JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

The Flavonol Quercetin-3-Glucoside Inhibits Cyanidin-3-Glucoside Absorption in Vitro

MICHAELA C. WALTON,*,† TONY K. MCGHIE,‡ GORDON W. REYNOLDS,† AND WOUTER H. HENDRIKS§

Institute of Food, Nutrition, and Human Health, Massey University, Private Bag 11 222, Palmerston North, New Zealand, The Horticulture and Food Research Institute of New Zealand Ltd., Private Bag 11 030, Palmerston North, New Zealand, and Animal Nutrition Group, Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands

At present, little is known about the mechanisms responsible for intestinal absorption of anthocyanins (ACNs). For example, it has not yet been established if ACNs are absorbed through an active transport mechanism, such as the sodium-dependent glucose transporter (SGLT1), or by passive diffusion. Previously, we found that the absorption of ACNs differs between regions of the digestive tract and is maximal in the jejunum, suggesting that an active transport mechanism is involved. In the present study, we examined the effect of D-glucose (main substrate of SGLT1), phloridzin (inhibitor of SGLT1), and quercetin-3-glucose (Q3G, a flavonol) on the absorption of cyanidin-3-glucoside (C3G; ~5 μmol/L) by mouse jejunum mounted in Ussing chambers. We found that the presence of either D-glucose (10, 20, and 40 mmol/L) or phloridzin (50, 100, and 200 μmol/L) resulted in a small but insignificant inhibition of C3G disappearance from the mucosal solution (decrease of disappearance with glucose, 33%; with phloridzin, 18%; NS). However, when the flavonol Q3G (50 μmol/L) was added to the mucosal solution together with the C3G, the disappearance of C3G was significantly decreased (74%; $p < 0.001$), and Q3G disappeared instead. In addition, we found phloretin and quercetin, the aglycones of phloridzin and Q3G, respectively, present in the mucosal solution and tissue extracts, indicating hydrolysis of these compounds by the enterocytes of the jejunum. In contrast, the aglycone cyanidin was not detected at all. Our results show that in the mouse small intestine, ACN absorption is not solely dependent on the activity of the SGLT1 transporter, as D-glucose and phloridzin had only a slight effect on uptake. Q3G, however, clearly inhibited C3G disappearance. These results suggest that there might be a competitive inhibition between C3G and Q3G absorption. It is possible that an absorption mechanism other than the SGLT1 is involved, which has a structural preference toward flavonols.

KEYWORDS: Anthocyanins; cyanidin-3-glucoside; quercetin-3-glucoside; phloridzin; D-glucose; Ussing chamber; SGLT1; absorption; jejunum

INTRODUCTION

Anthocyanins (ACNs) belong to a large and widespread group of water-soluble plant constituents, known collectively as flavonoids (*1*). They are glycosides and acylglycosides of anthocyanidins, which are polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium (flavylium) cation (*2*). ACNs are the red and blue pigments present in a large number of plant tissues including leaves, flowers, and fruits and, therefore, are consumed as normal components of the diet. The daily ACN consumption in humans has been estimated to be as much as 180−215 mg (*3*). Although the intake of ACNs seems reasonably high as compared to most other flavonoids (23 mg/day), including quercetin, kaempferol, myricetin, apigenin, and luteolin (*4*), their bioavailability, as indicated by the recovery in the plasma and urine after ingestion, has been shown to be extremely low (*5*−*11*). However, ACNs are rarely ingested on their own but rather as part of a fruit, vegetable, or meal, containing various other polyphenols and ingredients. Therefore, the amount of ACNs that is eaten together with other foods might be rather low. The ratio of flavonoids to ACNs in apples for example has been shown to be 10:1 (*12*).

More and more research has concentrated on the biological activities and possible health effects of ACNs. In particular, their antioxidant properties have been reported to protect against oxidative damage involved in a variety of diseases, such as

* To whom correspondence should be addressed. Tel: +64(06)356 9099 ext. 7372. Fax: +64(06)350 5657. E-mail: m.walton@massey.ac.nz.
† Massey University.
‡ The Horticulture and Food Research Institute of New Zealand Ltd.
§ Wageningen University.

10.1021/jf0607922 CCC: $33.50
Published on Web 06/06/2006

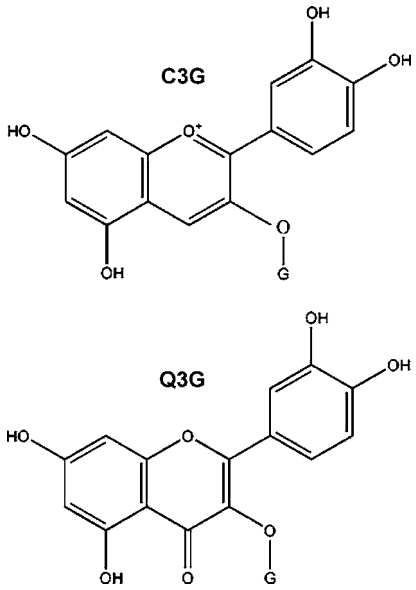

Figure 1. Chemical structures of C3G and Q3G.

diabetes (*13*), arteriosclerosis (*14*), neurodegeneration (*15*), and cancer (*16*−*18*). However, because the bioavailability, including absorption and metabolism, of ACNs has been shown to be very low, claimed health benefits of ACNs are questionable. To obtain a better understanding of the health-enhancing properties of ACNs, it is necessary to increase our knowledge of ACN absorption and metabolism. Once the absorption mechanisms for ACNs are better understood, their bioavailablilty could be increased and potential health benefits enhanced.

At present, the mechanisms of ACN absorption are still not fully understood. Whether ACNs are absorbed through passive diffusion or via an active transport mechanism remains to be determined. Several studies have shown that ACNs are absorbed as glycosides in humans and rats (*11*, *19*−*24*), and the intact forms have been recovered in plasma and urine after oral administration. The fact that ACNs are recovered in plasma and urine as the glycosidic forms implies that they are also absorbed as such. It is possible that the sugar moiety of the ACN molecule interacts with a sugar transport system, such as the intestinal sodium-dependent glucose transporter (SGLT1) and that ACNs are therefore absorbed via an active transport mechanism. We have recently shown that ACNs (cyanidin-3-glucoside; C3G) are mainly absorbed from the jejunum in mice (*25*). The fact that ACNs are not absorbed equally throughout the gastrointestinal tract suggests that an active transport mechanism is involved. In the case of other flavonoids, some reports indicate that quercetin-glucosides, as present in onions, show a higher bioavailability as compared to quercetin (aglycone) or quercetin-glucorhamnoside (rutin) (*26*−*29*). These results suggest that, due to the glucose moiety in the quercetin-3-glucoside (Q3G) molecule, the SGLT1 could be involved in Q3G absorption. Indeed, Wolffram et al. (*30*) have shown that the SGLT1 is involved in the uptake of the flavonol Q3G across the brush border membrane of rat small intestine. As Q3G and C3G have a similar chemical structure (**Figure 1**), it is possible that both compounds share the same transport mechanism.

The aim of the present study was to investigate a possible interaction of the ACN C3G with the SGLT1. Similar to our previous report, we used mouse jejunum mounted in Ussing chambers to investigate if D-glucose, phloridzin, and Q3G, all substrates of the SGLT1, affect C3G absorption. Inhibition of C3G absorption by any of these substances would provide evidence for an interaction between SGLT1 and C3G.

Table 1. Composition of RSs (mmol/L)[a]

compound	RS-A[b]	RS-B[c]
NaCl	120.00	114.00
KCl	5.00	5.00
$CaCl_2$	2.00	1.25
$MgCl_2$	1.00	1.10
NaH_2PO_4	1.80	0.30
Na_2HPO_4	0.20	1.65
$NaHCO_3$	25.00	25.00
glucose	0.00	10.00/20.00/40.00
glutamine	2.00	0.00
pyruvate Na	2.00	0.00

[a] Compositions are based on [b] Ref *50* (without glucose), and [c] Ref *51* (with glucose), with minor modifications.

MATERIALS AND METHODS

Animals. The study reported here was approved and followed the procedures set out by the Massey University Animal Ethics Committee (*31*). Sixteen male Swiss mice (age 6−8 weeks) obtained from the Small Animal Production Unit, Massey University (Palmerston North, New Zealand), were used. They were housed in rodent cages and kept in a room with controlled temperature (21 ± 1 °C), humidity (55 ± 5%), and lighting (12 h light and dark cycles with dawn and dusk transitional periods). The mice were fed a balanced standard rodent diet (as previously described elsewhere, *25*), prepared by the Food Processing Unit at Massey University, and were given access to both food and demineralized water ad libitum until the day of the experiment.

Experimental Setup. On the day of the experiments, mice were deprived of food for 2−3 h, anaesthetized with 6% halothane, and euthanized by cervical dislocation. Within 1 min of euthanasia, the abdomen was opened by a midline incision, and the intestine was dissected out and immersed in Ringer's solution (RS) (RS-A, **Table 1**) at room temperature. Two centimeter long pieces of the jejunum were opened longitudinally along the mesenteric border and mounted in individual Ussing chambers, which were then filled with RS. To investigate the effects of D-glucose on C3G absorption, the Ussing chambers were filled with RS containing either no D-glucose (RS-A, **Table 1**) or different concentrations of d-glucose (RS-B, **Table 1**). To investigate the influence of phloridzin and Q3G on C3G absorption, the Ussing chambers were filled with RS containing no D-glucose (RS-A, **Table 1**) and the phloridzin or Q3G was added simultaneously with the C3G at the start of each experiment. As ACNs are pH sensitive, all solutions were adjusted to pH 4.5 to stabilize the ACN during the experiments. pH values in the jejunum have been reported to be between 4.4 and 6.6 (*32*). For stabilizing reasons, we have chosen the lower range of these reported pH values. The area of jejunal tissue exposed to the RS was 0.5 cm^2. The tissues were kept at 37.5 °C, and the RS was aerated with Carbogen (95% O_2/5% CO_2). All tissues were voltage clamped throughout the experiments at 0 mV (*33*) using automatic clamp units (Campus Electronics and Mechanical, University of Otago, Dunedin, New Zealand). The RS was replaced at least three times before the start of each experiment to wash the tissues and remove any luminal contents. The volume of the RS on each side of the chambers was 6 mL.

After an equilibration period of at least 30 min, the test compounds C3G (dissolved in RS-A; final concentration, 5.01 ± 0.26 μmol/L), phloridzin (dissolved in RS-A; final concentrations, 50, 100, or 200 μmol/L), or Q3G [dissolved in dimethyl sulfoxide (DMSO); final concentration, 50 μmol/L; final concentration of DMSO in the Ussing chamber 0.25%] was added to the mucosal compartment. To keep the mucosal and the serosal compartments at an equal volume, the same amount of just RS-A was added to the serosal compartment at the beginning of each experiment. Chambers without tissue were used as controls to measure degradation of the test compounds due to experimental conditions during the 120 min of the experiment.

ACNs. The ACNs were prepared as previously described (*25*). Briefly, 600 g of frozen Boysenberries (*Rubus loganbaccus* × *baileyanus* Britt) was extracted twice with 1.5 L of acetone/water/acetic acid (70:29.5:0.5). Extracts were evaporated to 500 mL total volume at 40

°C, and lipids were removed by extraction three times with 500 mL of hexane. The aqueous layer was evaporated and then diluted to 20% methanol/water using methanol. Subsequently, the aqueous fraction was applied to a large LH-20 Sephadex column (50 mm ∅ × 370 mm height, 450 g of LH-20) and washed with 20% methanol/water. Boysenberry ACNs were eluted with 60% methanol/water, evaporated to dryness, and stored in the dark at 4 °C until use.

ACNs of boysenberries have previously been characterized as cyanidin-3-sophoroside, C3G, cyanidin-3-glucosylrutinoside, and cyanidin-3-rutinoside (*19*, *34*). The extract used in the present study contained predominantly C3G, the major monosaccharide ACN of boysenberries (*19*). For each experiment, a stock solution containing 2.5 mg of boysenberry extract/2 mL RS (RS-A, **Table 1**) was prepared.

Sample Preparation. Samples (400 μL) of RS from the mucosal and serosal compartments of the Ussing chambers were collected immediately after the addition of the C3G to the mucosal RS (0 min) and at 30, 60, 90, and 120 min. The mucosal RS samples were immediately acidified (1:1) with 5% formic acid/H_2O and stored at 4 °C until analysis. Serosal RS samples were not analyzed, as it has been found that test compounds do not enter the serosal compartment due to the presence of submucosal tissue layers (subepithelial tissue, muscle tissue), which hinder diffusion into the serosal bathing solution (*30*). This was consistent with our previous observations that ACNs were not detected in the serosal RS using similar experimental conditions (*25*). At the end of each experiment, the intestinal tissues were removed from the chambers and trimmed using iris scissors to leave only the part exposed to the test compounds (0.5 cm^2). The tissues were washed with ice-cold RS-A and kept at −20 °C until analyzed (usually not longer than 24 h). Determination of test compounds in the tissues was performed according to Tsuda et al. (*20*) with minor modifications. Frozen samples were blended with an Ultra-Turrax basic homogenizer in 2 mL of 0.4 M sodium phosphate buffer (pH 4.2), containing 0.1% (ethylenedinitrilo) tetraacetic acid (EDTA). Aliquots (400 μL) of homogenate were mixed with 2 mL of acetone, containing 0.1% trifluoroacetic acid (TFA), and centrifuged at 3000*g* for 5 min at 4 °C. The supernatant was collected, 20 μL of TFA was added, and the samples were evaporated under a stream of N_2 at ≤35 °C. The dried extracts were redissolved in 200 μL of methanol, containing 1% TFA, and transferred into a high-performance liquid chromatography (HPLC) vial for analysis.

HPLC Analysis. Concentrations of the test compounds in the mucosal RS and tissue extracts were determined by reversed phase HPLC with photodiode array (PDA) detection. The separation column used was a Phenomenex Aqua C_{18} (3 μm, 125 Å, 2.0 mm × 150 mm). Solvents A (5% formic acid/H_2O) and B (acetonitrile) were run at a flow rate of 1 mL/min. The solvent gradient started with a composition at 95% A, 5% B (for 5 min) and changed to reach 50% A, 50% B at 30 min (for 5 min). The composition then changed to reach 20% A, 80% B at 40 min (for 5 min), before returning to the starting conditions with a total run time of 50 min.

The sample injection volume was 10 μL for the mucosal RS samples and 50 μL for the tissue extracts. The PDA detector was used to collect spectral data (250−600 nm), and chromatograms were extracted at 520 and 280 nm. Chromatography data were collected and processed using a Waters Millennium Chromatography Manager version 4.0. C3G (Extrasynthese, Genay, France), and Q3G and phloridzin (Sigma, Sydney, Australia) concentrations were calculated using authentic standards with known concentrations.

Statistics. Data are presented as means ± standard errors of the mean (SEM). Statistical analysis was carried out using the SAS System for Windows, version 8. The residuals of each analysis were tested for normality. The significance of differences was assessed by repeated measures analysis of variance, and the least significant difference was used for comparison of individual means. Differences with $p \leq 0.05$ were considered significant.

RESULTS

After C3G was added to the mucosal RS of Ussing chambers containing mice jejunal tissue, the effect of the additional presence of D-glucose, phloridzin, or Q3G on C3G disappearance (indicative for absorption) from the mucosal RS was monitored over a period of 2 h. A control (Ussing chamber without tissue) was run with each experiment to determine the stability of C3G, Q3G, and phloridzin during the experimental period. The control experiments (no tissue) showed that there were no significant decreases in concentrations of C3G (**Figures 2**, **4**, and **8**), Q3G (**Figure 10**), or phloridzin (data not shown) during the 2 h experimental period, indicating no breakdown or destruction of these compounds due to the experimental conditions. The results of the controls verify that all three compounds were stable throughout the experiments and that any observed disappearance in tissue-mounted Ussing chambers indicates physiological activity of the tissue, such as absorption or metabolism.

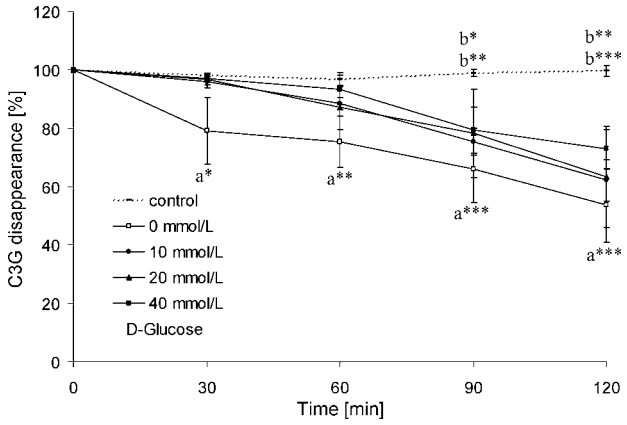

Figure 2. C3G disappearance from the mucosal solution over time without (0 mmol/L) and with the additional presence of D-glucose (10, 20, and 40 mmol/L). Values are means ± SEM ($n = 3$). The letter a indicates the significant difference to time point 0 without glucose; the letter b indicates the significant difference to time point 0 with D-glucose. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Figure 2 shows the disappearance of C3G from the mucosal RS over time, in the presence of varying concentrations of D-glucose. Without D-glucose in the RS (0 mmol/L D-glucose), there was a significant ($p < 0.05$) reduction in mucosal concentration of C3G after 30 min. The reduction became greater as the experiment progressed ($p < 0.01$ at 60 min; $p < 0.001$ at 90 and 120 min). The addition of D-glucose to the mucosal RS at all concentrations reduced but did not completely block C3G disappearance; a significant decrease in C3G concentration was still apparent at 90 (10, 20 mmol/L, $p < 0.01$; 40 mmol/L, $p < 0.05$) and 120 min (10, 20 mmol/L, $p < 0.001$; 40 mmol/L, $p < 0.01$). The C3G disappearance over the experimental period of 2 h without D-glucose was 44.8 ± 11.3%, with 40 mmol/L glucose 30.1 ± 6.5%, resulting in a 32.8% reduction (NS) of disappearance with the high D-glucose concentration (**Figure 3**). The slope (−0.33) of the regression equation between C3G disappearance and D-glucose concentration in the RS after 2 h was not significantly different from zero ($p = 0.255$).

The results for the experiments to determine the effects of phloridzin on the disappearance of C3G from the mucosal RS over time are shown in **Figure 4**. When no phloridzin was present in the RS (0 μmol/L phloridzin), C3G disappeared significantly from the mucosal solution between 60 and 120 min ($p < 0.001$). The addition of phloridzin did not appear to inhibit the disappearance of C3G; significant reductions in C3G concentrations in the mucosal RS were still apparent at 90 (50, 100 μmol/L, $p < 0.05$; 200 μmol/L, $p < 0.01$) and 120 min ($p < 0.001$), even in the presence of the highest concentration of

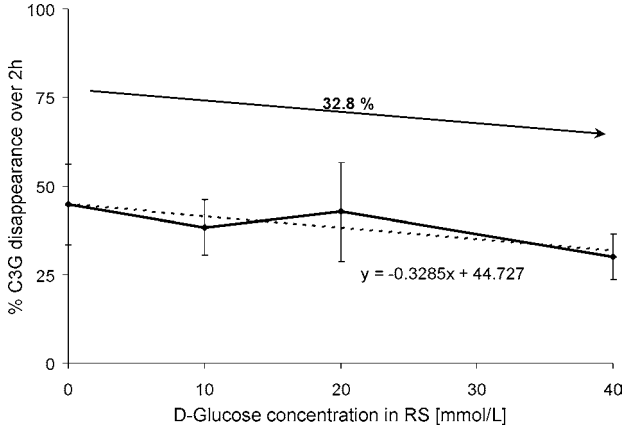

Figure 3. Percent C3G disappearance over 2 h without (0 mmol/L) and with the additional presence of D-glucose in the RS (10, 20, and 40 mmol/L). Values are means ± SEM ($n = 3$).

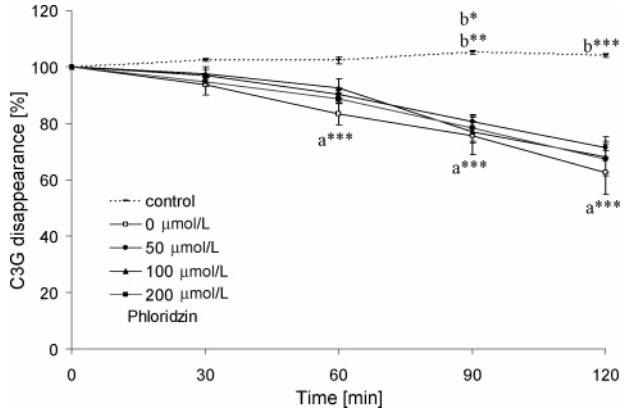

Figure 4. C3G disappearance from the mucosal solution over time without (0 μmol/L) and with the additional presence of phloridzin (50, 100, and 200 μmol/L). Values are means ± SEM ($n = 3$). The letter a indicates the significant difference to time point 0 without phloridzin; the letter b indicates the significant difference to time point 0 with phloridzin. *$p <$ 0.05, **$p < 0.01$, and ***$p < 0.001$.

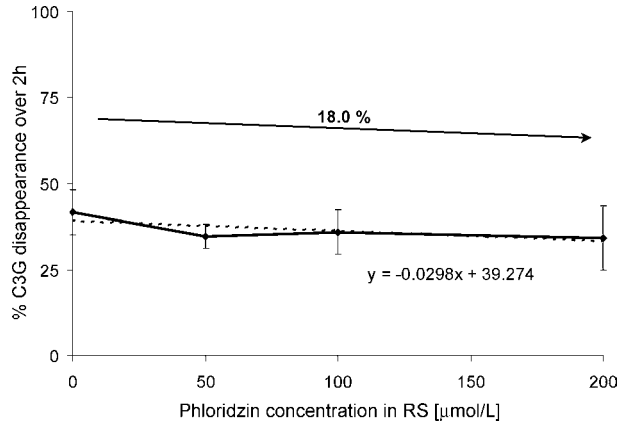

Figure 5. Percent C3G disappearance over 2 h without (0 μmol/L) and with the additional presence of phloridzin in the RS (50, 100, and 200 μmol/L). Values are means ± SEM ($n = 3$).

phloridzin. Increasing concentrations of phloridzin in the RS showed an insignificant effect on C3G disappearance over the experimental period of 2 h (without phloridzin, 41.7 ± 6.5%; with 200 μmol/L phloridzin, 34.2 ± 9.3%), resulting in a reduction (NS) of the disappearance by 18% (**Figure 5**). The slope (−0.03) of the regression equation between C3G disappearance and phloridzin concentration in the RS after 2 h was not significantly different from zero ($p = 0.489$). In addition, we detected phloretin, the aglycone of phloridzin in the mucosal RS, as well as in tissue extracts (**Figure 6C**,**D**). The disappearance of phloridzin from the mucosal RS over time (>60 min, $p < 0.001$) was paralleled by a significant increase of the aglycone phloretin in the mucosal RS (>60 min, $p < 0.001$) (**Figure 7**). Furthermore, the percentage conversion of phloridzin to phloretin was noticeably lower the higher the initial concentrations of phloridzin were.

The presence of Q3G in the mucosal RS significantly inhibited C3G disappearance. **Figure 8** shows the C3G disappearance in mucosal RS in the absence and presence of Q3G over time. While C3G decrease was highly significant over time without Q3G (60−120 min; $p < 0.001$), the amount of decrease was strongly reduced by the presence of 50 μmol/L Q3G (90 min, $p < 0.05$; 120 min, $p < 0.001$). Adding Q3G to the mucosal RS significantly decreased C3G disappearance over the experimental period of 2 h (without Q3G, 65.9 ± 2.3%; with 50 μmol/L Q3G, 17.2 ± 1.7%), resulting in a 73.9% reduction ($p < 0.001$) of the C3G disappearance (**Figure 9**). Interestingly, a significant decrease of Q3G from the mucosal RS over time (30 min, $p < 0.01$; 60−120 min, $p < 0.001$) could be detected instead (**Figure 10**). Additionally, like with phloridzin, the aglycone quercetin was detected in the mucosal RS and tissue extracts (**Figure 6A**,**B**). However, the aglycone cyanidin was not detected in mucosal RS or in tissue extracts.

DISCUSSION

There is increasing interest in the health-related effects of ACNs as they exhibit a range of biological activities and their frequent consumption may help to improve or at least maintain human health (*3*, *8*, *24*). However, to exert health effects, ingested ACNs need to be efficiently absorbed, circulate in sufficient concentrations in the human body, and reach the target tissues at concentrations sufficiently high to generate a biological effect. Further knowledge regarding absorption mechanisms is necessary to help understand details and to increase bioavailability of ACNs.

In our previous study, we showed that ACNs are predominantly absorbed from the jejunal tissue, which suggests the involvement of an active transport mechanism (*25*), rather than passive diffusion throughout the entire intestinal tract. As an example, the SGLT1 has been shown to be involved in the absorption of flavonols (*30*, *35*−*37*). This transporter is generally responsible for the "active" accumulation of sugars into cells. It has been established that there is one major SGLT1 that handles all hexoses, with D-glucose and D-galactose being the natural substrates (*38*). The function of the SGLT1 and its presence in the intestinal epithelium have been confirmed for numerous domestic animals and for humans (*39*). Rat SGLT1 is 87% identical to human SGLT1 (*40*).

Because of the close similarity of the chemical structure (**Figure 1**) between flavonols (Q3G) and ACNs (C3G), we expected a similar absorption mechanism for both compounds. If the SGLT1 were involved in ACN absorption, the simultaneous presence of D-glucose and ACN in the intestinal tract would cause a competition of the compounds for the binding site of the SGLT1. In the Ussing chambers used for these experiments, competition for a single transporter would result in less ACN disappearance from the mucosal RS. In the present study, up to 40 mmol/L D-glucose showed a slight, but not significant, inhibition of C3G disappearance (**Figures 2** and **3**). The SGLT1 has been shown to saturate at 30−50 mmol/L glucose in vivo (*41*), and it is therefore likely that at the

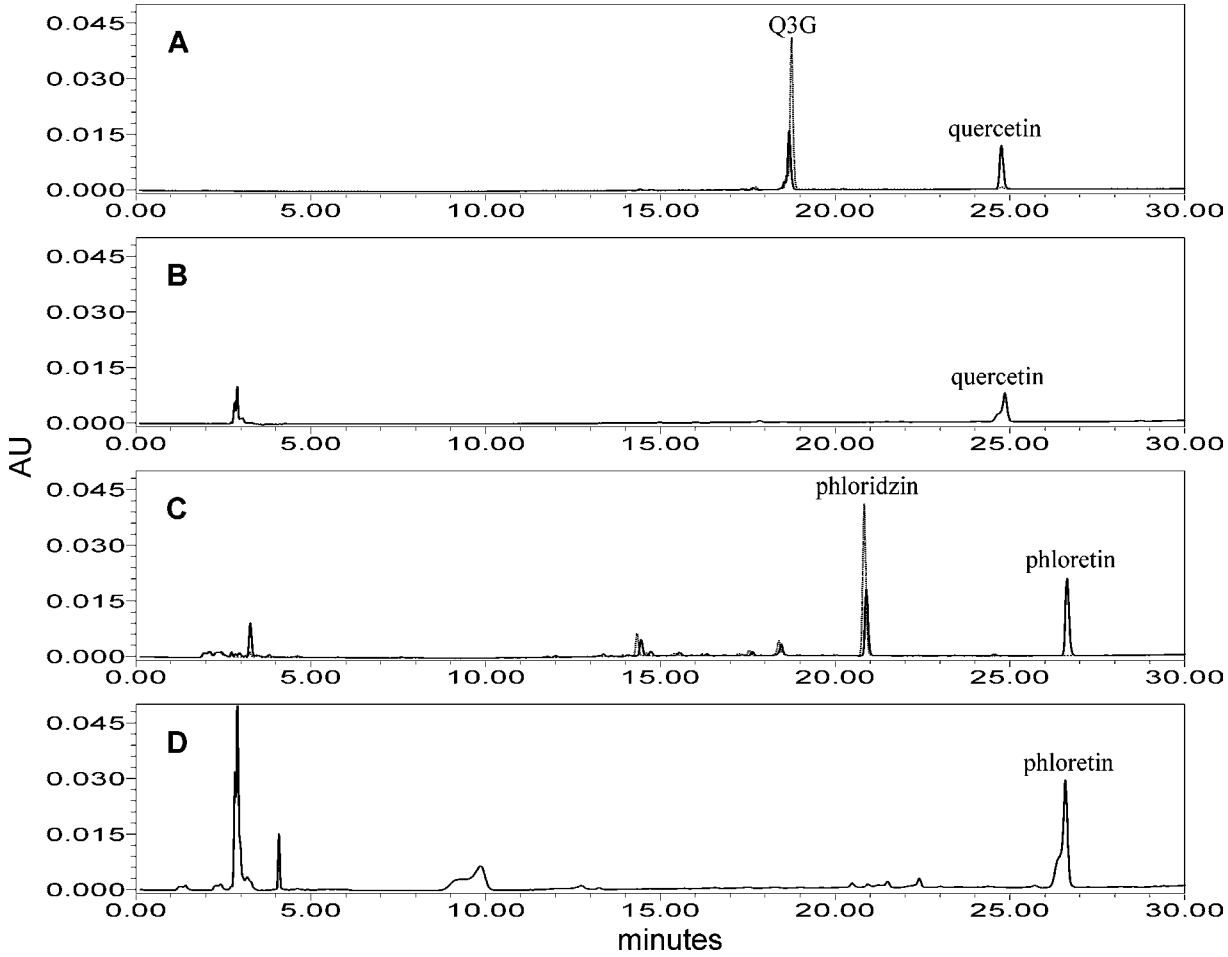

Figure 6. HPLC Chromatograms of mucosal solutions (**A** and **C**) and tissue samples (**B** and **D**). Dotted lines represent the start of the experiments (0 min), showing the original compounds Q3G and phloridzin; continuous lines represent the end of the experiments (120 min), showing the respective aglycones quercetin and phloretin. Detection is at 280 nm.

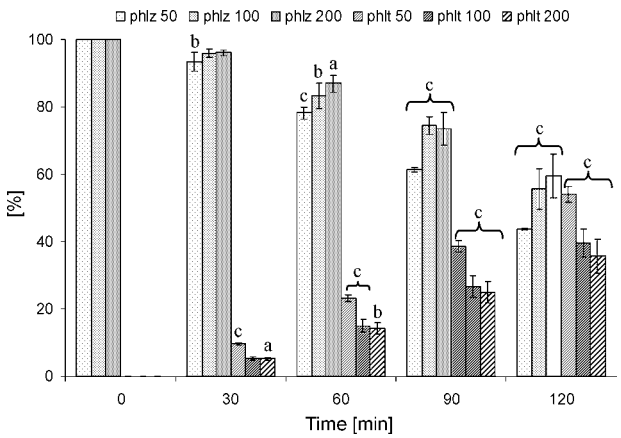

Figure 7. Phloridzin disappearance and phloretin appearance in mucosal solution over time. The bars show phloridzin (phlz) disappearance as well as phloretin (phlt) appearance after the simultaneous addition of either 50, 100, or 200 μmol/L phloridzin to the RS. Values are means ± SEM ($n = 3$). Shown is the significant difference to time point 0. a = $p < 0.05$, b = $p < 0.01$, and c = $p < 0.001$.

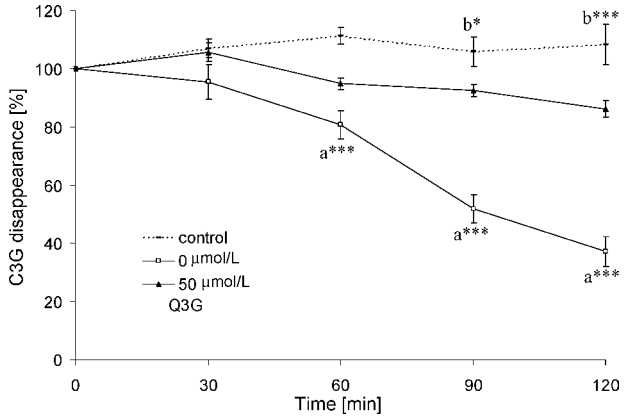

Figure 8. C3G disappearance from the mucosal solution over time without (0 μmol/L) and with the additional presence of Q3G (50 μmol/L). Values are means ± SEM ($n = 2$–4). The letter a indicates the significant difference to time point 0 without Q3G; the letter b indicates the significant difference to time point 0 with Q3G. *$p < 0.05$ and ***$p < 0.001$.

concentration of 40 mmol/L glucose the SGLT1 was saturated in the present study. If C3G would interact with the SGLT1, a saturation of the transporter would result in less disappearance of C3G. However, C3G did still significantly decrease from the mucosal RS, even with the high glucose concentration present (**Figure 2**), indicating no competition between the sugar and the ACN compound for the binding site at the transporter. Thus, it seems likely that C3G is not a substrate for the SGLT1. This is in contrast to a study by Mulleder et al. (*42*), where it was observed that urinary excretion of ACNs in humans was reduced when sucrose was ingested together with an elderberry concentrate, indicating that ACNs are associated with an intestinal sugar transport system. However, it is difficult to compare the two studies, as the present study used an in vitro

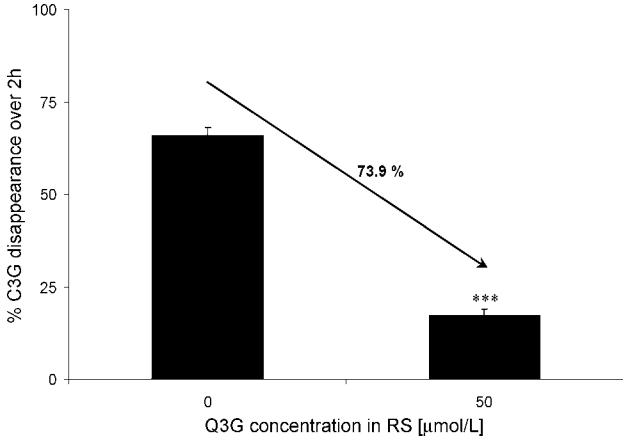

Figure 9. Percent C3G disappearance over 2 h without (0 μmol/L) and with the additional presence of Q3G in the RS (50 μmol/L). Values are means ± SEM (n = 2–4). ***p < 0.001.

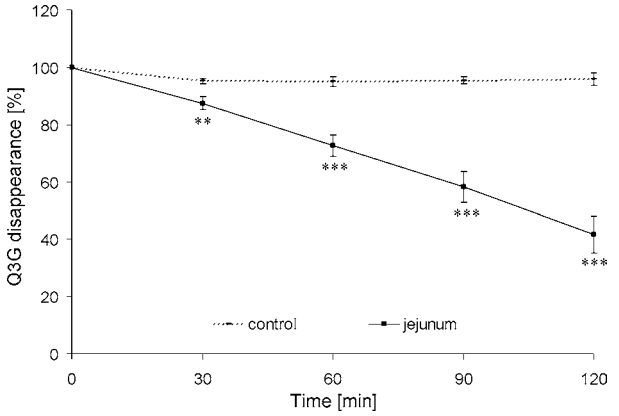

Figure 10. Q3G disappearance from the mucosal solution over time. Values are means ± SEM (n = 4). Indicated are significant differences to time point 0. **p < 0.01 and ***p < 0.001.

model to investigate the effect of D-glucose on ACN absorption at a molecular basis, whereas Mulleder et al. (*42*) conducted an in vivo study investigating urinal excretion of ACNs after oral administration to humans. In another study, Wolffram et al. (*30*) investigated the involvement of the SGLT1 on flavonol-absorption (Q3G) in rat jejunum mounted in Ussing type chambers. They found that a simultaneous addition of only 10 mmol/L D-glucose significantly reduced the disappearance of the flavonol Q3G from the mucosal solution, indicating competition for the transporter. It has further been shown that Q3G inhibits glucose uptake by SGLT1 in a specific and competitive manner, suggesting that Q3G is partly absorbed by SGLT1 (*43*). This implies that although flavonols and ACNs have some structural similarity, their absorption mechanisms appear to differ.

Phloridzin is a useful compound to study intestinal D-glucose transport, because it competitively binds to SGLT1 and inhibits its activity (*44*). In fact, Wolffram et al. (*30*) used phloridzin as a SGLT1 inhibitor and observed a significant reduced disappearance of the flavonol Q3G from the mucosal compartment when phloridzin (100 μmol/L) was simultaneously present. Thus, if ACNs are absorbed via the SGLT1 mechanism, the simultaneous presence of phloridzin would result in less disappearance of ACNs. We therefore investigated the effect of phloridzin (50−200 μmol/L) as an inhibitor of SGLT1 on C3G absorption. We found that phloridzin did not affect C3G disappearance, which supports the assumption that C3G is not a substrate and is not transported by the SGLT1.

In the present study, we also detected the aglycones phloretin and quercetin in the mucosal RS, indicating deglycosylation, whereas the aglycone cyanidin was not detected. The observed disappearance of Q3G and phloridzin from the mucosal RS, and the appearance of the algycones quercetin and phloretin in the mucosal RS, is most likely due to lactase phloridzin hydrolase (LPH) activity, a mammalian β-glucosidase present in the brush border of the small intestine (*45*). The appearance of the aglycone phloretin was expected, as phloridzin is a primary substrate for LPH. Interestingly, an increasing phloridzin concentration resulted in a decreased appearance of phloretin (**Figure 6**), which could imply a saturation of the enzyme. Besides phloridzin, LPH has been shown to be capable of hydrolyzing various flavonol and isoflavone glucosides (*45*). Deglycosylation by LPH and subsequent diffusion of the aglycone is also claimed to be the major route for Q3G absorption (*35*). Our detection of the aglycone quercetin in the mucosal RS as well as in jejunal tissue confirms the conclusions of Day et al. (*35*). The Q3G disappearance observed by Wolffram et al. (*30*) could therefore also be due to the activity of the LPH, as these authors detected a paralleled appearance of the aglycone quercetin in the mucosal solution. It is possible that the absorption mechanism for ACNs might be similar to the absorption of flavonols as suggested by Day et al. (*35*), involving deglycosylation by the LPH along the brush border membrane with subsequent diffusion into the enterocyte. In this study, the aglycone cyanidin was not detected in the mucosal solution or tissues extracts suggesting that deglycosylation does not occur. However, ACN aglycones have low stability and could therefore easily escape detection.

LPH has two distinct catalytic active sites. One for lactase activity, which has a broad substrate specificity with several glycosides as substrates, and the other for phloridzin hydrolase activity (*46*, *47*). The SGLT1 also possesses a sugar-binding site, with affinity to hexoses, such as D-glucose, as well as phloridzin (*38*). Thus, it is likely that in a system where both proteins (LPH and SGLT1) are present, a substrate for LPH is also attracted by the SGLT1. Indeed, the two SGLT1 substrates used in the present study (D-glucose and phloridzin) have also been reported to inhibit LPH (*48*, *49*). Therefore, the reduced Q3G disappearance observed by Wolffram et al. (*30*) could well be due to the inhibition of LPH instead of SGLT1. In our study, however, neither glucose nor phloridzin had an effect on C3G disappearance, eliminating the assumption of an interaction between C3G and LPH. Furthermore, this could explain the lack of detection of the aglycone cyanidin in the present study.

Interestingly, we found that C3G disappearance was significantly inhibited by the presence of the flavonol Q3G (**Figures 8** and **9**), and Q3G disappeared preferentially from the mucosal RS (**Figure 10**). These results show for the first time that a simultaneous ingestion of ACNs and flavonols might cause a competition for absorption, resulting in a reduced absorption of ACNs in the presence of flavonols. The competition between ACNs and flavonoids for intestinal absorption suggests that both compounds share the same transport mechanism. Moreover, our results indicate that this transport mechanism obviously has a preference toward flavonols instead of ACNs.

In conclusion, our results show that D-glucose and phloridzin had no effect on C3G disappearance, which proves that neither SGLT1 nor LPH is involved in ACN absorption. However, we have demonstrated for the first time that the flavonol Q3G significantly inhibited C3G absorption. Our results indicate that

ACNs and flavonols share a common absorption mechanism, with an obvious preference for the latter. We suggest that it is important to investigate ACN absorption together with other flavonoids or nutrients, as ACNs are rarely ingested entirely on their own. The occurrence of other flavonoids in fruit, vegetables, and wine or of other nutrient compounds in a meal might have a significant effect on ACN absorption and should be taken in account in future studies regarding ACN bioavailability. Further investigations are necessary to clarify interactions of different flavonoids regarding their absorption.

ABBREVIATIONS USED

ACNs, anthocyanins; C3G, cyanidin-3-glucoside; DMSO, dimethyl sulfoxide; LPH, lactase phloridzin hydrolase; PDA, photodiode array; Q3G, quercetin-3-glucoside; RS, Ringer's solution; SGLT1, sodium-dependent glucose transporter; TFA, trifluoroacetic acid.

LITERATURE CITED

(1) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*; CRC Press: Boca Raton, 1993.

(2) Strack, D.; Wray, V. The anthocyanins. In *The Flavonoids: Advances in Research Since 1986.*; Harborne, J. B., Ed.; Chapman & Hall: London, 1993; pp 1−22.

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

(4) Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr. Cancer* **1993**, *20*, 21−29.

(5) Cao, G.; Prior, R. L. Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clin. Chem.* **1999**, *45*, 574−576.

(6) Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **2002**, *50*, 7731−7737.

(7) Felgines, C.; Talavera, S.; Gonthier, M. P.; Texier, O.; Scalbert, A.; Lamaison, J. L.; Remesy, C. Strawberry Anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans. *J. Nutr.* **2003**, *133*, 1296−1301.

(8) Wu, X.; Cao, G.; Prior, R. L. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J. Nutr.* **2002**, *132*, 1865−1871.

(9) Bub, A.; Watzl, B.; Heeb, D.; Rechkemmer, G.; Briviba, K. Malvidin-3-glucoside bioavailability in humans after ingestion of red wine, dealcoholized red wine and red grape juice. *Eur. J. Nutr.* **2001**, *40*, 113−120.

(10) Netzel, M.; Strass, G.; Janssen, M.; Bitsch, I.; Bitsch, R. Bioactive anthocyanins detected in human urine after ingestion of blackcurrant juice. *J. Environ. Pathol. Toxicol. Oncol.* **2001**, *20*, 89−95.

(11) Felgines, C.; Texier, O.; Besson, C.; Fraisse, D.; Lamaison, J.-L.; Remesy, C. Blackberry anthocyanins are slightly bioavailable in rats. *J. Nutr.* **2002**, *132*, 1249−1253.

(12) McGhie, T. K.; Hunt, M.; Barnett, L. E. Cultivar and growing region determine the antioxidant polyphenolic concentration and composition of apples grown in New Zealand. *J. Agric. Food Chem.* **2005**, *53*, 3065−3070.

(13) Bonnefont-Rousselot, D. Glucose and reactive oxygen species. *Curr. Opin. Clin. Nutr. Metab. Care* **2002**, *5*, 561−568.

(14) Aviram, M.; Fuhrman, B. Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 146−161.

(15) Youdim, K. A.; Martin, A.; Joseph, J. A. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radical Biol. Med.* **2000**, *29*, 51−60.

(16) La Vecchia, C.; Chatenoud, L.; Altieri, A.; Tavani, A. Nutrition and health: epidemiology of diet, cancer and cardiovascular disease in Italy. *Nutr. Metab. Cardiovasc. Dis.* **2001**, *11*, 10−15.

(17) 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.

(18) Meiers, S.; Kemeny, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.* **2001**, *49*, 958−962.

(19) Cooney, J. M.; Jensen, D. J.; McGhie, T. K. LC-MS identification of anthocyanins in boysenberry extract and anthocyanin metabolites in human urine following dosing. *J. Sci. Food Agric.* **2004**, *84*, 237−245.

(20) Tsuda, T.; Horio, F.; Osawa, T. Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats. *FEBS Lett.* **1999**, *449*, 179−182.

(21) Miyazawa, T.; Nakagawa, K.; Kudo, M.; Muraishi, K.; Someya, K. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. *J. Agric. Food Chem.* **1999**, *47*, 1083−1091.

(22) Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J. Agric. Food Chem.* **2001**, *49*, 1546−1551.

(23) Cao, G.; Muccitelli, H. U.; Sanchez-Moreno, C.; Prior, R. L. Anthocyanins are absorbed in glycated forms in elderly women: A pharmacokinetic study. *Am. J. Clin. Nutr.* **2001**, *73*, 920−926.

(24) McGhie, T. K.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J. Agric. Food Chem.* **2003**, *51*, 4539−4548.

(25) Matuschek, M. C.; Hendriks, W. H.; McGhie, T. K.; Reynolds, G. W. The jejunum is the main site of absorption for anthocyanins in mice. *J. Nutr. Biochem.* **2006**, *17*, 31−36.

(26) Hollman, P. C.; de Vries, J. H.; van Leeuwen, S. D.; Mengelers, M. J.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62*, 1276−1282.

(27) Hollman, P. C.; van Trijp, J. M.; Buysman, M. N.; van der Gaag, M. S.; Mengelers, M. J.; de Vries, J. H.; Katan, M. B. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett.* **1997**, *418*, 152−156.

(28) Hollman, P. C. H.; Bijsman, M.; van Gameren, Y.; Cnossen, E. P. J.; de Vries, J. H. M.; Katan, M. B. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Res.* **1999**, *31*, 569−573.

(29) Olthof, M. R.; Hollman, P. C. H.; Vree, T. B.; Katan, M. B. Bioavailabilities of quercetin-3-glucoside and quercetin-4′-glucoside do not differ in humans. *J. Nutr.* **2000**, *130*, 1200−1203.

(30) Wolffram, S.; Block, M.; Ader, P. Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *J. Nutr.* **2002**, *132*, 630−635.

(31) Anonymous. *Code of Ethical Conduct for the Use of Live Animals for Teaching and Research*, revised ed.; Massey University: Palmerston North, New Zealand, 2003.

(32) Gray, V. A.; Dressman, J. B. Change of pH requirements for simulated intestinal fluid TS. *Pharmacopeial Forum* **1996**, *22*, 1943−1945.

(33) Ussing, H. H.; Zerahn, K. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **1951**, *23*, 110−127.

(34) Torre, L. C.; Barritt, B. H. Quantitative evaluation of Rubus fruit anthocyanin pigments. *J. Food Sci.* **1977**, *24*, 488−490.
(35) Day, A. J.; Gee, J. M.; DuPont, M. S.; Johnson, I. T.; Williamson, G. Absorption of quercetin-3-glucoside and quercetin-4′-glucoside in the rat small intestine: The role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochem. Pharmacol.* **2003**, *65*, 1199−1206.
(36) Ader, P.; Block, M.; Pietzsch, S.; Wolffram, S. Interaction of quercetin glucosides with the intestinal sodium/glucose cotransporter (SGLT-1). *Cancer Lett.* **2001**, *162*, 175−180.
(37) Walgren, R. A.; Lin, J. T.; Kinne, R. K.; Walle, T. Cellular uptake of dietary flavonoid quercetin 4′-beta-glucoside by sodium-dependent glucose transporter SGLT1. *J. Pharmacol. Exp. Ther.* **2000**, *294*, 837−843.
(38) Wright, E. M.; Turk, E.; Zabel, B.; Mundlos, S.; Dyer, J. Molecular genetics of intestinal glucose transport. *J. Clin. Invest.* **1991**, *88*, 1435−1440.
(39) Pajor, A. M.; Hirayama, B. A.; Wright, E. M. Molecular-biology approaches to comparative-study of na^{+}-glucose cotransport. *Am. J. Physiol.* **1992**, *263*, R489−R495.
(40) Lee, W. S.; Kanai, Y.; Wells, R. G.; Hedinger, M. A. The high affinity Na^{+}/glucose cotransporter. Reevaluation of function and distribution of expression. *J. Biol. Chem.* **1994**, *269*, 12032−12039.
(41) Debnam, E. S.; Levin, R. J. An experimental method of identifying and quantifying the active transfer electrogenic component from the diffusive component during sugar absortion measured *in vivo*. *J. Physiol.* **1975**, *246*, 181−196.
(42) Mulleder, U.; Murkovic, M.; Pfannhauser, W. Urinary excretion of cyanidin glycosides. *J. Biochem. Biophys. Methods* **2002**, *53*, 61−66.
(43) Cermak, R.; Landgraf, S.; Wolffram, S. Quercetin glucosides inhibit glucose uptake into brush-border-membrane vesicles of porcine jejunum. *Br. J. Nutr.* **2004**, *91*, 849−855.
(44) Kimmich, G. A.; Randles, J. α-Methylglucoside satisfies only Na+-dependent transport-system of intestinal epithelium. *Am. J. Physiol.* **1981**, *241*, C227−C232.
(45) Day, A. J.; Canada, F. J.; Diaz, J. C.; Kroon, P. A.; Mclauchlan, R.; Faulds, C. B.; Plumb, G. W.; Morgan, M. R.; Williamson, G. Dietary flavonoid and isoflavone glycosides are hydrolyzed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **2000**, *468*, 166−170.
(46) Fernandez, P.; Canada, F. J.; Jimenez-Barbero, J.; Martin-Lomas, M. Substrate specificity of small-intestinal lactase: Study of the steric effects and hydrogen bonds involved in enzyme−substrate interaction. *Carbohydr. Res.* **1995**, *271*, 31−42.
(47) Rivera-Sagredo, A.; Canada, F. J.; Nieto, O.; Jimenez-Barbero, J.; Martin-Lomas, M. Substrate specificity of small-intestinal lactase. Assessment of the role of the substrate hydroxyl groups. *Eur. J. Biochem./FEBS* **1992**, *209*, 415−422.
(48) Alpers, D. H.; Cote, M. N. Inhibition of lactose hydrolysis by dietary sugars. *Am. J. Physiol.* **1971**, *221*, 865−868.
(49) Ramaswamy, S.; Radhakrishnan, A. N. Lactase-phloridzin hydrolase complex from monkey small intestine: Purification, properties and evidence for two catalytic sites. *Biochim. Biophys. Acta* **1975**, *403*, 446−455.
(50) Butt, A. G.; Bowler, J. M.; McLaughlin, C. W. Villus and crypt cell composition in the secreting mouse jejunum measured with X-ray microanalysis. *J. Membr. Biol.* **1998**, *162*, 17−29.
(51) Sheldon, R. J.; Malarchik, M. E.; Fox, D. A.; Burks, T. F.; Porreca, F. Pharmacological characterization of neural mechanisms regulating mucosal ion transport in mouse jejunum. *J. Pharmacol. Exp. Ther.* **1989**, *249*, 572−582.

Received for review March 21, 2006. Revised manuscript received May 4, 2006. Accepted May 8, 2006.

JF0607922