APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1989, p. 1203–1208
0099-2240/89/051203-06$02.00/0

Vol. 55, No. 5

C-Ring Cleavage of Flavonoids by Human Intestinal Bacteria

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Received 12 December 1988/Accepted 7 February 1989

Four hitherto undescribed *Clostridium* strains capable of cleaving the C ring of quercetin, kaempferol, and naringenin at C-3–C-4 were isolated from the fecal flora of humans. None of the strains cleaved catechin. C-ring fission occurred when the substrate was either in solution or in suspension. Mixed cultures of flavonoid-hydrolyzing bacteria, flavonoid-cleaving bacteria, and *Escherichia coli*, which was used to provide the anaerobic environment, rapidly metabolized rutin to 3,4-dihydroxyphenylacetic acid, indicating that the intestinal half-life of the biologically active aglycone is short. The cleaving strains shared many phenotypic characteristics, including their inability to ferment sugars, but they differed sufficiently to indicate that they represent different species.

Flavonoid glycosides are synthesized by most if not all plants as an important component of their ecosystem. They are ingested in daily quantities of 1 to 2 g by humans who eat diets typical of those found in the Western world (23). Macdonald and Mader (25) have shown that cell extracts of feces and saliva (fecalase and salivase) hydrolyze flavonoid glycosides to their corresponding aglycones. Recently, we isolated three obligate anaerobic bacteria from the human intestinal flora that were capable of hydrolyzing glycosides to aglycones (27): *Bacteroides distasonis* hydrolyzed robinin to kaempferol, and *Bacteroides uniformis* and *Bacteroides ovatus* converted rutin to quercetin.

Ring fission of flavonoids in mammals was studied by Booth et al. (6) more than 30 years ago. They showed that the main metabolic products were hydroxyphenylacetic and phenylpropionic acids. Cheng et al. (7, 8) and Krishnamurty et al. (19) demonstrated that *Butyrivibrio* spp. from ruminal fluid cleaved the C ring of rutin and quercitrin but not the C ring of the aglycone quercetin. Quercetin was cleaved by a new bacterial species, *Eubacterium oxidoreducens*, which was recovered from the bovine rumen as reported by Krumholz and colleagues (20–22). Additional evidence of the central role of the intestinal resident flora in the catabolism of flavonoids has been provided by Das and Griffiths (9), who have shown that oral administration of aureomycin to guinea pigs suppresses the formation of ring fission products from ingested catechin. Moreover, Griffiths and Barrow (15) have noted that aglycones fed to germ-free rats did not appear in the urine as phenolic compounds, which was contrary to the case in conventional rats.

In this report we describe the isolation and tentative identification of human intestinal bacteria that cleave the C ring of flavonoids.

MATERIALS AND METHODS

Media. Supplemented peptone broth was purchased from Becton Dickinson and Co., Rutherford, N.J.; chopped meat (CM) broth was from Carr-Scarborough Microbiologicals, Decatur, Ga.; and prereduced brain heart infusion (BHI) broth (PR broth) was from Scott Laboratories, Inc., Fiskeville, R.I. Dehydrated BHI broth was obtained from BBL Microbiology Systems, Cockeysville, Md. Anaerobic broth (BHIC) was prepared with 37 g of BHI per liter and supplemented with 0.5 g of cysteine hydrochloride, 1 g of sodium bicarbonate, and 4 ml of 0.025% aqueous resazurine (J. T. Baker Chemical Co., Phillipsburg, N.J.). The media were distributed in 50-ml amounts, sterilized at 121°C for 20 min, and cooled to room temperature. At the time of use some vials of the anaerobic broth were further reduced biologically by adding 0.1 ml of a 24-h culture of a non-flavonoid-metabolizing strain of *Escherichia coli* (BHIC-*E. coli* broth) (29). The fast-growing facultative anaerobe lowered the E_h of the medium to −200 mV or less within 1 or 2 h. The presence of *E. coli* created the required anaerobic conditions without metabolizing the specific substrate.

Plated media were purchased from BBL. Aerobic cultures were incubated under standard conditions at 37°C, and anaerobic cultures were incubated in GasPak jars (BBL) at the same temperature.

Source of microorganisms. As the main purpose of the study was to detect intestinal organisms that cleave the C ring, fecal samples from healthy subjects on a diet typical of those found in the Western world constituted our main supply of bacteria. In addition, a number of the following bacterial strains that in earlier experiments were shown to synthesize enzymes that are active on the steroidal molecule were examined for their ability to cleave flavonoids: *Clostridium scindens* (27), *Eubacterium desmolans* (28), *Bifidobacterium adolescentis* (30), *Eubacterium lentum* (32), and *Clostridium paraputrificum* (4).

Substrates, reference compounds, and solvents. Substrates and chromatographic reference compounds (Fig. 1) were obtained partly from Pfaltz and Bauer, Stamford, Conn., and partly from Sigma Chemical Co., St. Louis, Mo. The products required no further purification. Reagent-grade and high-pressure liquid chromatographic (HPLC)-grade solvents were used throughout the experiments.

Isolation and identification of C-ring-cleaving microorganisms. The methodology for the isolation and identification of C-ring-cleaving microorganisms has been described previously (5). Briefly, fecal samples from healthy subjects on a Western diet were collected in stool cups. Within 30 min they were serially 10-fold diluted (10^2 to 10^{11}) in supplemented peptone broth. Portions (0.25 ml) from all dilutions were tested for bacterial cleavage of the C ring as described

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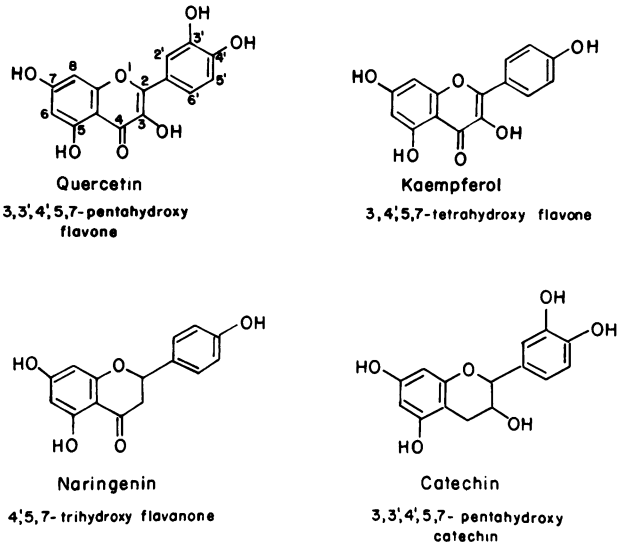

FIG. 1. Structures of pertinent flavonoids.

below. Samples of 0.1 ml of the three highest dilutions yielding positive results were seeded onto four sets of blood agar plates and incubated at 37°C for 48 h; two sets were incubated aerobically and two sets were incubated anaerobically. The colonies were counted, and the growth from one aerobic and one anaerobic set of plates was harvested in 2.5 ml of saline and tested for the ability to cleave the C ring. The information was designed to identify the sister plate (aerobic or anaerobic) with the fewest colonies that included at least one colony of a flavonoid-metabolizing organism. All colonies from this plate were isolated and tested for enzymatic activity. Pure strains of converting bacteria were identified by conventional phenotypic characterization (10–12, 16–18), susceptibility to high-potency antibiotic disks on petri dishes, polyacrylamide gel electrophoresis (26), and electron microscopy (28).

Conversion experiments. Unless otherwise stated, 50 ml of PR broth, BHIC broth, or BHIC-*E. coli* broth were supplemented with 1 to 15 mg of flavonoids dissolved in 0.5 ml of methanol or 0.25 ml of dimethyl sulfoxide. The final concentrations of substrate ranged from 20 to 300 μg per ml of medium. Methanol (1%) or dimethyl sulfoxide (0.5%) did not interfere with bacterial growth. The percent conversion was identical whether methanol (0.25 to 1%) or dimethyl sulfoxide (0.5%) was used. The supplemented media were seeded with 0.25 ml of the 18-h-old culture that was to be investigated or with 0.25 ml of progressively diluted fecal suspensions. The cultures were incubated at 37°C for from 4 h to 7 days; culture growth and flavonoid metabolism were checked at appropriate intervals as described previously (31). Bacterial growth was measured by determining the optical density at 430 nm with a spectrophotometer (model 250; Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Extraction and identification of flavonoid metabolites. For the extraction and identification of flavonoid metabolites, 5-ml portions of conversion cultures were used for thin-layer chromatographic analysis and 40-ml portions were used for HPLC. The portions were acidified to pH 2 and extracted with ethyl acetate for 30 s. The organic phase was collected, dried over sodium sulfate, and evaporated under nitrogen at 40 to 45°C.

Then, for thin-layer chromatography, the extracted residue was redissolved in 50 μl of methanol and spotted onto silica gel plates (IB2F Flex; J. T. Baker Chemical Co.). Good separation of quercetin, kaempferol, and naringenin and their corresponding metabolites was obtained with benzene-acetic acid-water (83:48:2; vol/vol/vol). The spots of flavonoids and metabolites were located under UV light (254 nm) and by iodine vapors. The chromatographic data are given in Table 1.

TABLE 1. Chromographic characteristics of flavonoids and their bacterial metabolites

Substrate	Metabolite[a]	R_f[b]	RT[c]
Quercetin		0.52	3.1
	3,4-DPA	0.56	1.04
Kaempferol		0.64	3.4
	4-HPA	0.72	1.12
Naringenin		0.64	1.34
	PLA	0.70	1.09
	Phloroglucinol[d]	0.34	1.50

[a] Abbreviations: 3,4-DPA, 3,4-dihydroxyphenylacetic acid; 4-HPA, 4-hydroxyphenylacetic acid; PLA, phenylacetic acid.
[b] Thin-layer chromatography was done on Bakerflex silica gel (IB2F), and the solvent system was benzene-acetic acid-water (83:48:2; vol/vol/vol).
[c] The HPLC column was MicroPak C_{18} 10 μm, and the solvent system was methanol-water (70:30; vol/vol) at a flow rate of 2 ml/min. RT, Retention time.
[d] Phloroglucinol was not seen in our experiments.

HPLC was performed on a high-pressure liquid chromatograph (5000; Varian Instrument Group, Walnut Creek, Calif.) equipped with a MicroPak C_{18} 10 μm column (Varian) and a UV detector set at 254 nm; the solvent system consisted of methanol-water (70:30; vol/vol); the flow rate was 2 ml/min. The products subjected to HPLC analysis were extracted from the cultures as described above, purified on thin-layer chromatographic plates, and extracted from the plate with 5 ml of ethyl acetate. After evaporation of the solvent, the residue was redissolved in 0.5 ml of the HPLC solvent system and 10 μl was injected into the column. Authentic standards were incorporated in all runs. In certain cases the metabolic products were purified on a SepPak C_{18} cartridge (Waters Associates, Inc., Milford, Mass.), followed by elution with ethyl acetate.

3,4-Dihydroxyphenylacetic acid (3,4-DPA) was identified also by HPLC and mass spectrometry.

RESULTS

Isolation of C-ring-cleaving bacteria from feces. C-ring cleavage of quercetin (20 μg/ml) was observed in BHIC-*E. coli* broth and PR broth seeded with progressively diluted feces from all five healthy subjects on a Western diet. Quercetin was completely metabolized up to a dilution of 10^9. Bacterial suspensions harvested from aerobically incubated blood agar plates did not cleave quercetin. In contrast, bacteria from anaerobically incubated plates seeded with feces diluted up to 10^9 metabolized quercetin to phenol carboxylic acids. All 113 colonies from a sister plate were isolated onto CM broth.

Batch testing followed by individual testing of the isolates yielded two different organisms, strains 257 and 258, that were capable of cleaving the C ring of quercetin. Two additional organisms, strains 264 and 265, were recovered in a later experiment.

Identification of C-ring-cleaving organisms. All four strains shared many characteristics. Colonies on rabbit blood agar incubated anaerobically were minute, slightly irregular, entire, convex, grey-white, shiny, smooth, and beta-hemolytic. Colonies on sheep blood agar were slightly larger but had no beta-hemolysis.

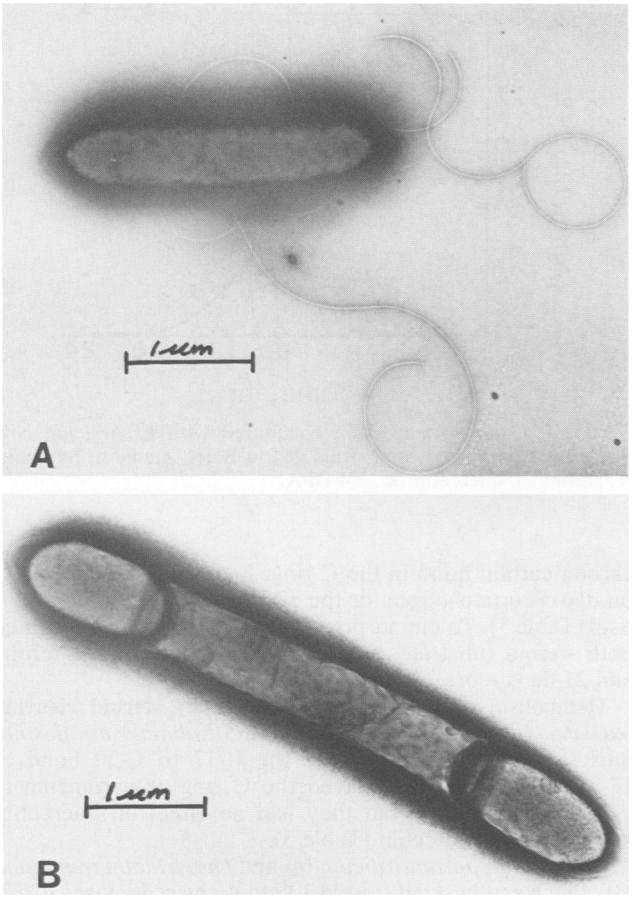

FIG. 2. Electron micrographs of strain 258 with peritrichous flagella (A) and with polar spores (B).

The organisms were variable by Gram staining but were mainly gram-negative rods, 2 to 7 μm in length, and motile with peritrichous flagella (Fig. 2A) but without capsules. Oval, polar spores were often observed (Fig. 2B); growth from CM agar slants survived heating at 80°C for 10 min. None of the four strains fermented amygdalin, arabinose, cellobiose, erythritol, fructose, glucose, glycogen, gum arabic, inositol, lactose, larch arabinogalactan, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose, xylan, or xylose. Neither esculin nor urea was hydrolyzed. Gelatin, meat, and casein were not digested. There was no reaction to milk. Nitrate was not reduced. Catalase, lecithinase, and lipase were not produced. The organisms were susceptible to cephalothin (30 U), kanamycin (1,000 U), and metronidazole (80 U) but were resistant to nalidixic acid (30 U), rifampin (15 U), and vancomycin (5 U). The strains were not pathogenic to mice, as demonstrated by intramuscular injection of a 0.1-ml mixture of equal parts of 48 h CM-glucose culture and 10% $CaCl_2$; nor were they toxic, as shown by intraperitoneal injection of 0.4 ml of the supernatant of a 48 h CM-glucose culture.

TABLE 3. Bacterial isolates with cleaving and desmolytic activities

Bacterial strain	Metabolism of the following substrates[a]:					
	Q[b]	K[b]	N[b]	C[b]	DOC[c]	F[d]
257	+	+	+	−	−	−
258	+	+	+	−	−	−
264	+	+	+	−	+	−
265	+	+	+	−	−	−
Clostridium scindens	−	−	+	−	−	+
Eubacterium desmolans	−	−	+	−	−	+
Clostridium cadaveris	−	−	−	−	−	+

[a] Abbreviations: Q, quercetin; K, kaempferol; N, naringenin; C, catechin; DOC, deoxycorticosterone; F, cortisol.
[b] C-ring cleavage.
[c] 21-Dehydroxylation.
[d] Side-chain cleavage of cortisol at C-17–C-20.

Despite these many similarities, the strains differed in their CO_2 requirements, indole production, susceptibility to penicillin, growth on bile agar, utilization of pyruvate and fumarate, and H_2S production (Table 2). Polyacrylamide gel electrophoresis patterns suggested that strain 257 and 258 belonged to different species.

Metabolism of flavonols by isolates. All four strains cleaved the C ring of quercetin and kaempferol between C-3 and C-4, forming 3,4-DPA and 4-hydroxyphenylacetic acid, respectively (Table 3). Both metabolites were derived from the B ring. The expected A-ring metabolite, phloroglucinol (Table 1), could not be detected in organic extracts (19, 20) or in the lyophilized culture. Attempts to release the metabolite by reflux hydrolysis at pH 2 or 10 for 8 h or following autoclaving of the culture failed. Additional experiments revealed that phloroglucinol was extractable from an aqueous solution for at least 72 h but was irreversibly bound to cysteine (0.05%) in buffer within 24 h. Similarly, it was irreversibly bound to unknown compounds in BHI broth within 7 h.

Metabolism of flavanone and catechin. Strains 257 and 258 cleaved the C ring of naringenin, with the formation of small amounts of phenylacetic acid; 85 to 90% of the substrate remained unmetabolized. Catechin was resistant to all four isolates.

Capacity to convert quercetin. C-ring cleavage by strains 257 and 264 was examined in PR broth supplemented with 20 to 300 μg of quercetin per ml. The lower concentration was prepared with methanol and the higher concentration was prepared with dimethyl sulfoxide. Regardless of concentration, quercetin was totally metabolized to 3,4-DPA within 24

TABLE 2. Phenotypic differences of C-ring-cleaving strains isolated from human fecal flora

Strain	H_2S	Acid products in PYG[a]	Fumarate utilization	Pyruvate utilization	CO_2 requirement	Indole production	Growth on bile agar	Penicillin susceptibility[b]
257	+	A, B	+	−	−	−	−	R
258	+	A, B, P	+	+	+	+	−	R
264	Weak	A, B, P	+	+	+	−	+	S
265	−	A, B, P	−	+	+	−	−	S

[a] Abbreviations: PYG, Peptone-yeast-glucose broth; A, acetic acid; B, butyric acid; P, propionic acid.
[b] Abbreviations: R, resistant; S, susceptible.

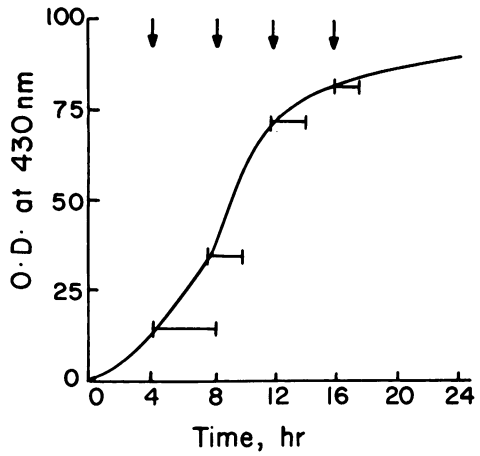

FIG. 3. Velocity of reaction at different growth phases. Inoculum strain 258 was used. The substrate was medium containing 100 μg of quercetin per ml. The arrow indicates the time at which quercetin was added to the medium; the bars indicate the incubation time needed for 100% conversion to 3,4-DPA. The growth curve is shown. The optical density (O.D.) was measured at 430 nm in a spectrophotometer (model 250; Gilford).

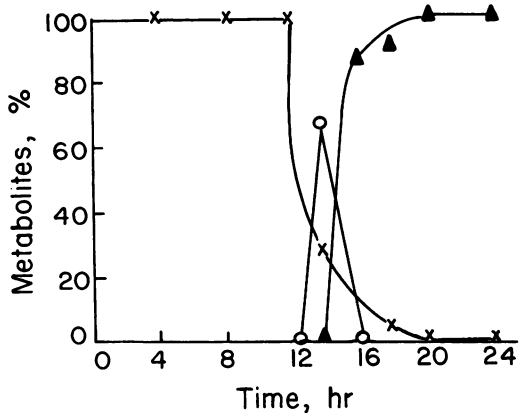

FIG. 4. Catabolism of rutin cocultured with *Escherichia coli*, *Bacteroides uniformis*, and strain 264 in BHIC medium. Symbols: ×, Rutin; ○, quercetin; ▲, 3,4-DPA.

h. Media, however, containing the higher concentrations of quercetin were slightly cloudy, suggesting that not only dissolved but also suspended quercetin is metabolized by bacteria. Accordingly, 50 ml of PR broth was supplemented with a 1-ml suspension of 15 mg of quercetin in water (final concentration of quercetin, 300 μg per ml of medium). It formed a yellow sediment, which disappeared within 1 or 2 days after inoculation of the medium with strain 257. The suspended substrate was totally metabolized to 3,4-DPA within 72 h.

Kinetic experiments. PR broth media supplemented with 20 and 100 μg of quercetin per ml were seeded with strain 257 or 258, incubated at 37°C, and sampled every second hour. Measurable conversion coincided with the end of the lag growth phase, was intense during the logarithmic growth phase, and was completed at the beginning of the stationary phase. 3,4-DPA was the only metabolite that was recovered. Phloroglucinol, the expected A-ring-derived metabolite, was not observed. C-ring fission was significantly faster in mature than in young cultures (Fig. 3), as shown by adding quercetin to converting cultures in different growth phases. Young cocultures of *Bacteroides uniformis* (hydrolyzing) and strain 264 (cleaving) in PR broth metabolized the glycoside rutin (100 μg/ml) to 3,4-DPA in 12 h. Trace amounts of quercetin were noted after 10 h of incubation but disappeared rapidly. If the substrate was added after 16 and 48 h of incubation of the cocultures, conversion was accomplished in 2 to 3 h.

In BHIC broth cultures of *Escherichia coli*, *Bacteroides uniformis*, and strain 264, conversion of rutin to 3,4-DPA was slower than that in PR broth. A distinct but short-lived peak of quercetin appeared after 14 h of incubation (Fig. 4).

Substrate competition. Strains 257 and 258 cleaved quercetin (100 μg/ml) and kaempferol (100 μg/ml) equally effectively in medium containing both substrates. Similarly, in PR broth containing quercetin (300 μg/ml) and deoxycorticosterone (20 μg/ml), the substrates were totally converted by strain 264 to 3,4-DPA and progesterone, respectively.

Metabolism of steroids by C-ring-cleaving bacteria. Strains 257, 258, and 265, all of which were capable of cleaving the carbon-carbon bond in the C ring, had no desmolase effect on deoxycorticosterone or the 17-hydroxylated steroid cortisol (Table 3). To our surprise, strain 264 21-dehydroxylated both steroid substrates, with the formation of progesterone and 21-deoxycortisol, respectively.

Metabolism of the flavonoid C ring by steroid cleaving bacteria. *Clostridium scindens* (1) and *Eubacterium desmolans* (5), both desmolytic for the C-17 to C-20 bond of 17-hydroxycorticoids, cleaved the C ring of naringenin in yields of 20 to 25%; but they had no effect on quercetin, kaempferol, or catechin (Table 3).

Clostridium paraputrificum (4) and *Clostridium innocuum* (3), which synthesized steroid 3-keto-4-ene reductases; *Clostridium cadaveris* (5) and *Bifidobacterium adolescentis* (30), which elaborated a 20-reductase; and *Eubacterium lentum* (29), which produced 21-dehydroxylase, had no effect on quercetin, kaempferol, naringenin, or catechin. *Bacteroides distasonis*, *Bacteroides uniformis*, and *Bacteroides ovatus* (2), which hydrolyze flavonoid glycosides, did not cleave the C ring.

DISCUSSION

Taxonomic position of C-ring-cleaving bacteria. The four clostridial isolates described here shared several characteristics. They synthesized enzymes that cleaved the C ring of flavonoids, were recovered from feces of normal individuals, and were assacharolytic clostridia with a paucity of conventional biochemical reactions. Differences in indole reaction, nitrate reduction, H_2S production, production of organic acids, and susceptibility to antibiotics, however, indicated that they should be assigned to separate species rather than to variants of the same species. DNA-DNA homology studies will eventually determine the classification. In the meantime, the organisms will be preserved under the code numbers used in this report.

Pathways of ring fission of flavonoids. In 1970, Krishnamurty et al. (19) reported that a *Butyrivibrio* species from ruminal fluid cleaved quercitrin to 3,4-DPA and phloroglucinol (Fig. 5). *Clostridium* sp. strains 257, 258, 264, and 265 also yielded 3,4-DPA; but phloroglucinol was not detected. Under similar circumstances Griffiths (14) also failed to isolate phloroglucinol, the expected A-ring metabolite. Griffiths thought that the compound might have been degraded further. Krumholtz and colleagues (20–22) reported that the

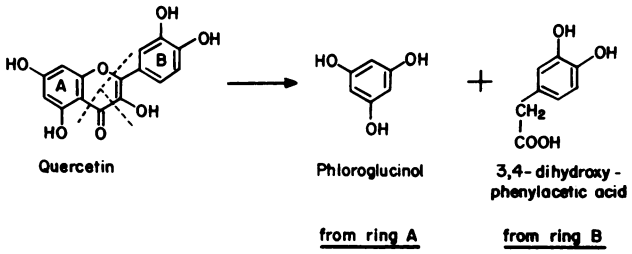

FIG. 5. Metabolic pathways of quercetin C-ring fission.

ruminal isolate *Eubacterium oxidoreducens* converted phloroglucinol to acetate and butyrate via dihydrophloroglucinol. In our conversion medium, phloroglucinol formed nondissociable compounds with cysteine hydrochloride and unknown agents in BHI broth. A similar aggregate has been observed in systems containing Δ-16-progesterone and cysteine. The two molecules formed a water-soluble product that escaped extraction with lipid solvents (13). We could not establish whether the phloroglucinol remained undetected because it was bound to cysteine hydrochloride or because it was further metabolized.

Flavonoid-metabolizing bacteria of the gut. Results of coculture experiments with *Escherichia coli* and hydrolyzing and cleaving bacteria indicated that the half-life in vitro of the biologically active aglycone is short. It is not unreasonable to assume that a similar situation exists in the gut; the presence of aglycones in the gut could be shortened further by absorption. Thus, beneficial or harmful effects of aglycones on intestinal structures, as surmised by analogy from in vitro studies, are likely modified by forces aimed at removing or catabolizing the compounds. Recent findings (2) and the data presented here begin to outline a picture of organized, collaborative bacterial catabolism of flavonoids. Bacterial synthesis of cleaving enzymes appears to be relevant only because of the presence in the intestinal flora of large numbers of *Bacteroides* spp. that are capable of hydrolyzing ingested glycosides (2). It is noteworthy that the flavonoid catabolic enzymes are synthesized by several bacterial species. A similar phenomenon is found in the degradation of steroidal structures. *Clostridium paraputrificum* is a potent reducer of the A ring of corticosteroids (2), *Eubacterium lentum* removes the 21-hydroxy group (32), and *Clostridium scindens* cleaves the side chain of 17-hydroxylated steroids (27). There is an ecologic advantage of a multibacterial participation in the catabolism of complicated structures.

In vivo studies give further credence to the central role of the bacterial flora in the catabolism of flavonoids. For example, flavonoid glycosides fed to germ-free animals undergo renal excretion, while they are absent in the urine of conventional animals (14). The segment of the gut in which glycoside hydrolysis and C-ring cleavage takes place is unknown. Our experiments indicate, however, that ample capacity for both catabolic functions exists. This is further supported by the observation that in vitro cleavage occurs rapidly in both the logarithmic and the stationary bacterial growth phases.

ACKNOWLEDGMENTS

We thank A. E. Ritchie from the National Animal Disease Center, Ames, Iowa, for the electron microscopic identification of strain 264 and Cedric Shackleton from Children's Hospital, Oakland, Calif., for the HPLC and mass spectroscopic identification of 3,4-DPA.

This investigation was supported by Public Health Service grant CA 25763 from the National Cancer Institute to St. Luke's-Roosevelt Institute for Health Sciences.

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