and H_2O and flow rate was set at 1.0 mL/min throughout the analysis.

Conditions of feeding experiments

Feeding experiment was performed for 39 days. Rats were divided into three groups (n= 6) and individually housed in a wire-bottomed stainless cage in a temperature- controlled room (23±1°C) with 12-h light/12-h dark cycle (light; 6:00~ 18:00). They were given free access to tap water and each experimental diet. Basal diet consisted of casein (20%), soy bean oil (5%), α-cornstarch (20%), cellulose powder (5%), DL-methionine (0.3%), choline bitartrate (0.2%), AIN-76^{TM} mineral mixture (3.5%), AIN-76^{TM} vitamin mixture (1.0%) and sucrose (45%). For the control diet, water (298 g/kg) was added to the diet during the pelleting process to adjust water content in the experimental diets. α-EG was incorporated into the diet at 10% (10% α-EG group) or 20% (20% α-EG group) on dry weight bases at the expense of sucrose. During the feeding period, drink and food intakes were measured every day and 24-hour urine was collected to check its volume once a week. α-EG in urine was determined by HPLC method as described above.

On the last day of the feeding period, blood was drawn from an abdominal aorta through a syringe treated with 1% heparin-Na under ether anesthesia. Plasma was obtained after centrifugation at 1,500x *g* for 20 min at 4°C with a refrigerated centrifuge RS-20 IV. Plasma uric acid and urea were determined using Wako kits.

Preparation of kidney specimen and histopathological test

Preparations of kidney specimen and histopathological test were kindly performed by Dr. Yanai (Faculty of Applied Biological Sciences, Veterinary Science of Gifu University).

Kidneys were fixed with 10% buffered formalin. The tissues were then embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (HE) (Yanai *et al.* 1993). Photomicrographs of renal specimens were used for histopathological test and evaluation of dilated level on renal (distal and collecting) tubules.

Statistical analysis

Statistical significance among means was estimated at $P< 0.05$ according to ANOVA and Duncan's multiple range test (Shinjo 2004).

Results

Transient diarrhea and vomiting were not observed and no mortality occurred in all rats during the feeding period. Table 4-1 summarizes total food and drink intakes, concentrations of uric acid and urea nitrogen in plasma and other growth parameters. Although final body weight and total food intake did not show significant differences among the three groups, body weight gain in the 20% α-EG group was significantly lower than that of the Control group. Feed efficiency showed significant decrease in the α-EG groups. Total drink intake was increased with increasing α-EG consumption. No increase in plasma uric acid and urea levels was observed in the α-EG groups. Table 4-2 summarizes organs weights including those relative to 100 g body weight. Kidneys weights in the α-EG groups were significantly higher and epididymal and abdominal fatty pad weights were significantly lower than those of the Control group. As shown in Fig. 4-1, the urine volumes in the 10 and 20% α-EG groups were higher about 1.2~ 1.9-fold and 2.1~ 4.2-fold than the Control group, respectively. Figure 4-2 shows the urinary excretion of α-EG in the feeding experiment. Approximately 60~ 90% of ingested α-EG was recovered from the urine. The photomicrographs of kidney specimen were shown in Fig. 4-3, which indicated typical dilation levels as "no significantly-dilated" in Fig. 4-3-(a), "slightly-dilated" in Fig. 4-3-(b)

and "moderately-dilated" in Fig. 4-3-(c). The number of the specimen evaluated in dilation level as "no significantly-dilated", "slightly-dilated" and "moderately-dilated", respectively, in each group were as follows; the Control group 2/6, 4/6 and 0/6; the 10% α-EG group, 0/6, 6/6 and 0/6; the 20% α-EG group, 0/6, 3/6 and 3/6. No necrosis, inflammation and edema of cell were observed in all specimens in terms of histopathological test.

Table 4-1 Final body weight, body weight gain, total food intake, total drink intake, feed efficiency and plasma uric acid and urea concentrations in the experimental rat groups

	Group		
	Control	10% α-EG	20% α-EG
Final body weight (g)	244 ± 8	236 ± 5	230 ± 6
Body weight gain (g)	156 ± 6 ^{a}	146 ± 4 ^{ab}	138 ± 6 ^{b}
Total food intake (g) *	775 ± 30	782 ± 30	811 ± 22
Total α-EG intake (g)	0 ± 0 ^{c}	77 ± 3 ^{b}	162 ± 4 ^{a}
Total water intake (g)	517 ± 55 ^{b}	601 ± 26 ^{b}	990 ± 32 ^{a}
Feed efficiency	0.200 ± 0.003 ^{a}	0.188 ± 0.004 ^{b}	0.170 ± 0.004 ^{c}
Plasma uric acid (mg/100 mL)	1.01 ± 0.11	0.94 ± 0.07	0.76 ± 0.07
Plasma urea (mg/100 mL)	17.1 ± 1.1 ^{a}	14.3 ± 0.5 ^{b}	14.9 ± 0.3 ^{ab}

Values are means ± standard error for six rats. Means in the same row not sharing the same superscript are significantly different at $P< 0.05$. Test diets were AIN-76™ based diet *ad libitum* (Control), 10% α-EG diet *ad libitum* (10% α-EG) and 20% α-EG diet *ad libitum* (20% α-EG).

* Wet weight in which water was contained at 298 g/kg diet.

Table 4-2 Organs and fatty pads weights in the experimental rat groups

	Group		
	Control	10% α-EG	20% α-EG
Liver (g)	10.6 ± 0.5	10.8 ± 0.3	11.3 ± 0.4
Liver (g/100 g BW)	4.34 ± 0.09 [b]	4.59 ± 0.12 [b]	4.90 ± 0.08 [a]
Kidneys (g)	1.61 ± 0.06 [b]	1.77 ± 0.02 [a]	1.89 ± 0.05 [a]
Kidneys (g/100 g BW)	0.66 ± 0.01 [c]	0.75 ± 0.01 [b]	0.82 ± 0.01 [a]
Spleen (g)	0.56 ± 0.02	0.60 ± 0.02	0.59 ± 0.03
Spleen (g/100 g BW)	0.230 ± 0.004 [b]	0.256 ± 0.005 [a]	0.257 ± 0.007 [a]
Epididymal fatty pad (g)	5.72 ± 0.45 [a]	4.30 ± 0.30 [b]	3.64 ± 0.19 [c]
Epididymal fatty pad (g/100g BW)	2.34 ± 0.14 [a]	1.82 ± 0.10 [b]	1.59 ± 0.11 [b]
Abdominal fatty pad (g)	5.65 ± 0.43 [a]	4.24 ± 0.26 [b]	3.84 ± 0.27 [b]
Abdominal fatty pad (g/100g BW)	2.31 ± 0.13 [a]	1.79 ± 0.09 [b]	1.67 ± 0.10 [b]

BW, body weight

Values are means ± standard error for six rats. Means in the same row not sharing the same superscript are significantly different at $P < 0.05$. For feeding conditions, see the legend for Table 4-1.

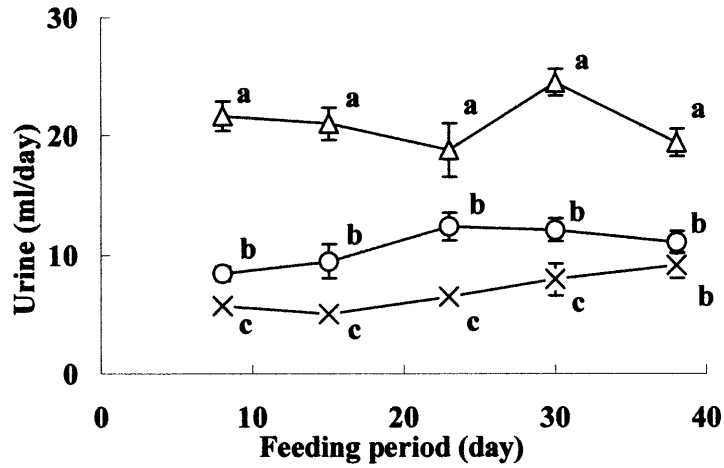

Fig. 4-1 Urine volume during the feeding period

Urine volume in the feeding period was plotted. The Control group (×) was given AIN-76^{TM} based diet *ad libitum*, the 10% α-EG group (○) was given 10% α-EG containing diet *ad libitum*, and 20% α-EG group (△) was given 20% α-EG containing diet *ad libitum*. Means in the same day not sharing the same alphabet are significantly different at $P< 0.05$. Each point represents mean ± standard error (n = 6).

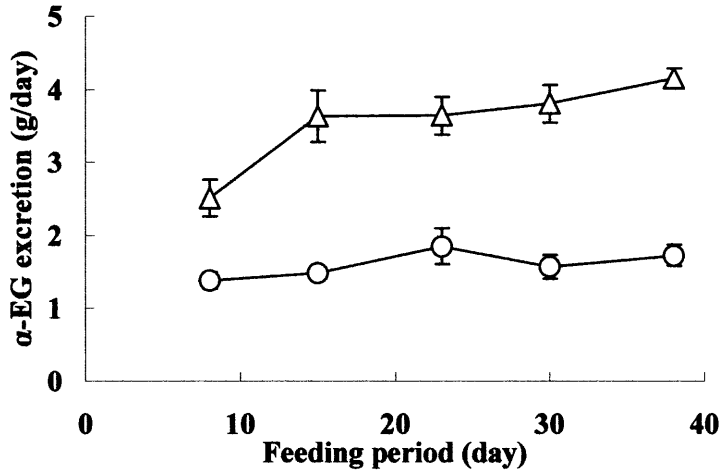

Fig. 4-2 Urinary excretion of α-EG in the feeding experiment

Urinary excretion of α-EG in the feeding experiment was plotted. α-EG was not detected in the urine of the Control group given AIN-76^{TM} based diet. The 10% α-EG group (○) was given 10% α-EG containing diet *ad libitum*, and 20% α-EG group (△) was given 20% α-EG containing diet *ad libitum*. Each point represents mean ± standard error (n = 6).

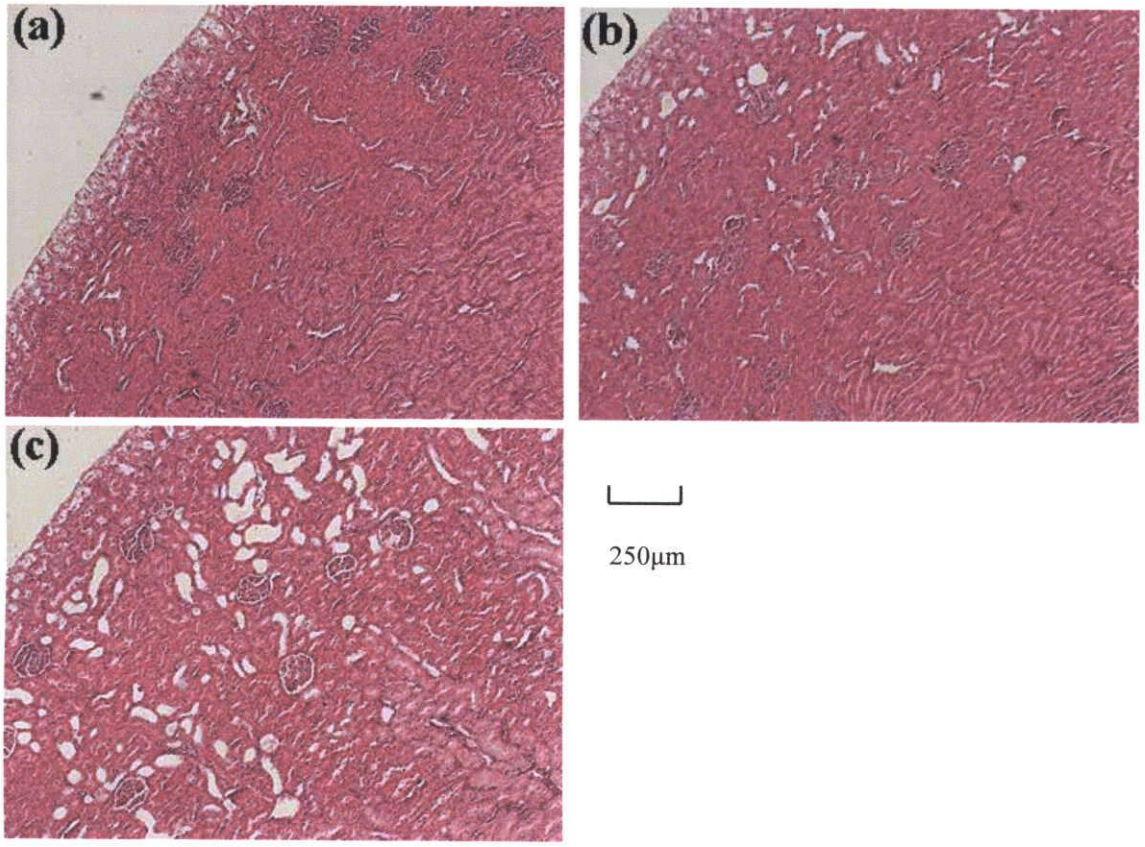

Fig. 4-3 Typical microphotographs of kidney specimens of the experimental rats

Fig. 4-3-(a) showed the one of the microphotographs of kidney specimen in the Control group evaluated in dilated level as "no significantly-dilated", Fig. 4-3-(b) showed the one of the microphotographs of kidney specimen in the 10% α-EG group evaluated in dilated level as "slightly-dilated" and Fig. 4-3-(c) showed the one of the microphotographs of kidney specimen in the 20% α-EG group evaluated in dilated level as "moderately-dilated". Kidneys were fixed with 10% buffered formalin. The tissues were embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (HE).

Discussion

During the α-EG feeding period, approximately 60~ 90% of ingested α-EG was recovered form the urine (Table 4-1 and Fig. 4-2). This result consisted with the previous study in which Wistar ST rat, 24 wks old, male, were received 0.46 M α-EG sample solution *ad libitum* (Chapter 3). Additionally, α-EG was absorbed through the small intestinal wall *via* SGLT1 (Chapter 2). These data suggested that α-EG was efficiently absorbed and excreted in urine easily as intact form. Feed efficiencies of the rats fed α-EG diets showed significantly low values (Table 4-1) depending on α-EG content in diets. This result was due to low hydrolyzing activity for α-EG of α-glucosidase (Chapter 1) and highly urinary excretion. Low energy value of α-EG was reflected on decreased fatty pad weights and decreases in weight gain and feed efficiency (Table 4-1). It might be probable that more protein from food was utilized for catabolism and resulted in decreases in weight gain and feed efficiency in the α-EG fed groups as compared to the Control group.

Urine volume of the rats fed α-EG increased with increasing α-EG consumption (Table 4-1 and Fig. 4-1). In general, high concentration of urinary excrements caused increase in urine volume by increasing osmotic pressure (Bär *et al.* 1995, Ogino *et al.* 1994,Til *et al.* 1996). In fact, α-EG concentrations in urine of the α-EG groups were more than 0.1 g/mL urine. Therefore it was suggested that α-EG excreted in urine increased urine volume by increasing osmotic diuresis. In several reports, α-EG was detected in urine of the man who took it from Sake (Imanari & Tamura 1971, Teague *et al.* 2004). From these results, it was surmised that a large amount of α-EG ingestion might contribute to osmotic diuresis in human as well as in rats.

Increases in kidneys weight and dilation level of renal tubules were related to the dietary α-EG level (Table 4-2 and Fig. 4-3). In terms of safety assessment on kidneys, it was important that there was no disorder of the renal cells even at the 20% dietary level (Fig.

4-3-(c)). Exposure at the 20% α-EG dietary level corresponded to ingestion of 27.5±1.1 g α-EG/kg body wt/day. If the renal exposure was calculated from the kidneys weights and the amounts of α-EG excreted, value was more than 1.5 g α-EG/g kidneys/day for rats fed diet containing 20% α-EG. The dilation of renal tubules might be caused by the increased diuresis and workload that was associated with the elimination of high amounts of absorbed α-EG. Increased kidneys weight was observed by ingestion of some soluble materials (Bär *et al.* 1995, Ogino *et al.* 1994, Til *et al.* 1996), such as erythritol that was efficiently absorbed and excreted unchanged into the urine. According to the calculation by Til *et al.* (1996), renal exposure even at 2.0 g erythritol/g kidneys/day did not cause detectable histopathological disorder in mice. The absence of any detectable histopathological damage on kidneys attested the lack of any adverse effects of α-EG up to 20% dietary level. As for renal functions, plasma uric acid and urea levels in the rats fed α-EG diets were decreased compared to the rats fed the control diet (Table 4-1) and glucosuria was not observed (data not shown). Increase in urine volume by α-EG might enhance urinary excretions of uric acid and urea from plasma. In general, fatal impairment by hyperuricemia was not observed in rodents which have uricase (EC 1.7.3.3) (Kang *et al.* 2002, Mazzali *et al.* 2001). However Bekairi *et al.* (1987) reported that plasma uric acid and urea levels were increased in ethanol-treated Wistar rat. Ethanol increases plasma uric acid level by enhancing purine nucleotide degradation and decreasing uric acid excretion by production of lactic acid (Nishimura *et al.* 1994, Yamamoto 2003, Yamanaka 1996) and it increases plasma urea level by change of amino acids metabolic patterns (Bekairi *et al.* 1987, Avogaro *et al.* 1986, Eriksson *et al.* 1980). Consequently, plasma uric acid and urea levels were not increased after high dose of α-EG (Table 4-1) differently from ethanol ingestion.

The exposure might disappear after discontinuation of α-EG because it was easily excreted into urine. α-EG might not be accumulated in body even if α-EG would be

reabsorbed by renal SGLTs in kidneys because of existence of α-EG hydrolyzing enzymes in kidneys (Chapter 1). Furthermore, α-EG did not cause severe renal impairment differently from drugs such as amino-glucosides (Chabner *et al.* 2001, Kahlmeter & Dahlager 1984).

In conclusion, ingested α-EG was excreted in urine as intact form with increasing urine volume. Kidneys weight was increased and renal tubules were dilated by α-EG ingestion without any detectable histopathological damage on renal cells and adverse effects on renal functions in terms of plasma uric acid and urea nitrogen levels in Wistar rats.

Chapter 5

Effects of discontinuation of ethyl α-D-glucoside feeding on kidneys and urine volume in rats

In Chapters 3 and 4, most of α-EG ingested from drink or diet was excreted into urine of Wistar rats. Furthermore, author reported that a large amount of α-EG ingestion caused osmotic diuresis and dilation of renal tubules with increased kidneys weight. Similar observations of osmotic diuresis and increased kidneys weight by ingesting indigestive soluble materials were reported. (Bär *et al.* 1995, Ogino *et al.* 1994, Til *et al.* 1996). The studies in Chapters 3 and 4 demonstrated that this renal morphological change by α-EG ingestion did not related to detectable histopathological damage on renal cells and adverse effects on renal functions in terms of plasma uric acid and urea levels (Chapter 4). Renal hypertrophy and glomerular hyperfiltration had relevance with hyperglycemia (Christiansen *et al.* 1981, Mogensen & Andersen 1973, Stalder & Schmid 1959), but some researchers showed that osmotic diuresis induced by diuretics was different from osmotic diuresis associated with hyperglycemia (Ishikawa *et al.* 1980, Sladek & Knigge 1977, Zerbe & Robertson 1983). Til *et al.* (1996) reported that erythritol ingestion caused osmotic diuresis and increased kidneys weight, and adverse effects on kidneys were not observed.

Whereas the author reported that α-EG showed osmotic diuresis (Chapters 3 and 4), duration of diuresis by α-EG remained unknown. In this Chapter, the author investigated whether osmotic diuresis and dilation of renal tubules by a large amount of α-EG ingestion could be normalized after discontinuation of α-EG ingestion and dilation of renal tubules was associated with increase of water elimination.

Materials and Methods

Reagents and Animals

α-EG sample solution was obtained from Ozeki Co. and other reagents were purchased from Nacalai Tesque Inc. Male Wistar ST clean rats (6 wks old, weighing 120~ 140 g) were purchased from Japan SLC Inc. Care and use of the rats in this study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals (1980), and animal experiments were approved by ethical committee of Faculty of Agriculture in Gifu University.

HPLC apparatus and analytical conditions

α-EG was kindly analyzed at Ozeki Co. as indicated in Chapter 3. In brief, the chromatographic system equipped with sugar column and evaporative light scattering detector was used. The column was eluted with a linear gradient of CH_3CN and H_2O and flow rate was set at 1.0 mL/min throughout the analysis.

Conditions of feeding experiments

Rats were individually housed in a wire bottom stainless steel cage in a temperature-controlled room (23±1 °C) with 12-h light/ 12-h dark cycle (light; 6:00~ 18:00), and were given free access to powder MF diet. In Experiment 1, rats were divided into three groups (n= 4), and were administered each test solution *ad libitum* for 26 days as indicated in parenthesis: Control-1 group (tap water), α-EG group (0.29 M α-EG), Glucose group (0.44 M glucose). In Experiment 2, rats were divided into two groups (n= 5), and were administered each test solution as indicated in parenthesis: Control-2 group (tap water for 42 days) and α-EGW group (0.29 M α-EG for 26 days followed by tap water for 16 days). During the feeding period, drink and food intakes were measured every day and 24-h urine was collected to check its volume once a week.

At the each end of the feeding period, rats were killed by withdrawing blood from ab-

dominal aorta using a syringe treated with 1% heparin- Na under ether anesthesia. Plasma was obtained after centrifugation of blood at 1,500x *g* for 20 min at 4 °C using a refrigerated centrifuge RS-20 IV. In Experiments 1 and 2, α-EG in plasma and urine was analysed by HPLC method as described above.

Preparation of kidney specimen and histopathological test

Preparation of kidney specimen and histopathological test were kindly performed by Dr. Yanai as described in Chapter 4. In brief, kidneys were fixed with 10% buffered formalin. The tissues were then embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (HE) (Yanai *et al.* 1993). Photomicrographs of renal specimens were used for histopathological test and evaluation of dilated level in renal (distal and collecting) tubules.

Statistical analysis

Statistical significance among means was estimated at $P< 0.05$ according to ANOVA followed by Duncan's multiple range test, student's *t*- test and Welch-test (Shinjo 2004).

Results

Transient diarrhea was not observed and no mortality occurred in all rats. Glucose was not detected from the urine of all rats during the experimental period.

Table 5-1 summarizes kidneys weight relative to 100 g body weight, plasma α-EG concentration and other growth parameters in Experiment 1. Total food intake in the Glucose and α-EG groups were decreased significantly compared to that in the Control-1 group, whereas final body weight did not show any significant difference among the three groups. Kidneys weight in the α-EG group was significantly increased compared to that of the Control and Glucose groups. α-EG was detected in the plasma of the α-EG group. Significantly higher drink intake and urine volume were observed in the α-EG and Glucose groups (Ta-

ble 5-1 and Fig. 5-1). Furthermore, these volumes in the Glucose group were significantly higher than those of the α-EG group. α-EG was detected in urine of the rats fed α-EG throughout the feeding period (Fig. 5-2).

Table 5-2 summarizes kidneys weight relative to 100 g body weight, plasma α-EG concentration and other growth parameters in Experiment 2. No significant difference was observed in kidneys weight between the Control-2 and α-EGW groups. α-EG was not detected in plasma at the end of the experiment (16 days after discontinuation of α-EG). Both drink intake (Fig. 5-3) and urine volume (Fig. 5-4) in the α-EGW group were significantly increased during the α-EG feeding period (up to day 26), however, they were normalized after discontinuation of α-EG (after day 27). In the α-EGW group, α-EG was excreted in urine through the α-EG feeding period, and α-EG was not detected in urine on day 28 (Fig. 5-5).

The details of dilation level as "no significantly-dilated", "slightly-dilated" and "moderately-dilated" are shown in Table 5-3. No necrosis, inflammation and edema were observed in all kidney specimens in terms of histopathological test.

Table 5-1 Body weight gain, final bodyweight, total food intake, total drink intake and plasma α-EG concentration and kidneys weight in Experiment 1

	Group		
	Control-1	EG	Glucose
Body weight gain (g)	154 ± 5	149 ± 8	155 ± 3
Final body weight (g)	285 ± 2	285 ± 10	292 ± 5
Total food intake (g)	480 ± 12 [a]	402 ± 15 [b]	299 ± 15 [c]
Total drink intake (g)	926 ± 57 [c]	1452 ± 89 [b]	2709 ± 134 [a]
Plasma α-EG (mM)	ND	4.49 ± 0.79	ND
Kidneys (g/100 g BW)	0.66 ± 0.02 [b]	0.74 ± 0.02 [a]	0.60 ± 0.02 [b]

ND, not detected; BW, body weight

Values are means ± standard error for four rats. Means in the same row not sharing the same superscript are significantly different at $P< 0.05$. The drinks were tap water *ad libitum* (Control-1), 0.29M α-EG solution *ad libitum* (EG), and 0.44M glucose solution *ad libitum* (Glucose).

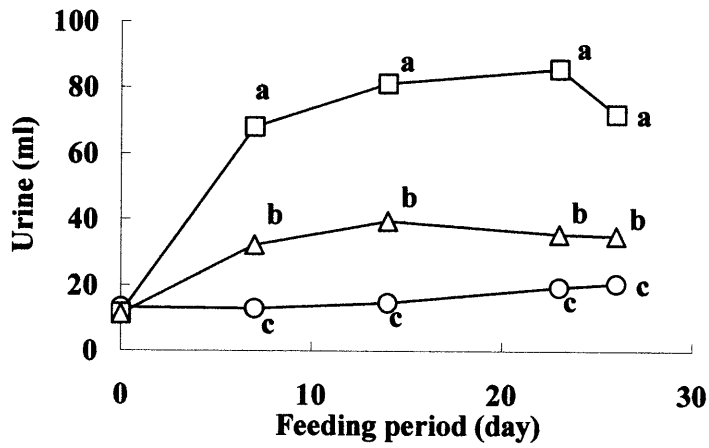

Fig. 5-1 Urine volume during the feeding period in Experiment 1

Urine volume in the feeding period was plotted. The Control-1 group (○) was given tap water *ad libitum*, the α-EG group (△) was given 0.29 M α-EG solution *ad libitum*, and the Glucose group (□) was given 0.44 M glucose solution *ad libitum*. Means in the same day not sharing the same alphabet are significantly different at $P< 0.05$. Each point represents mean ± standard error (n = 4).

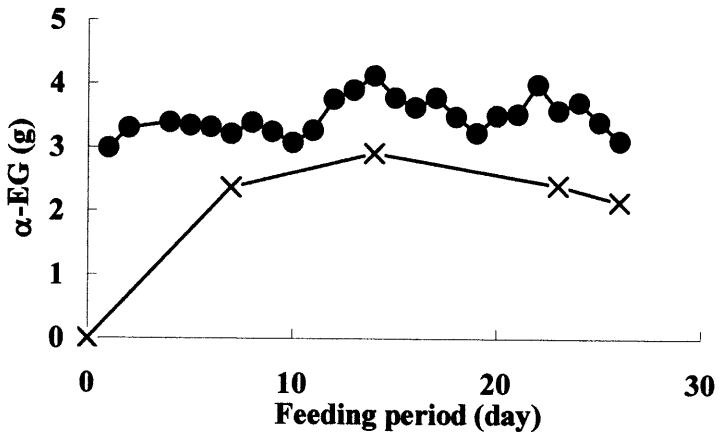

Fig. 5-2 α-EG intake and its urinary excretion during the feeding period in Experiment 1

α-EG intake and its urinary excretion in the feeding experiment were examined. α-EG intake (●) and α-EG excretion (×) in the α-EG group were plotted. The α-EG group was given 0.29 M α-EG solution *ad libitum*. Each point is mean ± standard error (n= 4).

Table 5-2 Body weight gain, final bodyweight, total food intake, total drink intake and plasma α-EG concentration and kidneys weight in Experiment 2

	Group	
	Control-2	α-EGW
Body weight gain (g)	198 ± 7	220 ± 7
Final body weight (g)	332 ± 8	353 ± 8
Total food intake (g)	793 ± 10	730 ± 19 *
Total drink intake (g)	1524 ± 94	2034 ± 109 *
Plasma α-EG (mM)	ND	ND
Kidneys (g/100 g BW)	0.58 ± 0.02	0.62 ± 0.01

ND, not detected; BW, body weight

Values are means ± standard error for five rats. *Significantly different from the Control-2 group *at* $P< 0.05$. The drinks were tap water *ad libitum* for 42 days (Control-2), and 0.29M α-EG solution for 26 days followed by tap water for 16 days *ad libitum* (α-EGW).

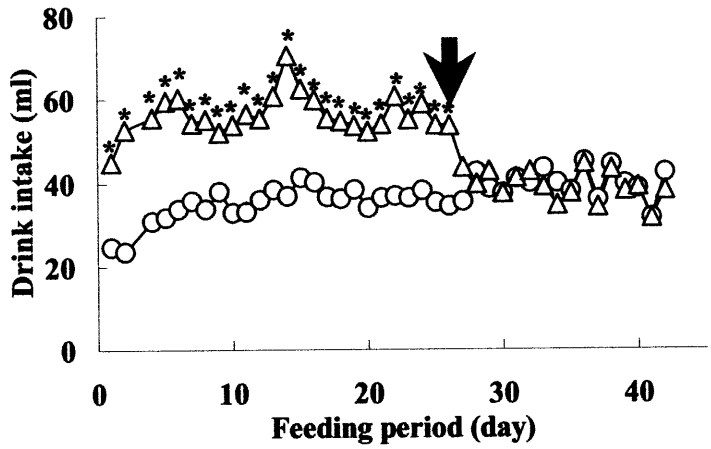

Fig. 5-3 Drink intake during the feeding period in Experiment 2

Drink intake in the feeding period was plotted. The Control-2 group (○) was given tap water for 42 days *ad libitum*, and the α-EGW group (△) was given 0.29 M α-EG solution for 26 days followed by tap water *ad libitum*. Each point represents mean ± standard error (n = 5). *Significantly different from the Control-2 group *at* $P< 0.05$. Arrow indicated the day when test solution was changed from α-EG solution to tap water in the α-EGW group.

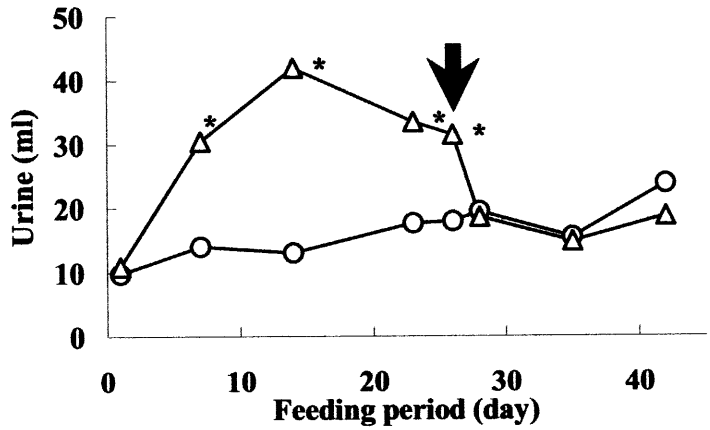

Fig. 5-4 Urine volume during the feeding period in Experiment 2

Urine volume in the feeding period was plotted. The Control-2 group (○) was given tap water for 42 days *ad libitum*, and the α-EGW group (△) was given 0.29 M α-EG solution for 26 days followed by tap water for 16 days *ad libitum*. Each point represents mean ± standard error (n = 5). *Significantly different from the Control-2 group *at* $P< 0.05$. Arrow indicated the day when test solution was changed from α-EG solution to tap water in the α-EGW group.

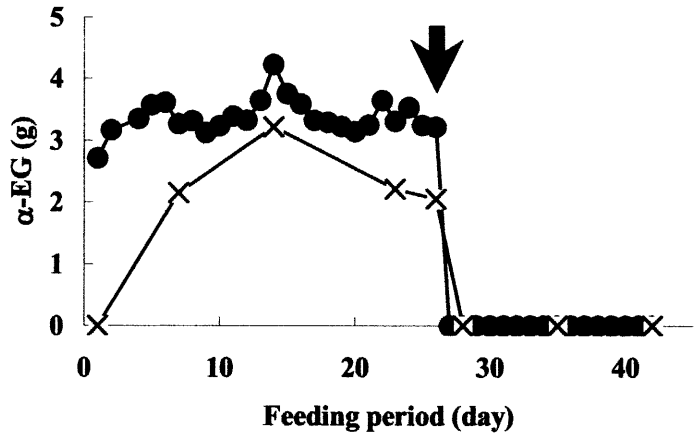

Fig. 5-5 α-EG intake and its urinary excretion during the feeding period in Experiment 2

α-EG intake and its urinary excretion of in the feeding experiment were examined. α-EG intake (○) and α-EG excretion (×) in the α-EGW group were plotted. The α-EGW group was given 0.29 M α-EG solution for 26 days followed by tap water for 16 days *ad libitum*. Each point is mean ± standard error (n= 5). Arrow indicated the day when test solution was changed from α-EG solution to tap water in the α-EGW group.

Table 5-3 Evaluation of morphological changes in kidneys in Experiments 1 and 2

	Group				
	Control-1	α-EG	Glucose	Control-2	α-EGW
Kidneys (n)	(4)	(4)	(4)	(5)	(5)
No significant	3	0	3	2	0
Slight	1	3	1	3	5
Moderate	0	1	0	0	0

Kidneys were evaluated in dilated level as "no significantly-dilated", "slightly-dilated" or "moderately-dilated". Kidneys were fixed with 10 % buffered formalin. The tissues were embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (HE). For feeding conditions, see legend for Tables 5-1 and 5-2.

Discussion

During the α-EG feeding period, rats in the α-EG and α-EGW groups received about 3.4 and 3.2 g α-EG/day, respectively. They were nearly equal to the amounts in the study of Chapter 4 in which rats were received α-EG containing diet (2.0~ 4.2 g α-EG/day). In the α-EG group, urine volume increased with increasing drink intake by α-EG ingestion, and α-EG was detected in urine at high concentrations (62~ 74 mg/mL urine). These results suggested that α-EG was associated with osmotic diuresis. Furthermore a large amount of α-EG ingestion induced dilation of renal tubules and increase in kidneys weight without adverse effects on kidneys in terms of histopathological test (Tables 5-1 and 5-3), which were consisted with previous study (Chapter 4). Some researchers reported that osmotic diuresis induced by mannitol or hypertonic sodium chloride solution was shown to be markedly different from osmotic diuresis associated with hyperglycemia (Christiansen *et al.* 1981, Mogensen & Andersen 1973, Sladek & Knigge 1977). On the other hand, in the Glucose group, drink intake was about 1.6-folds higher as compared to that of the α-EG group. When rats received carbohydrate solution *ad libitum*, such as glucose, its intake was markedly elevated (Mook *et al.* 1983). Consequently, increasing of drink intake increased urine volume (1.5~ 2.3-folds higher as compared to that of the α-EG group). Increase in urine volume in the Glucose group was simple water excretion and was not accompanied by diuresis differently from the α-EG group. Although more urine was excreted in the Glucose group as compared to the α-EG group, increase in kidneys weight (Table 5-1) and marked dilation of renal tubules were not observed (Table 5-3). These results suggested that dilation of renal tubules and increased kidneys weight were not induced by increased water elimination in kidneys. Til *et al.* (1996) reported that a large amount of erythritol ingestion increased kidneys weight and they suggested that increased kidneys weight might reflect the increased diuresis and workload that was associated with the elimination of a large

amount of absorbed erythritol. Furthermore, Ogino *et al.* (1994) reported that osmotic diuresis caused by urea infusion was observed in normal Wistar rats, and they concluded that increase in relative kidneys weight was not caused by fluid accumulation but renal hypertrophy/ hyperplasia because protein content in the kidneys was increased (Bär *et al.* 1995). Protein content in kidneys was not determined in this experiment, but the results in Experiments 1 and 2 indicated that the renal morphologic changes in the rats fed α-EG might be caused by workload that was not associated with simple water elimination but the elimination of a large amount of absorbed α-EG.

In the α-EG group, α-EG was not detected in the urine on day 28 of feeding period which was collected over a period of 24~ 48 h after discontinuation of α-EG. According to Teague *et al.* (2004) using the ^{1}H NMR spectroscopy method, α-EG was still detectable up to 28 h after ingestion in the urine of the man who took Sake. The difference might be drawn from the differences in limit of detection. However α-EG excretion into urine was rapidly declined within 24 h after discontinuation (Fig. 5-5), and drink intake and urine excretion in the α-EGW group were normalized after discontinuation of α-EG (Figs. 5-3 and 5-4). These results indicated that osmotic diuresis by α-EG was transient. Furthermore, between the α-EGW and Control groups, no significant differences in dilation level of renal tubules and kidneys weight were observed (Tables 5-2 and 5-3), which suggested that dilation of renal tubules would not progress after discontinuation of α-EG and tend to be normalized.

Active transport system for sugars (SGLT1) distributed in small intestine has been shown to play an important role in the transport of α-EG (Landau *et al.* 1962, Ramaswamy *et al.* 1976), and SGLTs distributed in renal proximal tubules transported alkyl glucosides (Kipp *et al.* 1996). Therefore a part of α-EG might be reabsorbed by these renal absorption systems. Plasma α-EG was detected in the α-EG group (Experiment 1), whereas it was not

detected in the α-EGW group in which α-EG ingestion was discontinued for 16 days (Experiment 2). In Chapter 1, the author showed that α-EG hydrolyzing activity was detected in crude enzyme solution prepared from rat kidneys. Therefore α-EG was hydrolyzed in kidneys even if α-EG might be reabsorbed in kidneys. Consequently, α-EG might not be retained in body. At present, the accurate information for retention time of α-EG in body remains unknown. However the history of α-EG ingestion might not be observed in blood at least over 16 days and in urine at least over 2 days.

In conclusion, α-EG disappeared from urine and plasma after its discontinuation, furthermore osmotic diuresis by α-EG was transient. Dilation of renal tubules with increasing kidneys weight by a large amount of α-EG ingestion was not caused by increasing water elimination and it was not progressed by its discontinuation.

Conclusion

Ethyl α-D-glucoside (α-EG) is a peculiar component in Sake, Japanese traditional alcohol beverage (Hayakawa *et al.* 2000, Imanari & Tamura 1971, Oka & Sato 1976, Oka *et al.* 1976). Therefore Japanese have been ingesting α-EG from Sake since ancient time. However few research papers dealing with the metabolic status of α-EG ingested has appeared. The purpose of this study was to reveal how α-EG is hydrolyzed, absorbed, excreted and how it affects animal body physiologically.

Hydrolyzing activity for α-EG was detected in crude enzyme solutions prepared from small intestinal mucosa and kidneys of Wistar rats (Chapter1). In the experiment using crude enzyme prepared from rat intestinal acetone powder, hydrolyzing activity for α-EG was much lower than those for disaccharides (Table 1-1). α-EG showed mixed-type inhibition for maltose and sucrose hydrolysis (Figs. 1-3 and 1-4), suggesting that α-EG might be hydrolyzed by maltase and sucrase in rat small intestine. Not all of ingested α-EG was hydrolyzed in small intestine, and α-EG exempted from hydrolysis in small intestine was absorbed into blood stream through the small intestinal wall (Fig. 2-1 and Table 3-1). Alkyl glucosides were transported on brash border membrane in small intestinal mucosa *via* sodium-dependent glucose transporter, type 1 (SGLT1). According to Landau *et al.* (1962), α-EG was extensively concentrated in everted hamster intestinal sac. Furthermore Ramaswamy *et al.* (1976) demonstrated that intestinal SGLT transported Ethyl β-D-glucoside (β-EG). As shown in Chapter 2, α-EG absorption was reduced by elimination of sodium-ion from experimental solution and addition of phlorizin in mucosal solution, (Figs. 2-3 and 2-4) using everted rat intestinal sac, suggesting that α-EG was absorbed through the small intestinal wall *via* SGLT1 in brush border membrane and facilitative glucose transporter, type 2 (GLUT2) in basolateral membrane, and consequently, α-EG was absorbed into blood

stream.

In renal proximal tubules, β-alkyl glucosides with short alkyl chain were transported by SGLTs (Kipp *et al.* 1996). Probably, a part of α-EG might be reabsorbed by these renal absorption systems. However, approximately 60~ 90% of α-EG ingested was excreted into urine of the rats fed α-EG (Chapters 3~ 5). Furthermore some researchers reported that α-EG was excreted into urine even when a minute amount of α-EG (1~ 7mg/mL) was ingested (Imanari & Tamura 1971, Teague *et al.* 2004). These observations suggested that α-EG filtered in kidneys was not completely reabsorbed back into blood stream.

In Chapters 3~ 5, α-EG was detected in urine of the rats fed α-EG at high concentration (more than 60 mg/mL urine). Furthermore urine volume of the rats fed α-EG increased with increase in α-EG consumption (Table 4-1 and Fig. 4-1). These results suggested that α-EG excreted into urine increased urine volume by osmotic diuresis. As described above, α-EG was excreted into urine of the man who took Sake (Imanari & Tamura 1971, Teague *et al.* 2004). From these results, it was surmised that a large amount of α-EG ingestion might contribute to osmotic diuresis in human as well as in rats. On the other hand, after discontinuation of α-EG ingestion, its concentration in urine declined rapidly within 24 h, and urine volume was normalized (Figs. 5-3 and 5-4), which indicated that osmotic diuresis by α-EG was transient.

A large amount of α-EG ingestion caused dilation of renal tubules and increase of kidneys weight (Tables 4-2 and 5-1). Increases in kidneys weight and dilation level of renal tubules were related to the dietary α-EG level (Table 4-1 and Fig. 4-2). The absence of any detectable histopathological damage in kidneys attested the lack of any adverse effects of α-EG up to 20% dietary level (Chapter 4). As for renal functions, plasma uric acid and urea levels were not increased after high dose of α-EG (Table 4-1). Thus a large amount of α-EG ingestion did not show adverse effect on kidneys. On the other hand, no increase in kidneys

weight (Table 5-1) and no marked dilation of renal tubules were observed (Table 5-3) in the rats fed glucose solution although urine volume was much higher (Fig. 5-1). Increased urine volume in the rats fed glucose solution was not associated with diuresis but simple water excretion. These results indicated that the renal morphologic changes in the rats fed α-EG might be caused by workload that was not associated with simple water elimination but the elimination of a large amount of absorbed α-EG.

α-EG was hydrolyzed by crude enzyme preparation from rat kidneys (Fig. 1-1). Therefore α-EG might not be retained in body even if α-EG was reabsorbed by renal SGLTs. The history of α-EG ingestion was not observed in blood and urine at least 16 days and 2 days after α-EG discontinuation, respectively. (Table 5-2 and Fig. 5-5).

α-EG was a low caloric food component because it was poorly hydrolyzed in the rat body and easily excreted into urine. In fact feed efficiencies of the rats fed α-EG containing diets showed significant low values (Table 4-1) depending on α-EG content in diets, and low energy value of α-EG reflected on decreased fatty pads weight and body weight gain (Table 4-1).

α-EG showed weak inhibition for hydrolysis of disaccharides (maltose and sucrose) by crude enzyme from rat intestinal acetone powder (Table 1-2). As shown in Table 1-2, IC_{50} values (concentration required for 50% inhibition of the reaction) of α-EG for maltose and sucrose hydrolysis were higher than that of arabinose and acarbose. Effect of α-EG as inhibitor for disaccharides hydrolysis was weak compared to that by arabinose and acarbose.

Kitamura *et al.* (1997) reported that α-EG applied to mouse skin showed physiological effect, and α-EG in medium was accelerated cell differentiation. In this investigation, intact form of α-EG was detected in plasma. This surmised the possibility that α-EG might affect skin cell differentiation by ingestion without direct application to skin.

In conclusion, only a part of α-EG ingested was hydrolyzed in small intestine, and

most of it was absorbed into blood stream as intact form through the small intestinal wall *via* SGLT1 in brush border membrane and GLUT2 in basolateral membrane. α-EG absorbed in blood stream was hydrolyzed in kidneys to some extent, but almost of plasma α-EG was excreted into urine with increase in urine volume by osmotic diuresis. Kidneys weight was increased and renal tubules were dilated by a large amount of α-EG ingestion without any detectable histopathological damage on renal cells and any adverse effects on renal functions in terms of plasma uric acid and urea nitrogen levels in Wistar rats. Osmotic diuresis by α-EG was not a permanent phenomenon, and α-EG disappeared from urine and plasma after its discontinuation. Furthermore dilation of renal tubules with increasing kidneys weight was caused by large amount of α-EG elimination and it did not get progressed when α-EG ingestion was discontinued. It was also found that α-EG was an insulin-independent, diuretic, and less-nutritive glucoside, which was safely applicable to food.

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References

Asano T, Yoshimura Y, Kunugita K. 1996. Sucrase inhibitory activity of D-xylose and effect on the elevation of blood glucose in rats. (in Japanese) Nihon Eiyo Shokuryo Gakkai Shi 49: 157-162.

Auwärter V, Sporkert F, Hartwig S, Pragst F, Vater H, Diefenbacher A. 2001. Fatty acid ethyl esters in hair as markers of alcohol consumption. Segmental hair analysis of alcoholics, social drinkers, and teetotalers. Clin Chem 47: 2114-2123.

Avogaro A, Cibin M, Croatto T, Rizzo A, Gallimberti L, Tiengo A. 1986. Alcohol intake and withdrawal: Effects on branched chain amino acids and alanine. Alcohol Clin Exp Res 10: 300-304.

Bär A, Til HP, Timonen M. 1995. Subchronic oral toxicity study with regular and enzymatically depolymerized sodium carboxymethylcellulose in rats. Food Chem Toxicol 33: 909-917.

Bekairi AM, Abulaban FS, Tariq M, Parmar NS, Ageel AM. 1987. Studies on ethanol and/or nicotine induced acute changes in the levels of plasma amino acids and other biochemical parameters of male Wistar rats. Alcohol Drug Res 7: 471-479.

Bormans GM, Oosterwyck GV, Groot TJ, Veyhl M, Mortelmans L, Verbruggen AM, Koepsell H. 2003. Synthesis and biologic evaluation of ^{11}C-methyl-D-glucoside, a tracer of the sodium-dependent glucose transporters. J Nucl Med 44:1075-1081.

Chabner BA, Ryan DP, Paz-Ares L, Garcia-Carbonero R, Calabresi P. 2001. *In*: Goodman and Gilman's the pharmacological basis of therapeutics, 10th edition (Hardman JG; Limbird LE; Gilman AG, eds) p1389-1459. McGrqw-Hill, New York.

Christiansen JS, Gammelgaard J, Frandsen M, Parving H-H. 1981. Increased kidney size, glomerular filtration rate and renal plasma flow in short-term insulin-dependent

diabetics. Diabetologia 20: 451-456.

Dahlqvist A. 1964. Method for assay of intestinal disaccharidases. Anal Biochem 7: 18-25.

Day AJ, Cañada FJ, Díaz JC, Kroon PA, Mclauchlan R, Faulds CB, Plumb GW, Morgan MRA, Williamson G. 2000. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. FEBS Lett 468: 166-170.

Eriksson T, Carlsson A, Liljequist S, Hagman M, Jagenburg R. 1980. Decrease in plasma amino acids in rat after acute administration of ethanol. J Pharm Pharmacol 32: 512-513.

Giudicelli J, Boudouard M, Delqué P, Vannier C, Sudaka P. 1985. Horse kidney neutral α-D-glucosidase: purification of the detergent-solubilized enzyme; comparison with the proteinase-solubilized forms. Biochim Biophys Acta 831: 59-66.

Giudicelli J, Delque-Bayer P, Sudaka P, Poiree JC. 1998. Renal neutral α-D-glucosidase has no role in transport of D-glucose derived from maltose hydrolysis. Am J Physiol Regul Integr Comp Physiol 274: R1150-R1157.

González Bosc LV, Vidal NA, Prieto R, Tur JA. 1998. Effect of atrial natriuretic peptide on α-methyl-D-glucoside intestinal active uptake in rats. Peptides 19: 1249-1253.

Gregory JF 3rd. 1998. Nutritional properties and significance of vitamin glycosides. Annu Rev Nutr 18: 277-296.

Gutschmidt S, Kaul W, Riecken EO. 1979. A quantitative histochemical technique for the characterisation of α-glucosidases in the brush-border membrane of rat jejunum. Histochemistry 63: 81-101.

Harmon DL, McLeod KR. 2001. Glucose uptake and regulation by intestinal tissues: Implications and whole-body energetics. J Anim Sci 79 (E. Suppl.): E59-E72.

Hayakawa K, Ando K, Yoshida N, Yamamoto A, Matsunaga A, Nishimura M, Kitaoka

M, Matsui K. 2000. Determination of saccharides in sake by high-performance liquid chromatography with polarized photometric detection. Biomed Chromatogr 14: 72-76.

Hirsh AJ, Yao SYM, Young JD, Cheeseman CI. 1997. Inhibition of glucose absorption in the rat jejunum: a novel action of α-D-glucosidase inhibitors. Gastroenterology 113: 205-211.

Ikeda TS, Hwang ES, Coady MJ, Hirayama BA, Hediger MA, Wright EM. 1989. Characterization of a Na^+/glucose cotransporter cloned from rabbit small intestine. J Membr Biol 110: 87-95.

Imanari T, Tamura Z. 1971. The identification of α-ethyl glucoside and sugar-alcohols in Sake. Agric Biol Chem 35: 321-324.

Ishikawa S, Saito T, Yoshida S. 1980. The effect of osmotic pressure and angiotensin II on arginine vasopressin release from guinea pig hypothalamo-neurohypophyseal complex in organ culture. Endocrinology 106: 1571-1578.

Jaakonmaki PI, Knox KL, Horning EC, Horning MG. 1967. The characterization by gas-liquid chromatography of ethyl β-D-glucosiduronic acid as a metabolite of ethanol in rat and man. Eur J Pharmacol 1: 63-70.

Kageyama S, Nakamichi N, Sekino H, Nakano S. 1997. Comparison of the effects of acarbose and voglibose in healthy subjects. Cli Ther 19: 720-729.

Kahlmeter G, Dahlager JI. 1984. Aminoglycoside toxicity - a review of clinical studies published between 1975 and 1982. J Antimicrob Chemother 13 (Supple. A): 9-22.

Kang D-H, Nakagawa T, Feng L, Watanabe S, Han L, Mazzali M, Truong L, Harris R, Johnson RJ. 2002. A Role for uric acid in the progression of renal disease. J Am Soc Nephrol 13: 2888-2897.

Kimmich GA, Randles J. 1981. α-Methylglucoside satisfies only Na^+-dependent trans-

port system of intestinal epithelium. Am J Physiol 241: C227-C232.

Kipp H, Lin J-T, Kinne RKH. 1996. Interactions of alkylglucosides with the renal sodium/D-glucose cotransporter. Biochim Biophys Acta 1282: 124-130.

Kitamura N, Ota Y, Haratake A, Ikemoto T, Tanno O, Horikoshi T. 1997. Effects of ethyl α-D-glucoside on skin barrier disruption. Skin Pharmacol 10: 153-159.

Koepsell H, Fritzsch G, Korn K, Madrala A. 1990. Two substrate sites in the renal Na(+)-D-glucose cotransporter studied by model analysis of phlorizin binding and D-glucose transport measurements. J Membr Biol 114: 113-32.

Kondo H. 1984. SAKE - a Drinker's Guide. Kodansha International. Tokyo.

Lameire N, Vanholder R. 2001. Pathophysiologic features and prevention of human and experimental acute tubular necrosis. J Am Soc Nephrol 12: S20-S32.

Landau BR, Bernstein L, Wilson TH. 1962. Hexose transport by hamster intestine *in vitro*. Am J Physiol 203: 237-240.

Lostao MP, Berjón A, Barber A, Ponz F. 1991. On the multiplicity of glucose analogues transport systems in rat intestine. Rev Esp Fisiol 47: 209-216.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Maeno M, Morimoto Y, Hayakawa T, Suzuki Y, Tsuge H. 1997. Feeding experiments of pyridoxine derivatives as vitamin B_6. Int J Vitam Nutr Res 67: 444-449.

Matsubara Y, Mizuno T, Sawabe A, Iizuka Y, Okamoto K. 1989. Structure and physiological activity of nitrogenous compounds and alkyl glycosides in lemon (*Citrus limon* BURM. *f.*), unshiu (*Citrus unshiu* MARCOV.), hassaku (*Citrus hassaku* HORT.), yuzu (*Citrus junos* SIEB) and Iyokan (*Citrus iyo*) peelings. (in Japanese) Nippon Nogeikagaku Kaishi 63: 1373-1377.

Mazzali M, Hughes J, Kim Y-G, Jefferson JA, Kang D-H, Gordon KL, Lan HY, Kiv-

lighn S, Johnson, RJ. 2001. Elevated uric acid increases blood pressure in the rat by a novel crystal-independent mechanism. Hypertension 38: 1101-1106.

Mogensen CE, Andersen MJF. 1973. Increased kidney size and glomerular filtration rate in early juvenile diabetes. Diabetes 22: 706-712.

Mook DG, Brane JA, Whitt JA. 1983. Effects of food deprivation on intake of solid and liquid sugars in the rat. Appetite 4: 259-268.

Muto N, Nakamura T, Yamamoto I. 1990. Enzymatic formation of a nonreducing L-ascorbic acid α-glucoside: Purification and properties of α-glucosidases catalyzing site-specific transglucosylation from rat small intestine. J Biochem 107: 222-227.

Nishimura T, Shimizu T, Mineo I, Kawachi M, Ono A, Nakajima H, Kuwajima M, Kono N, Matsuzawa Y. 1994. Influence of daily drinking habits on ethanol-induced hyperuricemia. Metabolism 43: 745-748.

Ogino Y, Okuda S, Ota Z. 1994. Effects of chronic, urea-induced osmotic diuresis on kidney weight and function in rats. Diabetologia 37: 225-231.

Oka S, Sato S. 1976. Contribution of ethyl α-D-glucoside to flavor construction in Sakè. (in Japanese) Nippon Nogeikagaku Kaishi 50: 455-461.

Oka S, Iwano K, Nunokawa Y. 1976. Formation of ethyl α-D-glucoside in Sakè brewing. (in Japanese) Nippon Nogeikagaku Kaishi 50: 463-468.

Olson AL, Pessin JE. 1996. Structure, function, and regulation of the mammalian facilitative glucose transporter. Annu Rev Nutr 16: 235-256.

Pragst F, Auwaerter V, Sporkert F, Spiegel K. 2001. Analysis of fatty acid ethyl esters in hair as possible markers of chronically elevated alcohol consumption by headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS). Forensic Sci Int 121: 76-88.

Ramaswamy K, Bhattacharyya BR, Crane RK. 1976. Studies on the transport of ali-

phatic glucosides by hamster small intestine *in vitro*. Biochim Biophys Acta 433: 32-38.

Reiss U, Sacktor B. 1982. Alteration of kidney brush border membrane maltase in aging rats. Biochim Biophys Acta 704: 422-426.

Sanai K, Seri K, Inoue S. 1997. Inhibition of sucrose digestion and absorption by L-arabinose in rats. (in Japanese) Nihon Eiyo Shokuryo Gakkai Shi 50: 133-137.

Sato K, Oku K, Yoneyama M, Tsujisaka Y. 1993. Glucosyl lactoside (O-β-D-galactopyranosyl-(1→4)-O-β-D-glucopyranosyl α-D-glucopyranoside) synthesized by the intermolecular transglucosylation using α-glucosidase. (in Japanese) Denpun Kagaku 40: 391-396.

Shimizu M, Kobayashi Y, Suzuki M, Satsu H, Miyamoto Y. 2000. Regulation of intestinal glucose transport by tea catechins. BioFactors 13: 61-65.

Shinjo A. 2004. Seibutu toukeigaku nyuumonn. (in Japanese) Asakura Shotenn Co. Tokyo, 4th edt.

Shinohara H, Goda T, Takase S, Sugawa-Katayama Y. 1993. Feeding medium-chain triglycerides to rats decreases degradation of sucrase-isomaltase complex in the jejunum. J Nutr 123: 1161-1167.

Sladek CD, Knigge KM. 1977. Osmotic control of vasopressin release by rat hypothalamo-neurohypophyseal explants in organ culture. Endocrinology 101: 1834-1838.

Stalder G, Schmid R. 1959. Severe functional disorders of glomerular capillaries and renal hemodynamics in treated diabetes mellitus during childhood. Ann Paediatr 193: 129-138.

Takenaka F, Uchiyama H. 2001. Effects of α-D-glucosylglycerol on the *in vitro* digestion of disaccharides by rat intestinal enzymes. Biosci Biotechnol Biochem 65: 1458-1463.

Tamai I, Tsuji A. 1996. Carrier-mediated approaches for oral drug delivery. Adv Drug Deliv Rev 20: 5-32.

Teague C, Holmes E, Maibaum E, Nicholson J, Tang H, Chan Q, Elliott P, Wilson I. 2004. Ethyl glucoside in human urine following dietary exposure: detection by ^{1}H NMR spectroscopy as a result of metabonomic screening of humans. Analyst 129: 259-264.

Til HP, Kuper CF, Falke HE, Bär A. 1996. Subchronic oral toxicity studies with erythritol in mice and rats. Regul Toxicol Pharmacol 24: S-221-S231.

Trumbo PR, Banks MA, Gregory JF 3rd. 1990. Hydrolysis of pyridoxine-5'-β-D-glucoside by a broad-specificity β-glucosidase from mammalian tissues. Proc Soc Exp Biol Med 195: 240-246.

Wilson TH, Wiseman G. 1954. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J Physiol 123: 116-125.

Wood IS, Trayhurn P. 2003. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. Br J Nutr 89: 3-9.

Wright EM. 1998. Genetic disorders of membrane transport I. Glucose galactose malabsorption. Am J Physiol 275: G879-G882.

Wright EM. 2001. Renal Na^{+}-glucose cotransporters. Am J Physiol Renal Physiol 280: F10-F18.

Yamamoto I, Muto N, Nagata E, Nakamura T, Suzuki Y. 1990. Formation of a stable L-ascorbic acid α-glucoside by mammalian α-glucosidase-catalyzed transglucosylation. Biochim Biophys Acta 1035: 44-50.

Yamamoto T. 2003. Effect of ethanol on uric acid level. (in Japanese) Nippon Rinsho 61 (Supple. 1): 143-147.

Yamanaka H. 1996. Alcohol ingestion and hyperuricemia. (in Japanese) Nippon Rinsho 54: 3369-3373.

Yanai T, Masegi T, Ueda K, Manabe J, Teranishi M, Takaoka M, Matsunuma N, Fukuda K, Goto N. 1993. Spontaneous globoid mineralization in the cerebellum of rats. J Comp Pathl 109: 447-451.

Yegles M, Labarthe A, Auwärter V, Hartwig S, Vater H, Wennig R, Pragst F. 2004. Comparison of ethyl glucuronide and fatty acid ethyl ester concentrations in hair of alcoholics, social drinkers and teetotallers. Forensic Sci Int 145: 167-173.

Zerbe RL, Robertson GL. 1983. Osmoregulation of thirst and vasopressin secretion in human subjects: effect of various solutes. Am J Physiol 244: E607-614.