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EVIDENCE FOR THE *IN VIVO* FORMATION OF ASCORBIC ACID 2-*O*-α-GLUCOSIDE IN GUINEA PIGS AND RATS

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Abstract—*In vivo* formation of ascorbic acid 2-*O*-α-glucoside (AA-2G) in guinea pigs and rats given ascorbic acid (AA) orally in combination with maltose was examined. A metabolite of AA which has the same HPLC retention characteristics as authentic AA-2G was detected in the blood, urine and liver of guinea pigs 1–2 hr after their administration. The metabolite was isolated from the urine by chromatographic procedures and identified as AA-2G by its UV spectrum, non-reducibility, susceptibility to α-glucosidase hydrolysis, HPLC profile and elementary analysis. The same glucoside was also synthesized by rats and found in the urine, although it could not be determined qualitatively in the blood. AA-2G-forming activities of tissue homogenates from both animals were apparently correlated with their α-glucosidase activities and, moreover, both activities were completely inhibited by a specific neutral α-glucosidase inhibitor. Thus, we conclude that AA-2G is a possible metabolite produced by enzymatic α-glucosidation after a combined administration of AA and maltose to guinea pigs and rats.

Ascorbic acid 2-*O*-α-glucoside (AA-2G‡) is an α-glucose conjugate of ascorbic acid (AA) at the C-2 position and a newly-found AA derivative [1]. This has been reported to be synthesized by regioselective transglucosylation with mammalian tissue [2, 3] and rise seed α-glucosidases [4]. In contrast to AA, AA-2G is characterized by its high stability toward thermal and oxidative degradation in aqueous solutions and its non-reducibility [1, 3, 4]. In addition, this glucoside has been shown not only to serve as an AA supplement when administered to experimental animals, but also to exert a potent therapeutic activity in scorbutic guinea pigs [5]. Furthermore, we have confirmed that AA-2G is able to stimulate the collagen synthesis in cultured human skin fibroblasts (manuscript in preparation). It can be, therefore, expected that AA-2G functions as vitamin C in man, who cannot synthesize AA in the liver, as well as guinea pigs.

So far, a number of 2-*O*-monosubstituted derivatives of AA including its sulfate [6, 7], phosphate [8] and methyl ether [9] were demonstrated to be stable *in vitro* and non-reducing. Among them, ascorbic acid 2-*O*-sulfate (AA-2S) [6, 10–12] and ascorbic acid 2-*O*-methyl ether (AA-2M) [13] were found as naturally occurring metabolites of AA. However, they were devoid of a substantial vitamin C activity in monkeys and guinea pigs [9, 14, 15]. On the other hand, ascorbic acid 2-*O*-phosphate (AA-2P) was demonstrated to exert an antiscorbutic activity in monkeys and guinea pigs [16], although it had not been equivocally identified as a metabolite of AA in these animals.

The enzyme which can synthesize AA-2G was first found in several tissues of rats and guinea pigs [2]. Therefore, it is necessary to ascertain whether AA-2G occurs as a metabolite of AA in these animals. Since there is a fundamental difference in the ability of AA biosynthesis between these animals, it is important to use both animals in order to understand the metabolism of AA in humans. In this work, we identified AA-2G as a metabolite of AA in guinea pigs and rats orally given AA together with maltose and then demonstrated that this glucoside might be produced by specific α-glucosidation catalysed by α-glucosidase.

MATERIALS AND METHODS

Materials. AA and its sodium salt (AA·Na) were purchased from Ishizu Pharmaceutical Co. (Osaka). Ascorbic acid 2-*O*-phosphate magnesium salt (AA-2P) and glucose assay kit were products of Wako Pure Chemical Co. (Osaka). Ascorbic acid 2-*O*-sulfate dipotassium salt (AA-2S) and rice seed α-glucosidase (EC 3.2.1.20) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2,6-Dichloroindophenol was from Tokyo Chemical Industry (Tokyo). Castanospermine was purchased from Boehringer Mannheim Yamanouchi (Tokyo). Maltose was from Hayashibara Biochemical Laboratories (Okayama). AA-2G and ascorbic acid 6-*O*-α-glucoside (AA-6G) were synthesized enzymatically and purified by HPLC in this laboratory, as described previously [2, 4]. All other reagents used were of analytical grade commercially available.

Administration study of AA. An aqueous solution (1.3 mL) containing 1 g of AA·Na and 0.5 g of maltose was given orally to male Hartley guinea pigs weighting 250–300 g (Kiwa Experimental Animal

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‡ Abbreviations: AA, ascorbic acid; AA-2G, ascorbic acid 2-*O*-α-glucoside; AA-2S, ascorbic acid 2-*O*-sulfate; AA-2P, ascorbic acid 2-*O*-phosphate; AA-2M, ascorbic acid 2-*O*-methyl ether; AA-6G, ascorbic acid 6-*O*-α-glucoside; HPLC, high performance liquid chromatography.

Table 1. Detection of AA-2G-like metabolite by HPLC in sera from guinea pigs given AA and maltose

Animal No.	Time (hr)	t_R (min) AA	t_R (min) AA-2G-like	Relative t_R AA-2G-like/AA
1	0	7.02 (13)	ND	—
	1	7.06 (3506)	8.50	1.204
	2	7.06 (563)	ND	—
2	0	6.96 (35)	ND	—
	1	6.94 (4204)	8.29	1.195
	2	6.94 (2332)	8.31	1.197
3	0	6.91 (33)	ND	—
	1	6.88 (1470)	8.27	1.202
	2	6.88 (455)	ND	—
4	0	6.84 (16)	ND	—
	0.7	6.87 (1702)	8.13	1.183
	1.5	6.88 (1151)	8.15	1.185
Standard		6.91	8.26	1.195

Guinea pigs were given orally a solution containing 1 g of AA·Na and 0.5 g of maltose, and blood samples were taken at the indicated times. The values in the parentheses represent serum AA level (nmol/mL).
ND, not detectable.

Lab., Wakayama) and male Wistar rats weighing 200–250 g (Japan SLC, Hamamatsu). In the case of guinea pigs, about 0.2 mL of blood was taken just before and at the indicated times after administration by heart puncture under light ether anesthesia and the animals were killed within 2 hr of administration. Each serum obtained by centrifugation was deproteinized by adding four volumes of ice-cold 1.06% metaphosphoric acid. At autopsy, the urine and bile samples were collected and an aliquot was mixed with four volumes of metaphosphoric acid. The liver was also excised and homogenized in four volumes of metaphosphoric acid with a glass–Teflon homogenizer. In the case of rats, urine was collected overnight with stainless steel metabolic cages and an aliquot was processed as described above. All samples were centrifuged at 5000 *g* for 10 min to obtain their supernatants.

Analysis and isolation of metabolite. For detection of metabolite, a Shimadzu LC-6A high performance liquid chromatograph was used with a Shimadzu SPD-6A spectrophotometric detector set at 240 nm and a C_{18} reverse-phase column (Shim-pack ODS, 6 × 150 mm, Shimadzu, Kyoto). The elution was carried out with 0.1 M phosphate buffer (pH 2.0) at a flow rate of 0.7 mL/min. The HPLC retention times (t_R, min) of AA metabolites were determined and relative t_R values were calculated as follows:

$$\text{relative } t_R = \frac{t_R \text{ of metabllite}}{t_R \text{ of AA}}.$$

The contents of AA and AA-2G were calculated on the basis of the individual standard curves plotted peak area and height, respectively, versus amount of each authentic compound.

To isolate a metabolite, the urine (1.5 mL) obtained from a guinea pig 2 hr after ingestion of AA·Na and maltose was applied to a Dowex 1 × 8 column (1.2 × 5 cm) equilibrated with distilled water. The eluate with 0.1 M ammonium formate was analysed by HPLC and the fractions containing a metabolite were collected and lyophilized. Further purification was achieved by HPLC under the same conditions described above. UV spectrum, reducibility toward 2,6-dichloroindophenol and susceptibility toward α-glucosidase and acid hydrolysis of the metabolite thus obtained were determined according to the method described previously [2].

Enzymatic formation of AA-2G by tissue homogenates. The liver, kidney, small intestine (proximal portion of jejunum) and spleen were removed from guinea pigs and rats and homogenized with nine volumes of 10 mM potassium phosphate buffer (pH 7.0) in a glass–Teflon homogenizer. The supernatants obtained at 1000 *g* for 20 min and each serum were used as enzyme sources. AA-2G-forming activity was determined by incubating 100 μL of tissue homogenates and 100 μL of substrate solution containing 35.6 μmol of maltose, 35.6 μmol of AA·Na, 2.6 μmol of thiourea and 0.2 M acetate buffer (pH 5.3) for 5 hr at 37°. AA-2G formed was quantitated by HPLC as described above. In another experiment, the activity was determined in a similar manner in the absence of maltose. α-Glucosidase activity was determined by measuring the rate of glucose formation from maltose by the method described previously [2]. Both activities were also measured in the presence of 0.5 mM castanospermine, a specific neutral α-glucosidase inhibitor [17].

RESULTS

Detection of AA-2G-like metabolite in guinea pigs and rats

Four guinea pigs which each received 1 g of

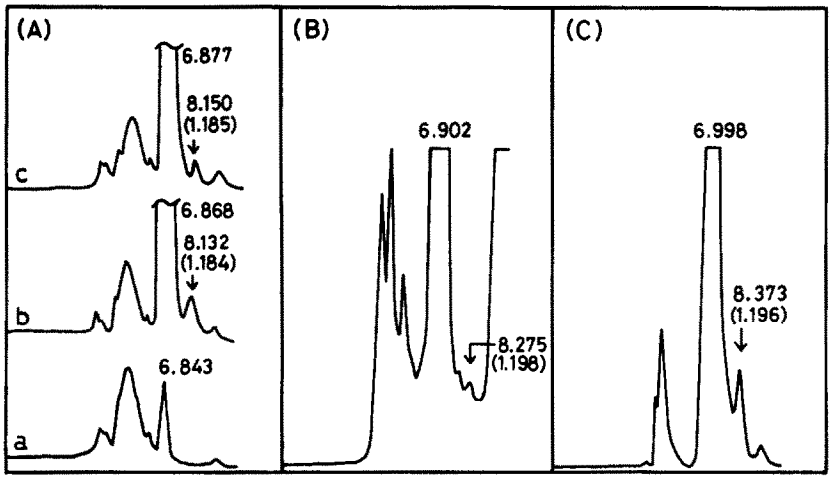

Fig. 1. Typical HPLC profiles of the serum (A), liver (B) and urine samples (C) from guinea pigs given AA and maltose. The blood was taken at 0 (a), 0.7 (b) and 1.5 hr (c) after administration and the liver and urine were removed at 1.5 hr after administration. A 10-μL aliquot of the processed sample was used for chromatographic analysis. The figures represent t_R values (min) of the peaks and the figures in the parentheses refer to their relative t_R values.

AA·Na in combination with 0.5 g of maltose were employed for detection of a new metabolite of AA. As summarized in Table 1, basal AA levels ranging from 13 to 35 nmol/mL of serum increased to 1470–4204 nmol/mL 1 hr after AA administration and simultaneously a new peak with a similar retention time to authentic AA-2G was detected in every serum. Two hours after administration the peak was still detectable in sera from two animals showing relatively high concentrations of serum AA. Since retention times were liable to change slightly with HPLC conditions such as temperature, pH and column deterioration, the relative t_R value, which was found to be almost constant, was used to determine the unknown peak. Based on this criterion, the peak appearing in sera showed relative t_R values remarkably similar to that of the authentic AA-2G. Typical HPLC profiles of sera just before and after AA administration are shown in Fig. 1A, together with those of the liver and urine from the same guinea pig. The peak with a similar relative t_R value was also detected in both samples (Fig. 1B and C), but not in the bile (data not shown). No corresponding peak appeared in the liver and urine from control guinea pigs. The relative t_R values of well-known derivatives of AA, AA-2P, AA-2S and AA-6G, were 0.91, 0.82 and 1.25, respectively, so at least two esters can be ruled out. These findings indicated that the AA-2G-like peak detected in the serum, liver and urine is the same product synthesized through the metabolism of AA.

The urine samples collected from control and AA-treated rats were also analysed by HPLC. Representative HPLC profiles from one of four animals are illustrated in Fig. 2. High levels of AA and an accompanying peak ($t_R = 7.77$ min) were detected in all samples from AA-administered rats. Such a peak was not observed in the urine of control rats. On the basis of relative t_R value and co-injection analysis with authentic AA-2G, this peak was also found to be the same as AA-2G-like substance detected in guinea pig samples. However, the corresponding peak could not be found in rat serum samples, because of a remarkable rise of serum AA concentration after its ingestion.

Isolation and identification of AA-2G from guinea pig urine

About 1.5 mL of urine containing the AA-2G-like substance was collected from guinea pig bladder 2 hr after AA administration. By HPLC analysis, the total amount of metabolite in the urine was calculated as corresponding to about 400 nmol (135 μg), equivalent to authentic AA-2G, simply indicating that urinary excretion is more than 0.008% of the given dose. The metabolite was isolated by chromatographic procedures to a purity of 97% on HPLC and analysed for its spectral and chemical properties (Fig. 3). Both bathochromicity and hyperchromicity observed in the UV spectra (Fig. 3A) were characteristic properties of AA and 2-monosubstituted AA derivatives [18], and the UV_{max}, 260 nm at pH 7.0 and 238 nm at pH 2.0, were consistent with those of AA-2G, but not AA, AA-2P, AA-2S and AA-6G [2]. The intact compound was not reducing towards a redox dye, but upon treatment with rice seed α-glucosidase it acquired reducing properties (Fig. 3B). Further evidence was obtained from its HPLC retention behaviour before and after acid hydrolysis (Fig. 3C). The compound heated for 3 min in boiling water showed the same retention characteristics, being heat-stable, as the reference compound, AA-2G. After mild acid hydrolysis in 1 N HCl at 100° for 3 min, the hydrolysate produced a new peak which appeared a little faster than the original one, and which really corresponded to AA. Simultaneously, glucose was

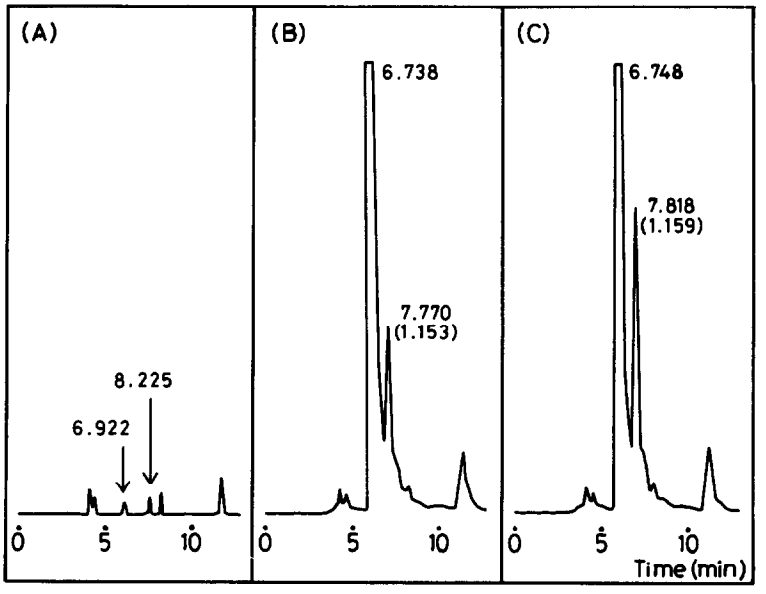

Fig. 2. Typical HPLC profiles of the urine samples from control (A) and AA-ingested rats (B). The urine was collected overnight from the rat given water or AA in combination with maltose. A 10-μL aliquot of the processed supernatant was injected alone (A and B) or co-injected with authentic AA-2G (C) to the column. The figures represent t_R values (min) of the peaks and the figures in the parentheses refer to their relative t_R values.

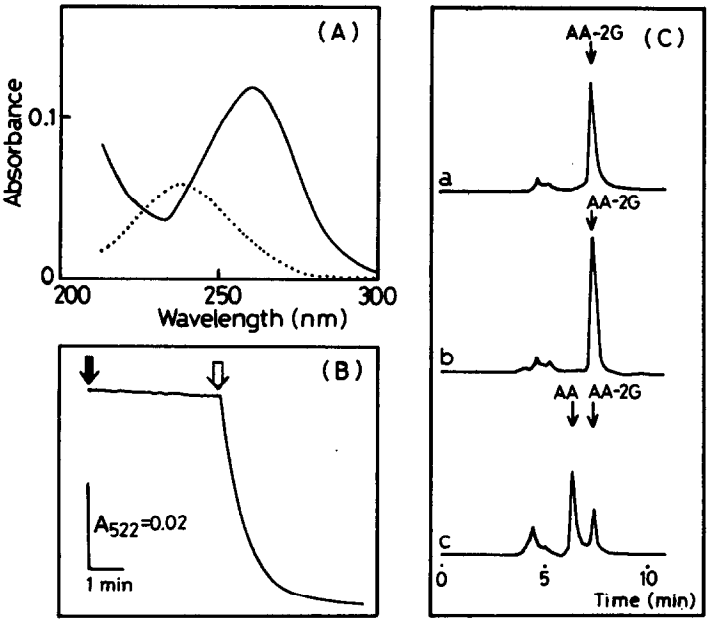

Fig. 3. Spectral and chemical properties of the AA-2G-like metabolite isolated from the urine of guinea pigs given AA and maltose. (A) UV spectra were measured in 0.1 M phosphate buffer [pH 7.0 (——) and pH 2.0 (·····)]. (B) Reducibility toward 2,6-dichloroindophenol was determined at 37° in 0.02 M acetate buffer (pH 5.3) and the decrease in absorbance was monitored at 522 nm. The metabolite and rice seed α-glucosidase were added at the positions indicated by black and white arrows, respectively. (C) Susceptibility to acid hydrolysis was examined by analysing the metabolite in HPLC after no treatment (a), heat treatment in water (b) and heat treatment in acid (c). The arrows indicate the elution positions of authentic AA and AA-2G.

Table 2. AA-2G-forming and α-glucosidase activities of tissue homogenates from guinea pigs and rats

Tissue	AA-2G formation (nmol/tube)		α-Glucosidase activity (units/mL)
	without maltose	with maltose	
Guinea pig			
Liver	0 ~ trace	768 (99)	0.205
Kidney	0 ~ trace	671 (99)	0.209
Jejunum	0	1860 (96)	0.764
Spleen	0	236 (100)	0.086
Serum	0	Trace	0.008
Rat			
Liver	104	492 (100)	0.195
Kidney	0 ~ trace	3854 (98)	2.700
Jejunum	0 ~ trace	2460 (98)	1.239
Spleen	0	287 (100)	0.073
Serum	0	328 (100)	0.289

Reaction mixture (200 μL) containing AA·Na and each tissue homogenate was incubated for 5 hr at 37° and at pH 5.3 in the presence or absence of maltose. The value in the parentheses shows the inhibition (%) of AA-2G formation by the α-glucosidase inhibitor, castanospermine.

detected in the solvent peak fraction by the glucose assay kit, as described in our previous paper [2].

AA-2G formation by tissue homogenates

Tissue homogenates and serum from guinea pigs were investigated for their ability to synthesize AA-2G (Table 2). When maltose was added to the reaction mixture, AA-2G-forming activity was found in small intestine, liver, kidney and spleen, but not serum. These activities were almost parallel to their α-glucosidase activities and, moreover, completely inhibited by the addition of 0.5 mM castanospermine, an inhibitor of α-glucosidase activity [5]. On the other hand, rat tissue homogenates and serum produced AA-2G more efficiently than guinea pig enzymes in the presence of maltose and these activities were also inhibited by castanospermine (Table 2). All enzyme preparations, except rat liver homogenates, exhibited little or no activity in the reaction mixture without maltose. Figure 4 shows the time course of AA-2G formation by rat liver homogenates in the presence or absence of maltose. AA-2G was apparently synthesized by these homogenates even in the absence of maltose.

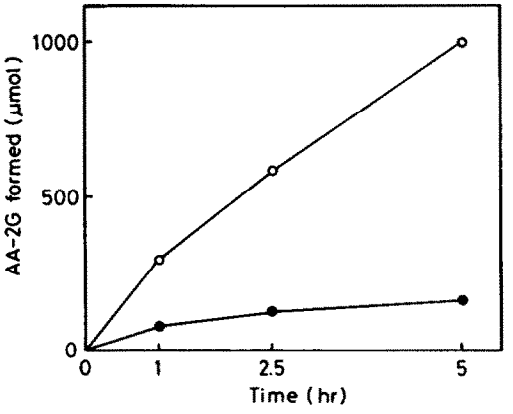

Fig. 4. Time-course of AA-2G formation by rat liver homogenates in the presence (○) or absence (●) of maltose. Enzyme reaction was carried out in the same way as described in Table 2 and the samples taken at the indicated times were analysed by HPLC for the quantitation of AA-2G.

DISCUSSION

Several results obtained for the new metabolite of AA support the theory that it is not a well-known AA derivative such as AA-2S, AA-2P or AA-2M, but a new one conjugated with α-glucose. Its non-reducibility and susceptibility to acid hydrolysis are characteristic of AA-2G, but not AA-6G [2]. Judging from these results, the metabolite detected in the blood and urine is concluded to be AA-2G. This is the first report of the occurrence of AA-2G in vertebrates. In spite of the high dose of AA given to the animal, the urinary excretion of AA-2G was very little. This is considered to be due to the saturable absorption of AA through the intestine, that is, the limited absorption at high dose levels. In AA-2G synthesis, the amount of product is considered to be proportional to that of AA as an acceptor. When the serum concentration of AA was elevated to 50–250 times that of the basal concentration after administration of 1 g of AA, the metabolic glucoside could be produced in the body and became detectable by our HPLC method. This means that it is difficult to detect the glucoside in normal animals by the present assay method. On the other hand, AA is widely consumed as a dietary supplement and gram amounts of AA are sometimes ingested with other carbohydrates by healthy individuals. In such a case, it may be possible to detect AA-2G in their sera or urine.

Smaller intestine, liver and kidney are the sites of absorption, metabolism and elimination and reabsorption of AA, respectively, and consequently these organs are exposed to high concentrations of AA after its ingestion [19]. These tissues were found to exhibit high AA-2G-forming activities *in vitro*. It seems reasonable to assume that AA-2G can be produced in at least one of these tissues. It was also demonstrated by the *in vitro* experiment that AA-2G was synthesized by a transglucosylation in which α-glucosidase utilized maltose as a glucose donor. Only rat liver homogenates had an ability to synthesize AA-2G moderately without the addition of maltose, though its endogenous glucose donor has not yet been identified. In addition, the possibility of its production by intestinal microorganisms was excluded by our previous finding that AA-2G could not be absorbed through the intestine after its oral administration to rats and guinea pigs [5]. Therefore, it appears that the occurrence of AA-2G in guinea pigs and rats results from the *in vivo* transglucosylation catalysed by α-glucosidase in these tissues. However, there is no direct evidence that the maltose given simultaneously with AA could be utilized for the production of AA-2G *in vivo*. Since our preliminary result indicates that guinea pigs cannot produce AA-2G after administration of AA without maltose, an α-glucose donor such as maltose must be required in this metabolic conversion of AA. The exact mechanism and site of this glucosidation of AA remain to be further elucidated.

Most glucoside conjugates so far reported are, to our knowledge, in the β-configuration. α-Glucosidation is a rare metabolic pathway, and only riboflavin, an endogenous material, has been reported to be conjugated with glucose in the α-configuration [20, 21]. Recently, Kamimura and coworkers [22, 23] have demonstrated the presence of α-glucoside conjugates of xenobiotics in rats. AA-2G is another example of α-glucoside formation *in vivo*. A limited amount of information is available on metabolites of AA in the body. AA-2S, first isolated from brine shrimp cysts [16], was shown to be a biliary [12] or urinary metabolite [10, 11] of AA in various species, while AA-2M was recovered from rat urine [13]. Although their physiological roles are not well understood, it is important to note that neither of these two compounds exhibited antiscorbutic activities in guinea pigs and monkeys [9, 14, 15]. Therefore, AA-2G is the first identified metabolite of AA which has a potential vitamin C activity. To clarify its physiological significance, it would be necessary to determine the exact amount of AA-2G in the tissues of animals at various experimental conditions by using a more sensitive assay method than the present HPLC.

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