Direct Intestinal Absorption of Red Fruit Anthocyanins, Cyanidin-3-glucoside and Cyanidin-3,5-diglucoside, into Rats and Humans

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We determined red fruit anthocyanins, cyanidin-3-glucoside (Cy-g) and cyanidin-3,5-diglucoside (Cy-dg), incorporated into plasma and liver of rats and human plasma by UV-HPLC. Fifteen minutes after an oral supplementation of a mixture of 320 mg of Cy-g and 40 mg of Cy-dg/kg of body weight, rats showed an increase to a maximum of 1563 μg (3490 nmol) of Cy-g/L and 195 μg (320 nmol) of Cy-dg/L in plasma and 0.067 μg (0.15 nmol) of Cy-g/g and a trace of Cy-dg together with methylated metabolites such as peonidin-3-glucoside in liver. In human plasma, 30 min after intake (2.7 mg of Cy-g and 0.25 mg of Cy-dg/kg of body weight), an average of 11 μg (24 nmol) of Cy-g/L and a trace of Cy-dg were found. Cyanidin as aglycone of Cy-g and Cy-dg was not found in such plasma samples, neither were conjugated and methylated anthocyanins. The results indicated that anthocyanins are incorporated keeping structurally intact glycoside forms, from the digestive tract into the blood circulation system in mammals.

Keywords: *Anthocyanins; cyanidin glucoside; catechin; absorption; metabolism*

INTRODUCTION

Anthocyanins are part of a very large and widespread group of plant constituents known collectively as flavonoids. The most common naturally occurring anthocyanins are 3-*O*-glycosidic or 3,5-di-*O*-glycosidic derivatives of 2-phenylbenzophyrylium (flavylium) structure, the corresponding aglycones being termed anthocyanidins. The structures of cyanidin-3-glucoside (Cy-g) and cyanidin-3,5-diglucoside (Cy-dg) and their aglycone, cyanidin (Cy), are shown in Figure 1. Cy is the most widespread anthocyanidin in the plant kingdom (Parkinson and Brown, 1981). It has been estimated that humans consume 180−215 mg of anthocyanins daily (Kuhnau, 1976).

During the past decade, anthocyanins have been revealed to have antioxidant activity postulated to relate to their antiatherosclerotic (Ghiselli et al., 1998; Laplaud et al., 1997; Satuegracia et al., 1997), anticarcinogenic (Bomser et al., 1996; Kamei et al., 1995) and antiinflammatoric (Lietti et al., 1976) properties. Cy-g and Cy have been reported to have effective antioxidant activity in linoleic acid autoxidation, liposome, rabbit erythrocyte membrane, and rat liver microsomal systems (Tsuda et al., 1994) and against radicals generated in the aqueous phase (Rice-Evans et al., 1995). However, it has yet to be elucidated whether anthocyanins, once ingested, are directly absorbed from the intestines into blood plasma and tissue organelles at sufficient levels to exert such beneficial pathophysiological properties in vivo as reported for in vitro experiments.

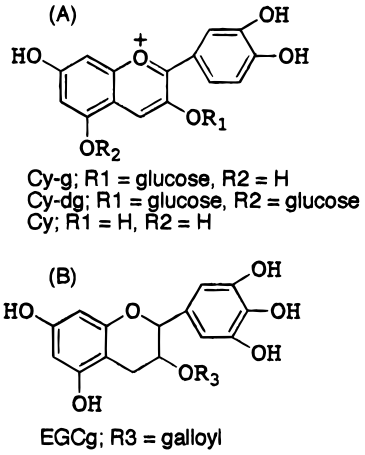

Figure 1. Structures of (A) anthocyanins, cyanidin-3-glucoside (Cy-g), cyanidin-3,5-diglucoside (Cy-dg), and their aglycone, cyanidin (Cy), and (B) tea catechin, (−)-epigallocatechin-3-gallate (EGCg).

At present, little is known as to the metabolic fate of anthocyanins (Parkinson and Brown, 1981; Scheline, 1991). Morazzoni et al. (1991) estimated at first that about 2.5 mg/L of anthocyanins could be incorporated into plasma of rat after 15 min of ingestion of a mixture of 15 kinds of anthocyanins (total 400 mg/kg of body weight). However, subsequent experiments have not been carried out and, therefore, the metabolism and concentration of individual anthocyanins in animals is yet to be studied. In addition, for humans, the pharmacokinetics of the absorption of anthocyanins have never been investigated.

There are several ways in which anthocyanins may undergo metabolic alteration. As anthocyanins are present in glycosidic forms, it has been speculated for a long time that bacterial digestion of the glycosidic

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linkage of anthocyanins by the gastrointestinal system may occur before absorption (Tamura et al., 1980), as has been demonstrated in the case of flavonol [rutin (quercetin-3-rutinoside); Bokkenheuser et al., 1987] and some drug glycosides (Friend and Chang, 1984). On the other hand, as metabolites of anthocyanins, glucuronide and sulfate conjugates have been expected to occur and play an important role in the metabolism as has been demonstrated for conjugation of several types of flavonoids such as tea catechin (epicatechin derivatives; Lee et al., 1995), flavonol (quercetin; Ueno et al., 1983), flavanone (naringenin; Fuhr and Kummert, 1995), and flavone (diosmetin; Boutin et al., 1993).

To date we have investigated the absorption and metabolism of tea catechins in rats and humans (Nakagawa et al., 1997; Nakagawa and Miyazawa, 1997a, 1997b). Tea catechins are derivatives of flavan-3-ol and differ structurally from many other types of flavonoids by their lack of a 4-keto group in the pyran ring. The anthocyanins are close relatives of such tea catechins because they have a 3-hydroxy group and lack a 4-keto group (Scheline, 1991). The molecular weight of tea catechin, (−)-epigallocatechin-3-gallate (EGCg; MW 458), is almost equivalent to that of anthocyanins (Cy-g; MW 448). Therefore, the metabolism of anthocyanins (especially for Cy-g) in mammals has been expected to be similar to that of tea catechin (EGCg).

In this study, we investigated using a UV-HPLC method the absorption of anthocyanins, Cy-g and Cy-dg, into rat and human body after oral ingestion, and compared their absorptivities with tea catechin, EGCg. As a result, we have confirmed that dietary anthocyanins are incorporated into plasma in structurally intact forms with no aglycones or conjugates of anthocyanins present, while tea catechin is partly metabolized to conjugates.

EXPERIMENTAL PROCEDURES

Chemicals. Red fruit anthocyanin extract (RAE), prepared by spray-drying the concentrated juice of such red fruits as elderberries (*Sambuscus nigra*) and black currants (*Ribes nigrum*), was donated by Lion Co. (Tokyo, Japan). Two types of RAE were used: one consisting of Cy-g 61 g/kg and Cy-dg 7.5 g/kg of RAE and other substances (gum arabic and maltodextrin) for the rat study and the other consisting of Cy-g 90.9 g/kg and Cy-dg 9.0 g/kg of RAE and other substances (gum arabic and maltodextrin) for ingestion in humans. Authentic Cy-g, Cy-dg, and Cy were all purchased from Funakoshi Co. (Tokyo, Japan). EGCg (above 95% purity) and Sunphenon DCF-1 capsule (containing 48.5 mg EGCg/capsule) extracted from green tea leaf were obtained from Taiyo Kagaku Co. (Yokkaichi, Japan). β-Glucuronidase (G-7896), sulfatase (S-9754), β-glucosidase (G-0395), and quercetin were purchased from Sigma (St. Louis, MO). Rutin (quercetin-3-rutinoside) was from Wako Pure Chemical Co. (Osaka, Japan). All other reagents and chemicals used were commercially available extra-pure grade products.

Rat Study. Male Sprague-Dawley rats were obtained at 9 weeks of age from Funabashi Farm Co. (Chiba, Japan; 270−290-g body weight, $n = 84$) and housed at 25 °C with a 12-h light−dark cycle with free access to commercial chow (F-2 pellet rations from Funabashi Farm Co.) and distilled water for 1 week. All animals were treated in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (NRC, 1985). After that, rats were unfed for 24 h and weighed. Body weight after food deprivation was 305 ± 10 g (mean ± SD). All rats were randomly assigned to three groups. In one group, 12 rats received by stomach tube either 0.8 g of RAE (equivalent to a mixture of 160 mg Cy-g and 20 mg Cy-dg/kg of body weight) dissolved in 3 mL of distilled water or 1.6 g of the same RAE or else were untreated (control). After a 30-min administration, all rats were anesthetized with ether, and blood was collected from the abdominal artery with a heparinized syringe. The second group of rats ($n = 48$) were killed similarly at 0 (untreated), 15, 30, 60, 120, and 240 min after dosing of 0.8 g of RAE (160 mg Cy-g and 20 mg Cy-dg/kg of body weight) or 1.6 g of RAE (320 mg Cy-g and 40 mg Cy-dg/kg of body weight), and their blood and liver were collected. The third group of rats ($n = 24$) were administered EGCg (320 mg/kg of body weight) dissolved in 3 mL of distilled water, and their blood and liver were collected at 0 (untreated), 15, 30, 60, 120, and 240 min after dosing.

Human Study. Seven male and five female adult volunteers (20−29 years, 40−70 kg) participated in this study. Human studies were performed according to the Helsinki Declaration. The subjects did not ingest any fruit juice and tea-related beverages for 12 h prior to the experiment. After fasting for 12 h, all volunteers were randomly separated into three groups. In one group ($n = 4$), volunteers orally ingested 1.6 g of RAE (equivalent to 2.7 mg Cy-g and 0.25 mg Cy-dg/kg of body weight) together with about 20 mL of water. Blood samples from the subjects were collected into heparinized collection tubes before and at 30 min after RAE consumption. From the second group ($n = 4$) of volunteers having been given the same amount of RAE, blood was collected before and 60 min after ingestion. The third group of volunteers ($n = 4$) orally ingested three capsules of Sunphenon DCF-1 (equivalent to 2.6 mg EGCg/kg of body weight). Blood samples were collected before and at 60 min after EGCg consumption.

Sample Preparation. Plasma was prepared by centrifuging the blood from both rats and humans at 1000*g* for 15 min at 4 °C. Rat liver was washed well with 0.15 mol/L NaCl aqueous solution. In the analysis of anthocyanins, a 1.0-mL aliquot of plasma sample was diluted with 0.2 mL of 0.44 mol/L trifluoroacetic acid (TFA) aqueous solution to avoid deterioration of anthocyanins. Liver (500 mg) was minced and homogenized in 2.5 mL of 0.44 mol/L TFA aqueous solution with a Teflon-glass homogenizer under ice-cold conditions. For analysis of tea catechin, 0.25 mL of plasma sample was diluted with the same amount of vitamin C−EDTA solution which consisted of 0.11 mol/L ascorbic acid and 2.8 mmol/L ethylenediaminetetraacetic acid disodium salt in 0.4 mol/L NaH_2PO_4 buffer at pH 3.9, and liver (500 mg) was homogenized in 2.5 mL of vitamin C−EDTA solution (Nakagawa et al., 1997; Nakagawa and Miyazawa, 1997a, 1997b).

Anthocyanins Analysis. For extraction of anthocyanins, an ODS solid phase extraction cartridge (Sep-Pak C_{18}, Waters, Milford, MA) was used. This extraction procedure followed the method of King et al. (1996) with modification. The cartridge was washed with 10 mL of methanol and equilibrated with 10 mL of 1.5 mol/L formic acid aqueous solution before use. After that, 1.0 mL of sample plasma diluted with 0.2 mL of 0.44 mol/L TFA solution or 1.0 mL of liver homogenate was applied to the cartridge. Water-soluble compounds, polar lipids, and neutral lipids were respectively eluted from sample plasma or liver with 10 mL of 1.5 mol/L formic acid aqueous solution, 10 mL of dichloromethane, and 10 mL of benzene. Anthocyanins were recovered finally with 5 mL of 0.44 mol/L TFA solution in methanol. The extract of anthocyanins in the methanol phase was evaporated to dryness with a rotary evaporator. The dried methanol extract was redissolved with 200 μL of 0.44 mol/L TFA aqueous solution and the 100-μL portion was subjected to the UV-HPLC analysis to determine anthocyanin concentration, as described below.

Anthocyanins were determined with a UV-HPLC system (Japan Spectroscopic Co., Tokyo, Japan) at 532-nm detection. An ODS column (Capcell Pak C18 type UG120, 4.6 mm × 250 mm; Shiseido Co., Tokyo, Japan) was used with a mixture of 1.5 mol/L formic acid aqueous solution and methanol (86:14, v/v) as column eluant at a flow rate of 1.0 mL/min. Column temperature was kept at 40 °C in a Jasco CO-965 column oven. A standard mixture was made by dissolving Cy-g, Cy-dg, and Cy in a 0.44 mol/L TFA aqueous solution. Cy-g, Cy-dg, and Cy were identified by comparing their retention times with those of standards. The concentration of Cy-g, Cy-dg, and Cy

in sample plasma and liver was calculated from a calibration curve made with standard solution. The peak identity for Cy-g, Cy-dg, and Cy was further confirmed from their characteristic UV−visible spectra recorded by HPLC combined with a Jasco MD-910 photodiode array detector (UV/vis-HPLC).

In the present extraction and determination procedures, when a mixture of 552 pmol of Cy-g and 555 pmol of Cy-dg was added to the plasma (1.0 mL) of anthocyanin-untreated control rat and the extract analyzed by UV-HPLC, 448 ± 8 pmol Cy-g (mean ± SD of three experiments) and 421 ± 3 pmol Cy-dg were confirmed to be recovered, indicating that the recovery was 81.1 ± 1.5% for Cy-g and 75.9 ± 0.5% for Cy-dg. Similarly for human plasma, the recovery was 79.0 ± 8.6% for Cy-g and 81.3 ± 8.0% for Cy-dg. When a mixture of 500 pmol of Cy-g and 500 pmol of Cy-dg was added to the rat liver homogenate (1.0 mL) from anthocyanin-untreated control rat, the recovery was 63.9 ± 5.2% for Cy-g and 76.2 ± 5.7% for Cy-dg.

Tea Catechin Analysis. EGCg in sample plasma and in liver homogenate was extracted and selectively determined by a chemiluminescence detection-HPLC method as previously reported (Nakagawa et al., 1997; Nakagawa and Miyazawa, 1997a,b).

Hydrolysis of Flavonoid Conjugates. The plasma (0.5 mL) prepared from anthocyanin- and EGCg-supplemented rat and human was mixed with 50 μL of 0.1 mol/L phosphate buffer (containing 2.9 mmol/L EDTA at pH 6.8) and 50 μL of β-glucuronidase solution (1250 unit/50 μL of distilled water) to hydrolyze glucuronide conjugates. For hydrolysis of sulfate conjugates, both 50 μL of 0.1 mol/L phosphate buffer (containing 2.9 mmol/L EDTA at pH 5.0) and 50 μL of sulfatase solution (100 unit/50 μL of distilled water) were added to plasma (0.5 mL). The sample mixture was then incubated at 37 °C for 60 min, and the reactant was extracted and analyzed as mentioned above. A blank was also tested using enzyme-free distilled water in the same manner.

Digestibility of Flavonoid to Aglycone in Vitro. RAE (containing 820 nmol of Cy-g and 60 nmol of Cy-dg) was incubated at 37 °C in 1.0 mL of 0.02 mol/L acetate buffer, pH 5.0, together with 530 unit of β-glucosidase (1 unit liberates 1.0 μmol of glucose from salicin per minute at pH 5.0 and 37 °C). The same amount of RAE was also incubated at 37 °C with homogenized rat feces (0.35 g/1.0 mL of 0.01 M phosphate buffer, pH 7.0). The feces were obtained from male Sprague-Dawley rats maintained on a commercial chow (F-2 pellet rations). After incubation at various times, an aliquot (0.1 mL) was removed and mixed with methanol (0.1 mL). The mixture was centrifuged at 1000*g* for 15 min, and a 50 μL of supernatant portion was subjected to UV-HPLC to determine anthocyanins and their aglycone (Cy) concentrations. The digestibility of rutin (820 nmol) to its aglycone (quercetin) was also tested in the same manner. Rutin and quercetin were determined simultaneously by the UV-HPLC method (Bokkenheuser et al., 1987).

Statistics. The data were expressed as the mean ± SD. Statistical comparisons were made with Student's *t* test. All comparisons were made at the two-sided 0.05 significance level. The values of kinetic parameters were determined from blood level time profile data in rats using standard formulas.

RESULTS

UV-HPLC Chromatogram of Anthocyanins. Figure 2 shows the UV-HPLC chromatogram of a mixture of standard Cy-g (injected amount, 880 pmol), Cy-dg (780 pmol), and Cy (800 pmol). Under the present HPLC conditions, Cy-g, Cy-dg, and Cy were separately detected. The peak area of Cy-g, Cy-dg, and Cy was proportional to their concentrations in the range from 5 to 1000 pmol. The retention time and peak sharpness of these anthocyanins were not affected by the injection volume (5−100 μL). For the three determinations of the standard Cy-g, Cy-dg, or Cy at each concentration, the

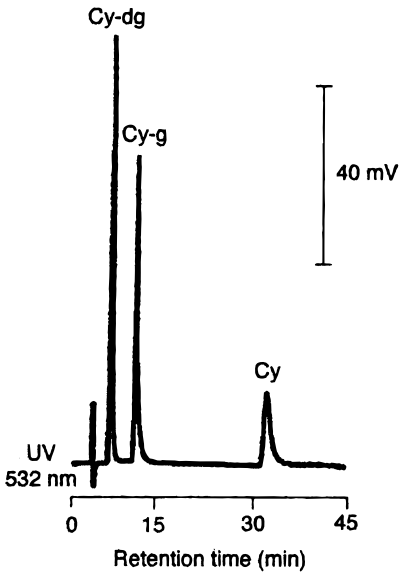

Figure 2. UV-HPLC chromatograms of standard anthocyanins, Cy-g (880 pmol), Cy-dg (780 pmol), and Cy (800 pmol). Detection: 532 nm.

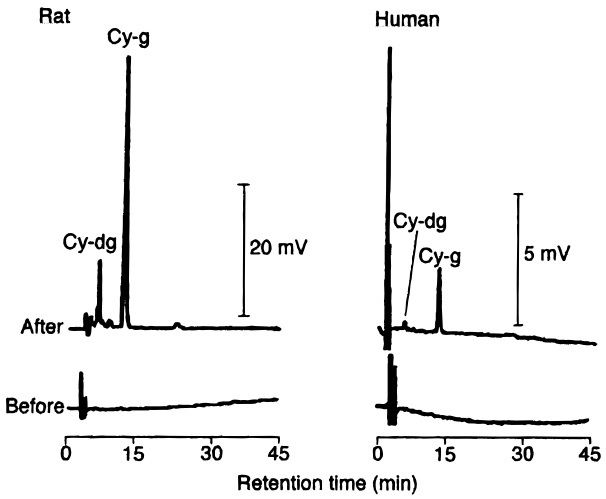

Figure 3. UV-HPLC chromatograms of plasma extracts of anthocyanin-supplemented rat and human plasma. Left, rat plasma methanol extract before and 30 min after a single oral administration of anthocyanins (160 mg of Cy-g and 20 mg of Cy-dg/kg of body weight); right, human plasma methanol extract before and 30 min after intake of anthocyanins (2.7 mg of Cy-g and 0.25 mg of Cy-dg/kg of body weight).

standard error was all within 1.5% of the mean value. The detection limit was 2 pmol for Cy-g and Cy-dg and 4 pmol for Cy at 3 in the signal-to-noise ratio. Figure 3 shows the chromatograms of plasma extracts of rats and humans before and 30 min after anthocyanin administration. In both rat and human plasma, no anthocyanins were found before administration, but two peaks respectively ascribed to Cy-g and Cy-dg appeared after the oral supplementation. However, a Cy peak was not detected in any plasma sample even after anthocyanin administration. No interference peaks were observed on the chromatograms of the plasma extracts. Cy-g (12 min of retention time) and Cy-dg (7 min) peaks were respectively identical to the retention times of standard Cy-g and Cy-dg. The peak components detected by the UV-HPLC of plasma extract were further identified as anthocyanins by UV/vis-HPLC. The photodiode array spectra of standard Cy-g and Cy-dg were completely identical to those of corresponding peak components (at

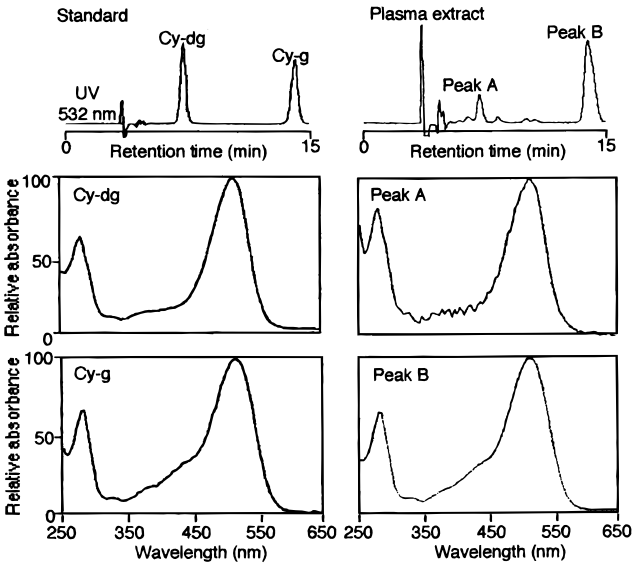

Figure 4. UV/vis-HPLC chromatograms and UV/vis spectra of standard anthocyanins and plasma extracts of anthocyanin-supplemented rat. Rat plasma methanol extract 30 min after a single oral administration of anthocyanins (160 mg of Cy-g and 20 mg of Cy-dg/kg of body weight) was analyzed. A, Cy-dg; B, Cy-g.

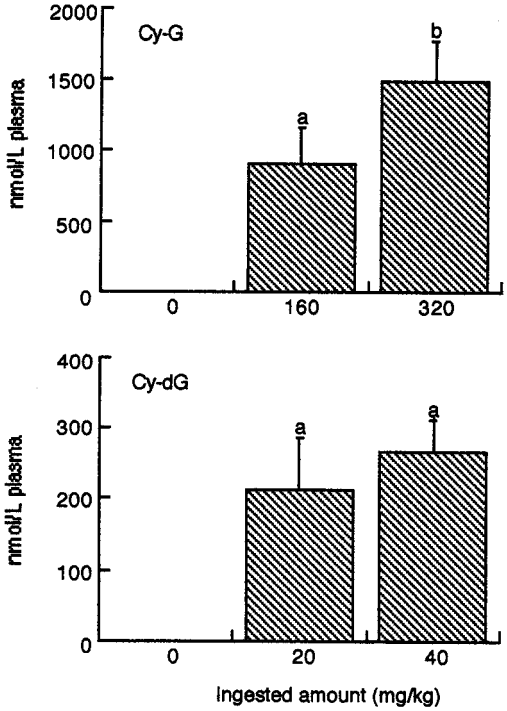

Figure 5. Plasma Cy-g and Cy-dg concentrations in the rat after a single oral administration of graded amounts of anthocyanins (corresponding to 0, 160, and 320 mg of Cy-g and 0, 20, and 40 mg of Cy-dg/kg of body weight). Values are the mean ± SD of four rats. (a, b) Values with different letters are significantly different at $P < 0.05$.

12 min and 7 min of retention time, respectively) of plasma extracts (Figure 4). Although the data are not shown, the appearance of a Cy peak was confirmed in such UV-HPLC when anthocyanin-administered rat and human plasma were treated with 6 mol/L HCl to hydrolyze the glycosidic linkage in Cy-g and Cy-dg. The chromatograms of liver extracts of rat after anthocyanin administration were similar to that of plasma, but the Cy-dg peak was at a trace level in liver. The Cy peak was not found in any liver sample even after anthocyanins ingestion.

Anthocyanins in Rat. Figure 5 shows rat plasma Cy-g and Cy-dg concentrations at 30 min after a single oral administration of anthocyanins. Plasma Cy-g and Cy-dg levels before the administration were below the detection limit (<2 nmol/L plasma), but 30 min after the intake, plasma Cy-g increased to 907 nmol (406 μg)/L in Cy-g-administered rats (160 mg/kg of body weight) and 1493 nmol (669 μg)/L in rats given 320 mg of Cy-g/kg of body weight. Cy-dg also showed an increase to 212 nmol (129 μg) and 266 nmol (162 μg)/L in the rats that received 20 and 40 mg of Cy-dg/kg of body weight, respectively. The results suggested a dose-dependent incorporation of anthocyanins, especially of Cy-g, into rat plasma. Figure 6A shows time-course changes in anthocyanins in rat plasma after a single oral administration. The plasma anthocyanin level reached a maximum [3490 nmol (1563 μg) of Cy-g/L and 320 nmol (195 μg) of Cy-dg/L] at 15 min after the oral intake (320 mg of Cy-g and 40 mg of Cy-dg/kg of body weight), then gradually decreased to 140 nmol of Cy-g/L and 50 nmol of Cy-dg/L at 240 min. No Cy peak was found in the plasma throughout the experiment. In the liver, Cy-g increased to a maximum level [0.15 nmol (0.067 μg)/g of liver] at 15 min after supplementation (320 mg of Cy-g and 40 mg of Cy-dg/kg) and thereafter attenuated rapidly (Figure 6B). A Cy peak was not detected in liver samples. The time to reach maximal concentration in plasma and liver was shorter in anthocyanins than that in tea catechin, EGCg (Figures 6 and 7). The EGCg level in free form in plasma and liver reached a maximum at 30 min after the oral intake (320 mg of EGCg/kg of body weight). Comparing the pharmacokinetic parameters of Cy-g with that of EGCg at the same dosage (320 mg/kg of body weight), the total area under the concentration time curve (AUC) calculated from plasma EGCg concentrations was 2.8-fold higher than that of Cy-g (Table 1). These may indicate that the bioavailability of tea catechin is more predictable than that of anthocyanins.

Anthocyanins in Humans. Human plasma did not contain any anthocyanins before the intake. Plasma Cy-g in the subjects 30 min after supplementation (2.7 mg of Cy-g and 0.25 mg of Cy-dg/kg of body weight) was an average of 24 nmol (11 μg)/L (Table 2). At 60 min after the intake, plasma Cy-g was 29 nmol (13 μg)/L. Plasma Cy-dg was found in several volunteers, but the concentrations were too small to evaluate (below 2 nmol/L). In humans, Cy was not detected in any plasma samples (Figure 3). On the other hand, at 60 min after a single oral intake of EGCg (2.6 mg EGCg/kg body weight), plasma EGCg in free form attained to relatively higher concentration (725 nmol/L) than that of anthocyanins (Table 2). Thus, it seems likely in humans that the absorption rate of tea catechin is higher than that of anthocyanins.

Metabolites of Anthocyanins. In anthocyanin-supplemented rat plasma, the chromatographic profile of plasma extract showed no change regardless of β-glucuronidase or sulfatase treatment (Figure 8). Similarly, human plasma extract showed no change despite the enzymatic hydrolysis of anthocyanins. These results indicated no glucuronide or sulfate of anthocyanins is present in rat and human plasma. In contrast to anthocyanins, the enzymic treatment of EGCg-supplemented plasma from rats and humans resulted in an increase of the concentration of EGCg in free form

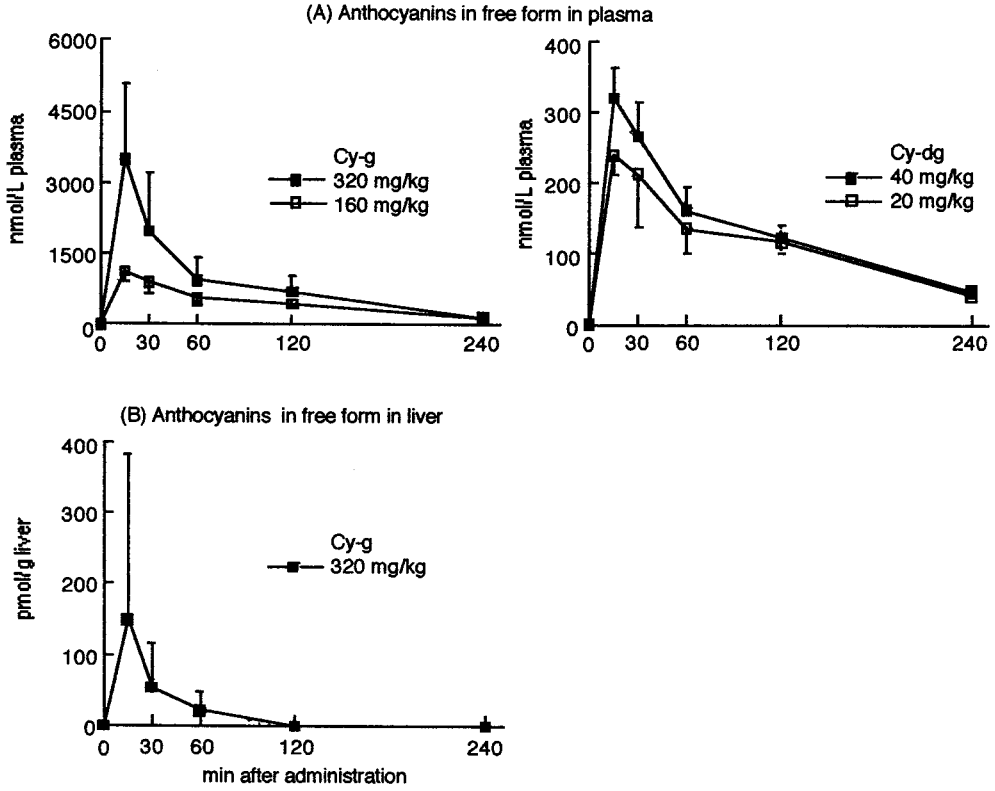

Figure 6. Time-course changes in Cy-g and Cy-dg concentrations in plasma (A) and liver (B) of rats treated with a single oral dose of anthocyanins (160 or 320 mg of Cy-g and 20 or 40 mg of Cy-dg/kg of body weight). Values are the mean ± SD of four rats. Liver Cy-g and Cy-dg concentrations in free form were below detection limit when 160 mg of Cy-g and 20 or 40 mg of Cy-dg/kg of body weight were ingested.

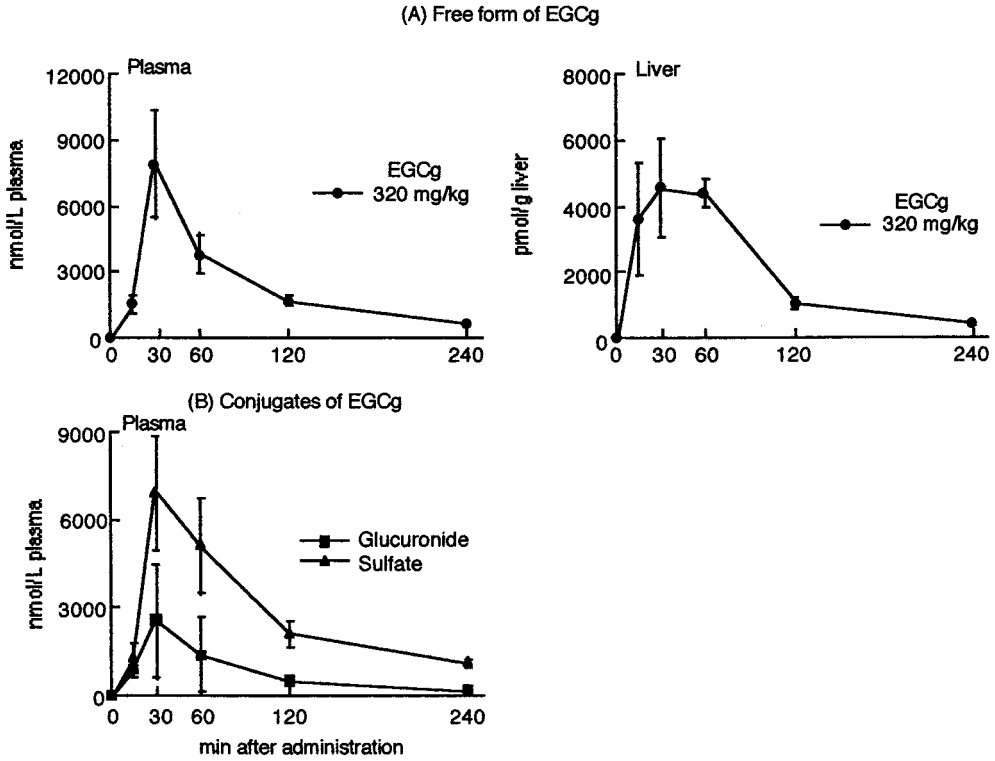

Figure 7. Time-course changes of EGCg concentrations in free form and in conjugates in plasma and liver of rats after a single oral administration of tea catechin (320 mg of EGCg/kg of body weight). (A) Free form of EGCg in plasma and liver. (B) Glucuronide and sulfate conjugates of EGCg in plasma. Values are the mean ± SD of four rats.

(Figure 7 and Table 2). In both rat and human plasma, the concentrations of EGCg metabolites were on the order of sulfate conjugates ≥ free form > glucuronide conjugates (Figure 7 and Table 2). Time-course changes in concentrations of conjugates of EGCg in rat plasma were proportional to free form EGCg (Figure 7).

In Vitro Testing. In this study, oral administration of Cy-g and Cy-dg did not yield detectable amounts of

Table 1. Pharmacokinetic Parameters of Cy-g and EGCg in Rats[a]

parameters	Cy-g	EGCg
C_{max}, nmol/L	3490	7910
C_{max}, μg/L	1560	3620
T_{max}, h	0.25	0.50
AUC, μg·h·mL^{-1}	1.62	4.58
rel bioavailability[b]	2.83	

[a] Rats were treated with a single oral dose of either anthocyanin preparation containing Cy-g (320 mg/kg of body weight) or tea catechin (320 mg of EGCg/kg of body weight). C_{max}, maximum plasma concentration; T_{max}, time to reach maximal plasma concentration; AUC, total area under the concentration time curve. [b] Relative bioavailability was calculated as follows: (AUC of EGCg)/(AUC of Cy-g).

Table 2. Cy-g and EGCg Concentrations in Human Plasma after Administration of Red Fruit Anthocyanins and Tea Catechin[a]

subject	before administration	30 min after administration	60 min after administration
		Plasma Cy-g (nmol/L)	
A	<2[b]	22	
B	<2	22	
C	<2	17	
D	<2	34	
E	<2		23
F	<2		43
G	<2		23
H	<2		27
		24 ± 7[c]	29 ± 10[c]
		Plasma EGCg (nmol/L)	
		free form EGCg	725 ± 78[c]
		glucuronide form	284 ± 92[c]
		sulfate form	803 ± 138[c]

[a] Each subject received either Cy-g (2.7 mg/kg of body weight) or EGCg (2.6 mg/kg of body weight) as a single oral supplementation after 12 h of fasting. [b] Below the detection limit (<2 nmol/L). [c] Mean ± SD of four subjects.

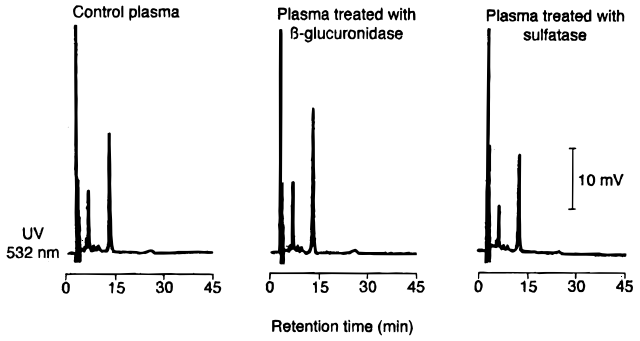

Figure 8. UV-HPLC chromatograms of rat plasma anthocyanin extract after hydrolysis with β-glucuronidase or sulfatase. Rat plasma 30 min after a single oral administration of anthocyanins (160 mg of Cy-g and 20 mg of Cy-dg/kg of body weight) was reacted with either β-glucuronidase, sulfatase, or enzyme-free water (control).

their aglycone (Cy) in plasma and liver of rats and human plasma. However, flavonoid glycosides generally are known to yield their aglycone by hydrolysis in the mammalian gut, as in the case of rutin (quercetin-3-rutinoside) (Bokkenheuser et al., 1987). Thus, in vitro digestibility of anthocyanins to their aglycone was investigated, and compared with that of rutin. Incubation of RAE (containing 820 nmol of Cy-g and 60 nmol of Cy-dg) with either commercial β-glucosidase or rat fecal homogenate entirely failed to liberate cyanidin from cyanidin glycosides (Table 3). On the contrary, rutin was hydrolyzed slightly by β-glucosidase and fecal

Table 3. Hydrolysis of Anthocyanins or Rutin to Corresponding Aglycone Using β-Glucosidase and Fecal Homogenate

RAE[a]	% cyanidin liberated from RAE (incubation time, h)			
incubated with				
β-glucosidase	nd[b] (0)	nd (0.5)	nd (1.5)	
fecal homogenate	nd (0)	nd (1)	nd (4)	nd (22)

rutin	% quercetin liberated from rutin (incubation time, h)			
incubated with				
β-glucosidase	nd (0)	0.4 ± 0.1 (0.5)	0.8 ± 0.1 (1.5)	
fecal homogenate	nd (0)	nd (1)	0.7 ± 0.3 (4)	16.1 ± 1.6 (22)

[a] Red fruit anthocyanin extract (RAE) contained 820 nmol of Cy-g and 60 nmol of Cy-dg. [b] Not detected (below 2 pmol for Cy-g and Cy-dg).

homogenate with prolonged incubations and liberated aglycone (quercetin) (Table 3). The results indicated that anthocyanins are rather stable against hydrolytic cleavage in the gut.

DISCUSSION

The present study demonstrated direct intestinal incorporation of red fruit anthocyanins in the glycoside form into the plasma and liver of rats and into the plasma of humans after ingestion. It was confirmed that aglycones and conjugates of anthocyanins were not present in rat and human plasma.

To date there have been several reports on the metabolism of anthocyanins. According to an early study by Horwitt (1933), considerable amounts of anthocyanins dosed by subcutaneous injection (100 mg/kg of body weight) to rats were excreted in urine, apparently in intact form, but no anthocyanins were detected in urine when about twice this dose was administered orally. On the other hand, the same paper indicated that only a small portion of anthocyanin dosed was excreted in the urine of rabbits after oral ingestion. Subsequent studies by Griffiths and Smith (1972a,b) showed that some anthocyanins are metabolized when given orally to rats or incubated with rat intestinal microorganisms; malvidin-3,5-diglucoside gave rise in vivo to three unidentified neutral urinary metabolites but none of these were detected in the in vitro incubation experiments. Lietti and Forni (1976) have estimated that anthocyanins administered by intravenous or intraperitoneal injection undergo a rapid wide distribution to rat tissues such as plasma, bile, urine, heart, kidneys, liver, lungs, and skins but did not refer to the effect when anthocyanins are orally supplemented. It remains unknown to what extent anthocyanins are absorbed from the intestine into the animal body following oral ingestion, although the previous papers mentioned above suggest that glycoside hydrolysis and ring fission occur to a limited extent in absorption and metabolism of anthocyanins in animals.

In this study, an appreciable amount of orally administered anthocyanin, both Cy-g and Cy-dg, is confirmed to be absorbed from the digestive tract into rat and human bodies (Figure 3). Calculating from the AUC of Cy-g (Table 1), about 1% less of the ingested Cy-g was estimated to be incorporated into rat plasma. In plasma samples of fasted rats and humans, both anthocyanin levels were below the detection limit (<2 nmol/L). In the rat study, plasma Cy-g and Cy-dg concentrations

significantly increased with increasing amount of anthocyanin ingested (Figure 5), indicating dose-dependent incorporation of anthocyanins into the animal body. The rat plasma anthocyanin concentrations determined by the present UV-HPLC method were roughly comparable to the plasma total concentrations of anthocyanins estimated by Morazzoni et al. (1991). Both plasma Cy-g and Cy-dg levels reached a maximum just 15 min after oral intake, then gradually decreased (Figure 6A). In the liver, Cy-g also increased to a maximum level at 15 min after intake and then declined rapidly (Figure 6B). The results suggest that in the pharmacokinetics of absorption, anthocyanins have a relatively short half-life. Comparing pharmacokinetics and bioavailability of anthocyanins with those of tea catechin in rats, the absorption of Cy-g and Cy-dg seemed somewhat faster than that of EGCg (Figures 6 and 7), and consequently the relative bioavailability of Cy-g against EGCg was estimated to be 2.8 times less (Table 1). In the present study, the human plasma Cy-g level was relatively lower (29 nmol Cy-g/L at 60 min after ingestion of 2.7 mg Cy-g/kg body weight) than the values expected from the rat study (Table 2), whereas a much higher concentration of EGCg in free form (725 nmol/L plasma) than Cy-g was found at 60 min after oral intake (2.6 mg EGCg/kg body weight) (Table 2). For other flavonoids, the concentration of quercetin (classified as flavonols) in human plasma has been reported to be 649 nmol/L at 2.9 h after a 64-mg oral intake (Hollman et al., 1996). Therefore, the absorption rate of anthocyanins in humans may be relatively lower than that of other flavonoids, though the possibility was considered that part of absorbed anthocyanins is metabolized to some noncolored forms which would escape from detection under the present conditions.

On considering metabolism of anthocyanins, it is helpful that many types of flavonoids are changed to some extent into glucuronide and sulfate conjugates in vivo (Scheline, 1991). UDP-glucuronosyl transferase and sulfotransferase in livers would be responsible for this conjugation (Boutin et al., 1993; Hackett, 1986). In the present study, no conjugates of anthocyanins were found in rat and human plasma (Figure 8), while tea catechin, EGCg, was metabolized to some extent into conjugates (Figure 7). Thus, the flavylium cation structure of anthocyanins seems to impart resistance against such enzymic conversion into conjugates. On the other hand, no detectable amounts of Cy in rat plasma and liver and in human plasma were found even after oral administration of Cy-g and Cy-dg. This suggests that bacterial hydrolysis of the glycosidic bond in Cy-g and Cy-dg does not occur in the gastrointestinal tract. In contrast to anthocyanins, flavonoids in glycosidic form such as rutin, naringin, and hesperidin have been reported to be hydrolyzed to aglycones in mammals (Booth et al., 1958; Griffiths and Smith, 1972a; Manach et al., 1997). β-Glucosidase activity found in intestinal bacteria would be most responsible for such hydrolysis as observed in flavonoid-glycosides (Bokkenheuser et al., 1987). In in vitro tests, we found that both Cy-g and Cy-dg could not be hydrolyzed to aglycone by commercial β-glucosidase or fecal homogenate, while rutin was converted to some extent into its aglycone after those treatments (Table 3). Thus, the flavylium cation structure of anthocyanins would be metabolically much more stable against bacterial hydrolysis than in the case of other flavonoids lacking the cation group.

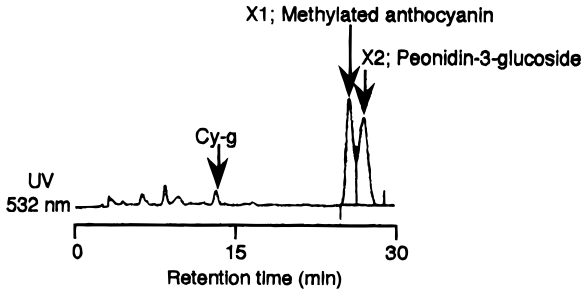

Figure 9. UV-HPLC chromatogram of liver extracts of anthocyanin-supplemented rats. Rat liver extract at 30 min after a single oral administration of anthocyanins (320 mg of Cy-g and 40 mg of Cy-dg/kg of body weight) was analyzed with UV-HPLC. An ODS column (YMC ODS-H80, 4.6 mm × 250 mm; YMC, Tokyo, Japan) was used with a mixture of 1.5 mol/L formic acid aqueous solution and acetonitrile (92:8, v/v) as column eluant at a flow rate of 1.0 mL/min. Peak component X2 was identified as peonidin-3-glucoside, and X1 was postulated as the methylated anthocyanin.

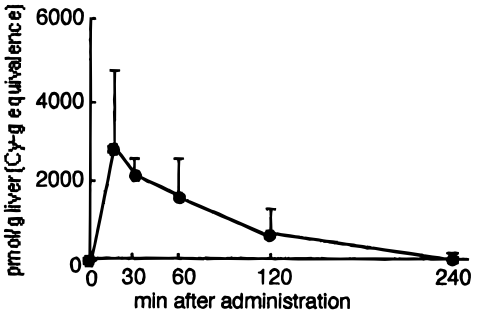

Figure 10. Time-course changes in the concentration of anthocyanin metabolites in livers of rats treated with a single oral dose of anthocyanins (320 mg of Cy-g and 40 mg of Cy-dg/kg of body weight). Values are the mean ± SD of four rats. The concentration was the sum of peak component X1 and X2 described in Figure 9 and expressed as Cy-g equivalence with its calibration curve.

During this study, we recently became aware of the presence of two anthocyanin metabolites in rat liver. The liver extract after ingesting anthocyanins (320 mg of Cy-g and 40 mg of Cy-dg/kg of body weight) was analyzed with UV-HPLC (Figure 9). One of these metabolites was isolated and identified as methylated Cy-g, i.e., peonidin-3-glucoside (Peo-g), by comparing its retention time, UV/vis spectrum, and mass spectrum with that of standard Peo-g (Funakoshi Co.). Another metabolite was presently postulated to be the methylated anthocyanin due to its reactivity with aluminum chloride (Harborne, 1958). The concentration of these methylated metabolites present in liver was about 20 times higher than Cy-g in liver, and such methylated products disappeared from liver after 240 min of ingestion (Figures 6B and 10). Therefore, a large portion of absorbed Cy-g accumulated in the liver would be methylated. Probably, this methylation reaction would consequently result in the substantially low anthocyanin concentration in rat plasma and human plasma as described above (Tables 1 and 2). On the other hand, since methylated metabolites such as Peo-g and other methylated compounds were scarcely found in the plasma of rats and humans, these metabolites may be excreted from liver directly into bile.

The antioxidant function of flavonoids including anthocyanins could account for many of their health-promoting and disease-preventing effects (Brouillard et al., 1997; Kandaswami and Middleton, 1997). Epide-

miological studies have suggested that individuals who consume flavonoids have a lower risk of cardiovascular disease (Hertog et al., 1993; Keli et al., 1996; Renaud and de Lorgeril, 1992). Flavonoids have been suggested to reduce the risk of cardiovascular disease by preventing the oxidation of plasma low density lipoprotein (LDL). The oxidative modification of LDL is recognized to be an important step in the formation of atherosclerotic plaques and subsequent cardiovascular disease (Steinberg et al., 1989).

In conclusion, the present results demonstrated the direct intestinal absorption of anthocyanins in the intact glycoside form, Cy-g and Cy-dg, into rat and human bodies after oral supplementation. No aglycones or conjugates of anthocyanins occurred in plasma, suggesting the glycoside form is rather the responsible intermediate for the bioavailability of anthocyanins in human.

ABBREVIATIONS USED

AUC, total area under the concentration time curve; Cy-dg, cyanidin-3,5-diglucoside; Cy-g, cyanidin-3-glucoside; EGCg, (−)-epigallocatechin-3-gallate; Peo-g, peonidin-3-glucoside; RAE, red fruit anthocyanin extract; TFA, trifluoroacetic acid.

LITERATURE CITED

Bokkenheuser, V. D.; Shackleton, C. H. L.; Winter, J. Hydrolysis of dietary glycosides by strain of intestinal *Bacteroides* from humans. *Biochem. J.* **1987**, *248*, 953−956.

Bomser, J.; Madhavi, D. L.; Singletary, K.; Smith, M. A. In vitro anticancer activity of fruit extracts from *Vaccinium* species. *Planta Med.* **1996**, *62*, 212−216.

Booth, A. N.; Jones, F. T.; DeEds, F. Metabolic fate of hesperidin, eriodictyol, homoeriodictyol, and diosmin. *J. Biol. Chem.* **1958**, *230*, 661−668.

Boutin, J. A.; Meunier, F.; Lambert, P. H.; Hennig, P.; Bertin, D.; Serkiz, B.; Volland, J. P. In vivo and in vitro glucuronidation of the flavonoid diosmetin in rats. *Drug Metab. Disp.* **1993**, *21*, 1157−1166.

Brouillard, R.; George, F.; Fougerousse, A. Polyphenols produced during red wine ageing. *Biofactors* **1997**, *6*, 403−410.

Friend, D. R.; Chang, G. W. A colon-specific drug-delivery system based on drug glycosides and the glycosidases of colonic bacteria. *J. Med. Chem.* **1984**, *27*, 261−266.

Fuhr, U.; Kummert, A. L. The fate of naringin in humans: a key to grapefruit juice-during interactions? *Clin. Pharmacol. Ther.* **1995**, *58*, 365−373.

Ghiselli, A.; Nardini, M.; Baldi, A.; Scaccini, C. Antioxidant activity of different phenolic fractions separated from an italian red wine. *J. Agric. Food Chem.* **1998**, *46*, 361−367.

Griffiths, L. A.; Smith, G. E. Metabolism of apigenin and related compounds in the rat. *Biochem. J.* **1972a**, *130*, 141−151.

Griffiths, L. A.; Smith, G. E. Metabolism of myricetin and related compounds in the rat. *Biochem. J.* **1972b**, *128*, 901−911.

Hackett, A. M. The metabolism of flavonoid compounds in mammals. *Progr. Clin. Biol. Res.* **1986**, *213*, 177−194.

Harborne, J. B Spectral method of characterizing anthocyanins. *Biochem. J.* **1958**, *70*, 22−28.

Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007−1011.

Hollman, P. C.; v.d. Gaag, M.; Mengelers, M. J.; van Trijp, J. M.; de Vries, J. H.; Katan, M. B. Absorption and disposition kinetics of dietary antioxidant quercetin in man. *Free Radical Biol. Med.* **1996**, *21*, 703−707.

Horwitt, M. K. Observations on behavior of the anthocyanin pigment from Concord grapes in the animal body. *Proc. Soc. Exp. Biol. Med.* **1933**, *30*, 949−951.

Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. Suppression of tumor cell growth by anthocyanins in vitro. *Cancer Invest.* **1995**, *13*, 590−594.

Kandaswami, C.; Middleton, E., Jr. Flavonoids as antioxidants. In *Natural antioxidants*; Shahidi, F., Ed.; AOCS Press: Champaign, IL, 1997; pp 174−203.

Keli, S. O.; Hertog, M. G. L.; Feskens, E. J. M.; Kromhout, D. Dietary flavonoids, antioxidant vitamins and incidence of stroke. The Zutphen study. *Arch. Intern. Med.* **1996**, *154*, 637−642.

King, R. A.; Broadbent, J. L.; Head, R. J. Absorption and excretion of the soy isoflavone genistein in rats. *J. Nutr.* **1996**, *126*, 176−182.

Kuhnau, J. The flavonoids. A class of semi-essential food components. Their role in human nutrition. *World Rev. Nutr. Diet* **1976**, *24*, 117−191.

Laplaud, P. M.; Lelubre, A.; Chapman, M. J. Antioxidant action of Vaccinium myrtillus extract on human low density lipoproteins in vitro: initial observations. *Fundam. Clin. Pharm.* **1997**, *11*, 35−40.

Lee, M.-J.; Wang, Z.-Y.; Li, H.; Chen, L.; Sun, Y.; Gobbo, S.; Balentine, D. A.; Yang, C. S. Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol. Biomark. Prevent.* **1995**, *4*, 393−399.

Lietti, A.; Cristoni, A.; Picci, M. Studies on *Vaccinium myrtillus* anthocyanosides. Vasoprotective and antiinflammatory activity. *Arzneim. Forsch.* **1976**, *26*, 829−832.

Lietti, A.; Forni, G. Studies on *Vaccinium myrtillus* anthocyanosides. II. Aspects of anthocyanins pharmacokinetics in the rat. *Arzneim. Forsch.* **1976**, *26*, 832−835.

Manach, C.; Morand, C.; Demigne, C.; Texier, O.; Regerat, F.; Remesy, C. Bioavailability of rutin and quercetin in rats. *FEBS Lett.* **1997**, *409*, 12−16.

Morazzoni, P.; Livio, S.; Scilingo, A.; Malandrino, S. Vaccinium myrtillus anthocyanosides pharmacokinetics in rats. *Arzneim. Forsch.* **1991**, *41*, 128−131.

Nakagawa, K.; Okuda, S.; Miyazawa, T. Dose-dependent incorporation of tea catechins, (−)-epigallocatechin-3-gallate and (−)-epigallocatechin, into human plasma. *Biosci., Biotechnol. Biochem.* **1997**, *61*, 1981−1985.

Nakagawa, K.; Miyazawa, T. Chemiluminescence−high-performance liquid chromatographic determination of tea catechin, (−)-epigallocatechin-3-gallate, at picomole levels in rat and human plasma. *Anal. Biochem.* **1997a**, *248*, 41−49.

Nakagawa, K.; Miyazawa, T. Absorption and distribution of tea catechin, (−)-epigallocatechin-3-gallate, in the rat. *J. Nutr. Sci. Vitaminol.* **1997b**, *43*, 679−684.

National Research Council Guide for the Care and Use of Laboratory Animals. Publication No. 85-23 (rev.), National Institutes of Health; Bethesda, MD, 1985.

Parkinson, T. M.; Brown, J. P. Metabolic fate of food colorants. *Annu. Rev. Nutr.* **1981**, *1*, 175−205.

Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* **1995**, *22*, 375−383.

Satuegracia, M. T.; Heinonen, M.; Frankel, E. N. Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. *J. Agric. Food Chem.* **1997**, *45*, 3362−3367.

Scheline, R. R. Metabolism of oxygen heterocyclic compounds. In *CRC handbook of mammalian metabolism of plant compounds*; CRC Press: Boca Raton, FL, 1991; pp 243−305.

Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol: Modifications of low density lipoprotein that increase its atherogenicity. *New Engl. J. Med.* **1989**, *320*, 915−924.

Tamura, G.; Gold, C.; Ferro-Luzzi, A.; Ames, B. N. Fecalase: a model for activation of dietary glycosides to mutagens by intestinal flora. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 4961−4965.

Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Choi, S. W.; Kawakishi, S.; Osawa, T. Antioxidative activity of the anthocyanin pigments cyanidin 3-*O*-*β*-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 2407−2410.

Ueno, I.; Nakano, N.; Hirono, I. Metabolic fate of [14C]-quercetin in the ACI rat. *Jpn. J. Exp. Med.* **1983**, *53*, 41−50.

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