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Quercetin Derivatives Are Deconjugated and Converted to Hydroxyphenylacetic Acids but Not Methylated by Human Fecal Flora in Vitro

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By using a batch in vitro anaerobic fecal fermentation model, we have shown that the fecal microflora can rapidly deconjugate rutin, isoquercitrin, and a mixture of quercetin glucuronides. High levels of β,D-glucosidase, α,L-rhamnosidase, and β,D-glucuronidase were present. Rutin underwent deglycosylation, ring fission, and dehydroxylation. The main metabolite, 3,4-dihydroxyphenylacetic acid, appeared rapidly (2 h) and was dehydroxylated to 3-hydroxyphenylacetic acid within 8 h. The pattern of in vitro fermentation of rutin was not changed by changing the pH (6.0 or 6.9), fermentation scale (10 or 1000 mL), or donors of the inoculum. Hydroxyphenylacetic acids were not methylated by colon flora in vitro. The colonic microflora has enormous potential to transform flavonoids into lower molecular weight phenolics, and these might have protective biological activities in the colon. The site of absorption of flavonoids and the form in which they are absorbed are critical for determining their metabolic pathway and consequent biological activities in vivo.

KEYWORDS: Rutin; isoquercitrin; quercetin glucuronides; metabolism; deconjugation; ring fission; dehydroxylation

INTRODUCTION

Polyphenols in fruits and vegetables are thought to have a protective role in reducing the risk of chronic diseases such as cardiovascular disease and certain cancers (*1*, *2*). Flavonoids are the most abundant polyphenols in our diets and occur in many plants in the form of glycosides, which are water-soluble and chemically stable (*3*). The linked sugar is often glucose or rhamnose but can also be galactose, arabinose, xylose, or other sugars (*4*).

The first stage in metabolism is likely to be the deglycosylation of flavonoid glucosides by β-glucosidases in the cells of the gastrointestinal mucous by lactase phlorizin hydrolase, a β-glucosidase on the outside of the brush-border membrane, before absorption (*5*). After passing across the small intestine brush border, falvonoids undergo methylation, sulfation, glucuronidation, or a combination in the small intestine or liver (*6*). Conjugated flavonoids pass into the bile through enterohepatic circulation (*7*) and therefore may reach the colon as glucuronides or other metabolites via this route. Hollman and co-workers (*7*) showed that flavonol glycosides from onions, such as rutin, have a delayed absorption (t_{max} 9.3 h) compared with that of flavonol glucosides (t_{max} 0.7 h), suggesting hydrolysis of rutin by colon microflora before absorption.

McDonald and co-workers (*8*) showed that rutin and quercitrin were hydrolyzed by bacteria in the human gut to quercetin. A fecal suspension was capable of cleaving the C-ring of rutin to yield 3,4-dihydroxyphenylacetic acid with the transient appearance of quercetin (*9*). Isolated *Eubacterium ramulus* cultures were capable of cleaving the C-ring of quercetin-3-glucoside to phloroglucinol and 3,4-dihydroxyphenylacetic acid (*10*). Oral administration of rutin to humans led to the detection of 3-hydroxyphenylacetic acid, 3,4-dihyroxyphenylacetic acid, and 3-methoxy-4-hydroxyphenylacetic acid in the plasma after 4 h, showing a maximal detection between 8 and 12 h and a reduction to baseline at 20−35 h (*11*). Hollman and Katan (*12*) proposed a scheme for ring cleavage of flavonols in body tissues and colon (**Figure 1**). However, direct experimental proof of the relative proportions of the ring fission metabolites of flavonols by human colon flora alone within a physiological time frame remains to be elucidated.

In vitro fermentation methods for dietary fiber and proteins (*13*, *14*) cannot be readily adopted for studies of flavonoids. Because fecal matter is derived from the components of the diet, the fecal inoculum contains a background level of phenolic

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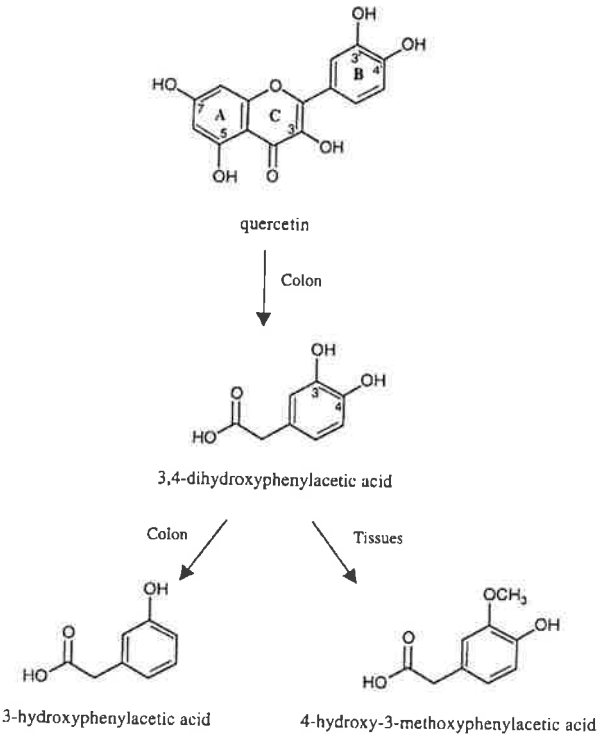

Figure 1. Proposed scheme of quercetin metabolism to hydroxyphenylacetic acid derivatives (modified from ref *12*).

compounds. The detection limits of the substrates and metabolites from this background in the analysis determines the substrate levels applied to the in vitro colon fermentation. On the other hand, the chosen concentration of the substrate should be as near as possible to the dietary level, because the inoculum might be affected by the antimicrobial properties of phenolic compounds (*15*), the substrate, or its metabolites.

The aim of this study was to use in vitro fecal fermentation to verify the role of colon flora in the metabolism of rutin, to compare the rates of deconjugation of rutin, isoquercitrin, and a mixture of quercetin glucuronides, and to measure activities of the relevant deconjugating enzymes in the fecal inoculum. Important parameters relating to the substrate-to-inoculum ratio and pH and incubation times in terms of deglycosylation and formation of ring fission products are discussed.

MATERIALS AND METHODS

Materials. Rutin (quercetin-3-*O*-rhamnoglucoside) and isoquercitrin (quercetin-3-*O*-glucoside), used as substrates in the fermentation experiments, and high-performance liquid chromatography (HPLC)-grade rutin trihydrate, a flavonoid standard, were purchased from Extrasynthese (Genay, France). Apigenin, used as an internal standard in the deconjugation study, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, phloroglucinol, 3,4-dihydroxybenzoic acid, and 4-hydroxybenzoic acid, used as standards of fermentation metabolites, were Sigma products. 3,4-Dihydroxyphenylpropionic acid, 3-(4-hydroxyphenyl)propionic acid, and 3-hydroxybenzoic acid, fermentation metabolite standards, were purchased from Aldrich. Resorcinol, an internal standard of fermentation metabolites, was a Merck (Germany) product, and a fermentation metabolite standard 3-(3-hydroxyphenyl)-propionic acid was purchased from Apin Chemicals (U.K.). A mixture of quercetin glucuronides was prepared as described previously (*16*). The composition of the quercetin glucuronide mixture was 85% quercetin-4′-*O*-glucuronide, 6.5% quercetin-3′-*O*-glucuronide, and 6.5% quercetin-3/7-*O*-glucuronide.

Preparation and Spiking of the Fecal Slurry. Fecal slurry (16.7%, w/v) was prepared under strictly anaerobic conditions with use of feces from four healthy donors, who usually ingested a normal diet, presented no digestive disease, and had not received antibiotics for at least 3 months. Freshly passed feces were immediately taken in an anaerobic chamber, pooled and homogenized with the same weight of carbonate-phosphate buffer according to Karppinen and co-workers (*13*), filtered through a 1-mm sieve, and diluted with the same buffer. Fecal slurry (5%, w/v) was prepared similarly, in the same buffer at pH 6.0 (A) or pH 6.9 (B). Fecal slurry (B; 5%, w/v) was spiked with 0.5, 1.0, 1.5, and 2.0 μmol of rutin in methanol.

Fermentation Conditions. In small-scale experiments, rutin or isoquercitrin (1 μmol) or a mixture of quercetin glucuronides (0.20 μmol) in methanol were added to 50-mL headspace bottles. The methanol evaporated spontaneously, after which the bottles were inoculated with the fecal slurry (10 mL), resulting in concentrations 100 μmol/L for rutin or isoquercitrin or 20 μmol/L for the mixture of quercetin glucuronides. The flasks were inoculated and closed tightly in the anaerobic chamber and incubation was performed at 37 °C with magnetic stirring (250 rpm) for 0−24 h.

The large-scale experiment was conducted with two identical 1-L laboratory fermenters. The substrate-to-inoculum ratio was as described above and the fecal inoculum in carbonate-phosphate buffer pH 6.9 (5%, w/v) was divided into two portions of 1000 mL, which were transferred under strictly anaerobic conditions to 1-L transfer bottles. One of the bottles contained 100 μmol/L rutin. The fermenters were sterilized in situ with water and cooled to 37 °C, after which the water was emptied out and the fermenters flushed with nitrogen through the sterile gas inlet line. The inocula were then transferred anaerobically to the fermenters.

After inoculation, the fermenters were again flushed with nitrogen to ensure strictly anaerobic conditions. Temperature was maintained at 37 °C and pH was controlled at >6.9 with 0.2 mol/L NaOH. To ensure even suspension of the slurry and good control of temperature, a gentle agitation of 100 rpm was used. The level of anaerobicity was monitored by continuous measurement of the redox potential of the cultures. Samples of the culture slurry were taken under strictly anaerobic conditions.

Assays of (α,L)-Rhamnosidase, (β,D)-Glucosidase, and (β,D)-Glucuronidase Enzyme Activities. Enzyme solution was prepared as follows. The cells of 1 mL of fecal inoculum (5%, w/v) were washed three times with a 0.1 mol/L phosphate buffer (pH 6.0), the cells were lysed with lysozyme (Sigma L-6876, 1 g/L at 37 °C for 1 h) and the reaction was stopped with sodium azide (2 mg/L). Finally, centrifuged (13 000g for 5 min) cell-free extract was used as a source of enzymes. The reaction mixture (total volume of 0.5 mL) contained 1 mmol/L of the substrate [*p*-nitrophenyl-(α,L)-rhamnopyranoside, -(β,D)-glucopyranoside, or -(β,D)-glucuronide], 0.1 mol/L phosphate buffer (pH 6.9), and the enzyme solution (0.1 mL). The reaction mixture was incubated for 10 min at 37 °C, stopped by addition of 0.01 mol/L sodium hydroxide (3 mL) and the absorbance at 405 nm was measured by U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) using *p*-nitrophenol as a standard. Protein concentration was measured according to the method of Bradford (*17*).

Treatment of the Fecal Samples before the Analysis. The contents of the bottles were either directly freeze-dried and weighed before the extraction and analysis, or in the deconjugation study, the fermentation was finished by addition of methanol up to a concentration of 70% (v/v). Methanol was evaporated under nitrogen before freeze-drying and weighing.

Extraction and Analysis of Rutin from the Directly Freeze-Dried Samples. The standard stock solution of rutin was prepared by dissolving the standard in methanol containing 0.2% (v/v) ortho-phosphoric acid to a concentration of 300 μmol/L. For the calibration curve, ranging from 0.3 to 30 μmol/L, the standard stock solution was diluted to final concentrations with 75% methanol in water containing 0.2% (v/v) ortho-phosphoric acid. Freeze-dried samples were extracted in 25 mL of 75% methanol in water containing ortho-phosphoric acid (0.2%, v/v), filtered (0.45 μm), and analyzed with a Waters HPLC system comprising a Millenium^{32} (version 3.05.01) chromatography manager, a Waters 712 WISP automatic sample injector, a Waters 2487

Dual Wavelength Absorbance Detector, and two Waters 6000A pumps. Reverse-phase separations were performed at room temperature with use of a 150 × 3.9 mm i.d., 5-μm C_{18} Symmetry column (Waters) fitted with a 20 × 3.9 mm i.d., 5-μm C_{18} Symmetry guard column (Waters). The mobile phase was a 25 min, 20−60% gradient of methanol in water with 300 μL/L trifluoroacetic acid, eluted at flow rate of 0.8 mL/min. After each analysis the column was washed with 100% methanol for 2 min, returned to 20% methanol in water, and re-equilibrated for 10 min before the next analysis. The eluted components were monitored at 280 and 340 nm.

Extraction and Analysis of Quercetin Derivatives in the Deconjugation Experiment. Methanol (15 mL) containing 1 mmol/L ascorbic acid and an internal standard (apigenin, 60 μmol/L) were added to the freeze-dried samples. The samples were placed on ice and disrupted by pulse sonication with a Status 70 homogenizer (Philip Hams Scientific) at 13 A/s. All sonications were performed for 15 min with the samples at <4 °C. Samples were centrifuged at 13500*g* for 10 min at 4 °C to precipitate the protein, concentrated to dryness by rotary evaporation, and resuspended in 1 mL of methanol/water (80:20, v:v). Samples were filtered through 0.22-μm filter units before analysis by HPLC.

A modified version of the previously published analytical HPLC method was used (*18*). Solvents A (water/tetrahydrofuran/trifluoroacetic acid, 98:2:0.1, v/v/v) and B (acetonitrile) were run at a rate of 1 mL/min, with a gradient of 17% B (2 min), increasing to 25% B (5 min), 35% B (8 min), 50% B (5 min), and then to 100% B (5 min). A column-cleanup stage was performed at 100% B (5 min) followed by a re-equilibration at 17% B (15 min). The column was packed with Prodigy 5 μmol/L ODS3 reverse-phase silica, 250 × 4.6 mm i.d. (Phenomenex, Macclesfield, U.K.). Diode array detection monitored the eluent at 270 and 370 nm. An external standard of rutin was run approximately every six runs. Comparisons of flavonol standards with formed metabolites in the fecal slurries were based on retention time and UV spectra.

Extraction and Analysis of the Fecal Metabolites. Hydroxyphenylacetic acid metabolites were extracted with resorcinol as an internal standard in methanol/water (90:10, v/v) with 0.4% (v/v) orthophosphoric acid, filtered (Macherey-Nagel 615 1/4), concentrated with a rotary evaporator (30 °C) to about 5 mL, and refiltered [poly(vinylidene difluoride), 0.45 μm]. Liquid chromatographic experiments were conducted by using a high-performance liquid chromatograph (HP 1090, Hewlett-Packard Co., Palo Alto, CA) in combination with an autoinjector, an autosampler, a diode array detector (205 and 220 nm), and a column (ODS Hypersil, 5 μm, 100 × 2.1 mm, Hewlett-Packard Co., Palo Alto, CA). The separation of hydroxyphenylacetic acids, hydroxyphenylpropionic acids, and hydroxybenzoic acids was performed by using a water/methanol gradient, 20−30% (6 min) and 30−100% (9 min) eluted at a flow rate of 0.4 mL/min. The column was washed with 100% methanol for 4 min and equilibrated for 3 min before the next analysis. Separations of hydroxyphenylacetic acid derivatives (experiment using inoculum B) were performed by using a water/methanol gradient, 3−20% (1.7 min) and 20−50% (6 min) eluted at a flow rate of 0.4 mL/min. After each analysis the column was washed with 50% methanol in water for 4 min, equilibrated for 3 min before the next analysis. Detection limit for 3-hydroxybenzoic acid was 30 μg/g fecal d.w. and for all the other fermentation metabolites, 10 μg/g fecal d.w.

HPLC-MS. An HP Series 1100 with Spheri-5 RP-18 100 × 1 mm column (Applied Biosystems) was used. A gradient elution (mobile phases: A, 10 mmol/L ammonium acetate in water with 0.2% (v/v) acetic acid, pH 3.9; B, 10 mmol/L ammonium acetate in methanol with 0.2% (v/v) acetic acid. Gradient: 0 min, 5% B; 2 min, 20% B; 8 min, 50% B; 8.1 min, 5% B; 30 min, 5% B. Flow rate: 40 μL/min) was used for the separation of the metabolites. Methanol with 0.1% (v/v) ammonium hydroxide was added postcolumn (30 μL/min) by an HPLC pump (HP1090) to enhance the ionization process in the mass spectrometer (Micromass Quattro II triple quadruple, Manchester, U.K., with electrospray interface-MS). The measurements were performed in negative-ionization mode. The capillary voltage was −3 kV, cone voltage 18 V, and the source temperature, 120 °C. Nitrogen was used as drying and nebulizer gas. In the product ion spectra measurements (MS-MS) argon was used as collision gas. Collision energy used was 17 V. Analyses were conducted in multiple reaction mode, where, on the basis of the product ion spectra of the metabolites, one or two diagnostic fragment ions were used for identification of the metabolites.

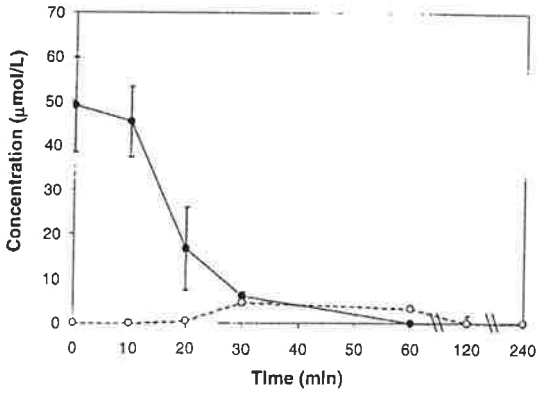

Figure 2. Formation of quercetin (○) from rutin (●) (100 μmol/L) in vitro with 5% (w/v) fresh human fecal slurry in carbonate-phosphate buffer with micronutrients, pH 6.0, at 37 °C. Error bars represent the standard error of the mean of three experiments.

RESULTS

Preliminary experiments were performed to determine the optimum concentration of fecal slurry used as an inoculum of colon bacteria and the substrate-to-inoculum ratio. Rutin concentrations of 0.03, 0.30, or 3.0 μmol were incubated with 10 mL of a 16.7% (w/v) fecal slurry and only the highest concentration was clearly detectable in analysis above the background level of the inoculum (results not shown). However, this concentration (300 μmol/L of rutin) was considered too high to be given as a dietary level and it may also have affected the bacterial population of the slurry. When the inoculum concentration was decreased to 5% (w/v) and spiked with 0.5−2.0 μmol of rutin per 10 mL of fecal slurry (50−200 μmol/L), a concentration of 50 μmol/L could only just be detected above the background. Thus, a rutin concentration of 100 μmol/L in 5% fecal slurry was chosen for the detection of metabolites from fecal samples.

With use of the batch in vitro anaerobic fecal fermentation model, we investigated the action of bacterial β-glucosidases, β-glucuronidases, and α-rhamnosidases in the turnover of flavonoid conjugates. At 0 h, specific enzyme activities of β-glucosidase, β-glucuronidase, and α-rhamnosidase in a 5% fecal slurry were 6.2 ± 0.1, 2.0 ± 0.0, and 1.2 ± 0.4 nmol·min^{-1}·mg^{-1} protein, respectively. The low α-rhamnosidase activity suggests slower deconjugation of rutin than the deconjugation of the mixture of quercetin glucuronides or isoquercitrin. Complete degradation of isoquercitrin by bacterial enzymes occurred after 20 min of incubation. Similarly, the mixture of quercetin glucuronides was completely hydrolyzed by the colonic bacteria after a 10-min incubation period. Rutin was metabolized more slowly than the other two substrates but was completely hydrolyzed after a 60-min incubation period (**Figure 2**).

To determine the metabolites of rutin in the colon, the experiment above was repeated, but time points were taken over a longer time (**Figure 3a**). The primary metabolite, 3,4-dihydroxyphenylacetic acid, was formed during the first 2 h and a lag-phase was observed in the formation of the dehydroxylation product 3-hydroxyphenylacetic acid, which appeared concomitantly with a decrease in the primary metabolite. Both

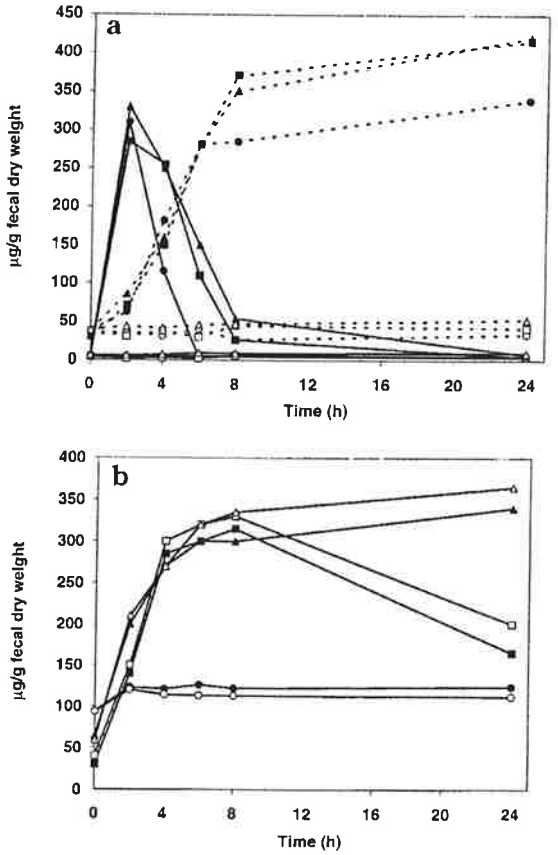

Figure 3. (a) Formation of the hydroxyphenylacetic acid metabolites in vitro in the presence (closed symbols) and absence (open symbols) of rutin (100 μmol/L) by using fecal slurry (5%; w/v) in a carbonate-phosphate buffer. 3,4-Dihydroxyphenylacetic acid (—); 3-hydroxyphenylacetic acid (- - -); ●, ○, 10-mL scale, pH 6.0, inoculum A; ■, □, 10-mL scale, pH 6.9, inoculum B; ▲, △, 1000-mL scale, pH 6.9, inoculum B. (b) Formation of the background metabolite 3,4-dihydroxyphenylpropionic acid (—) in vitro in the presence (closed symbols) and absence (open synbols) of rutin (100 μmol/L) by using fecal slurry (5%; w/v) in a carbonate-phosphate buffer. ●, ○, 10-mL scale, pH 6.0, inoculum A; ■, □, 10-mL scale, pH 6.9, inoculum B; ▲, △, 1000-mL scale, pH 6.9, inoculum B.

these metabolites were detected in the fecal background incubated without rutin, but their level was just above the detection limits for the metabolites (10 and 30 μg/g fecal d.w. for 3,4-dihydroxypenylacetic acid and 3-hydroxyphenylacetic acid, respectively). Of the original rutin (100 μmol/L), 61% and 60% was converted to phenylacetic acid metabolites at 2 and 24 h, respectively, calculated by using the average dry weight of the samples (0.268 ± 0.005 g). Low levels of 3-methoxy-4-hydroxyphenylacetic acid (7.6−12.9 μg/g fecal d.w.) were found both in the fecal matter in the presence and absence of rutin. Presence of 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid was confirmed by HPLC-MS.

A similar pattern was observed for the metabolite formation regardless of the scale (10 or 1000 mL) or different inocula (A in buffer, pH 6.0, and B in buffer, pH 6.9) obtained from different donors (n = 4) (**Figure 3a**). Because of the 20-min stabilization period of the large-scale equipment, deconjugation of rutin had already occurred before the first sampling. Therefore, the fermenter was not a suitable vessel for investigating the initial deglycosylation of the flavonoids by the bacterial enzymes, but it is suitable for studies on the ring fission of flavonoids. Anaerobic conditions were also maintained throughout all experiments, and monitoring redox potential and pH gave additional information on fermentation conditions, which could not have been obtained on a smaller scale. The redox potential showed slight decrease to −222 mV at 3 h compared with 202 ± 1 mV at the start and after 10−24 h of large-scale fermentation in vitro. The pH (7.6) of the culture (B) in the fermenter did not vary during the time course of the experiment. Rapid conversions could be more accurately detected by using rapid freezing with liquid nitrogen, by which the levels of metabolites in the 0-h samples were below the detection limits (**Figure 3a**).

A third metabolite was detected as 3,4-dihydroxyphenylpropionic acid according to retention time and UV spectrum in our HPLC analysis. Different origins of the fecal inoculum gave rise to different levels of background metabolites: maximum levels of 3,4-dihydroxyphenylpropionic acid formation were 120 and 330 μg/g fecal d.w. in experiments with inocula A and B, respectively. Similar metabolite formation occurred, however, both in large and small scale when the same inoculum (B) was used (**Figure 3b**). According to HPLC-MS data, the background metabolite was 3-hydroxyphenylpropionic acid, although some dehydroxylation of dihydroxyphenyl metabolites may have occurred during sample storage (2 months, −20 °C) between HPLC analysis and HPLC-MS detection. HPLC-MS analysis also showed different ratios for 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid at the 2-h time point compared with the ratios obtained by HPLC analysis from the same sample.

DISCUSSION

In the human colon, the presence of bacterial enzymes such as β-glucosidases, β-glucuronidases, and α-rhamnosidases enables hydrolysis of rutin, isoquercitrin, and the mixture of quercetin glucuronides, to release the quercetin aglycone. Deconjugation of isoquercitrin and the mixture of quercetin glucuronides occurred very rapidly in the in vitro colonic fermentation, whereas rutin was deglycosylated at a slower rate. Similarly, the amount of α-rhamnosidase activity detected in the fecal material was also lower than that of β-glucosidase and β-glucuronidase, suggesting that the hydrolysis of rutin would occur at a slower rate than hydrolysis of the other substrates. The resulting quercetin aglycone appeared only transiently, before further metabolism.

Intracellular α-rhamnosidase and β-glucosidase activities have been reported to be 0.061 and 0.083 μmol·min^{-1}·mg^{-1} protein, respectively, from *Bacteroides* JY-6 human intestinal bacteria (*19, 20*). In a study performed by Gråsten and co-workers (*21*), sonicated and centrifuged fecal slurry (10%, w/w) contained 3.4 ± 2.4 and 2.3 ± 1.0 nmol·min^{-1}·mg^{-1} protein β-glucuronidase and 7.1 ± 2.1 and 4.9 ± 0.9 nmol·min^{-1}·mg^{-1} protein β-glucosidase activities for women and men, respectively, consuming rye bread diets (*21*). In our study, despite lower fecal slurry concentration (5%, w/v), removal of background protein, color, and extracellular enzymes, specific activities of β-glucuronidase and β-glucosidase (2.0 and 6.2 nmol·min^{-1}·mg^{-1} protein, respectively) agree with those shown by Gråsten and co-workers (*21*). Our results also represent the enzyme activities of a mixed bacterial population as found in vivo and not just from one species.

Primary and secondary metabolites were formed in stoichiometric amounts during the course of the experiment. Most of the rutin (60%) was converted to hydroxyphenylacetic acids, 3,4-dihydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid. With a mixture of human intestinal bacteria, Winter and

co-workers (*9*) also demonstrated rapid cleavage of rutin, resulting in the formation of 3,4-dihydroxyphenylacetic acid. Their results indicated that the intestinal half-life of the biologically active aglycone was short (*9*). Ring fission and dehydroxylation of 3,4-dihydroxyphenylacetic acid in vivo has been demonstrated qualitatively using paper chromatography from urine of rats, rabbits, guinea pigs, and humans after quercetin administration (*22*), but the dynamics and stoichiometry of metabolite formation by human fecal flora in vitro has not been shown previously in a physiological time frame (24 h), based on in vivo results (*11*).

The scheme proposed by Hollman and Katan (*12*) in **Figure 1** is based on in vivo data and represents the overall metabolism of rutin in both the colon and the tissues. 3-Hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, and 3-methoxy-4-hydroxyphenylacetic acid were also detected in blood after rutin administration or in urine after quercetin administration in humans, respectively (*11*, *23*). 3-Methoxy-4-hydroxyphenylacetic acid was detected both in the presence and absence of rutin in our study, indicating fecal excretion of this tissue metabolite in vivo, which does not undergo rutin metabolism by fecal bacteria.

The remainder of the rutin substrate (40%) was either converted to other metabolites, which were below the limits of detection or were bound to the fecal material and were thus unextractable by solvents or converted to gaseous fermentation products. Walle and co-workers (*24*) found 52% of oral dose of ^{14}C-quercetin as ^{14}C-carbon dioxide in exhalation gases. They suggested that it was a major metabolite and was from the intestine because of its late appearance after dose (*24*). We investigated the presence of other expected metabolites [4-hydroxyphenylacetic acid, phloroglucinol, 3-(4-hydroxyphenyl)-propionic acid, 3,4-dihydroxybenzoic acid, 3-hydroxybenzoic acid, and 4-hydroxybenzoic acid] but these also were below our limits of detection (10 μg/g fecal d.w.). Phloroglucinol, an expected metabolite from cleavage of the A ring of quercetin, could not be quantitatively extracted from our freeze-dried fecal samples (results not shown). Similarly, Griffiths and Smith (*25*) could not detect the presence of this metabolite in fecal samples and proposed that it be rapidly metabolized to acetate and butyrate. However, phloroglucinol and 3,4-dihydroxybenzaldehyde could be detected from *Enterococcus* (fecal bacterium) culture by a different method of analysis (*10*).

The hydroxyphenylpropionic acids appeared both in the absence and presence of rutin, and the level of concentration was dependent on the inoculum. The inoculum represents an average flora because it is prepared by pooling four samples from different donors. The metabolites were either derived from the dietary background in the fecal inoculum or the were endogenous from the donors. Also, dehydroxylation rates can vary from one inoculum to another because of varying dehydroxylating enzyme activities present in the slurries. Hollman and Katan (*12*) proposed that 3,4-dihydroxyphenylpropionic acid could be formed in the colon and dehydroxylated to 3-hydroxyphenylpropionic acid as metabolites of flavanones, of which, for example, hesperidin is present in significant quantities in orange juices, which are commonly consumed (*12*, *26*). Formation of 3,4-dihydroxyphenylpropionic acid was slightly inhibited in inoculum B by rutin, suggesting possible antibacterial activity of its metabolites.

Substrate concentrations 1 and 5 mmol/L have been used in fecal fermentation studies in vitro for proanthocyanidins and catechins, respectively (*27*, *28*). The substrate concentration used in this study (100 μmol/L) was the lowest reported and represents a concentration closer to the dietary intake level of flavonols [6−64 mg/day (*29*)]. This substrate concentration was suitable for both after the disappearance of substrate and detection of metabolites from the fecal background, because it was below the saturation point of rutin [205 μmol/L for anhydrous rutin in water (*30*)].

According to Guyton and Hall (*31*) the volume of chyme entering the *cecum* through the ileocecal valve is 1500 mL, and between 80 and 200 mL of the daily load of chyme is lost in the feces. In the in vitro study, fecal matter was diluted to cecal concentration [5−13% (v/v); calculated from volumes of feces and cecal chyme]. Several groups have reported the usage of 5% (w/v) fecal slurry for degradation and biotransformation of flavonoids (*28*, *32*), and also 1% (w/v) fecal slurry has been applied in the catabolism of proanthocyanidins (*27*). The slurry concentration used in this study (5%, w/v) is within the limits of human physiology.

We could observe little change in the pH throughout the large-scale fermentation in vitro. According to radiotelemetric recordings of physiological pH from the mouth to the large bowel, a pH value of 5.43 was reported in the transverse colon (*33*). The initial pH of the medium could therefore be adjusted to as low as 5.5 without losing the physiological conditions. Variation in pH of the buffer (6.0 or 6.9) did not affect the pattern of metabolite formation in our study.

The formation of metabolites of rutin only slightly affected the redox potential measured during the fermenter experiment. A significant amount of cysteine hydrochloride (0.5 g/L in the medium) and strictly anaerobic conditions resulted in a low redox potential in the fermenter.

In conclusion, quercetin derivatives were first deconjugated by fecal enzymes and then metabolized by C-ring cleavage and further dehydroxylated, but not methylated by colon flora in vitro. The pattern of metabolism was not changed by altering the initial pH of the medium (6.0 or 6.9) or the fermentation scale (10 or 1000 mL), or by using different inocula. The results were shown in reference to the background metabolites, hydroxyphenylpropionic acids. The levels of background metabolites varied because of different inocula, but the polyphenol metabolism was reproducible, consistent throughout the study and in agreement with the in vivo studies.

SAFETY

Only healthy volunteers were qualified to be donors of the fecal samples. Disposable plasticware was used whenever possible, and all the glassware, equipment, and tables were disinfected by using VIRKON (Antec Int. Ltd., Suffolk, U.K.) before standard cleaning procedures.

ABBREVIATIONS USED

Rutin, quercetin-3-*O*-rhamnoglucoside; isoquercitrin, quercetin-3-*O*-glucoside; t_{max}, timepoint of maximal detected absorption; quercitrin, quercetin-3-*O*-rhamnoside; HPLC, high-performance liquid chromatography; d.w., dry weight; MS, mass spectrometry.

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