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# Metabolic Pathway of Cyanidin 3-*O*-*β*-D-Glucopyranoside in Rats

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For better understanding of the physiological function of anthocyanins, the absorption and metabolism of cyanidin 3-*O*-*β*-D-glucopyranoside (Cy3G), which is one of the major anthocyanins in colored food materials, were precisely investigated. Combining two modalities newly developed, that is, highly sensitive semi-micro-HPLC and vein cannulation, Cy3G and its four major metabolites (M1−M4) were detected in the blood plasma of rats after oral administration of Cy3G (100 mg/kg of body mass). The plasma concentration of Cy3G reached its maximum at 15 min after the ingestion. Metabolite 2 (M2) and metabolite 3 (M3) showed their maximum plasma levels at 15 and 30 min, respectively, whereas metabolite 1 (M1) and metabolite 4 (M4) showed their maximum levels at 60 and 120 min, respectively. The maximum plasma concentrations of the four metabolites were in the following order: M3 (21 nM) > M4 (20 nM) > M1 (8.5 nM) > M2 (5 nM). When Cy3G was directly injected into the neck vein, only M2 and M3 were detected in the plasma, indicating that both M1 and M4 were produced during absorption from the gastrointestinal tract. Tandem MS analysis of the metabolites showed that M2 and M3 were monomethylated Cy3G, while M1 and M4 were glucuronides of Cy and methylated Cy, respectively. M3 was assigned as peonidin 3-*O*-*β*-D-glucopyranoside (Pn3G) from the comparison of the retention time of authentic Pn3G.

**KEYWORDS: Cyanidin 3-*O*-*β*-D-glucopyranoside; peonidin 3-*O*-*β*-D-glucopyranoside; 4′-*O*-methylcyanidin 3-*O*-*β*-D-glucopyranoside; absorption; metabolic pathway; glucuronide**

## INTRODUCTION

Anthocyanins are red pigments that are widely distributed in many edible plants such as blueberries (*1*), blackcurrants (*2*), strawberries (*3*), and purple black rice (*4*). Many functions of anthocyanins have been reported, such as antioxidant activity (*4*−*7*), anticancer activity (*8*, *9*), improvement of vision (*10*, *11*), and induction of apoptosis (*12*). We have shown unique antioxidant properties of anthocyanin in vitro (*13*−*15*), but further information including uptake, tissue distribution, and metabolic fate are essential to elucidate its physiological functions.

We previously showed that delphinidin 3-*O*-*β*-D-glucopyranoside (Dp3G) is metabolized to form 4′-*O*-methyldelphinidin 3-*O*-*β*-D-glucopyranoside (MDp3G) in rats (*16*). However, only a little is known about the metabolic fate of other anthocyanins (*17*−*19*), although uptake of anthocyanins from various sources was studied in both experimental animals and humans (*20*−*27*). Two things restricting in vivo studies of anthocyanins were the limited number and quantity of available anthocyanin standards.

In the present study, we developed a method to cannulate via a silicon tube the carotid vein of rats, which allowed us to inject anthocyanins and also to sample blood plasma to determine the anthocyanin concentration. Combining this sampling method with the highly sensitive semi-micro-HPLC, we determined plasma profiles of cyanidin 3-*O*-*β*-D-glucopyranoside (Cy3G) (**Chart 1**) purified from blackcurrant after oral and intravenous administration in rat, together with its metabolites.

## MATERIALS AND METHODS

**Chemicals.** Cy3G was purified from a blackcurrant (*Ribes nigrum* L.) extract according to a previously described method (*2*). All other reagents were purchased from Wako Pure Chemical Industries. An authentic sample of peonidin 3-*O*-*β*-D-glucopyranoside (Pn3G) was

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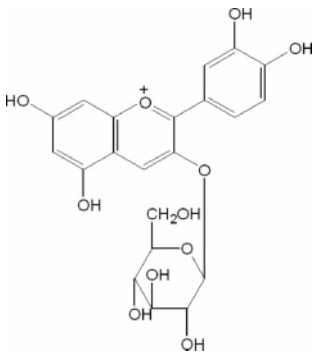
**Chart 1.** Structure of Cyanidin 3-*O*-$\beta$-D-Glucopyranoside




purified from a bilberry species (*Vaccinium myrtillus* L.) according to previously described methods with a slight modification (*28*). Briefly, bilberry extract was separated on an open column packed with MCI gel (4.5 cm × 45 cm) by $H_2O$ as the elution solution with increased amounts of methanol (0:1 to 1:0). The Pn3G-containing fraction thus obtained was further purified by semipreparative HPLC with a Develosil ODS HG-5 column (20 mm × 250 mm, Nomura Chemical Co. Ltd., Aichi, Japan) using 0.5% TFA containing 13% acetonitrile solution as the elution solvent. The peak fraction was evaporated to dryness in vacuo and stored at −80 °C until use.

**Animals and Diets.** SPF male Wistar ST rats (5 weeks of age, 120 g body mass) were purchased from Japan SLC Inc. (Hamamatsu, Japan) and individually housed in stainless steel wire-mesh cages at 23 ± 1 °C for conditioning under a 12 h light/dark cycle. The rats were allowed free access to tap water and a control diet for 7 days before the experiment. The rats were treated in accordance with the guidelines of the Niigata University of Pharmacy and Applied Life Sciences.

**Experimental Design and Plasma Preparation.** After the 7 day conditioning period, four rats were cannulated with a polyethylene tube (PE-50) in a neck vein under anesthesia with diethyl ether. The tube was guided out from the back of the rat. After starvation for 24 h, Cy3G (100 mg/kg of body mass) dissolved in 0.1% citric acid was orally administered. During the experiment, the rats were allowed to move freely in the cages.

For intravenous administration, Cy3G (2 mg/kg of body mass) dissolved in saline was injected via the polyethylene tube. On the basis of the detection limit of the present HPLC method, the dose of Cy3G administered was determined for both orally and intravenously administered experiments as described above. Blood samples were collected via the cannulated tube using a heparinized syringe at 15, 30, 60, 120, 240, 360, and 480 min after the Cy3G administration. Donor blood was obtained from the inferior vena cava of other healthy rats using a sodium citrate (500 $\mu$L of 10% sodium citrate for 8 mL of blood) containing needle and syringe under anesthesia with diethyl ether. After withdrawal of the blood sample (600 $\mu$L), the same volume of donor blood was injected through the cannulated vein tube. Each blood sample was immediately centrifuged at 3000*g* for 5 min at 4 °C for HPLC analysis.

**Determination of Cy3G and Its Metabolites in the Plasma.** Extraction of Cy3G and its metabolites was carried out using a Sep-Pak $C_{18}$ cartridge light column (Waters), essentially according to a previously reported method (*16*). Briefly, the plasma samples (300 $\mu$L) were applied to Sep-Pak $C_{18}$ cartridges conditioned with methanol (2 mL) and 3% trifluoroacetic acid (TFA) aqueous solution (2 mL). After the sample application, the cartridges were washed successively with 2 mL of 3% TFA aqueous solution, dichloromethane, and benzene, and then Cy3G and its metabolites were eluted with 50% acetonitrile containing 1% TFA aqueous solution (1 mL). The eluates were evaporated to dryness in vacuo and dissolved in 150 $\mu$L of distilled water containing 0.5% TFA. The TFA solution was passed through Centricut (0.45 $\mu$m, Kurabou Co. Ltd., Japan) before HPLC injection. HPLC was performed according to our previous report with a slight modification (*1*). Briefly, aliquots (100 $\mu$L) of the solutions were injected into an HPLC system (Hitachi 7200). HPLC was carried out on a Develosil ODS HG-5 column (Nomura, 1.0 mm × 150 mm) using 18% methanol containing 0.5% TFA as the elution solvent at a flow rate of 0.1 mL/min, and the elution peaks were monitored at 520 nm with a UV−vis detector (Hitachi, Japan). The recovery of Cy3G and its metabolites in this method was checked using authentic Cy3G and found to be 81.2%.

**Identification of Cy3G and Its Metabolites.** Cy3G metabolites were separated by HPLC as described above using an ODS column (Nomura, 4.6 mm × 150 mm), and the Cy3G metabolites were identified using TOF MS−MS spectrometry. Each sample was dissolved in methanol, and 20 $\mu$L was subjected to mass spectrometry performed with a Q-Tof Ultima (Waters, Manchester, U.K.). The conditions for TOF MS−MS are as follows: A syringe pump (single syringe pump, KD Science Inc.) was used to provide a constant infusion (300 $\mu$L/h) of the sample into the MS ion source. MS parameters used were as follows: 3.2 kV for capillary; 9.1 kV for reflection. Argon gas was used for collision at a pressure of 11 psi, and the applied voltage was 24 V.

## RESULTS

**Figure 1** shows typical HPLC chromatograms of plasma at 15 and 30 min after oral administration of Cy3G (**B**, **C**), together with that of authentic Cy3G (**A**). Four metabolite peaks (M1−

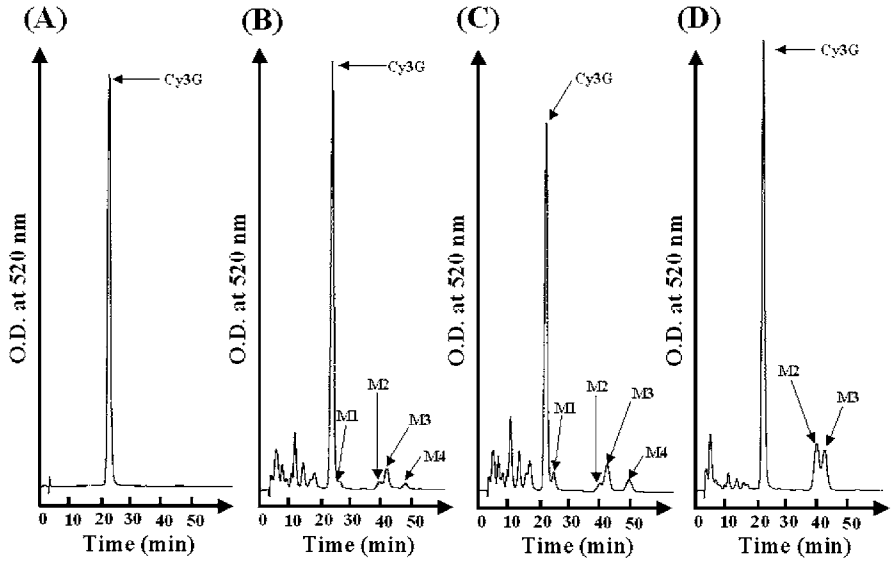



**Figure 1.** Typical HPLC chromatograms of rat plasma after administration of Cy3G: (**A**) authentic Cy3G, (**B**) plasma at 15 min after oral administration, (**C**) plasma at 30 min after oral administration, (**D**) plasma at 15 min after intravenous injection.

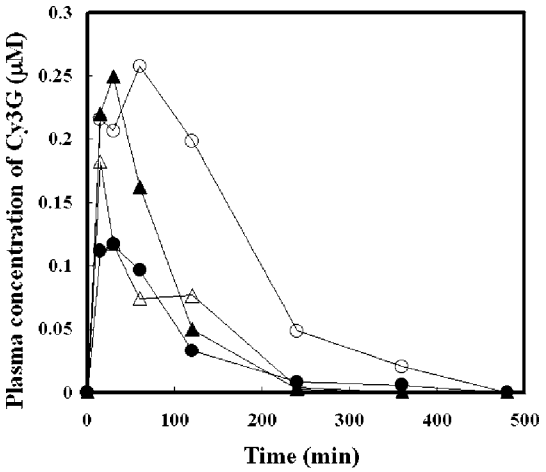



**Figure 2.** Plasma concentration profiles of Cy3G in four individual rats: ○, rat 1; ●, rat 2; △, rat 3; ▲, rat 4.

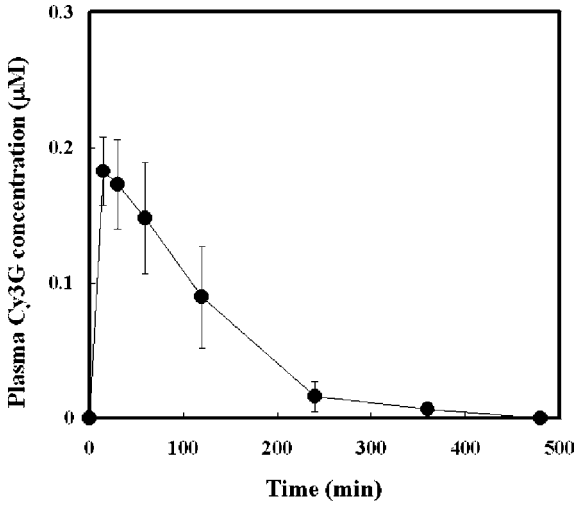



**Figure 3.** Plasma concentration profiles of Cy3G. Values are means ± SEM of four rats.

M4) were detected in the blood plasma at 15 min after oral administration, although several hydrophilic peaks were also observed. **Figure 2** shows the plasma Cy3G profiles obtained independently for each of four rats. It was remarkable that both the peak time and plasma level diverged considerably from rat to rat. The peak plasma level appeared in the time range from 15 to 60 min after oral administration, and the maximum concentration in the plasma varied from 0.12 to 0.26 $\mu$M among the four rats. **Figure 3** shows the averaged plasma profile of Cy3G in the rats. Cy3G showed its maximum concentration of 0.18 $\mu$M at 15 min, and then the concentration decreased with time.

**Figure 4** shows the averaged plasma concentration profile of the four detected metabolites. Metabolite 2 (M2) and metabolite 3 (M3) showed their plasma peaks at 15 and 30 min, respectively, whereas metabolite 1 (M1) and metabolite 4 (M4) showed their peaks at 60 and 120 min, respectively. The maximum plasma concentrations as Cy3G equivalents calculated from the peak areas determined at 520 nm absorbance were as follows: M3 (21 nM) > M4 (20 nM) > M1 (8.5 nM) > M2 (5 nM).

When Cy3G was directly injected into the neck vein (2 mg/kg of body mass), only M2 and M3 were observed in the plasma, indicating that M1 and M4 were produced during the process of gastrointestinal absorption (**Figure 1D**). MS analysis of the

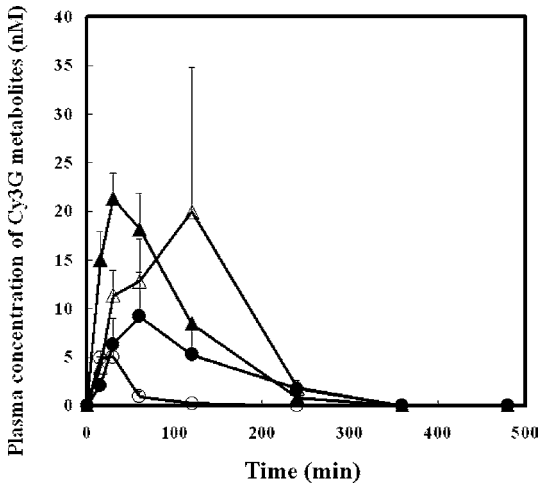



**Figure 4.** Plasma concentration profiles of Cy3G metabolites: ●, M1; ○, M2; ▲, M3; △, M4. Values are means ± SEM of four rats.

four metabolites revealed their *m*/*z* (mass-to-charge) ratios as follows: 463 for M1, 463 for M2, 463 for M3, and 477 for M4. These results indicated that M1, M2, and M3 were methylated Cy3G or the glucuronide of the Cy aglycon, and that M4 was the glucuronyl conjugate of methylated Cy. For further analysis of their structures, MS−MS analysis was performed. From the MS fragmentation pattern showing *m*/*z* 463 for the molecular ion and 287 for the aglycon, respectively, M1 was assigned as cyanidin glucuronide (**Figure 5A**). In the same way, M2 and M3 were assigned as Pn3G and an isomer of Pn3G (463 for the molecular ion and 301 for the aglycon) (**Figure 5B**,**C**). From comparison with the retention time of authentic Pn3G, it was determined that M3 was Pn3G. Further, M4 was assigned as a glucuronide of methylated Cy from the fragmentation pattern, although the methylated site remained unclear (**Figure 5D**).

## DISCUSSION

Anthocyanins are a family of flavonoids which have a variety of physiological functions (*4*−*12*). However, few studies have been carried out on the biotransformation of anthocyanins after oral administration, although catechins were reported to be taken up after a glycolytic process, and are thus determined as the glucuronate or sulfate in the plasma (*29*, *30*). It has recently been reported that a glucuronyl conjugate was formed from Cy3G, and both glucuronyl and sulfate conjugates were formed from pelargonidin (*17*, *18*). On the other hand, we showed that Dp3G was absorbed as an intact glucoside form and metabolized to 4′-*O*-methyl-Dp3G (*16*). These results indicate that the metabolic fate of anthocyanins may differ depending on their aglycon structure. Among the anthocyanins, the metabolism of Cy3G has been studied most extensively because it is rich in nature, and thus, and authentic sample is available. However, the results obtained to date are not consistent (*17*, *19*). In the present study, we determined four metabolites of Cy3G in plasma, and their structures were assigned by tandem MS analysis.

Since the plasma level of orally administered anthocyanins is relatively low compared to that of catechins, the rats must be sacrificed for each data point to collect a sufficient blood sample to analyze the metabolites. Moreover, large amounts of anthocyanins (400 mg/kg of body mass) had to be administered. In the present study, a highly sensitive method for determining

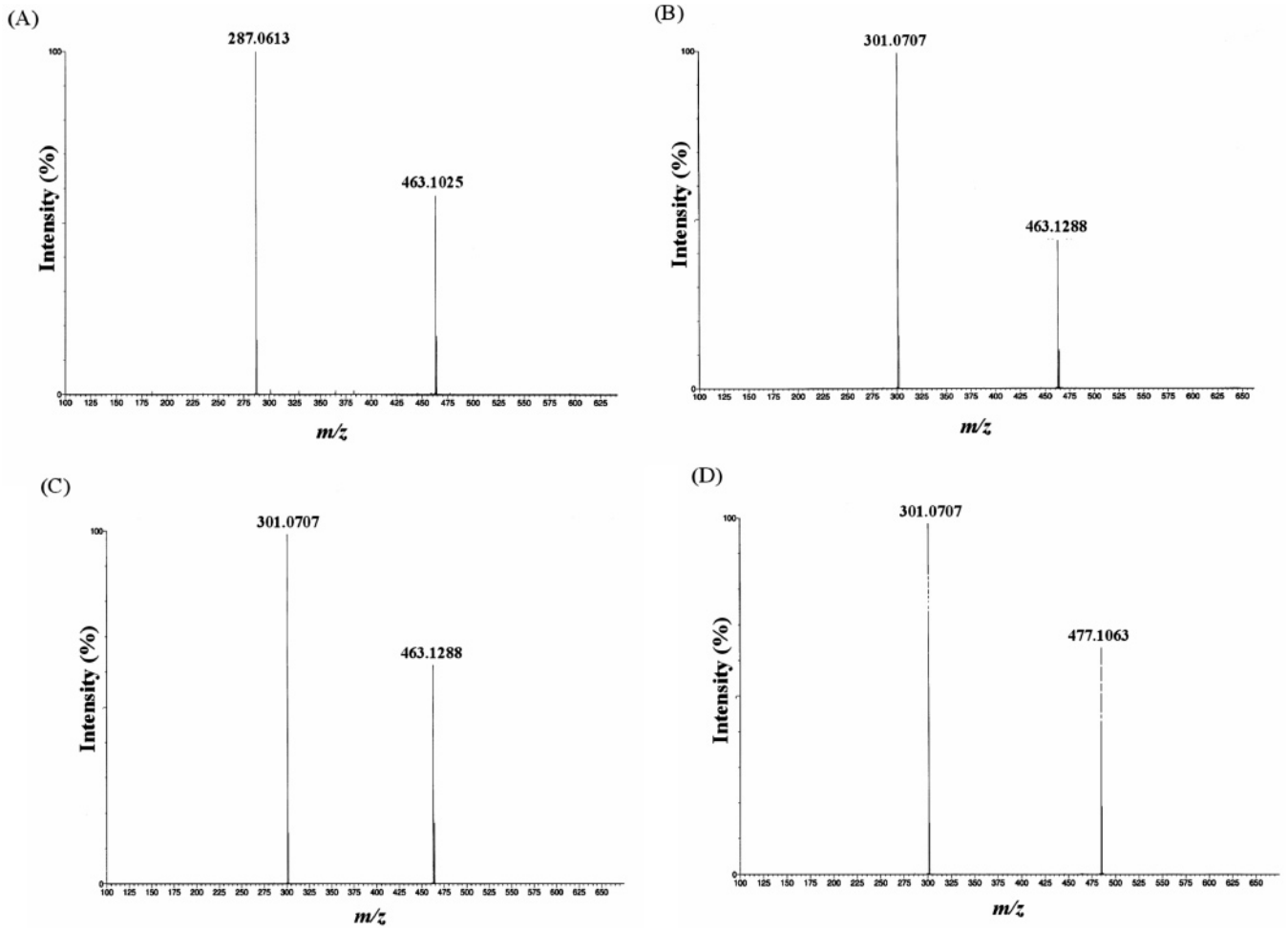



**Figure 5.** MS–MS spectra of Cy3G metabolites: (**A**) M1, (**B**) M2, (**C**) M3, (**D**) M4.

anthocyanin and its metabolites was developed for blood plasma using an HPLC system equipped with a semi-microcolumn. Moreover, by combining the carotid vein cannulation for both anthocyanin injection and plasma sampling, we were able to determine the time course change of the plasma level of orally administered anthocyanin in individual rats. The present study revealed that the plasma profile of the anthocyanin in each rat differed widely from rat to rat (**Figure 2**), but the averaged plasma profile of Cy3G obtained from the four individual rats was almost the same as those reported elsewhere, and the maximum plasma level was approximately 0.18 $\mu$M at 15 min after oral administration (**Figure 3**).

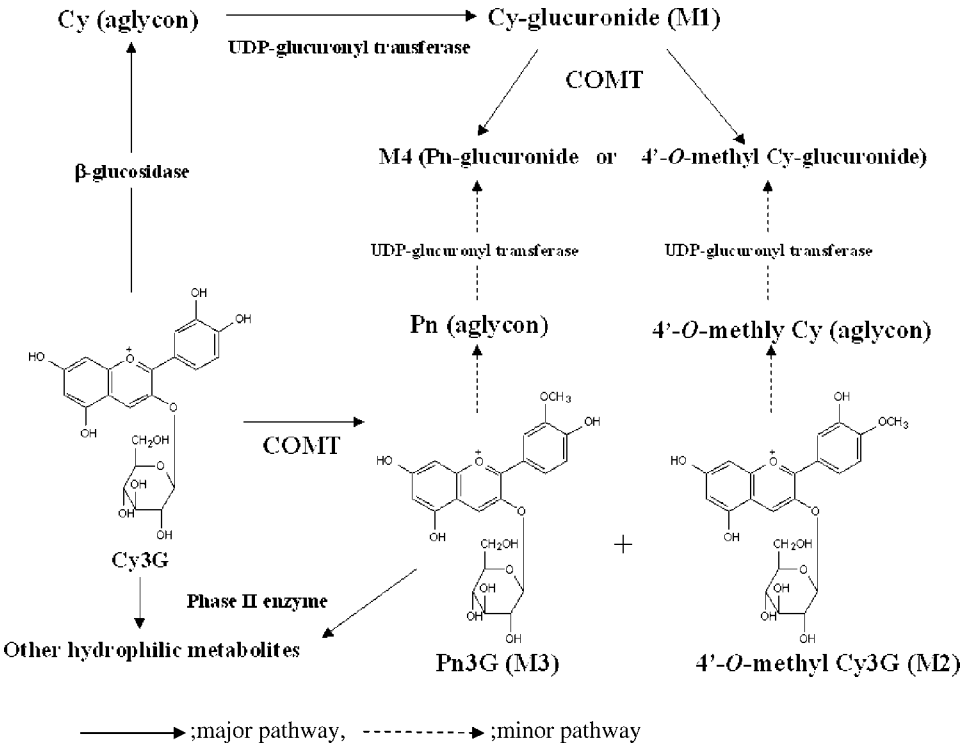
In the plasma, we found two groups of metabolite peaks, the metabolites that migrated faster than Cy3G and those that migrated slower. We first focused our attention, in the present study, on the four hydrophobic metabolites (M1−M4) that were eluted more slowly than Cy3G.

When Cy3G was directly injected into the neck vein, only M2 and M3 were detected (**Figure 1D**), indicating that M1 and M4 were formed during the absorption process in the gastrointestinal tract. The slower plasma peak appearance of these metabolites (M1 and M4) also supports this idea. Indeed, the MS analysis revealed that M1 and M4 are glucuronyl conjugates of Cy and methylated Cy, respectively. On the other hand, both M2 and M3 are methylated metabolites of Cy3G.

To deduce the methylation site in M2 and M3, they were cochromatographed with authentic Pn3G. The results clearly showed that M3 is Pn3G. This is consistent with the observation reported by Miyazawa et al. (*19*). On the other hand, M2 was assigned as 4′-*O*-methyl-Cy3G on the basis of the following discussion. A spectroscopic shift after methylation is similar to that for Dp3G when it was transformed to 4′-*O*-methyl-Dp3G was observed (*16*). The retention time of 4′-*O*-methyl-Dp3G was shorter than that of 3′-*O*-methyl-Dp3G (petunidin 3-*O*-$\beta$-D-glucopyranoside (Pt3G), our observation). Zimman et al. also observed a similar HPLC behavioral change for Pt3G (*31*).

In the present study, several hydrophilic peaks were also found to be generated even in the plasma at 15 min after intravenous injection. It was noted that the numbers of these metabolites were essentially the same for the plasma samples obtained from orally administered rats (**Figure 1B−D**), although the produced amounts of these peaks were smaller in the case of intravenous administration. Further, these peaks became larger at 30 min than at 15 min after oral administration, indicating the intestinal tract contributed to the production of these metabolites. Although the data are not shown, we have observed that about 10% of anthocyanin intravenously administered was excreted in bile. Hence, the hydrophilic metabolites detected in the plasma of intravenously administered rats were expected to be produced either in the liver or in the intestinal tract. Therefore, it is strongly indicated that they were extended glucuronide or sulfate conjugates of Cy3G and its methylated metabolites. Recently, Wu et al. reported the production of the extended glucuronyl conjugate of Cy3G, which might correspond to one of the hydrophilic peaks observed here (*17*). Further studies are needed to clarify the chemical structures of these metabolites. Taking all these pieces of information

**Scheme 1.** Possible Pathway of Cy3G Metabolism




together, we summarized a metabolic fate of orally administered Cy3G as shown in **Scheme 1**.

Cy3G orally administered was absorbed in the gastrointestinal tract, appeared in the plasma, and then was metabolized to form Pn3G (M3) and 4′-*O*-methyl-Cy3G (M2), probably by catechol *O*-methyltransferase (COMT) in the liver. At the same time Cy3G reaching the intestine was cleaved to a certain extent by *β*-glucosidase to release the aglycon. So, it is reasonable to deduce that the released aglycon might be subjected to glucuronidation by UDP glucuronyltransferase in the intestinal tract to form metabolite M1 and then taken up. The cyanidin glucuronide thus taken up in the plasma was further metabolized to M4 (peonidin glucuronide or 4′-*O*-methylcyanidin glucuronide) by COMT in the liver. It is hard to consider that released anthocyanidin, the aglycon, was absorbed and glucuronidated in the liver because the aglycon is very unstable under physiological conditions, and no aglycon was observed in the blood plasma in the present study. Another possible mechanism for the production of M4 is that methylated Cy3G (M2 and M3) produced in the liver was excreted into the jejunum by enterohepatic circulation via the bile duct and further glucuronidation occurred similarly to the case of Cy3G discussed above. This was supported by the observation that the plasma peak time of M4 was slower than those for M2 and M3. We also confirmed in our previous study that part of the Dp3G moved down to the small intestine after 15 min of oral administration of 100 mg/kg Dp3G. This also suggests that the glucuronides formed in the intestinal tract from Cy and methyl-Cy, aglycons formed by glycolysis, are absorbed from the intestine even after 15 min of oral administration.

As we previously showed, Dp3G was metabolized to only one metabolite which was assigned as 4′-*O*-methyl-Dp3G (*28*), but neither Dp (the aglycon) nor its glucuronyl conjugates were detected (*16*). Therefore, we proposed a hypothesis that flavonoids having pyrogallol in the B ring are predominantly methylated at 4′-OH compare to those having a catechol B ring structure. In the present study, we showed that Cy3G orally administered gave rise to both 3′ and 4′-*O*-methyl-Cy3G as the metabolites, but the amount of 3′-*O*-methyl metabolite was greater than that of 4′-*O*-methyl metabolite, consistent with our previous suggestion. However, it was noted that the amount of 4′-*O*-methyl metabolites found in the plasma after intravenous administration of Cy3G was as high as that of 3′-*O*-methyl metabolites. These results suggest that the methylation site and the extent might be affected by not only the anthocyanin B ring structure but also the tissue where anthocyanin is methylated such as the liver or intestine. An additional factor affecting the metabolic fate of anthocyanin is the stability of the aglycon. The present study, thus, indicated that metabolic studies are essential to elucidate functional roles of anthocyanins in vivo.

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