ABSORPTION AND METABOLISM OF FLAVONOIDS IN THE CACO-2 CELL CULTURE MODEL AND A PERFUSED RAT INTESTINAL MODEL

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ABSTRACT:

The purpose of present study was to determine the intestinal absorption and metabolism of genistein and its analogs to better understand the mechanisms responsible for their low oral bioavailability. The Caco-2 cell culture model and a perfused rat intestinal model were used for the study. In both models, permeabilities of aglycones (e.g., genistein) were comparable to well absorbed compounds, such as testosterone and propranolol. In the Caco-2 model, permeabilities of aglycones were at least 5 times higher \( (p < 0.05) \) than their corresponding glycosides (e.g., genistin), and the vectorial transport of aglycones was similar \( (p > 0.05) \). In contrast, vectorial transport of glucosides favored excretion \( (p < 0.05) \). Limited hydrolysis of glycosides was observed in the Caco-2 model, which was completely inhibited \( (p < 0.05) \) by 20 mM glucuronolactone, a broad specificity glycosidase inhibitor. In the perfused rat intestinal model, genistin was rapidly hydrolyzed (about 40% in 15 min) in the upper intestine but was not hydrolyzed at all in the colon. Aglycones were rapidly absorbed \( (P_{eff} > 1.5) \), and absorbed aglycones underwent extensive (40% maximum) phase II metabolism via glucuronidation and sulfation in the upper small intestine. Similar to the hydrolysis, recovery of conjugated genistein was also region-dependent, with jejunum having the highest and colon the lowest \( (p < 0.05) \). This difference in conjugate recovery could be due to the difference in the activities of enzymes or efflux transporters, and the results of studies tend to suggest that both of these factors were involved. In conclusion, genistein and its analogs are well absorbed in both intestinal models, and therefore, poor absorption is not the reason for its low bioavailability. On the other hand, extensive phase II metabolism in the intestine significantly contributes to its low bioavailability.

Genistein, a soy isoflavone, is being tested for its ability to prevent prostate cancer (NCI, 1996). Its efficacy is supported by epidemiological evidence and in vitro mechanistic studies (Kurzer and Xu, 1997). However, the bioavailability of genistein and its glucoside analog (i.e., genistin, the main natural glucoside form of genistein in soy products such as tofu and soy milk) are poor (Xu et al., 1994, 1997). However, the bioavailabilities of genistein and its glucoside analogs have been reported to be poor (Xu et al., 1994, 1997). Genistein is absorbed after first-pass metabolism of genistein may be one of the main reasons for its poor bioavailability. In rats, the main metabolites are 7-OH-glucuronic acid and 4'-OH-sulfate (King et al., 1996). In humans, 7-OH-glucuronic acid and genistin is the major metabolite (about 90%), whereas 4'-OH-sulfate (10%) is the minor metabolite (King and Bursill, 1998; Setchell et al., 2001). Other pathways (e.g., bacteria-mediated metabolism) may also be involved, based on the variety of metabolites recovered in urine (King and Xu, 1997; King and Bursill, 1998; Watanabe et al., 1998; Setchell et al., 2001). Liver conjugates of genistein are partially excreted into the urine or eliminated through the bile (King et al., 1996). Recently, it was shown that flavone glucuronides and isoflavone glucuronides were secreted by the rat enterocytes (Crespy et al., 1999; Liu et al., 1999; Andlauer et al., 2000), thereby identifying another pathway for the disposition of flavones. This phenomenon of intestinal secretion of glucurononlated metabolites of xenobiotics has rarely been shown previously (Fischer et al., 1996).

Published studies have suggested that genistein-7-O-glucoside (or genistin) is not absorbed from the intestine, which contributes to its poor oral bioavailability. For example, a recent study has suggested that genistin is not transported across the Caco-2 model (Walle et al., 1999). Walle and his coworkers further suggested that the presence of a multidrug resistant protein 2 (MRP2) counteracted the absorption of genistin in the Caco-2 model (Walle et al., 1999; Walgren et al., 2000). The same research group later showed that a glucose transporter (SGLT1) facilitated the transport of quercetin-4'-β-glucoside (a flavone glucoside) (Walgren et al., 2000), especially when the function of MRP2 was suppressed. Since genistein is absorbed after administration of genistein (either in soy products or as pure genistein), absorption and metabolism of genistein and its analogs to better understand the mechanisms responsible for their low oral bioavailability. The Caco-2 cell culture model and a perfused rat intestinal model were used for the study. In both models, permeabilities of aglycones (e.g., genistein) were comparable to well absorbed compounds, such as testosterone and propranolol. In the Caco-2 model, permeabilities of aglycones were at least 5 times higher \( (p < 0.05) \) than their corresponding glycosides (e.g., genistin), and the vectorial transport of aglycones was similar \( (p > 0.05) \). In contrast, vectorial transport of glucosides favored excretion \( (p < 0.05) \). Limited hydrolysis of glycosides was observed in the Caco-2 model, which was completely inhibited \( (p < 0.05) \) by 20 mM glucuronolactone, a broad specificity glycosidase inhibitor. In the perfused rat intestinal model, genistin was rapidly hydrolyzed (about 40% in 15 min) in the upper intestine but was not hydrolyzed at all in the colon. Aglycones were rapidly absorbed \( (P_{eff} > 1.5) \), and absorbed aglycones underwent extensive (40% maximum) phase II metabolism via glucuronidation and sulfation in the upper small intestine. Similar to the hydrolysis, recovery of conjugated genistein was also region-dependent, with jejunum having the highest and colon the lowest \( (p < 0.05) \). This difference in conjugate recovery could be due to the difference in the activities of enzymes or efflux transporters, and the results of studies tend to suggest that both of these factors were involved. In conclusion, genistein and its analogs are well absorbed in both intestinal models, and therefore, poor absorption is not the reason for its low bioavailability. On the other hand, extensive phase II metabolism in the intestine significantly contributes to its low bioavailability.

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1 Abbreviations used are: MRP2, multidrug resistant protein 2; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; MPA, mobile phase A; MPB, mobile phase B; ANOVA, analysis of variance; pH, para-amino hippuric acid; LPH, lactase phlorizin hydrolase.
an activation mechanism must be present. Previously, the proposed mechanism responsible for this activation is hydrolysis of glucosides by bacteria glucosidase. Results of recent studies have suggested the involvement of intestinal glycosidases (Day et al., 1998, 2000; Andlauer et al., 2000).

The purpose of the present studies was to determine how intestinal disposition of genistein and its glucoside (genistin or genistein-7-O-glucoside; Fig. 1) contributes to their low bioavailability by investigating their transport across the Caco-2 cell monolayer (Hidalgo et al., 1989) and their absorption and metabolism in the rat intestine (Hu et al., 1988). Both model systems were used here because they have complementary characteristics (Kim et al., 1993; Zheng et al., 1994) and none of these two models when used alone is sufficient to achieve the goals of the study. For example, the Caco-2 model is excellent for studying the mechanism of transepithelial transport but often lacks or poorly expresses phase I and phase II enzymes. On the other hand, the rat perfusion model, an in situ model with intact circulation, is very suited to study regional absorption and metabolism but not secretory processes.

Genistein and genistin were chosen because they are the main active ingredients in a formulation used for chemoprevention trials and many soy isoflavone products. Apigenin and apigetrin (apigenin-7-O-glucoside; Fig. 1), which are flavonoid analogs of genistein and genistin, respectively, were chosen to gain some perspective on the effects of structural change on the intestinal transport and metabolism processes. Lastly, studies were performed to identify the glucosidase responsible for hydrolysis of glucosides.

Experimental Procedures

Materials. Cloned Caco-2 cells (TC7) were a kind gift from Dr. Monique Rouset (Institut National de la Santé et de la Recherche Médicale U178, Villejuif, France). Genistein, genistin (genistein-7-O-glucoside), apigenin, and apigetrin (apigenin-7-O-glucoside) were purchased from Indofine Chemicals (Somerville, NJ). β-Glucuronidase, α-glucosidase, β-glucosidase, sulfatase, and Hanks’ balanced salt solution (HBSS; powder form) were purchased from Sigma (St. Louis, MO). [14C]PEG4000 was obtained from PerkinElmer Life Sciences (Boston, MA). All other materials (typically analytical grade or better) were used as received.

Animals. Male Sprague-Dawley rats aging between 70 to 110 days old and weighing between 260 to 350 g (Simonsen Laboratory, Gilroy, CA) were used. The rats were fed with Teklad F6 rodent diet (W) from Harlan Bioproducts for Science (Indianapolis, IN). The rats were fasted overnight before the date of the experiment.

Cell Culture. The culture conditions for growing Caco-2 cells have been described previously (Hu et al., 1994a,b). The seeding density, growth medium, and quality control criteria were all implemented in the present study as described previously (Hu et al., 1994a,b). TC7 cells were maintained in 10% fetal bovine serum and fed every other day. The cells were ready for experiments from 19 to 22 days after seeding.

Animal Surgery. The surgical procedures were similar to those described in earlier publications with minor modification (Hu et al., 1988, 1995). In the present study, the cannulation to the jejunum was connected to a short cannula, which was 1.5 to 2 cm long and can be easily disconnected or reconnected to the main tube. The main tube was attached to a syringe driven by an infusion pump (Model PHD 2000; Harvard Apparatus, Cambridge, MA).

Transport Experiments in the Caco-2 Cell Culture Model. Experiments in triplicate were performed in HBSS (Hu et al., 1994a,b). Isoflavone or isoflavonoid transport experiments were performed using pH 6.0 (which is jejunal pH) at the apical side and pH 7.4 (which is the serosal pH) at the basolateral side. The protocols for performing cell culture experiments were similar to those described previously (Hu et al., 1994a,b). Briefly, the cell monolayers were washed three times with HBSS, pH 7.4, at 37°C. The transepithelial electrical resistance values were measured, and those with transepithelial electrical resistance values less than 500 ohms × cm² were not used. The monolayers were loaded with a solution containing the compound of interest, and the amounts of transepithelial transported were followed as a function of time by HPLC. Four samples were taken at different times, which were immediately acidified to pH 2.0 by the addition of 20 μl of 0.9 N phosphoric acid to stabilize the test compounds, and the amounts transported were determined by HPLC.

Transport and Metabolism Experiments in the Perfused Rat Intestinal Model. This was a single-pass perfusion method, and the procedures were similar to those described previously (Hu et al., 1988, 1995). After the rats were anesthetized, they underwent the following procedures, which were illustrated here using jejunal perfusion. Briefly, after a 10- to 12-cm intestinal segment was cannulated, it was washed for 30 min with HBSS, pH 6.5, using an infusion pump (Model PHD 2000) at a flow rate of 0.382 ml/min for 30 min. For the experiments, two flow rates were used. For aglycones, the flow rate was 0.382 ml/min, and four samples were collected every 10 min; for glucosides, the flow rate was 0.191 ml/min, and four samples were collected every 20 min. In general, steady-state transport was usually achieved within 30 min after the perfusion of a solution (pH 6.0) containing the compound of interest and PEG4000 (as a water flux marker) began, and it was maintained throughout the experimental period. After perfusion, the length of the intestine was measured as described previously (Hu et al., 1988, 1995). The outlet concentrations of test compounds in the luminal perfusate (or perfusate) were determined by HPLC, and the radioactivity of labeled PEG4000 in the perfusate was determined by liquid scintillation spectrophotometry.

Hydrolysis Experiments. Hydrolysis of flavonoid-conjugates by glucuronidase. A portion of the perfusate was used for direct measurement of the outlet aglycone concentrations by HPLC. The remaining intestinal perfusate sample was incubated with glucuronidase (500 μl of perfusate + 5 μl of glucuronidase) at 37°C for 1 h to reconvert conjugated test compounds to their respective aglycone forms, which were also analyzed by HPLC.

Stability of glucosides in HBSS solution and perfusate solution. In this experiment, genistin and apigetrin were put into an HBSS buffer or a blank perfusate freshly collected from a perfused intestinal segment. The final concentration of each compound was 20 μM in the test solution. The solutions were incubated at 37°C, and samples (500 μl) were taken at 0, 30, and 60 min. A 200-μl portion of acetonitrile was added to each sample, which was then centrifuged at 13,000 rpm for 15 min and analyzed by HPLC.

Hydrolysis of genistin and apigetrin by various forms of glucosidases. Various forms of glucosidases, including 5 μl of isomaltase, 0.25 and 5 μl of α-glucosidase, and 0.1, 0.25, and 5 μl of β-glucosidase, were used in a pilot study to determine which enzyme may hydrolyze the glucosides. Each enzyme was used in a 500-μl solution containing 2.5 to 100 μM genistin or apigetrin at 37°C. The reaction was allowed to proceed for 1 h and was stopped by addition of 200 μl of acetonitrile. For the inhibition study, hydrolysis experiments were performed in the presence of the α-glucosidase inhibitor acarbose at concentrations of 0.1 and 100 μM.

Determination of kinetic parameters of glucosidase-catalyzed hydrolysis. The study was done similar to those described above. However, the initial rate of hydrolysis was measured by taking multiple samples at early time points.

Sample Analysis. The HPLC conditions are listed as follows: HPLC system, H-P 1090 series II with a diodearray detector (Wilmington, DE); column, Chromax Spherisorb (particle size, 3 μm; dimension, 4 × 100 mm); mobile phase A (MPA), 80% 20 mM NaH₂PO₄ + 30 mM NH₄H₂PO₄, pH 3.0, 10% CH₂OH, and 10% CH₂CN; mobile phase B (MPB), 90% CH₂CN in H₂O. A gradient method was used for the analysis of the test compound at a flow rate 1 ml/min. The wavelengths were 254 and 338 nm for isoflavonoids and flavonoids, respectively. For genistein and genistin, the percentage of MPA was 92%, 0 to 4 min; 65%, 4 to 5 min; and 65%, 5 to 9 min. The retention
times for genistein and genistin were approximately 7.6 and 4.2 min, respectively (Fig. 2A). For apigenin and apigetrin, the percentage of MPA was 88%, 1 to 4 min; 60%, 4 to 5 min; and 50%, 5 to 7 min. The retention times for genistein and genistin were approximately 7.6 and 4.2 min, respectively (Fig. 2B). There was a 5-man recovery time between the injections.

**Determination of Total Original Compound Remaining in the Perfusate.** Shown in Fig. 2, A and B, are typical HPLC chromatograms of outlet perfusate before or after metabolite reversion with glucuronidase/sulfatase. The results indicated that additional peaks not seen in the blank perfusate before or after metabolite reversion with glucuronidase. In both panels, the first peak in the solid elution profile is a conjugate of a test compound, the second peak the glycoside, and the third peak the aglycone. The conjugate of a test compound can be converted back to the test compound upon hydrolysis with glucuronidase.

**Observed ABS total** was used to calculate the relative errors of our estimation:

\[
\delta^2 = \left( \frac{\text{Calculated ABS}_{\text{total}} - \text{Observed ABS}_{\text{total}}}{\text{ABS}_{\text{ave}}} \right) \cdot 100\% ^2
\]

where ABSave is the average of calculated ABS total and observed ABS total. A similar procedure was used to calculate total amount of genistin, apigenin, and apigetrin remaining in the relevant perfusate using the additional mathematical relationships shown in Table 1. The concentration of compound in the perfusate before hydrolysis was used to calculate an effective permeability (P*eff) and an unbiased intestinal wall permeability (P*w), which were designated as calculated permeability values. On the other hand, the concentration of compound in the perfusate after hydrolysis using glucuronidase was used to calculate corrected P*eff and P*w, which were designated as corrected permeability values.

**Data Analysis: Perfusion Study.** P*eff and P*w were obtained as described previously (Hu et al., 1988, 1995). The mathematical principle of this analysis method is well established (Hu et al., 1988, 1995). This method measured the steady-state uptake of test compounds to calculate P*w. At steady-state, P*w was calculated using the following equations:

\[
P*_{\text{w}} = \frac{P*_{\text{eff}}}{1 - \frac{P*_{\text{eff}}}{P*_{\text{eff}}}}
\]

where

\[
P*_{\text{eff}} = 1 - \frac{C_{\text{w}}/C_{\text{o}}}{4Gz}
\]

\[
P*_{\text{w}} = \left( A \cdot (Gz)^{1/3} \right) - 1
\]

\[
A = 4.5 \text{ Gz} + 1.065
\]

In eqs. 3 to 6, Cw and Co are inlet and outlet concentrations, respectively; Gz or the Graetz number, a scaling factor that incorporates flow rate and intestinal length to make the permeability dimensionless, is the same as previously defined (Hu et al., 1988, 1995); and A is a correction factor for aqueous resistance of the intestine (Hu et al., 1988). Cw was adjusted for water flux as indicated by the concentration of [14C]PEG4000, a nonabsorbable marker compound, and data points were discarded if the water flux exceeded 0.55%/cm of intestine (Hu et al., 1988, 1995).

The P* values are a better representation of the intestinal membrane permeability than P*eff because the contribution of the unstirred water layer (P*uw) is factored out (Hu et al., 1988, 1995). Previous publications have indicated that compounds with a P*uw larger than 1 are generally well absorbed (>75%) (Johnson and Amidon, 1988). However, when drug permeates rapidly, P*uw could approach infinity as P*eff dominates the P*uw. Under such circumstance, a change in P*uw does not significantly impact the overall permeation, whereas a change in P*eff does.

**Enzyme-Catalyzed Hydrolysis.** Rates of hydrolysis of genistin and apigenin by β-glucosidase may be described by the classical Michaelis-Menten equation. The values of Km and Vmax were obtained through nonlinear regression (Sigma Plot) by using the following equation:

\[
\text{Initial Rates of Hydrolysis} = \frac{V_{\text{max}} \cdot C}{(K_{\text{m}} + C)}
\]

In eq. 7, Vmax is the maximum rate of hydrolysis, K_m is the affinity constant, and C is the initial concentration of the substrate.

One-way ANOVA or a paired Student’s t test (Microsoft Excel; Redmond, WA) was used to analyze the data. The prior level of significance was set at 5% or p < 0.05.

**Results**

**Absorption and Metabolism Studies in the Caco-2 Model.** Vectorial transport of genistin and apigenin in Caco-2 cell culture model. Vectorial transport experiments are used to determine the involvement of carrier-mediated transport, and a difference in absorptive and secretory transport often signals the involvement of a transport carrier. The results showed that absorptive transport of aglycones...
(i.e., genistein and apigenin) was the same as the secretory transport (Fig. 3). Both of these compounds have high absorptive permeabilities with values similar to that of propranolol and testosterone (not shown). Propranolol and testosterone are well absorbed in humans (Artursson and Karlsson, 1991).

**Vectorial transport of genistin and apigetrin in Caco-2 cell culture model.** Secretory transport of genistin and apigetrin was much faster than \( \rho < 0.05 \) their absorptive transport (Fig. 3). In addition, about 1 to 2% genistin and apigetrin were metabolized to their aglycone forms, genistein and apigenin (not shown). Very small amounts (detectable but not quantifiable) of the glucuronide metabolite of both compounds were also observed (not shown). Previously, monoglycosides of flavone and isoflavone were also found to be poorly absorbed (Table 2). However, significant amounts (25% of original) of highly permeable aglycones were found in jejunal lumen as conjugated metabolites. Without correcting for the secreted metabolites, calculated \( P^* \text{eff} \) values were slightly lower than calculated \( P^{*\text{eff}} \) values in jejenum and duodenum.

**Absorption and metabolism of genistin and apigenin.** The results indicated that the aglycones genistein and apigenin are rapidly absorbed (Table 2). However, significant amounts of phase II conjugates of these metabolites were found in the perfusate (Table 2). These metabolites had similar UV spectral properties as the parent compound and were reconverted into parent compounds upon hydrolysis by glucuronidase. At a (single-pass perfusion) flow rate of 0.382 ml/min, approximately 45% of genistin was taken up by jejunum, of which about 30% was secreted back to jejunal lumen as conjugated metabolites. Without correcting for the secreted metabolites, calculated \( P^* \text{eff} \) of genistein and apigenin were 2.99 ± 0.61 and 4.41 ± 0.52, respectively. After correcting for the metabolism of both compounds during the perfusion, corrected \( P^* \text{eff} \) were 1.62 ± 0.43 and 2.02 ± 0.77, respectively (Table 3).

We also determined the absorption and metabolism of genistin in different segments or regions of the intestinal tract. The results indicated that genistin is equally and highly permeable in all segments of the intestine (Fig. 5A). There was metabolism of genistin in all segments of the intestine except the colon (Fig. 5B). However, metabolism by the ileum was much slower than that in the duodenum and jejunum, which could account for 30% of the absorbed amount (Table 2). Corrected \( P^* \text{eff} \) values were slightly lower than calculated \( P^{*\text{eff}} \) values in jejunum and duodenum.

**Absorption and metabolism of genistin and apigetrin.** Genistin and apigetrin were expected to be absorbed slowly in the jejunum based on Caco-2 transport data. Therefore, a flow rate of 0.191 ml/min was chosen for the determination of glucoside permeabilities in the rat model. Surprisingly, the apparent permeabilities of genistin and apigetrin were not low in jejunum (Table 3). In addition, significant amounts (25% of original) of highly permeable aglycones were found in the perfusate (Table 4). After correcting for metabolites found in perfusate, jejunal \( P^* \text{eff} \) and \( P^* \text{w} \) of genistin were found to be 0.623 ±
of glucoside in the presence of gluconolactone and pAH. The concentration of gluconolactone (20 mM) was also used in a glucose-free media. The concentration of 0.379 and 1.160/H11006 was quantified by using glucuronidase-free sulfatase. The rate, 0.382 ml/min). Glucuronide was quantified by using sulfatase-free glucuronidase, and analyze the data and showed that there is significant (p < 0.05) inhibition of glucoside transport as the glucose concentration increased from 0 to 25 mM and that there is a significant increase in uptake (p < 0.05) but not in the transport (P > 0.1) of glucoside in the presence of gluconolactone and pAH.

TABLE 2
Recovery of original and metabolites of perfused compounds (i.e., genistein and apigetrin) as a function of time

<table>
<thead>
<tr>
<th>Compound</th>
<th>Perfusate Sample</th>
<th>Aglycone</th>
<th>Glucuronide</th>
<th>Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>0–10</td>
<td>50 ± 7</td>
<td>10 ± 4</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>10–20</td>
<td>60 ± 10</td>
<td>12 ± 3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>20–30</td>
<td>52 ± 14</td>
<td>14 ± 5</td>
<td>2 ± 2</td>
</tr>
<tr>
<td></td>
<td>30–40</td>
<td>40 ± 11</td>
<td>16 ± 3</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Apigenin</td>
<td>0–10</td>
<td>36 ± 6</td>
<td>12 ± 3</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>10–20</td>
<td>42 ± 8</td>
<td>11 ± 2</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>20–30</td>
<td>41 ± 3</td>
<td>11 ± 2</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>30–40</td>
<td>40 ± 4</td>
<td>11 ± 2</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not detected.

TABLE 3
Effective (P\textsuperscript{eff}) and wall permeability (P\textsubscript{u,w}) of genistein and analogs before (calculated) and after correction (corrected) for the excretion of phase II metabolites

<table>
<thead>
<tr>
<th></th>
<th>P\textsuperscript{eff}</th>
<th>P\textsubscript{u,w}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated</td>
<td>Corrected</td>
</tr>
<tr>
<td>Genistein</td>
<td>2.99 ± 0.61</td>
<td>1.62 ± 0.43</td>
</tr>
<tr>
<td>Apigenin</td>
<td>4.41 ± 0.52</td>
<td>2.02 ± 0.77</td>
</tr>
<tr>
<td>Genistein</td>
<td>1.85 ± 0.26</td>
<td>0.62 ± 0.38</td>
</tr>
<tr>
<td>Apigetrin</td>
<td>6.17 ± 0.53</td>
<td>0.49 ± 0.18</td>
</tr>
</tbody>
</table>

0.379 and 1.160 ± 0.872, respectively; and those of apigetrin were 0.493 ± 0.181 and 0.717 ± 0.360, respectively (Table 3).

We also determined the absorption and metabolism of genistein in other segments of the small intestine (Fig. 6). The results indicated that apparent permeabilities of genistein were much higher (p < 0.05) in duodenum and jejunum than ileum and colon (Fig. 6A). This pattern of permeability difference is matched by amount of genistein found in the perfusate (Fig. 6B).

**In Vitro Stability and Hydrolysis Studies.** Stability of genistin and apigetrin in blank perfusate. Stability studies of genistin and apigetrin in HBSS buffer and in blank perfusate were performed to minimize the bias that may have existed when the disappearance rates of glycosides were used to calculate the permeability. The results indicated that genistin was stable in HBSS buffer solution and in the perfusate for at least 4 h. The results also indicated that apigetrin was stable in HBSS buffer but not in the perfusate buffer. Additional studies suggested that the hydrolysis was not affected by centrifugation at 13,000 rpm for 15 min. This suggested that the enzyme was not associated with whole cells, eliminating the possibility that intact bacteria are responsible for the hydrolysis. Thus, it is likely that a small amount of glucosidase sloughed off the enterocytes and was responsible for the hydrolysis of apigetrin. To stabilize the apigetrin, perfusate samples were immediately acidified to pH 2.0, which eliminated all hydrolysis.
recovered in the perfusate (p small intestine changed significantly after correction for metabolites formed and metabolism adjustment). Furthermore, the permeability values of genistin in the upper gated genistein formed in different regions of the intestine (with or without metabolism adjustment). According to a one-way ANOVA analysis, there were statistically significant differences in the apparent permeability values of genistin, the amounts of free genistein, and conjugated genistein formed in different regions of the intestine (with or without metabolism adjustment). Furthermore, the permeability values of genistin in the upper small intestine changed significantly after correction for metabolites formed and recovered in the perfusate (p < 0.05).

![Graph](image1.png)

**Fig. 6.** Absorption and metabolism of genistin in different regions of the small intestine (n = 4).

The study was performed using genistin at a concentration of 100 µM in buffer at 37°C, pH 6.5. The absorption parameters were reported as Papp values before (solid bars) and after correcting (slashed bars) for metabolites of genistin, if any. Shown in the lower panel are the hydrolytic metabolites of genistin (expressed as percent genistin) and phase II conjugates of genistein (percent metabolized). According to a one-way ANOVA analysis, there were statistically significant differences in the apparent permeability values of genistin, the amounts of free genistein, and conjugated genistein formed in different regions of the intestine (with or without metabolism adjustment). Furthermore, the permeability values of genistin in the upper small intestine changed significantly after correction for metabolites formed and recovered in the perfusate (p < 0.05).

**TABLE 4**

Recovery of original and metabolites of perfused compounds (i.e., genistin and apigetrin) as a function of time

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample time</th>
<th>Conjugates</th>
<th>Glucosides</th>
<th>Aglycones</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistin</td>
<td>0–20</td>
<td>15 ± 9.5</td>
<td>56 ± 9</td>
<td>22 ± 7</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>20–40</td>
<td>15 ± 6.4</td>
<td>55 ± 11</td>
<td>24 ± 7</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>40–60</td>
<td>16 ± 5.7</td>
<td>57 ± 10</td>
<td>24 ± 4</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>60–80</td>
<td>15 ± 3.8</td>
<td>60 ± 7</td>
<td>21 ± 7</td>
<td>95</td>
</tr>
<tr>
<td>Apigetrin</td>
<td>0–20</td>
<td>43 ± 3</td>
<td>18 ± 19</td>
<td>21 ± 6</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>20–40</td>
<td>45 ± 4</td>
<td>16 ± 18</td>
<td>24 ± 7</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>40–60</td>
<td>44 ± 5</td>
<td>11 ± 18</td>
<td>22 ± 12</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>60–80</td>
<td>40 ± 4</td>
<td>18 ± 26</td>
<td>24 ± 13</td>
<td>82</td>
</tr>
</tbody>
</table>

**Hydrolysis of genistin and apigetrin by glucosidases.** In the perfusion study, genistin and apigetrin were hydrolyzed to their corresponding aglycones in the intestinal perfusate. To determine which type of glucosidase may be responsible for their hydrolysis, three glycosidases, isomaltase, α-glycosidase, and β-glycosidase, were chosen for our study. It was found that isomaltase (5 U) could not hydrolyze either compound in a 2-h study. In contrast, β-glucosidase (5 U) hydrolyzed both compounds very quickly. Lastly, α-glucosidase (5 U) seemed to hydrolyze both genistin and apigetrin. When a specific α-glucosidase inhibitor (0.1 and 100 µM), was used, however, the hydrolysis was not inhibited at all.

To further characterize β-glucosidase-catalyzed hydrolysis, enzymatic reactions were carried out at concentrations of 2.5, 5, 7.5, 10, 20, 30, 50, and 100 µM, respectively. The K_m was found to be 18.5 ± 5.9 and 27.0 ± 0.24 µM for genistin and apigetrin, respectively, and V_max was found to be 5.42 ± 0.65 and 19.2 ± 0.7 nmol/(min mg of protein) for genistin and apigetrin, respectively (Fig. 7).

**Discussion**

Isoflavones undergo complex biotransformation and transport processes after oral administration (Fig. 8). Presently, the vast majority of information in the literature is based on analysis of blood and urine samples (Xu et al., 1994, 1995; King et al., 1996; Kurzer and Xu, 1997; King and Bursill, 1998; Setchell et al., 2001). That work established the importance of biotransformation in the disposition of genistin and other isoflavones. However, these studies could not be used to differentiate the contribution of intestine versus liver and other organs in the biotransformation of genistein and its isoflavone analogs.

Intestinal contribution to this complex network of biotransformation and disposition pathways has started to emerge only recently. For example, we first reported on the importance of intestinal conjugation in the disposition of genistin in 1999 (Liu et al., 1999), which were confirmed independently by a different research group (Andlauer et al., 2000). Walle et al. (1999) first reported the importance of MRP2 in the transport of genistin. However, none of the reports so far has focused on integrating all the intestinal disposition pathways. Therefore, one of the main goals of our research was to characterize various intestinal disposition processes that may affect the biotransformation and bioavailability of genistin and its isoflavone analogs.
As depicted in Fig. 8, small intestinal disposition of genistein is a complex network of various absorption, metabolism, and efflux processes, which cannot be described accurately via the use of systemic and urine sampling. In the large intestine, microflora metabolism may introduce additional complexity to the intestinal disposition and bio-transformation in vivo.

For isoflavone glucosides, hydrolysis of glucosides to release the aglycones by intestinal glycosidase is a critical first step in their disposition because it serves as the initiator of all subsequent disposition processes. Our data further suggest that intestinal hydrolysis of glucosides is rapid (about 50% in 15 min) when compared with intestinal transit times of 2 to 4 h. If this hydrolysis in humans occurs as fast as we had observed in the rats, the role played by intestinal microflora in hydrolyzing monoglycosides of isoflavones could diminish significantly. Previously, the role of this intestinal glycosidase was not well recognized, and therefore, glucosides were not expected to be absorbed until activated by intestinal microflora (NCI, 1996; Kurzer and Xu, 1997). To characterize the enzymes responsible for the hydrolysis in rat intestine, activities of glycosidase were determined in all segments of the intestine, and it was found that more glucosides were hydrolyzed in the upper small intestine than in the colon. Previously, regional differences among all the intestinal segments were not reported. Since glucosides seemed to be poorly permeable (Fig. 2), these results tend to suggest that intestinal glycosidases are brush-border enzymes. A search of the literature suggests that lactase phlorizin hydrolase or LPH is probably the enzyme responsible since it is the only brush-border glycosidase described to hydrolyze flavonoid glycosides (Day et al., 2000, 1998). LPH has been shown to hydrolyze flavonoid glucosides (Day et al., 1998, 2000; Ioku et al., 1998) and is expressed by humans and rats.

The reasons for poor uptake of glucosides, such as genistin and apigenin, are not entirely clear. Possible mechanisms include 1) very slow passive diffusion, 2) poor substrate of glucose transporters, and 3) efflux by intestinal efflux transporters, such as MDR1 and MRP1. Poor lipid solubility and the presence of multiple hydroxyl groups (for hydrogen bonds) (Conradi et al., 1991) are possible reasons for poor passive diffusion. In the absence of efficient uptake via passive diffusion, uptake of glucosides is dependent on the presence of a carrier transporter. A glucose transporter (e.g., SGLT1) has been proposed to be responsible for the transport of glucosides in Caco-2 cells (Walgren et al., 2000b). However, transport of glucosides is very slow even in the absence of glucose (Fig. 3). The maximal permeabilities of these glucosides in the Caco-2 model are much less than mannitol, a paracellular leakage marker with 15% absorption (Artursson and Karlsson, 1991). Since the permeability of labeled glucose was at least 100 times faster in the Caco-2 cells (not shown), the amounts of glucosides transported via SGLT1 in the Caco-2 cells are fairly limited. It is possible that MRP2-mediated efflux makes a larger contribution to the poor uptake of glucosides by the intestinal cells (Walle et al., 1999). For example, uptake and transport of glucosides were increased up to 100% in the presence of tested MRP2 inhibitors (Walgren et al., 2000a). However, the maximal permeability, even after doubling, is still less than the permeability of mannitol. Taken together, slow passive diffusion, poor uptake via SGLT1, and the presence of an efflux carrier for glucosides render the intestinal hydrolysis as the critical first step in the intestinal disposition of glucosides.

Our results clearly showed that all aglycones are rapidly absorbed with permeabilities comparable to compounds with 100% absorption. Therefore, absorption is not a critical step in the intestinal disposition of aglycones. Rather, phase II conjugation of aglycones and subsequent secretion of these phase II conjugates into the lumen are the most important because approximately 30% of absorbed aglycones are conjugated and subsequently excreted into the intestinal lumen. Hydrolysis and subsequent conjugation were very efficient processes, considering the fact that the average residence time of the perfusate in the intestinal segment (10 cm on average) was less than 15 min. Although these data alone do not prove the relative importance of gut versus liver metabolism of orally dosed genistein, they clearly suggest that intestinal glucuronidation is more important than previously thought.

In the rat perfusion studies, the amounts of phase II conjugates recovered from the perfusate were region-dependent, with the highest recovery in the jejenum, followed closely by the duodenum, the terminal ileum at distant third, and none in the colon. Therefore, the amounts of conjugates recovered in the intestinal perfusate are directly related to the amounts of conjugates formed in that intestinal segment. Second, the amounts of conjugate recovered were mainly dependent on the activities of efflux transporters that secrete phase II conjugates in that segment. Obviously, a combination of these two mechanisms could also be responsible.

MRP2 is present in the intestinal tract (Taipalensuu et al., 2001) and has been shown to excrete phase II conjugates (e.g., glucuronidated metabolites) in liver, intestine, and kidneys (Ayrton and Morgan, 2001). We hypothesized that MRP2 is involved in the excretion of phase II isoflavone conjugates. This is supported by the fact that the recovery pattern of genistein conjugate in the rat small intestine agrees with the expression pattern of MRP2, which has the highest expression level in jejunum and lowest in the terminal ileum (Mottino et al., 2001). It has also been supported by the fact that the addition of 100 μM verapamil (a potent p-glycoprotein inhibitor) to the jejunal perfusate did not change the recovery pattern of glucuronide metabolites (data not shown). Therefore, this hypothesis is well supported as long as the formation rates of glucuronidated metabolites followed the same pattern or were equal in all parts of the small intestine.

Reverse transcription-polymerase chain reaction analysis indicated that rat intestine expresses UGT1A1, UGT1A2, UGT1A6, and
UGT1A7 (Grams et al., 2000). Although the distribution pattern of UGT1A1s in the gastrointestinal tract is isoform specific, of the four isoforms found in the rat intestine, only UGT1A6 has a slightly higher expression level in the rectum. Otherwise, all UGT1A levels either decreased (UGT1A2 and UGT1A7) or stayed relatively unchanged (UGT1A2 and UGT1A6) from duodenum to colon. The distribution of some UGT2B isoforms might follow that of UGT1s. For example, UGT2B7 also had higher expression in the upper small intestine than colon (Czernik et al., 2000). Taken together, we believe that our data strongly support the hypothesis that absence or reduced level of MRP2 expression in the terminal ileum and colon is the most likely reason why there was significant difference in the recovery of genistein conjugates in the perfuse of proximal versus distal intestinal segments. Further studies are necessary and are underway to test and confirm this hypothesis.

Finally, glucosidase of bacteria origin ordered from Sigma can rapidly hydrolyze genistin and apigenitin to their respective aglycones, suggesting that the roles played by the microflora should not be totally discounted. An interesting side note about our studies is that β-glucosidase was presented in glucuronidase and glucuronidase/sulfatase preparations purchased from Sigma. This may provide some explanations why previous studies have not detected intact glycosides in various biological samples after sample treatment with β-glucuronidase.

In conclusion, our data clearly showed that intestinal glycosidases play a more important and prominent role than previously thought in the intestinal disposition of isoflavone and flavone glycosides. Our data also clearly showed that intestinal secretion of glucuronidated isoflavone and flavone was very rapid and efficient. Since conjugated compounds are poorly permeable, these metabolites were expected to be reabsorbed by the intestinal microflora into their aglycone forms in the colon, which could then either be reabsorbed or metabolized. This “intraintestinal” recycling process may play a significant role in the disposition of flavonoids and isoflavonoids. Coupled with known enterohepatic recycling of genistein and other flavonoids (King and Bursill, 1998; Watanabe et al., 1998), these processes could explain why flavonoids in general and genistein in particular have poor oral bioavailability.

References


