Degradation of Quercetin and Luteolin by *Eubacterium ramulus*

ANNETT BRAUNE,1,2∗ MICHAEL GÜTSCHOW,3† WOLFRAM ENGST,2 AND MICHAEL BLAUT1,2

Abteilung Gastrointestinale Mikrobiologie,1 Deutsches Institut für Ernährungsforschung,2 D-14558 Bergholz-Rehbrücke, and Institut für Pharmazie, Universität Leipzig, D-04103 Leipzig.3 Germany

Received 15 June 2001/Accepted 27 September 2001

The degradation of the flavonol quercetin and the flavone luteolin by *Eubacterium ramulus*, a strict anaerobe of the human intestinal tract, was studied. Resting cells converted these flavonoids to 3,4-dihydroxyphenylacetic acid and 3-(3,4-dihydroxyphenyl)propionic acid, respectively. The conversion of quercetin was accompanied by the transient formation of two intermediates, one of which was identified as taxifolin based on its specific retention time and UV and mass spectra. The structure of the second intermediate, alphitonin, was additionally elucidated by 1H and 13C nuclear magnetic resonance analysis. In resting-cell experiments, taxifolin in turn was converted via alphitonin to 3,4-dihydroxyphenylacetic acid. Alphitonin, which was prepared by enzymatic conversion of taxifolin and subsequent purification, was also transformed to 3,4-dihydroxyphenylacetic acid. The coenzyme-independent isomerization of taxifolin to alphitonin was catalyzed by cell extract or a partially purified enzyme preparation of *E. ramulus*. The degradation of luteolin by resting cells of *E. ramulus* resulted in the formation of the intermediate eriodictyol, which was identified by high-performance liquid chromatography and mass spectrometry analysis. The observed intermediates of quercetin and luteolin conversion suggest that the degradation pathways in *E. ramulus* start with an analogous reduction step followed by different enzymatic reactions depending on the additional 3-hydroxyl group present in the flavonol structure.

Flavonoids are polyphenolic compounds which are present in foods and beverages of plant origin. The daily intake of flavonoids calculated on the basis of the aglycones was estimated to range from approximately 3 to 70 mg in different countries, and it may well exceed these values in regions with a very high intake of tea and vegetables (5, 10, 13). In vivo data on absorption and metabolism after oral intake are contradictory. However, a major part of ingested flavonoids are not absorbed and are largely degraded by the intestinal microflora.

It was shown in vitro that flavonoids are potent antioxidants and inhibitors of ubiquitous enzymes, and their anticarcinogenic properties were demonstrated with different cell lines (for a review, see reference 8). Due to these properties, flavonoids are reported to protect against cancer, coronary heart disease, and stroke. In order to judge the potential beneficial health effects of flavonoids in humans, studies on their fate in the gastrointestinal tract, including transformation by bacteria, are necessary. Intestinal bacteria play important roles not only in deconjugation of flavonoids but also in their further degradation. The bacterial metabolites, which possibly exert biological activities different from those of the original flavonoids, may be absorbed and further metabolized in the human body. Therefore, it is essential to study their conversion by intestinal bacteria and to identify and characterize the fermentation products formed. Although some flavonoid-degrading species, their substrates, and some of the final products are known (2, 15, 18, 22), information on the anaerobic degradation pathways, intermediates, and the enzymes involved is lacking.

*Eubacterium ramulus*, a strict anaerobe resident in the human intestinal tract, grows with quercetin-3-glucoside (isoquerectin) as the sole carbon and energy source. The only intermediates detected in this degradation were quercetin and phloroglucinol, the fermentation products being 3,4-dihydroxyphenylacetic acid, butyrate, and acetate (20). Furthermore, *E. ramulus* was found to be able to split the ring system of several other flavonols and flavones, forming the corresponding hydroxyphenylacetic and hydroxyphenylpropionic acids, respectively. Degradation pathways of flavonols and flavones were proposed, which include reduction of the heterocyclic C-ring of the aglycon, yielding dihydroflavonols and flavanones, respectively, followed by ring fission. Cleavage of the resulting chalcones might subsequently give rise to the respective phenolic acids (19). *E. ramulus* was detected in fecal samples from each of 20 persons tested at cell numbers which average 0.16% of the total flora (21). Therefore, *E. ramulus* may be considered a common inhabitant of the human intestine and a key organism for flavonoid degradation in this habitat.

In order to test the proposed flavonoid degradation pathways, the fermentation of the flavonol quercetin and the flavone luteolin by resting cells of *E. ramulus* was studied. In this report we describe the detection and identification of intermediates of quercetin and luteolin degradation, respectively.

MATERIALS AND METHODS

**Organism.** *E. ramulus* strain wK1, previously isolated from a human fecal sample (20), was used throughout the study. The organism will be made available upon request.

**Chemicals.** Quercetin, luteolin, and eriodictyol were purchased from Roth (Karlsruhe, Germany), taxifolin was purchased from Sigma (Deisenhofen, Germany), and 3,4-dihydroxyphenylacetic acid and 3-(3,4-dihydroxyphenyl)propionic acid were purchased from Fluka (Deisenhofen, Germany). High-pressure
liquid chromatographic (HPLC)-grade methanol (Fuka) was used throughout the experiments.

Growth media and anoxic techniques. The anoxic techniques were essentially those of Hnagte (11) and Bryant (3). A gas phase of N\textsubscript{2}CO\textsubscript{2} (80:20, vol/vol) was used. The anoxic workststak (MK 3; DW Scientific, Shipley, Great Britain) had a gas phase of N\textsubscript{2}CO\textsubscript{2} (80:20, vol/vol). E. ramulus strain W21 (20) was grown under strictly anoxic conditions in tubes fitted with butyl-rubber stoppers and screw caps. The medium (ST medium) contained the following compounds per liter: 9 g of tryptically digested meat peptone, 1 g of proteose peptone, 3 g of meat extract, 4 g of yeast extract, 6 g of glucose, 3 g of NaCl, 2 g of Na\textsubscript{2}HPO\textsubscript{4}, 0.5 ml of Tween 80, 0.25 g of cysteine, 0.25 g of L-cysteine-HCl, 0.1 g of MgSO\textsubscript{4} \cdot 7 H\textsubscript{2}O, 5 mg of FeSO\textsubscript{4} \cdot 7 H\textsubscript{2}O, and 3.4 mg of MnSO\textsubscript{4} \cdot 2 H\textsubscript{2}O. The pH after autoclaving at 121°C for 20 min was between 6.8 and 7.1.

Preparation of resting cell suspensions and degradation experiments. The E. ramulus cultures grown overnight in ST medium were transferred into the anoxic workststak and were prepared for centrifugation (10,000 × g, 15 min). After centrifugation, the cells were washed once with 50 mM potassium phosphate buffer (pH 6.9) containing and the preparation of alphitonin.

Transformation of the results. To elucidate the pathways of flavonol and flavone transformation by E. ramulus, the fermentation of quercetin and luteolin by resting cells was investigated. Flavonoid conversion was followed by analysis of samples by HPLC. The individual samples were centrifuged, and the pellets were lyophilized and dissolved in methanol. Dimethylformamide was less suitable as the solvent. Both the resulting solution and the lyophilized and dissolved in the same solvents for further analysis by HPLC.

Preparation of cell extracts and partially purified enzyme preparations. The cell extracts were prepared in the presence or absence of oxygen at 4°C from E. ramulus cultures grown overnight in ST medium supplemented with 0.1 mM quercetin. The cells were centrifuged (10,000 × g, 15 min), washed once with 50 mM potassium phosphate buffer (pH 6.9), and resuspended in the same buffer supplement with DNase, and ruptured by twofold passage through a French pressure cell at 130 MPa (SLM Instruments, Rochester, N.Y.). Cell extracts (average, 15 mg of protein/ml) were obtained by centrifugation at 18,000 × g for 20 min. The cytoplasmic fraction was prepared by centrifugation at 100,000 × g for 45 min.

The enzyme enrichment was performed at 4°C under aerobic conditions using a fast-performance liquid chromatography system (Amersham Pharmacia Bio tech, Freiburg, Germany). The cytoplasmic fraction (average, 13 mg of protein/ml) was loaded onto a DEAE-Sepharose (Amersham Pharmacia Biotech) column (6 by 2.5 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.2). Elution was done with a gradient of KCl (0 to 1 mM) in 50 mM potassium phosphate buffer (pH 7.2) at a flow rate of 2 ml/min. Fractions with taxifolin-transforming activity were used for the characterization of the taxifolin transformate and the preparation of alphitonin.

Determination of the taxifolin-transforming activity. Taxifolin transformation was detected by HPLC analysis. The assay contained 60 μM taxifolin (added from a 1.2 mM stock solution in DMSO) in 50 mM potassium phosphate buffer (pH 6.9). The final DMSO concentration was 5%. The reaction was started by the addition of cell extract (average, 150 μg of protein), soluble enzyme fraction (average, 130 μg of protein), or partially purified enzyme preparation (average, 42 μg of protein), respectively. The assay was performed in the presence or absence of oxygen at room temperature. For HPLC analysis, samples were taken at different times and mixed with one volume of methanol-H\textsubscript{2}O-acetic acid (50:45.5, vol/vol/vol) to stop the reaction. Control reactions were devoid of enzyme or contained enzyme preparations inactivated by incubation for 1 h at 50°C.

HPLC. Flavonoids and aromatic metabolites were measured using an HPLC system with a diode array detector (Gynkotek, Munich, Germany). The HPLC system was equipped with a pump Model 480, degasser ERC-5535, autosampler GINA 160, a column oven, a diode array detector UVD-320, and a reversed-phase C\textsubscript{18} column (LiChroCART 250-4 LiChropher 100 RP-18, 5 μm; 250 by 4 mm; Merck) and a 996 PDA detector. The mobile phase was a gradient of aqueous formic acid and methanol similar to those used for isolation of the metabolites (described above) with a flow rate of 0.5 ml/min and was split 6.1 prior to introduction into the mass spectrometer. MS analyses were carried out in either positive or negative ionization mode. The temperature of the ion source was maintained at 150°C. The desolvation temperature was 350°C, and the desolvation gas N\textsubscript{2} had a flow rate of 400 liters/min. The temperature and capillary voltage used for the analysis of quercetin and luteolin were 50 V and 3.7 kV, respectively, and 20 V and 3.0 kV for the analysis of the metabolites. Product ion scans of [M + H\textsuperscript{+}]\textsuperscript{+} were performed at low-energy collisions (15 to 30 eV) using argon as the collision gas (1.5 × 10\textsuperscript{4} to 2.8 × 10\textsuperscript{5} m\textsuperscript{2} Pa) for the PHR-ESI-MS and mass spectra were compared to those of reference substances.

Samples from a liquid chromatographic (HPLC) grade methanol and taxifolin preparations were subjected to MS with electron impact ionization (EI-MS, 70 eV) using a Varian MAT CH\textsuperscript{6} spectroometer (Varian, Palo Alto, Calif.). EI-MS of taxifolin (m/z, percent): 304 (M\textsuperscript{+}, 28), 275 (32), 153 (92), 123 (68), EI-MS of alphitonin (m/z, percent): 304 (M\textsuperscript{+}, 12), 126 (100), 123 (75). Preparation of alphitonin for NMR analysis. Taxifolin (8.8 mg) was incubated with 4 ml of the partially purified taxifolin-transforming preparation until no further alphitonin formation was observed (210 min). Samples of maximally 250 μl each were injected onto the HPLC column. Alphitonin and the nontransformed taxifolin were separated using the TFA-methanol gradient. The fractions of both substances were manually collected, pooled, and dried by vacuum centrifugation (RC 10.22; Jouan, Saint-Nazaire, France).

NMR analysis. 1H NMR spectra (300 MHz) and 13C NMR spectra (75 MHz) were recorded on a Varian Gemini 300 in DMSO-\textit{d}_6. 13C NMR signals were assigned on the basis of attached proton test (APT). Nontransformed taxifolin, which was obtained from the alphitonin preparation as described above, and commercially available taxifolin gave identical 1H NMR spectra. 1H NMR of taxifolin: δ 4.51 (dd, J = 11.2, 6.1 Hz, 1H, 3-H), 5.00 (d, J = 11.2 Hz, 1H, 2-H), 5.76 (d, J = 6.1 Hz, 1H, 3-OH), 5.88, 5.93 (each d, J = 2.0 Hz, 6-H, 8-H), 6.76 (s, br, 2H, 5'-H, 6'-H), 6.89 (s, br, 1H, 2'-H), 8.97, 9.03, 10.12, 9.91 (each 4, s, 4H, OH), 11.26, 11.28 (each 4, m, 2H, 6-H, 8-H). 13C NMR of alphitonin: δ 28.85 (each 2, d, J = 13.9 Hz, 2H, CH\textsubscript{2}), 5.73, 5.79 (each d, J = 1.8 Hz, 2H, 5'-H, 7'-H), 6.39 (d, J = 8.2 Hz, 1H, 6'-H) 6.51 (d, J = 9.1 Hz, 1H, 6'-H). 13C NMR of alphitonin: δ 61.55 (CH\textsubscript{3}), 90.39, 96.39 (C-5, C-7), 101.98 (C-2), 106.19 (C-3a), 115.65, 118.59 (C-2', C-5'), 121.93 (C-6'), 125.66 (C-1'), 144.45, 154.08 (C-3', C-4'), 158.64 (C-7a), 168.69, 172.56 (C-4', C-6'), 193.60 (C-3').

RESULTS

In order to elucidate the pathways of flavonol and flavone transformation by E. ramulus, the fermentation of quercetin and luteolin by resting cells was investigated. Flavonoid conversion was followed by analysis of samples by HPLC. The individual samples were centrifuged, and the pellets were lyophilized and dissolved in methanol. Dimethylformamide was less suitable as the solvent. Both the resulting solution and the supernatant were manually collected and examined. This method was found to be advantageous compared to the lyophilization of the whole sample because of a better recovery. The detected metabolites were identified by their specific retention times using two different HPLC gradients, and their UV spectra were recorded in the TFA-methanol system. In addition, mass spectrometry was applied for confirmation of the results.
In the first fermentation experiments using resting cells, only the final products of flavonoid degradation were observed (data not shown). However, intermediates could be observed by taking the samples immediately after adding the substrates and decreasing the cell density and the incubation temperature.

**Quercetin degradation by resting cells of E. ramulus.** Resting-cell suspensions of *E. ramulus* degraded 1 mM quercetin to produce 0.8 mM 3,4-dihydroxyphenylacetic acid within 4.8 h (Fig. 1A). Retention times of quercetin were 31.5 min with the TFA-methanol gradient (UV spectrum, $\lambda_{max} = 260$ nm) and 31.1 min with the FA-methanol gradient. Because of its low
solubility in buffer, only small amounts of quercetin were recovered from the supernatant of the cell suspension. In contrast, 3,4-dihydroxyphenylacetic acid appeared exclusively in the supernatant. The identity of the latter compound was confirmed on the basis of its retention times (11.6 min, TFA-methanol gradient; 10.5 min, FA-methanol gradient) and UV spectrum ($\lambda_{\text{max}} = 286$ nm) compared to the reference substance. MS analysis (flow injection) of the product gave the expected [M+H]$^+$ of m/z 169 in the positive mode and [M-H]$^-$ of m/z 167 in the negative mode. The loss of (CO$_2$) resulting in the in-source fragment ion m/z 123 was observed in the negative mode and represents a typical fragmentation of phenolic acids (12). The degradation of quercetin was accompanied by the formation of two transient soluble intermediates (Fig. 1B): one of these was identified as taxifolin based on its retention times (25.4 min, TFA-methanol gradient; 23.9 min, FA-methanol gradient) and UV spectrum ($\lambda_{\text{max}} = 294$ nm), which were identical to those of the reference substance. ESI-MS analysis showed the respective molecular ion peak of m/z 305 [M+H]$^+$ of this second intermediate with relatively different retention times of 17.8 min (TFA-methanol gradient) and 16.3 min (FA-methanol gradient) exhibited a UV spectrum ($\lambda_{\text{max}} = 295$ nm) similar to that of taxifolin. However, in comparison to taxifolin a high rate of in-source fragmentation occurred, characterized by relatively high intensities of m/z 287 and m/z 259, indicating the loss of water and CO, respectively (data not shown). For further characterization, the compound was purified and the structure was elucidated by $^1$H and $^{13}$C NMR analysis (see below). It was unambiguously identified as alphitonin [2-(3,4-dihydrobenzyl)-2,4,6-trihydroxybenzofuran-3-one], an isomeric form of taxifolin. The MS/MS spectrum of alphitonin is shown in comparison to that of taxifolin (Fig. 2). A reference substance was not available.

**Degradation of taxifolin and alphitonin by resting cells of E. ramulus.** Since taxifolin was identified as an intermediate in the quercetin degradation pathway, its transformation was studied with resting cells of E. ramulus (Fig. 3). The conversion of 1 mM taxifolin resulted in the formation of 3,4-dihydroxyphenylacetac acid. Small amounts of alphitonin were formed transiently. Besides the HPLC retention times and UV spectra, LC-MS measurements were utilized to prove the identity of both substances. Incubation of 0.26 mM alphitonin with resting cells of E. ramulus led to the formation of 3,4-dihydroxyphenylacetic acid (Fig. 4). The identity of this compound was confirmed by its retention times and UV and mass spectra. Using LC-MS/MS, the same pattern of daughter ions of m/z 169 [M+H]$^+$ (77, 123, 105) was observed for the product formed from both taxifolin and alphitonin. The same pattern was obtained for the reference substance of 3,4-dihydroxyphenylacetic acid.

**Transformation of taxifolin by cell extracts of E. ramulus.** The transformation of taxifolin (60 $\mu$M) was also catalyzed by cell extracts, the soluble enzyme fraction, and a partially purified enzyme prepared from E. ramulus extracts (Fig. 5), respectively. No transformation was observed without the enzyme preparation or with an inactivated enzyme preparation. The relatively small amount of taxifolin used in these experiments was completely transformed to alphitonin as the final product in the presence or absence of oxygen without the addition of any coenzyme. The enriched enzyme preparation was also tested for transformation of the dihydroflavone eriodictyol. However, neither with nor without oxygen was a conversion observed.

**Structural elucidation of alphitonin.** The alphitonin formed by taxifolin transformation and purified by HPLC was highly pure as judged by NMR analysis. Taxifolin was completely absent from this preparation. By using the APT technique, alphitonin could be distinguished from taxifolin due to the CH$_2$ signal in the $^{13}$C NMR spectrum of alphitonin (Fig. 6). The structure of alphitonin could be unequivocally deduced from the NMR data. In particular, $^{13}$C and $^1$H NMR signals indicated that the ring A was being nonsymmetrically substituted. Thus, a ring-open chalcone structure could be excluded. According to the structure of alphitonin, a signal for the benzylic carbon (41.55 ppm) was assigned; the diastereotopic hydrogens gave two doublets (2.82, 2.88 ppm; $J = 14.0$ Hz).

**Luteolin degradation by resting cells of E. ramulus.** Resting cells of E. ramulus were also used to investigate the degradation of the flavone luteolin, whose structure lacks the 3-hydroxy group present in quercetin. Luteolin (0.5 mM; TFA-methanol and FA-methanol gradients, 32.4 min, $\lambda_{\text{max}} = 348$ nm) was transformed via the intermediate eriodictyol to 0.4 mM 3-(3,4-dihydroxyphenyl)propionic acid within 120 min (Fig. 7). Luteolin was found exclusively in the pellet of the centrifuged samples. Eriodictyol was predominantly recovered from the supernatant, whereas the pellet contained approximately 10% of this compound. The final product, 3-(3,4-dihydroxyphenyl)propionic acid, was almost exclusively found in the supernatant. The identity of eriodictyol was confirmed by its retention times (29.8 min, TFA-methanol gradient; 29.2 min, FA-methanol gradient) and UV spectrum ($\lambda_{\text{max}} = 294$ nm). Similarly, the identity of 3-(3,4-dihydroxyphenyl)propionic acid as the product of the luteolin degradation was demonstrated on the basis of its retention times (16.3 min, TFA-methanol gradient; 14.5 min, FA-methanol gradient) and UV spectrum ($\lambda_{\text{max}} = 286$ nm). MS analysis (flow injection) gave the expected molecular ion peaks of eriodictyol (m/z 289 [M+H]$^+$) and 3-(3,4-dihydroxyphenyl)propionic acid (m/z 183 [M+H]$^+$, m/z 181 [M-H]$^-$).

**DISCUSSION**

These investigations were done in order to get insight into the pathway of flavonoid degradation by a relevant bacterial species of the human intestinal tract, E. ramulus. The flavonol quercetin was chosen because quercetin glycosides are highly abundant dietary flavonoids. The ability of E. ramulus to grow on quercetin-3-glucoside was previously shown. Quercetin and phlorogluconol were detected as intermediates in the transformation of quercetin-3-glucoside. The formation of phloroglucinol indicated that E. ramulus is capable of splitting the heterocyclic C-ring of quercetin (20). The degradation of quercetin was also reported for other human intestinal bacteria and for species from the bovine rumen. Examples include Butyribrio sp. C3 (4), Clostridium orbiscindens (22), Pediococcus Q-05 (16), and Eubacterium oxidoreducens (17). In contrast to...
E. oxidoreducens, which is able to grow on the aglycon quer-
cetin as the sole carbon and energy source in the presence of
hydrogen or formate as reductants (17), the growth of E. ram-
ulus with quercetin was strictly dependent on glucose, which
could be replaced neither by hydrogen nor by formate (20).

However, as described herein, resting cells of E. ramulus are
able to convert quercetin and its flavone analogue, luteolin.
This offered the opportunity to study these transformations
quantitatively in the absence of glucose and other media com-
ponents.

FIG. 2. Product ion spectra (MS/MS) of the m/z 305 [M+H]^+ of alphitonin (A) and taxifolin (B). The MS/MS spectrum of taxifolin shows a reverse-Diels-Alder fragment ion of m/z 153, with high intensity. This fragment was not observed in the case of alphitonin.
In accordance with previous reports using growing cultures (19, 20), quercetin was transformed by resting cells of *E. ramulus* to 3,4-dihydroxyphenylacetic acid (Fig. 1 and structures in Fig. 8). In the course of fermentation, the enol carbon C-3 is transformed to the carboxyl group of 3,4-dihydroxyphenylacetic acid. Thus, this bacterial degradation of quercetin does not occur via reverse reactions of its biosynthesis in plants. In the quercetin synthesis pathway of plants (for a review, see reference 6), the intermediate taxifolin is formed by hydroxylation of eriodictyol (structure in Fig. 9).

Two intermediates of the quercetin degradation were identified, taxifolin and alphitonin (Fig. 1). Separate experiments...

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**FIG. 3.** Time course of taxifolin degradation by resting cells of *E. ramulus* (optical density at 600 nm, 2.6; temperature, 19°C). The initial concentration of taxifolin was 1 mM. Concentrations of taxifolin (○), alphitonin (▲), and 3,4-dihydroxyphenylacetic acid (▼) are given.

**FIG. 4.** Time course of alphitonin degradation by resting cells of *E. ramulus* (optical density at 600 nm, 6; temperature, 19°C). The initial concentration of alphitonin was 0.26 mM. Concentrations of alphitonin (▲) and 3,4-dihydroxyphenylacetic acid (▼) are given.
showed that both taxifolin and alphitonin were transformed to 3,4-dihydroxyphenylacetic acid (Fig. 3 and 4). The degradation of taxifolin to 3,4-dihydroxyphenylacetic acid is in accordance with previous results using growing cells of *E. ramulus* (19).

Alphitonin was identified as an intermediate of the conversion of taxifolin to 3,4-dihydroxyphenylacetic acid (Fig. 3). From these data, the pathway of the quercetin degradation shown in Fig. 8 could be deduced. It starts with the reduction of the...
double bond in the 2,3-position of quercetin, resulting in the formation of taxifolin. The following ring contraction to the identified isomeric alphitonin probably occurs by a ring opening-recyclization mechanism via a chalcone or diketone structure. However, this postulated chalcone (or tautomeric diketone) could not be observed, presumably because of the fast cyclization to alphitonin. We cannot distinguish whether this cyclization is part of an enzyme-catalyzed reaction or whether it occurs spontaneously. Neither the \( \alpha \)-hydroxychalcone [2-hydroxy-3-(3,4-dihydroxyphenyl)propenone] nor the diketone [3-(3,4-dihydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propane-1,2-dione] have been described in the literature so far. As a result of the ring contraction to alphitonin, a benzylic \( \text{CH}_2 \) group is already formed as it finally appears in the product, 3,4-dihydroxyphenylacetic acid. An oxidative decarboxylation step is postulated for the conversion of alphitonin to phloroglucinol and 3,4-dihydroxyphenylacetic acid. Whereas 3,4-dihydroxyphenylacetic acid was identified as a final product, phloroglucinol was shown to undergo further degradation to butyrate and acetate (L. Schoefer, personal communication).

This is the first report describing alphitonin as an intermediate of bacterial metabolism. The elucidation of structure became necessary because a reference substance for this compound was not available, and the molecular masses of alphitonin and the corresponding chalcone structure are identical. The structure of alphitonin, isolated from the heartwood of *Alphitonia excelsa*, was reported in 1960 (1). The compound was also identified in the wood of *Alphitonia petriei* but not in that of *Alphitonia whitei* (7). Remarkably, no studies on the biological activity of alphitonin have been reported so far. Our results, however, indicate that alphitonin appears as an intermediate of intestinal metabolism of the abundant flavonoid quercetin and might be absorbed in the human intestinal tract.

We also investigated the degradation of the flavone luteolin by resting cells of *E. ramulus*. The proposed degradation pathway is shown in Fig. 9. Initial reduction led to the formation of eriodictyol, similar to the transformation of quercetin to taxifolin. Eriodictyol was identified as an intermediate in the resting-cell fermentation (Fig. 7). By the ensuing ring cleavage, a chalcone structure could be formed which may be further reduced to a dihydrochalcone. However, neither of these compounds nor a fused five-membered structure similar to alphitonin was observed. The final product of luteolin degradation was 3-(3,4-dihydroxyphenyl)propionic acid, which was identified in fermentation experiments with resting cells (Fig. 7), comparable to the results obtained previously with growing cells of *E. ramulus* (19), which fermented luteolin and eriodictyol in the presence of glucose. Phloroglucinol is certainly another intermediate, as postulated for several flavonoid degradation pathways (9, 18, 22), but its further degradation occurred instantly. Similar to our results, it was reported that eriodictyol is converted to 3-(3,4-dihydroxyphenyl)propionic acid by a strain of *Clostridium butyricum* (18).

The comparison of the pathways (Fig. 8 and 9) reveals that degradation of both quercetin and luteolin by *E. ramulus* starts with the reduction of the double bond in the 2,3-position prior to
to the C-ring fission. The steps that follow differ due to the 3-hydroxyl group in the quercetin molecule, which is the only difference from the luteolin structure. This hydroxyl group seems to be a prerequisite for the formation of the alphitonin structure following C-ring cleavage. In contrast, the fission of the heterocyclic ring of eriodictyol, which results from luteolin, would lead directly to a chalcone structure, and for further transformation a second reduction step is assumed. These postulated intermediates could not be observed during luteolin conversion by resting cells, although such chalcones and dihydrochalcones are known to be stable (6). It was shown previously that the dihydrochalcone phloretin is also degraded by growing cells of *E. ramulus* in the presence of glucose (19). The final products resulting from the B-ring of quercetin and luteolin, respectively, differ in one carbon atom within the side chain, indicating an additional decarboxylation step in the case
of quercetin degradation. The notion that the degradation of flavonols and flavones by E. ramulus occurs by two different pathways and involves different enzymes is supported by the finding that the taxifolin-transforming enzyme preparation did not transform eriodictyol, either in the presence or in the absence of oxygen (data not shown).

In conclusion, the fermentation by resting cells constitutes an advantageous method for the detection of intermediates of flavonoid degradation by intestinal bacteria. These bacterial metabolites should be included in investigations concerning the flavonoid effects after ingestion by humans. However, the characterization of the involved enzymes and the reaction mechanisms requires further studies with cell-free systems. This approach has already been initiated for the taxifolin isomerization as described above, and it will be continued in ongoing studies in our laboratories.

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REFERENCES