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Orally Administered Crocetin and Crocins Are Absorbed into Blood Plasma as Crocetin and Its Glucuronide Conjugates in Mice

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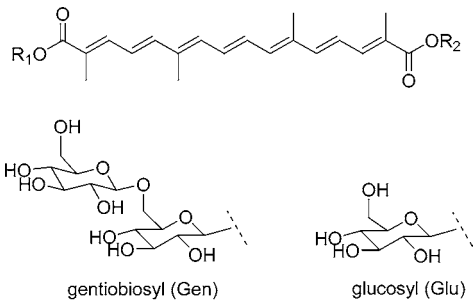
A series of crocetin glycosides (crocins) are the main pigment of the stigmas of saffron (*Crocus* sativus L.) and the fruits of gardenia (*Gardenia jasminoides* Ellis). Although numerous studies have demonstrated that crocetin and crocins have a variety of biological functions, the metabolism of dietary crocetin and crocins remains unknown. In the present study, we investigated the intestinal absorption of orally administered crocetin and crocins in mice. Orally administered crocetin was rapidly absorbed into the blood circulation and was present in plasma as an intact free form and as glucuronide conjugates (crocetin-monoglucuronide and -diglucuronide). Crocetin and its glucuronide conjugates were also found in crocins-administered mouse plasma, whereas intact crocins (glycoside forms) were not detected. These results indicate that orally administered crocins are hydrolyzed to crocetin before or during intestinal absorption, and absorbed crocetin is partly metabolized to mono- and diglucuronide conjugates.

KEYWORDS: Crocetin; crocin; *Crocus sativus*; *Gardenia jasminoides*; carotenoid; apocarotenoid; intestinal absorption; glucuronide; mouse

INTRODUCTION

Crocetin (**Figure 1**) is a unique carotenoid because of its short carbon chain length (C_{20} apocarotenoid) and because of the two carboxyl groups at both ends of the carbon chain. A series of the glycosides of crocetin, named crocins (**Figure 1**), are found in the stigmas of saffron (*Crocus sativus* L.) and in the fruits of gardenia (*Gardenia jasminoides* Ellis) (*1*, *2*). In these plants, crocetin is postulated to be synthesized from zeaxanthin by enzymatic cleavage and then glycosylated to crocins (*3*, *4*). Saffron stigmas and gardenia fruits have been widely used as yellow food colorings and medicinal herbs (*5*).

Numerous studies have demonstrated a variety of pharmacological actions for crocetin and crocins, e.g., enhancement of oxygen diffusivity (*6*, *7*), increment of ocular blood flow (*7*), inhibition of tumor cell proliferation (*8*−*13*), and protective effects against atherosclerosis (*14*), hepatotoxicity (*15*), bladder toxicity (*16*), and ethanol-induced hippocampal disorders (*17*−*19*). However, in contrast to these investigations aimed to evaluate biological activities, there are few studies on the absorption and metabolism of crocetin and crocins. In the present study, we investigated the absorption of orally administered crocetin and crocins into the blood circulation in mice. Information on the absorption and metabolism of crocetin and crocins would serve to elucidate the molecular mechanisms of the biological functions.



crocetin: R_1=R_2=H (**1**)
crocin-1: R_1=R_2=Gen (**2**)
crocin-2: R_1=Gen, R_2=Glu (**3**)
crocin-3: R_1=Gen, R_2=H
crocin-4: R_1=Glu, R_2=H

Figure 1. Chemical structures of crocetin and crocins in saffron stigmas and gardenia fruits.

MATERIALS AND METHODS

Crocetin and Crocins. Crocins [a mixture of crocin-1 (**2**) and crocin-2 (**3**)] were prepared from gardenia fruits as follows. Dried and cut gardenia fruits were extracted three times with 50% aqueous

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methanol. The combined aqueous methanol extracts were then concentrated in vacuo and applied onto an Amberlite XAD-7 resin (Rohm and Haas, Philadelphia, PA) column. The column was washed with water, and the pigments were eluted with 70% ethanol. The eluate was evaporated to dryness in vacuo, dissolved in water, and subjected to medium-pressure liquid chromatography on an Ultra Pack ODS-50B (Yamazen, Osaka, Japan). After they were washed with water and 15% acetonitrile, crocins were eluted with 20% acetonitrile. The eluted crocins were concentrated in vacuo and then lyophilized.

Crocetin (**1**) was prepared from the Amberlite resin-eluted pigments by saponification. In brief, the pigments were saponified with 10% sodium hydroxide aqueous solution at 65 °C for 3.5 h. The solution was then acidified with phosphoric acid, and the yielded precipitate was washed with water twice and then with methanol. Crocetin was then crystallized from dimethylformamide. The crystallized crocetin was washed with methanol and dried in vacuo.

Oral Administration of Crocetin and Crocins to Mice. Male ICR mice (8 weeks old, weighing 32−38 g; Clea Japan Inc., Tokyo, Japan) were housed at 25 °C with a 12 h light/dark cycle and acclimated with free access to an MF standard rodent diet (Oriental Yeast, Tokyo, Japan) and tap water. After 7 days of feeding, mice were deprived of food for 15 h before the oral administration described below.

A mixed micelle solution (0.2 mL) containing crocetin or crocins (40 nmol each) was administered to each mouse by direct stomach intubation. The solution containing 10 mmol/L sodium taurocholate, 10 mmol/L sodium oleate, 1.25 mmol/L lysophosphatidylcholine (1-palmitoyl-*sn*-glycero-3-phosphocholine), 1.0 mmol/L monoolein, 0.5 mmol/L cholesterol, and 0.2 mmol/L crocetin or crocins was prepared as previously described (*20*). The micelle composition chosen was based on the micellar phase composition of the duodenal content of human subjects given a fat-rich meal (*21*). The mixed micelle solution was used as a common vehicle for crocetin and crocins, although crocins are soluble in water without micelles. The molar amount of crocins was determined spectrophotometrically using an authentic crocetin solution after crocins were hydrolyzed to crocetin with potassium hydroxide (*22*).

At 0 (untreated), 0.25, 0.5, 1, 2, 4, or 8 h after the administration, each mouse was anesthetized with diethyl ether, and blood was collected from the caudal vena cava with a heparinized syringe. Plasma was prepared by centrifugation of blood at 1000*g* for 15 min at 4 °C and stored at −80 °C. All procedures were conducted in accordance with the Guidelines for Experimental Animals of the National Food Research Institute, Japan.

Extraction from Plasma. Plasma (50 μL) was mixed with 50 μL of 0.1 mol/L sodium phosphate buffer (pH 7.0) and 0.5 mL of methanol. The mixture was vortexed for 1 min and centrifuged at 2200*g* for 10 min at 4 °C. A portion of the supernatant (0.4 mL) was mixed with 1.0 mL of 0.2% ammonium acetate aqueous solution. The mixture was then applied to a Bond Elut C18 (50 mg) solid phase extraction cartridge (Varian, Harbor City, CA), which was washed with methanol (1.0 mL) and equilibrated with water/methanol (3:1, v/v) containing 0.2% ammonium acetate (2.0 mL) before use. After the sample was loaded, the cartridge was washed with water/methanol (3:1, v/v) containing 0.2% ammonium acetate (2.0 mL), 0.2% ammonium acetate aqueous solution (2.0 mL), and hexane (2.0 mL) in this order. After that, methanol (1.0 mL) was loaded, and the eluate was collected. The eluate was evaporated to dryness in vacuo. The residue was dissolved in 80 μL of water/methanol (1:1, v/v) and filtrated with an Ultrafree-MC centrifugal filter device (0.2 μm; Millipore, Bedford, MA). An aliquot (20 μL) of the filtrate was subjected to the chromatographic analyses described below.

Samples were handled under dim yellow light to minimize the degradation and isomerization of carotenoids by light irradiation. In the extraction procedure, the recoveries of crocetin and crocins (**1**−**3**) spiked to untreated mouse plasma (∼100 pmol/mL) were 95.3 ± 3.5, 93.4 ± 8.7, and 91.9 ± 5.8%, respectively (mean ± SD of triplicate).

Enzymatic Hydrolysis of Crocetin-Glucuronides. Plasma (50 μL) was mixed with 50 μL of β-glucuronidase (type VII-A; Sigma-Aldrich, St. Louis, MO) solution (100 U) in 0.1 mol/L sodium phosphate buffer (pH 7.0). The mixture was incubated for 60 min at 37 °C. After the incubation, crocetin was extracted as described above. The difference between the amount of free crocetin measured after the β-glucuronidase treatment (total crocetin in plasma) and the amount measured without the treatment (free crocetin in intact plasma) was defined as the amount of crocetin-glucuronides in the sample.

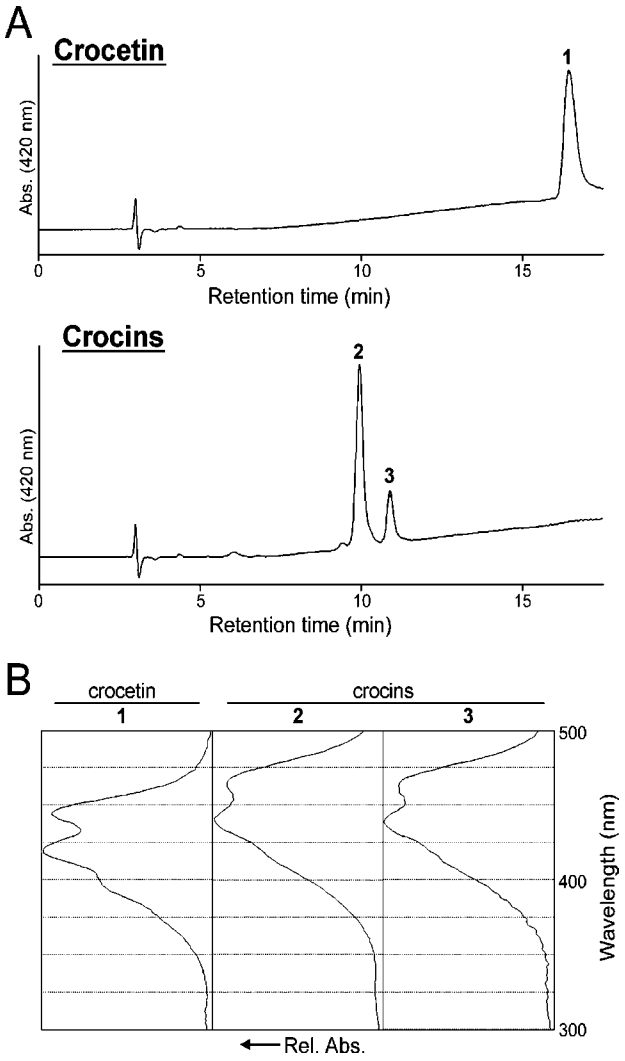

Figure 2. HPLC chromatograms (**A**) and UV–visible absorption spectra (**B**) of crocetin (**1**) and a mixture of crocins (**2** and **3**) used in the present oral administration trials.

Analytical Methods. High-performance liquid chromatography (HPLC) was carried out with an HP-1100 system (Agilent Technologies, Palo Alto, CA). A TSK-gel ODS 80Ts column (2 mm × 250 mm; Tosoh, Tokyo, Japan) attached to an ODS guard column (2 mm × 10 mm; Tosoh) was used with a linear gradient elution of 0.1% ammonium acetate aqueous solution (solvent A) and methanol containing 0.1% ammonium acetate (solvent B) at a flow rate of 0.2 mL/min. The gradient elution was programmed by changing solvent B as follows: 0−1 min, 50% B; 1−25 min, linear gradient from 50 to 100% B; 25−30 min, 100% B. The eluate was monitored with a photodiode array detector (300−500 nm). Crocetin was quantified from the peak area at 420 nm by use of the calibration curve of an authentic crocetin standard. The HPLC chromatograms and UV−visible absorption spectra of crocetin and crocins are shown in **Figure 2**. For liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis, the HPLC system was connected to a 4000 Q Trap mass spectrometer (Applied Biosystems, Foster City, CA). The electrospray interface was used in the negative ion mode.

RESULTS AND DISCUSSION

The aim of this work was to investigate the metabolic fate of dietary crocetin and crocins. In the present study, we

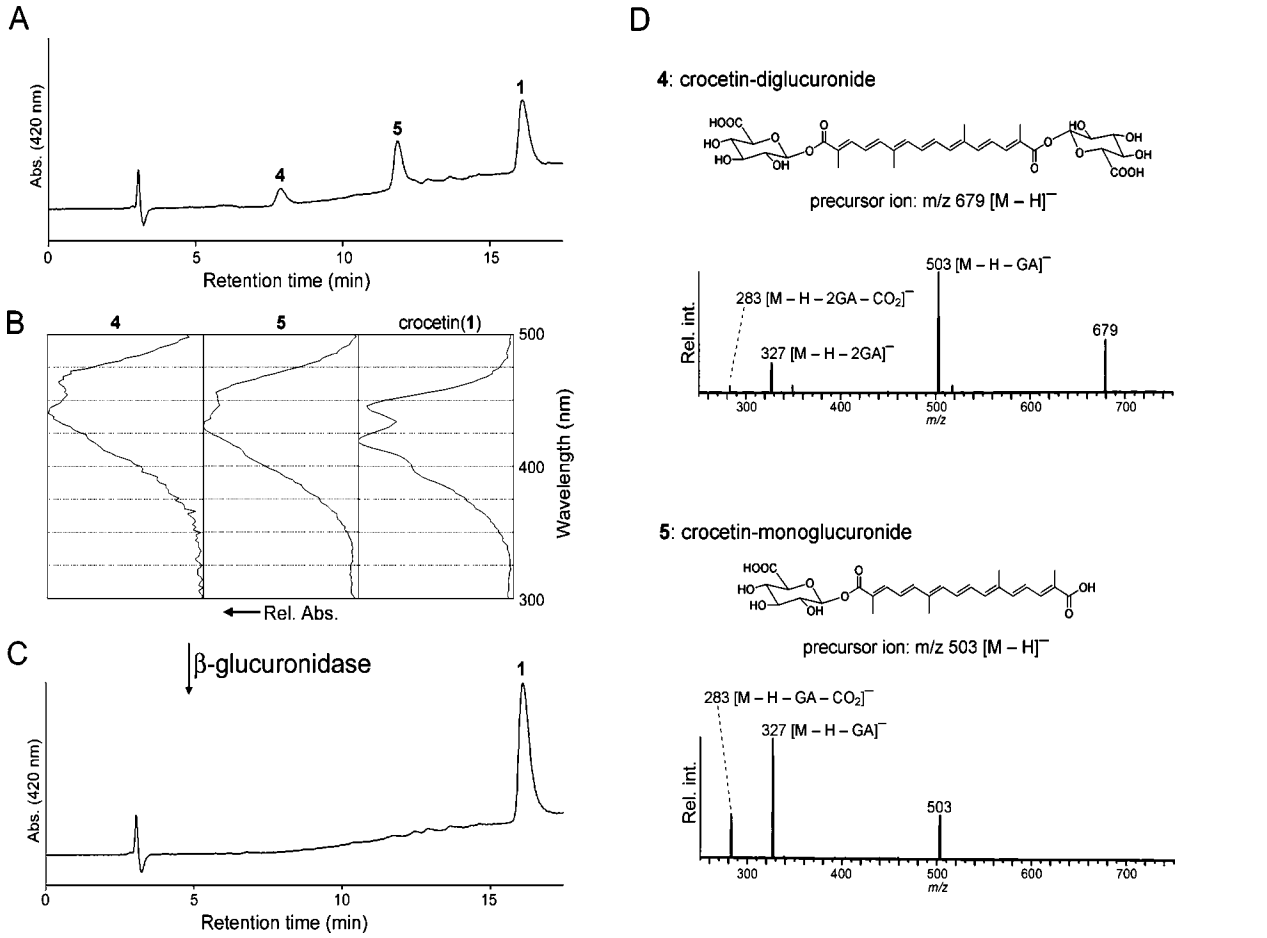

Figure 3. Analysis of crocetin-administered mouse plasma (0.5 h after dose). (**A**) HPLC chromatogram of the plasma extract without β-glucuronidase treatment. (**B**) Visible absorption spectra of metabolite **4** (crocetin-diglucuronide), metabolite **5** (crocetin-monoglucuronide), and intact crocetin (**1**) shown in chromatogram **A**. (**C**) HPLC chromatogram of the plasma extract after β-glucuronidase treatment. (**D**) LC-MS/MS product ion spectra of **4** (crocetin-diglucuronide; precursor ion at *m*/*z* 679) and **5** (crocetin-monoglucuronide; precursor ion at *m*/*z* 503). GA, glucuronic acid moiety.

administered crocetin- or crocins-containing mixed micelle solution orally to mice. Thereafter, plasma metabolites were recovered by solid phase extraction and analyzed by HPLC equipped with a photodiode array detector and a mass spectrometer. β-Glucuronidase treatment was also performed to verify the conjugated metabolites in plasma.

Figure 3A shows the representative HPLC chromatogram of the extract of crocetin-administered mouse plasma without β-glucuronidase treatment. In addition to crocetin (intact free form; **1**), two metabolite peaks at 8.0 (**4**) and 11.9 (**5**) min of the retention times were found in the HPLC profile. The metabolites were identified as crocetin-diglucuronide (**4**) and crocetin-monoglucuronide (**5**) in the present study as described below. The results indicate that orally administered crocetin is partially metabolized to the ester type glucuronides in the intestinal mucosa (during absorption), in the liver (after absorption), or in both.

The identification of metabolites **4** and **5** was performed as follows. In comparison with crocetin (**1**), **4** and **5** showed earlier elution time and bathochromic UV−visible absorption shift accompanied by the loss of the spectral fine structure (**Figure 3A**,**B**). These characteristics, similar to those shown in crocins (**Figure 2**), suggested that the carboxyl group(s) of crocetin were esterified with polar compound(s) in **4** and **5**. In addition, the disappearance of the metabolite peaks by β-glucuronidase treatment (**Figure 3C**) indicated that **4** and **5** are glucuronide conjugates of crocetin. The chemical structures of **4** and **5** were finally confirmed as crocetin-diglucuronide and crocetin-monoglucuronide, respectively, by the LC-MS/MS product ions attributable to the neutral loss of glucuronic acid moieties (**Figure 3D**).

In crocins-administered mice, crocetin (**1**) and its glucuronide conjugates (**4** and **5**) were also found in the plasma (**Figure 4**), whereas no intact crocins (neither **2** nor **3**) were detected throughout the 8 h period after the dose. The result suggests that crocins are hydrolyzed to crocetin before or during absorption and then undergo the metabolic pathway of crocetin. Hence, orally ingested crocins could not act as bioactive molecules by themselves in vivo except in the gastrointestinal tract. Free form crocetin, crocetin-glucuronides, or other metabolites derived from them may act as bioactive molecules even when crocins are orally taken. To date, numerous studies have been reported on the hydrolysis of glycosidic linkage in the gastrointestinal tract. Among them, the hydrolysis of glycosylated flavonoids is well-investigated (*23*−*25*). β-Glucosidase in intestinal epithelial cells can hydrolyze some glycosylated flavonoids after the flavonoids are absorbed into the cells as glycoside forms by sodium-dependent glucose transporter 1. In addition, lactase phlorizin hydrolase catalyzes extracellular hydrolysis of some glycosides. After the extracellular hydrolysis, aglycones would be absorbed into the epithelial cells by passive diffusion. These endogenous enzymes may be involved in the hydrolysis of crocins. Intestinal microflora may also participate in the hydrolysis. β-Glucosidases from some intestinal microflora are reported to hydrolyze the gentiobiose moiety of ginsenosides in addition to the glucose moiety (*26*).

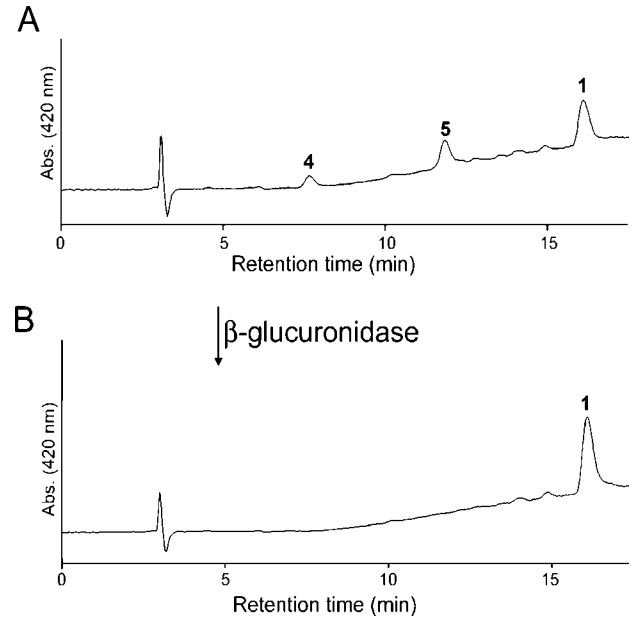

Figure 4. HPLC chromatograms of crocins-administered mouse plasma extracts before (**A**) and after (**B**) β-glucuronidase treatment (0.5 h after dose). Key: **1**, crocetin; **4**, crocetin-diglucuronide; and **5**, crocetin-monoglucuronide.

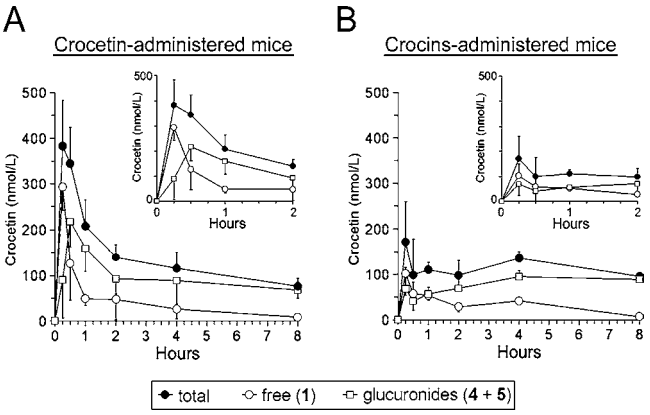

Figure 5. Concentration–time profiles of plasma crocetin after an oral administration of crocetin (**A**) and crocins (**B**). Insets in both panels illustrate the first 2 h of the main graph. Values are means ± SD ($n = 4$).

Figure 5 shows the concentration−time profiles of plasma crocetin after an oral administration of crocetin and crocins. In crocetin-administered mice, plasma crocetin reached its maximum concentration within 0.5 h after the dose and then decreased gradually (**Figure 5A**). The rapid increase of the crocetin concentration suggests that crocetin is absorbed into the blood circulation via the portal vein. More hydrophobic common carotenoids (e.g., β-carotene and lutein), which are known to be absorbed via the lymph duct as the component of chylomicrons, have been reported to reach the maximum plasma concentration more slowly (~2 h) in mice after an oral dose (*27*). In blood plasma, free form crocetin may be bound to albumin by occupying the fatty acid binding site as reported in vitro (*28*). In crocins-administered mice (**Figure 5B**), the concentration−time profile of plasma crocetin was considerably different from that in crocetin-administered mice (**A**), especially in the early period (0−2 h after dose). The lower plasma crocetin concentration of crocins-administered mice in comparison with that of crocetin-administered mice in the early period may reflect the gradual hydrolysis of crocins into crocetin in the gastrointestinal lumen (before absorption), in the intestinal mucosa (during absorption), or in both.

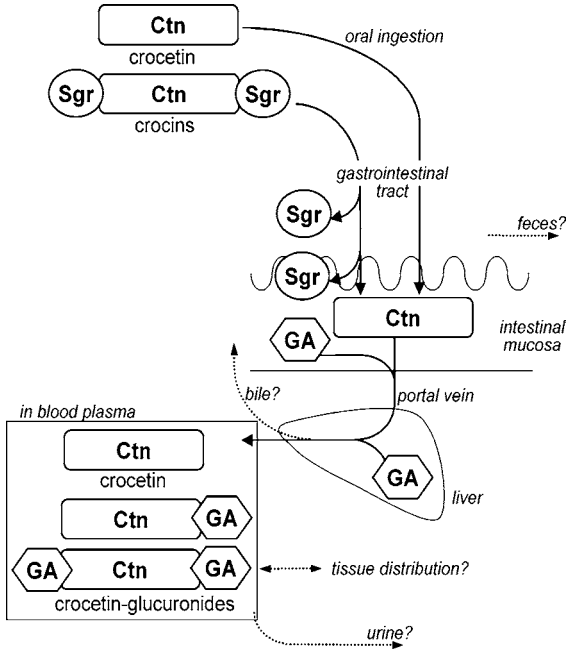

Figure 6. Proposed metabolic fate of orally administered crocetin and crocins in mice. Ctn, crocetin moiety; GA, glucuronic acid moiety; and Sgr, sugar moiety (gentiobiose or glucose).

To our knowledge, this is the first report on the glucuronide conjugation of apocarotenoid. Liu and Qian (*29*) indicated that orally administered crocetin was absorbed into rat plasma. Levy et al. (*30*) also demonstrated the presence of bixin and norbixin, apocarotenoids in annatto (*Bixa orellana* L.), in human plasma after the ingestion of annatto extracts. However, in these studies (*29*, *30*), the glucuronide conjugation of apocarotenoid was not taken into account. Although glucuronide conjugates are generally considered to be metabolic waste products that are eventually excreted in bile or urine, some glucuronide conjugates have been reported to be biologically active (*24*, *31*). For instance, a glucuronide conjugate of retinoic acid (retinoyl β-glucuronide) plays several roles in the functions of vitamin A (*31*), although retinoyl β-glucuronide may be hydrolyzed to retinoic acid in target tissues before exerting its biological activities. In the present study, crocetin-glucuronides were retained in the mouse plasma even 8 h after the administrations of both crocetin and crocins, whereas free form crocetin almost disappeared at 8 h after the doses (**Figure 5**). Hence, crocetin-glucuronides may act as bioactive molecules by themselves or may act as vehicles for the transport of crocetin to the target tissues.

We postulate the metabolic pathway of orally administered crocetin and crocins in mice as in **Figure 6**. Orally administered crocins would be hydrolyzed to crocetin before being incorporated into blood circulation. Thereafter, crocetin would be partly metabolized to mono- and diglucuronide conjugates. As reported for other xenobiotics such as polyphenols (*23*, *25*), crocetin is likely to be metabolized to the glucuronide conjugates both in the intestinal mucosa and in the liver of mice. However, further research should be needed to reveal in what tissues the glucuronidation of crocetin occurs actually. The rapid absorption and glucuronidation of crocetin indicate that the metabolic fate of crocetin is quite different from those of common C_{40} carotenoids. The present findings would be useful in further investigations of the metabolism and disposition in vivo of

crocetin and crocins, as well as in elucidating the molecular mechanisms of their biological functions in animals and humans.

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