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ARTICLE

Green Tea Flavan-3-ols: Colonic Degradation and Urinary Excretion of Catabolites by Humans

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Following the ingestion of green tea, substantial quantities of flavan-3-ols pass from the small to the large intestine (Stalmach et al. *Mol. Nutr. Food Res.* **2009**, *53*, S44−S53; *Mol. Nutr. Food Res.* **2009**, doi: 10.1002/mnfr.200900194). To investigate the fate of the flavan-3-ols entering the large intestine, where they are subjected to the action of the colonic microflora, (−)-epicatechin, (−)-epigallocatechin, and (−)-epigallocatechin-3-*O*-gallate were incubated in vitro with fecal slurries and the production of phenolic acid catabolites was determined by GC-MS. In addition, urinary excretion of phenolic catabolites was investigated over a 24 h period after ingestion of either green tea or water by healthy volunteers with a functioning colon. The green tea was also fed to ileostomists, and 0−24 h urinary excretion of phenolic acid catabolites was monitored. Pathways are proposed for the degradation of green tea flavan-3-ols in the colon and further catabolism of phenolic compounds passing into the circulatory system from the large intestine, prior to urinary excretion in quantities corresponding to ca. 40% of intake compared with ca. 8% absorption of flavan-3-ol methyl, glucuronide, and sulfate metabolites in the small intestine. The data obtained point to the importance of the colonic microflora in the overall bioavailability and potential bioactivity of dietary flavonoids.

KEYWORDS: Green tea; flavan-3-ols; catabolites; colonic degradation; urinary excretion

INTRODUCTION

Green tea is an especially rich source of a variety of flavan-3-ol monomers with the levels in young leaves of *Camellia sinensis* comprising up to 30% of the dry weight (*1*). Typically, the main components are (−)-epigallocatechin and (−)-epigallocatechin-3-*O*-gallate, together with smaller but still substantial quantities of (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, (+)-gallocatechin, and (+)-catechin and low levels of (+)-catechin-3-*O*-gallate (**Figure 1**) (*2*, *3*). Green tea infusions have been reported to exert a wide range of beneficial effects in vitro having antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, and antihypertensive properties (*4*−*7*). These effects are attributed to the flavan-3-ol content of the teas and, as a consequence, there is interest in the bioavailability and metabolism of flavan-3-ols as they pass through the body following ingestion.

Recently, HPLC with multistage mass MS detection (i.e., MS^2 and MS^3) has been used to analyze flavan-3-ols in plasma and urine, and this has facilitated the identification of a range of metabolites (*8*, *9*). After acute ingestion of green tea by human subjects, Stalmach et al. (*10*) detected a total of 10 metabolites in plasma, in the form of *O*-methylated, sulfated, and glucuronide conjugates of (epi)catechin and (epi)gallocatechin, with 29−126 nM peak plasma concentrations (C_{max}) occurring 1.6−2.3 h after ingestion, indicative of absorption in the small intestine. Plasma also contained unmetabolized (−)-epigallocatechin-3-*O*-gallate and (−)-epicatechin-3-*O*-gallate with respective C_{max} values of 55 and 25 nM. Urine excreted 0−24 h after the consumption of green tea contained 15 metabolites of (epi)catechin and (epi)gallocatechin, but (−)-epigallocatechin-3-*O*-gallate and (−)-epicatechin-3-*O*-gallate were not detected. Overall flavan-3-ol metabolite excretion was equivalent to 8% of intake; however, urinary (epi)gallocatechin metabolites corresponded to 11% of (epi)gallocatechin ingestion, whereas (epi)catechin metabolites were detected in amounts equivalent to 28.5% of (epi)catechin intake. These findings, along with those of Auger et al. (*11*), indicate that (epi)catechins are highly bioavailable, being absorbed and excreted to a much greater extent than most other flavonoids.

Subsequently, a similar green tea feeding study was carried out using human volunteers with an ileostomy (*12*). The 0−24 h plasma and urinary profiles were very similar to those obtained with subjects with an intact, functioning colon, confirming that the flavan-3-ol metabolites were absorbed principally in the small intestine. The ileal fluid contained ca. 70% of the ingested flavan-3-ols in the form of parent compounds (33%) and 23 metabolites (37%). The main metabolites effluxed back into the lumen of the

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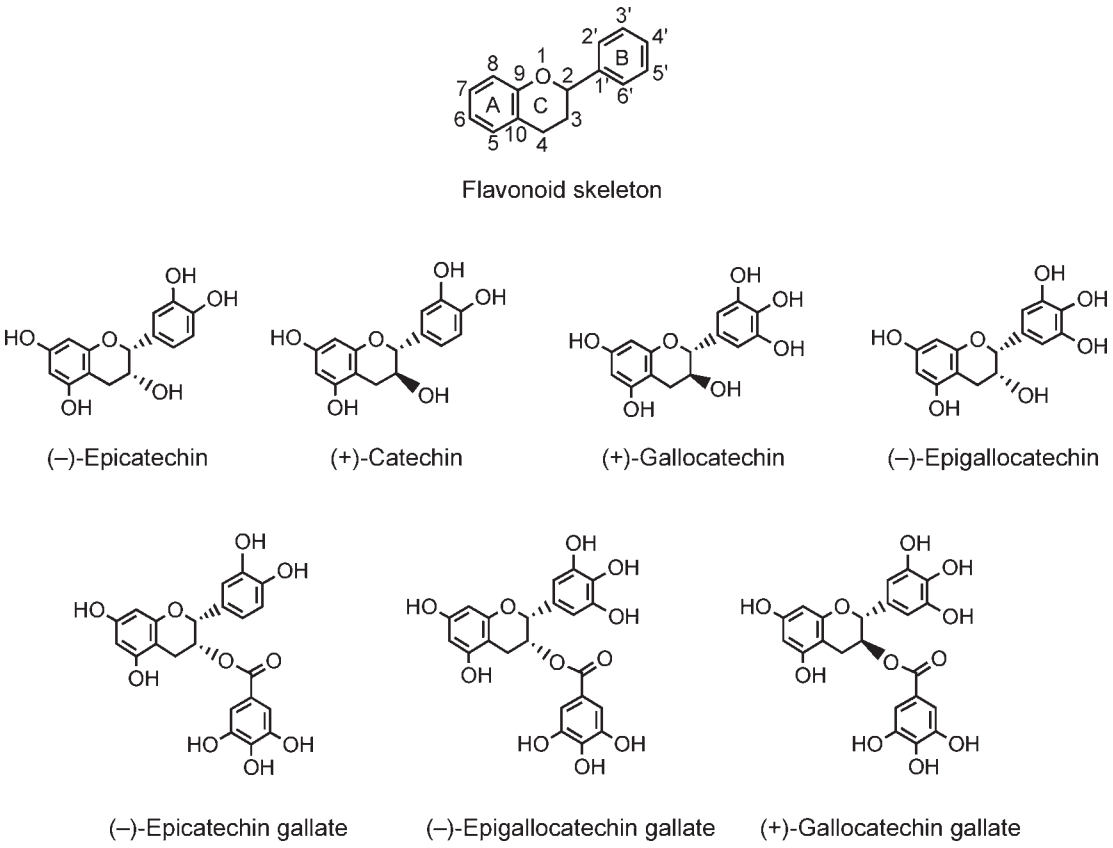

Figure 1. Structures of green tea flavan-3-ols.

small intestine were *O*-linked sulfates and methyl sulfates of (epi)catechin and (epi)gallocatechin. This indicates that in subjects with a functioning colon substantial quantities of flavan-3-ols pass from the small to the large intestine.

On entering the large intestine, most flavonoids are broken down to phenolic acids by the action of colonic microflora (*13*, *14*). The colon typically contains $10^{11}-10^{12}$ bacterial cells/g, of which the majority, such as *Bacteroides* sp., *Eubacterium* sp., *Bifidobacterium* sp. *Clostridium*, *Lactobacillus*, anaerobic cocci, and *Fusobacterium* spp., are strict anaerobes (*15*, *16*). These bacteria are capable of producing several enzymes such as glycosidases and sulfatases (*17*−*19*) that hydrolyze and remove conjugating moieties, such as glucosides, glucuronides, and sulfates, from the flavonoid skeleton. The released aglycones are then catabolized to ring fission products and low molecular weight phenolic acids (*20*, *21*). For instance, in the presence of human intestinal bacteria, flavan-3-ols undergo reductive cleavage to yield epimers such as 1-(3′,4′)-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxy)propan-2-ol (*22*), which is converted to (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone (*23*). The available evidence suggests that the phenylvalerolactones are then broken down to simpler phenolic acids (*24*).

In this paper we report the degradation and subsequent excretion of flavan-3-ols known to enter the large intestine after the consumption of green tea, adding to information obtained on small intestine absorption and urinary excretion of metabolites obtained by Stalmach et al. (*12*). (−)-Epicatechin, (−)-epigallocatechin, and (−)-epigallocatechin-3-*O*-gallate were incubated in vitro with human fecal slurries cultured under anaerobic conditions, and the production of phenolic acid catabolites was monitored by GC-MS. In addition, urinary excretion of catabolites was investigated over a 24 h period after the ingestion of green tea by both healthy volunteers and subjects with an ileostomy.

MATERIALS AND METHODS

Materials. Pyrocatechol, pyrogallol, *p*-coumaric acid, hippuric acid, 2-, 3-, and 4-hydroxybenzoic acid, 3-methoxy-4-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, (−)-gallocatechin, (+)-catechin, (−)-gallocatechin-3-*O*-gallate, resazurin, and tryptone were obtained from Sigma-Aldrich (Poole, Dorset, U.K.). 3-Hydroxycinnamic acid was purchased from Fisher (Leicestershire, U.K.). Ferulic acid and 3,4-dihydroxybenzoic acid were obtained from AASC Chemicals (Southampton, U.K.). 3-(3-Hydroxyphenyl)propanoic acid was supplied by Fluorochem (Derby, U.K.). Derivatization reagent [(*N*,*O*-bis(trimethylsilyl)acetamide (BSTFA) + 1% trimethylchlorosilane (TMCS)] was purchased from Sigma-Aldrich. HPLC grade ethyl acetate and methanol were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland). (−)-Epigallocatechin, (−)-epigallocatechin-3-*O*-gallate, and (−)-epicatechin were gifts from Finlay Tea Solutions U.K. Ltd. (London, U.K.). All other chemicals including phenolic acid standards were purchased from Sigma-Aldrich.

Green Tea. Brewed green tea was prepared by adding 300 mL of boiled distilled water to 3 g of Indonesian green tea leaves (Tetley GB Ltd., Greenford, Middlesex, U.K.), which were left to brew for 3 min with continuous stirring before filtering. The green tea brew was then cooled to room temperature prior to analysis of 5 μL volumes in triplicate by HPLC-PDA-MS, as described by Stalmach et al. (*2*), and GC-MS.

Human Feeding Studies. Five healthy volunteers (four males, one female) and five volunteers (two males, three females) with an ileostomy, and hence with no colon, participated in this study. This study protocol was approved by the University of Glasgow Royal Infirmary Ethics Committee (REC reference no. 04/S070/48). The volunteers were nonsmokers, not pregnant, aged between 41 and 54 years, and were not under any medication. The ileostomy volunteers have had an ileostomy for 16.6 ± 9.8 years and were otherwise healthy. All subjects were required to follow a diet low in flavonoids for 2 days prior to the study and for 24 h after supplementation, avoiding fruits, vegetables, high-fiber products, and beverages such as tea, coffee, fruit juice, and wine. On the day of the study, after an overnight fast, each subject drank 300 mL of green tea. In a separate study, the volunteers with an intact colon drank 300 mL of water instead of green tea. Urine was collected 0−2, 2−5, 5−8, and 8−24 h after

Table 1. GC Retention Time and Characteristic Ions of Phenolic Acids and Catabolites in Green Tea, Urine, and Fecal Slurries[a]

phenolic acids and catabolites	t_R (min)	base ion (*m*/*z*)	qualifier ion (*m*/*z*)	identification	location
pyrocatechol	12.62	254	239; 73	standard, NIST	FS, U
pyrogallol	14.98	239	342; 73	standard, NIST	FS, U
4-hydroxybenzoic acid	16.86	267	223; 193	standard, NIST, Olthof et al. (*28*)	GT, U
4-hydroxyphenylacetic acid	17.02	296	281; 252	standard, NIST, Olthof et al. (*28*)	FS, U
3-(3-hydroxyphenyl)propionic acid	21.88	310	205; 192	standard	FS, U
3-methoxy-4-hydroxyphenylacetic acid	22.07	326	209; 179	standard, Olthof et al. (*28*), Jenner et al. (*31*)	U
3-hydroxycinnamic acid	23.00	308	293; 147	standard, Zadernowski et al. (*27*)	GT
hippuric acid	25.25	105	206; 236	standard, NIST, Olthof et al. (*28*)	U
3-(3-hydroxyphenyl)-3-hydroxypropionic acid	25.88	267	207; 147	NIST	U
4-coumaric acid	28.70	308	293; 219	standard, NIST	GT
gallic acid	29.96	458	281; 443	standard, NIST	GT
ferulic acid	33.18	338	249; 323	standard, NIST	GT
5-(3,4-dihydroxyphenyl)-γ-valeric acid	33.90	267	426; 205	NIST	FS
(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	34.44	352	267; 73	Gonthier et al. (*30*)	FS
(−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	34.95	352	268; 73	NIST	FS, U

[a] FS, fecal slurry; GT, green tea; U, urine; t_R, retention time.

Table 2. Flavan-3-ol Content of a 300 mL Infusion of Green Tea[a]

flavan-3-ol	μmol/300 mL
(−)-epicatechin	69 ± 1
(+)-catechin	18 ± 1
(−)-epigallocatechin	190 ± 2
(+)-gallocatechin	50 ± 1
(−)-epicatechin-3-*O*-gallate	64 ± 1
(+)-gallocatechin-3-*O*-gallate	4.6 ± 0.0
(−)-epigallocatechin-3-*O*-gallate	238 ± 6
total flavan-3-ols	634 ± 6

[a] Data expressed as mean values ± SE (*n* = 3).

Table 3. Phenolic Acid Content of a 300 mL Infusion of Green Tea[a]

phenolic acid	free	conjugated
4-hydroxybenzoic acid	0.1 ± 0.0	0.1 ± 0.0
3-hydroxycinnamic acid	0.0 ± 0.0	1.6 ± 0.2
3,4-dihydroxybenzoic acid	0.5 ± 0.1	nd
4-coumaric acid	0.1 ± 0.0	7.4 ± 0.8
gallic acid	17.3 ± 1.4	nd
ferulic acid	nd	0.1 ± 0.0
total	18.1 ± 2.3	9.2 ± 1.0

[a] Data expressed as mean values in μmol ± SE (*n* = 3). nd, not detected.

supplementation, in both studies. The volumes excreted were measured and aliquots stored at −80 °C prior to analysis by GC-MS.

Flavan-3-ol Fecal Fermentations. Fecal samples were obtained from three healthy volunteers (two males, one female). They were nonsmokers, aged from 30 to 38 years, had not consumed antibiotics for at least 3 months prior to the study, and had avoided alcohol and polyphenol-rich foods for 48 h before fecal collection. Fecal samples were collected and incubated within 30 min of passage.

Fermentation Medium. The fermentation medium was prepared by mixing 2.0 g of tryptone in 200 mL of buffer solution (consisting of 2 g of $NH_3 \cdot CO_3$, 17.5 g of $Na_2 \cdot 2CO_3$, and 500 mL of distilled water), 200 mL of macromineral stock solution (2.85 g of $Na_2HPO_4 \cdot H_2O$, 3.1 g of $KH_2PO_4 \cdot H_2O$, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 500 mL of distilled water), 100 μL of micronutrient stock solution (consisting of 13.2 g of $CaCl_2 \cdot 2H_2O$, 10.0 g of $MnCl_2 \cdot 4H_2O$, 1.0 g of $CoCl_2 \cdot 6H_2O$, 8.0 g of $FeCl_3 \cdot 6H_2O$, and distilled water up to 100 mL), and 1.0 mL of 1% (w/v) resazurin solution (as a redox indicator). The medium was adjusted to pH 7 using HCl, boiled, and allowed to cool under oxygen-free nitrogen to remove oxygen. Twenty milliliters of reducing solution (consisting of 312 mg of cysteine hydrochloride, 2 mL of 1 M NaOH, 312 mg of sodium sulfide, and 47.5 mL of distilled water) was added to the medium.

In Vitro Fermentation. The in vitro fermentation method used in this study was adapted from Jaganath et al. (*25*, *26*). For each volunteer, 6.4 g of freshly voided fecal sample was homogenized with 20 mL of Sorensen's phosphate buffer, pH 7, to make a 32% fecal slurry. Five milliliters of the slurry was added to 44 mL of fermentation medium and 1 mL of substrate in 100 mL fermentation bottles to make a total volume of 50 mL. The substrates used were as follows: 50 μmol of either (−)-epicatechin, (−)-epigallocatechin, or (−)-epigallocatechin-3-*O*-gallate in the presence of 0.5 g of glucose. As a control, incubations with human fecal slurries were carried out without the addition of flavan-3-ols. After addition of the substrate, the fermentation bottles were purged with oxygen-free nitrogen and sealed airtight. All samples were placed horizontally in a shaking water bath at 60 strokes/min and incubated at 37 °C for 48 h, aiming to simulate conditions in the colonic lumen. Aliquots of fermented fecal samples (4.0 mL) were collected from each bottle after 0, 4, 6, 24, 30, and 48 h and stored immediately at −80 °C. The average initial pH of the cultures was 7.2, and at the end of the 48 h incubation period this had typically dropped to pH 6.7.

Extraction of Free and Conjugated Phenolic Acids in Green Tea. Extraction, fractionation, purification, and formation of trimethylsilyl derivatives of free phenolic acids in the green tea was carried out using a method developed by Zadernowski et al. (*27*). The aqueous green tea infusion was centrifuged at 2000*g* for 20 min, and the supernatant was acidified to pH 2 with 6 M HCl and extracted three times with ethyl acetate. The ethyl acetate extracts, which contained free phenolic acids, were combined, dried with anhydrous sodium sulfate, and reduced to dryness in vacuo at 40 °C. The residual aqueous phase containing conjugated phenolic acids was adjusted to pH 7.0 with 2 M NaOH and reduced in volume in vacuo at 40 °C, after which 20 mL of 4 M NaOH was added and the solution placed under an atmosphere of N_2 for 4 h at room temperature. The solution was acidified with 6 M HCl to pH 2.0 and partitioned three times against ethyl acetate (1:1 v/v). The ethyl acetate extracts were combined, dried, and reduced to dryness in vacuo.

Both extracts were dissolved in ethyl acetate, dried using activated molecular sieves (Sigma-Aldrich), transferred into glass vials, and dried under N_2 gas. The dried extracts were silylated in sealed vials using 300 μL of *N*,*O*-BSTFA + 1% TMCS heated at 80 °C for 80 min on a heating block. The sealed vials were vortexed every 30 min to achieve complete silylation. Care was taken during preparation as TMCS is highly sensitive to moisture. Samples were cooled in a closed dry container prior to analysis by GC-MS (Thermo Finnigan and Trace DSQ).

Extraction of Phenolic Acids in Human Fecal Slurries and Urine. Fecal slurries and urine were prepared as described previously (*28*, *29*). After thawing, 1 mL samples of urine and fecal extracts were added to 4 mL of 0.2 M HCl containing 100 μg of 2,4,5-trimethoxycinnamic acid as an internal standard. A styrene divinyl benzene solid phase extraction

Table 4. Quantities of Catabolites in Fecal Slurries from Three Donors Incubated for 4, 6, 24, 30, and 48 h with 50 μmol of either (−)-Epicatechin, (−)-Epigallocatechin, or (−)-Epigallocatechin-3-*O*-gallate[a]

substrate	catabolite	donor	4 h	6 h	24 h	30 h	48 h
(−)-epicatechin	4-hydroxyphenylacetic acid	I	nd	nd	2.3 ± 0.5	nd	1.4 ± 0.2
		II	0.3 ± 0.0	0.3 ± 0.0	nd	0.2 ± 0.0	0.1 ± 0.0
		III	0.2 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.6 ± 0.1
	3-(3-hydroxyphenyl)propionic acid	I	nd	nd	27.4 ± 4.3	nd	nd
		II	nd	nd	nd	0.3 ± 0.0	0.2 ± 0.0
		III	nd	0.3 ± 0.0	1.2 ± 0.1	4.4 ± 0.8	2.7 ± 0.4
	5-(3,4-dihydroxyphenyl)-γ-valeric acid	I	nd	nd	nd	nd	nd
		II	nd	nd	nd	2.6 ± 0.2	5.3 ± 0.1
		III	nd	0.1 ± 0.0	4.1 ± 0.5	16.3 ± 3.0	5.1 ± 0.2
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	I	nd	nd	2.2 ± 0.5	nd	nd
		II	nd	1.8 ± 0.2	0.5 ± 0.1	19.1 ± 1.1	1.8 ± 0.0
		III	0.3 ± 0.1	1.0 ± 0.1	nd	nd	nd
(−)-epigallocatechin	4-hydroxyphenylacetic acid	I	nd	nd	2.3 ± 0.3	3.1 ± 0.5	3.7 ± 0.9
		II	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
		III	0.2 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
	(−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	I	nd	nd	10.3 ± 2.2	20.6 ± 4.3	5.6 ± 1.5
		II	nd	nd	5.2 ± 3.4	5.7 ± 1.0	5.3 ± 1.7
		III	1.9 ± 0.1	7.8 ± 2.0	3.0 ± 0.2	3.2 ± 0.5	2.3 ± 0.0
(−)-epigallocatechin-3-*O*-gallate	pyrocatechol	I	nd	nd	9.0 ± 0.5	12.9 ± 1.3	19.1 ± 0.9
		II	0.8 ± 0.1	19.0 ± 0.2	33.9 ± 1.1	31.0 ± 9.0	19.3 ± 7.5
		III	nd	nd	nd	nd	nd
	pyrogallol	I	nd	nd	20.9 ± 1.0	4.1 ± 0.4	nd
		II	0.1 ± 0.0	2.0 ± 0.1	nd	nd	nd
		III	nd	nd	nd	nd	nd
	4-hydroxyphenylacetic acid	I	nd	nd	0.7 ± 0.1	1.6 ± 0.3	3.1 ± 0.2
		II	nd	nd	0.4 ± 0.2	0.3 ± 0.1	0.1 ± 0.0
		III	nd	nd	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
	(−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	I	nd	nd	10.3 ± 2.2	20.6 ± 4.3	5.6 ± 1.5
		II	nd	nd	5.2 ± 3.4	5.7 ± 1.0	5.3 ± 1.7
		III	1.9 ± 0.1	7.8 ± 2.0	3.0 ± 0.2	3.2 ± 0.5	2.3 ± 0.0
blank	4-hydroxyphenylacetic acid	I	nd	nd	0.2 ± 0.3	0.1 ± 0.0	0.2 ± 0.0
		II	nd	nd	nd	nd	0.1 ± 0.0
		III	nd	nd	nd	nd	0.2 ± 0.0
	3-(3-hydroxyphenyl)propionic acid	I	nd	nd	nd	nd	nd
		II	nd	nd	nd	nd	nd
		III	nd	nd	0.7 ± 0.2	0.4 ± 0.1	0.5 ± 0.1

[a] Data expressed as mean values in μmol ± standard error (n = 3). nd, not detected.

cartridge (Phenomenex, Macclesfield, U.K.) was used for sample purification. Before loading with the acidified samples, the cartridge was preconditioned with 5 mL of ethyl acetate, followed by 5 mL of methanol, and finally 5 mL of 0.1 M HCl. After the urine or fecal extract was added, the cartridge was washed with 5 mL of 0.1 M HCl and eluted in 3 mL of ethyl acetate. The upper ethyl acetate phase was separated from the lower aqueous phase and dried using an activated molecular sieve prior to being reduced to dryness in vacuo. The extracts were then silylated as described above in preparation for analysis by GC-MS.

GC-MS Analysis. Phenolic acids in silylated extracts of green tea, urine, and fecal slurries were analyzed by GC-MS (Trace DSQ, Thermo Finnigan) using a ZB-5MS 30 m × 0.25 i.d. × 0.25 μm capillary column (Phenomenex, Cheshire, U.K.) with helium as a carrier gas (1.0 mL/L). The GC-MS conditions were as follows: injection volume (1 μL), initial temperature at 80 °C for 5 min raised to 160 °C at 10 °C/min for 10 min and to 235 °C at 5 °C/ min for 10 min, injector temperature (280 °C), MS transfer line (290 °C), ion source (200 °C), and split ratio (1:100). Mass spectra were scanned at m/z 50−650 at 0.82 scans/s. Electron impact energy was 70 eV. Phenolic compounds were identified according to their retention time, mass spectra of authentic standards, and NIST 98 library screening. Quantifications were based on a standard curve of 2,4,5-trimethoxycinnamic acid (internal standard) with typical recoveries being ca. 80%. All standards and samples were analyzed in triplicate.

Statistical Analysis. All samples were analyzed in triplicate, and quantitative estimates are presented as mean values ± standard error (n = 3). Data on healthy human volunteers who drank green tea and water were subjected to statistical analysis using paired t test with Minitab software, version 13 (Minitab Inc., Addison-Wesley Publishing, Reading, MA).

RESULTS

GC-MS-Based Identification of Phenolic Acids and Catabolites. GC-MS was used to identify and quantify phenolic acids and related compounds in green tea, urine, and fecal slurries.

Table 5. Urinary Excretion of Catabolites by Five Human Subjects over a 24 h Period after Drinking 300 mL of either Green Tea or Water[a]

catabolite	drink	0−2 h	2−5 h	5−8 h	8−24 h	total
pyrocatechol	W	nd	n.d	nd	nd	nd
	GT	0.6 ± 0.6	0.1 ± 0.1	0.1 ± 0.1	1.4 ± 1.1[c]	2.2 ± 2.0[b]
pyrogallol	W	nd	nd	nd	nd	nd
	GT	0.6 ± 0.6	2.9 ± 1.6	8.5 ± 4.3	57 ± 32[c]	69 ± 29[b]
4-hydroxybenzoic acid	W	2.9 ± 1.6	3.2 ± 0.9	3.8 ± 1.9	3.5 ± 1.7	13 ± 2
	GT	1.6 ± 1.2	2.9 ± 1.6	4.5 ± 2.1	15 ± 4[c]	24 ± 5[b]
4-hydroxyphenylacetic acid	W	79 ± 47	70 ± 26	57 ± 23	64 ± 13	270 ± 106
	GT	64 ± 52	48 ± 22	56 ± 18	605 ± 306[c]	773 ± 330
3-methoxy-4-hydroxyphenylacetic acid	W	4.3 ± 1.9	4.6 ± 2.0	3.6 ± 1.6	5.6 ± 1.8	18 ± 7
	GT	5.1 ± 2.7	4.3 ± 1.7	5.5 ± 2.3	32 ± 10[c]	47 ± 10
hippuric acid	W	26 ± 10	29 ± 10	26 ± 8.6	56 ± 21	137 ± 43
	GT	31 ± 17	38 ± 23	88 ± 39	721 ± 187[c]	878 ± 238
3-(3-hydroxyphenyl)-3-hydroxypropionic acid	W	1.8 ± 0.7	1.1 ± 0.5	1.2 ± 0.5	2.7 ± 0.8	6.8 ± 1.8
	GT	3.1 ± 2.6	2.5 ± 0.8	4.7 ± 2.3	37 ± 18[c]	47 ± 18[b]
(−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	W	nd	nd	nd	nd	nd
	GT	nd	nd	0.6 ± 0.6	20 ± 15[c]	21 ± 16[b]

[a] Data expressed as mean values in μmol ± standard error ($n = 5$). GT, green tea; W, water; nd, not detected. [b] Catabolites, based on a paired t test, that are excreted in significantly higher amounts when volunteers drank green tea compared to water ($p < 0.001$). [c] Catabolites that are excreted in significantly higher amounts when volunteers drank green tea compared to water ($p < 0.05$).

Identifications were based on cochromatography with reference compounds, coupled with mass spectrometric fragmentation patterns and, when authentic standards were not available, by reference to published data (*27*, *28*, *30*, *31*) and the NIST 98 MS library. A summary of the 16 compounds identified in this manner is presented in **Table 1**.

Flavan-3-ols and Phenolic Acids in Green Tea. HPLC-PDA-MS analysis revealed that 300 mL of the green tea infusion contained a total of 634 μmol of flavan-3-ols with the main components being (−)-epigallocatechin-3-*O*-gallate (238 μmol) and (−)-epigallocatechin (190 μmol) (**Table 2**). In comparison, the free and conjugated phenolic acid content of the tea measured, with GC-MS, was extremely low, with free gallic acid (17.3 μmol) and conjugated 4-coumaric acid (7.4 μmol) being the principal constituents (**Table 3**).

Catabolism of Flavan-3-ols in Fecal Slurries. In our earlier study in which green tea was fed to volunteers with an ileostomy, although some absorption occurred in the small intestine, particularly of (epi)catechins, there was a substantial recovery of flavan-3-ols and their metabolites in ileal fluid (*12*), indicating that in subjects with an intact function colon, these compounds would pass to the lower bowel and be subjected to the action of colonic bacteria. To investigate the potential fate of these compounds in the large intestine, 50 μmol of key green tea flavan-3-ol monomers was incubated with fecal samples from three subjects for 48 h, under anaerobic conditions.

Incubation of the fecal slurries with (−)-epicatechin resulted in the appearance of four catabolites. 4-Hydroxyphenylacetic acid was present in only small amounts, whereas 3-(3-hydroxyphenyl)propionic acid, (−)-5-(3′,4′-dihydroxyphenyl-γ-valerolactone, and 5-(3,4-dihydroxyphenyl)-γ-valeric acid appeared, albeit transitorily, after 24−48 h incubation periods in amounts, which on a mole basis, correspond to 32−54% of the (−)-epicatechin substrate (**Table 4**). As is typical with this model system, there were substantial differences in the catabolite profiles of the individual subjects, presumably reflecting variations in their colonic microflora (*25*, *26*).

Breakdown of (−)-epigallocatechin by the fecal bacteria also yielded small quantities of 4-hydroxyphenylacetic acid along with much larger quantities of (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, in one instance 20.6 μmol, equivalent to 41% of the added (−)-epigallocatechin (**Table 4**). In contrast to the incubations with (−)-epicatechin when 5-(3,4-dihydroxyphenyl)-γ-valeric acid was produced, catabolism of (−)-epigallocatechin did not yield the equivalent compound, 5-(3,4,5-trihydroxyphenyl)-γ-valeric acid, in detectable quantities.

Fecal incubation of (−)-epigallocatechin-3-*O*-gallate also resulted in conversion of the (−)-epigallocatechin moiety to (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and trace amounts of 4-hydroxyphenylacetic acid, whereas cleavage of the 3-*O*-gallate group resulted in the appearance of pyrogallol and its dehydroxylation product pyrocatechol (**Table 4**). At its highest level, 33.9 μmol, in fecal material from volunteer II after 24 h, the pyrocatechol content corresponded to release of the 3-*O*-gallate group from 68% of the 50 μmol of (−)-epigallocatchin-3-*O*-gallate added to the incubate. Fecal material incubated for 48 h without the addition of flavan-3-ols contained only minor quantities of 4-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)-propionic acid (**Table 4**).

Urinary Excretion of Flavan-3-ol Catabolites after the Ingestion of Green Tea and Water. Data on the presence of phenolic acids and related compounds in urine collected over the 24 h period after the consumption of either green tea or water by healthy subjects with an intact colon are presented in **Table 5**. Three compounds, pyrocatechol, pyrocatechol, and (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, all of which were products of flavan-3-ol degradation in fecal slurries (**Table 4**), were detected exclusively in urine collected after the consumption of green tea. Pyrocatechol and pyrogallol are both derived from the gallic acid moiety of 3-*O*-gallated flavan-3-ols, whereas (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone is a colonic catabolite of (epi)gallocatechins (**Table 4**). Other compounds present in 0−24 h urine after drinking water but excreted in significantly higher quantities after green tea consumption were 4-hydroxybenzoic acid and 3-(3-hydroxyphenyl)-3-hydroxypropionic acid. Larger amounts of 4-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, and hippuric acid were detected in the 0−24 h urine, but the quantities excreted after drinking green compared to water were not statistically different (**Table 5**). In general, the quantities of the individual catabolites excreted after drinking water changed little during the 0−2, 2−5, 5−8, and 8−24 h periods after collection. In contrast, following green tea consumption, the level of catabolites increased markedly in the 8−24 h urine, which is in keeping with the colonic origin of these compounds. As with flavan-3-ol degradation in the fecal slurries, there were substantial variations in the urinary excretion of

Table 6. Urinary Excretion of Catabolites by Five Human Subjects with an Ileostomy over a 24 h Period after Drinking 300 mL of Green Tea[a]

catabolite	0−2 h	2−5 h	5−24 h	total
pyrocatechol	nd	nd	nd	nd
pyrogallol	nd	nd	nd	nd
4-hydroxybenzoic acid	nd	nd	nd	nd
4-hydroxyphenylacetic acid	1.4 ± 0.6	2.7 ± 1.0	51 ± 33	55 ± 34
3-methoxy-4-hydroxyphenylacetic acid	nd	nd	nd	nd
hippuric acid	0.8 ± 0.5	1.4 ± 1.4	70. ± 42	72 ± 43
3-(3-hydroxyphenyl)-3-hydroxypropionic acid	nd	nd	nd	nd
(−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone	nd	nd	nd	nd

[a] Data expressed as mean values in μmol ± standard error (n = 5). nd, not detected.

catabolites originating from the large intestine probably reflecting, as observed previously (*25*, *26*), variations in the colonic microflora.

Urine collected from subjects with an ileostomy over the 24 h period after the consumption of green tea contained a much simpler spectrum of catabolites compared to urine from subjects with an intact colon, with the only compounds excreted being substantially reduced quantities of 4-hydroxyphenylacetic acid and hippuric acid (**Table 6**).

DISCUSSION

In an earlier separate investigation, ileostomists drank 300 mL of the same green tea used in the current study. Of the ingested 634 μmol of flavan-3-ol monomers, 206 μmol of the parent compounds and 232 μmol of their methylated, sulfated, and glucuronide metabolites were recovered in the 0−24 h ileal fluid (*12*). More specifically, the ileal fluid contained 117 μmol of (epi)catechins and 321 μmol of (epi)gallocatechins and their metabolites, of which 153 μmol was 3-*O*-gallated flavan-3-ols that included 123 μmol of (epi)gallocatechin-3-*O*-gallates. In total there was a 69% recovery in ileal fluid, and in subjects with a functioning large intestine this represents flavan-3-ols that would pass to the lower bowel, where they will be subjected to ring fission as a result of the catabolic action of colonic microflora.

To assess colonic catabolism of flavan-3-ols, the fate of 50 μmol of (−)-epicatechin, (−)-epigallocatechin, and (−)-epigallocatechin-3-*O*-gallate was investigated using an in vitro fecal fermentation model. In vitro fermentation has been used by other researchers to study colonic catabolism of flavonoids (*31*−*35*), but, in most cases, the experimental approach used varied with respect to the inoculum size, type of media, anaerobic conditions, and choice of buffer. In this study a relatively large amount of inoculum, 320 g/L, was used to provide a large initial bacterial population, which ensured a better survival rate of the bacterial species, with glucose being used as a carbon source (*36*). The main limitation of in vitro fermentation is that it may not fully depict the in vivo conditions. The removal of fecal material may alter the bacterial composition and thus may not represent the microflora in the colonic lumen and on the colonic mucosa where catabolism occurs in vivo. The accumulation and retention of the degradation products in the fermentation vessel make collection, identification, and quantification of the metabolites easier, but is not necessarily representative of the events that occur in vivo, when the actual concentration of a metabolite at any time interval is dependent on the combined rates of catabolism and absorption. and this cannot be simulated in vitro. However, use of this in vitro model provides information on the types of breakdown products formed and helps elucidate the pathways involved, and the rate of catabolism can be determined in a quick, reliable, and affordable manner (*36*).

The predominant fecal degradation products of (−)-epicatechin were (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3,4-dihydroxyphenyl)-γ-valeric acid, and 3-(3-hydroxyphenyl)propionic acid, which at specific time points accumulated in amounts corresponding to 38, 32, and 54%, respectively, of the substrate dose (**Table 4**). Trace quantities of 3-hydroxyphenylacetic acid were also produced. In keeping with studies by Toshiyuki et al. (*37*), this indicates that the breakdown of (−)-epicatechin by colonic bacteria leads to the production of (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone with 1-(3′,4′)-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxy)propan-2-ol, which did not accumulate in detectable amounts in the fecal slurries, acting as a transient intermediate, as illustrated in **Figure 2**. The dihydroxyvalerolactone is then converted to 5-(3,4-dihydroxyphenyl)-γ-valeric acid that is further metabolized to 3-(3-hydroxyphenyl)propionic acid which, in turn, yields low levels of 4-hydroxyphenylacetic acid.

Incubation with 50 μmol of (−)-epigallocatechin-3-*O*-gallate led to the appearance of pyrogallol and pyrocatechol in quantities indicating a >60% cleavage of the 3-*O*-galloyl group (**Table 4**) and release of (−)-epigallocatechin (**Figure 2**). As a consequence, except for pyrogallol and pyrocatechol, incubations with (−)-epigallocatechin-3-*O*-gallate and (−)-epigallocatechin resulted in the production of very similar catabolite profiles (**Table 4**). This implies the operation of a degradative route parallel to that observed with (−)-epicatechin as illustrated in **Figure 2**, although, presumably because of a rapid rate of turnover, neither 1-(3′,4′,5′)-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxy)propan-2-ol nor 5-(3,4,5-trihydroxyphenyl)-γ-valeric acid was produced in detectable quantities.

Three hundred milliliters of the same green tea fed previously to ileostomists in the study in which the flavan-3-ol content of ileal fluid was determined (*12*), was, in the present investigation, ingested by healthy human subjects with an intact colon, after which 0−24 h urine was collected. For comparative purposes in a crossover study the healthy subjects also drank 300 mL of water, following which urine was collected for 24 h. Data on the urinary catabolites excreted following the intakes of green tea and water are presented in **Table 5**. Pyrogallol and pyrocatechol were detected in urine only after green tea consumption and in a combined quantity, 71.2 μmol, which is equivalent to 47% of the 153 μmol of 3-*O*-galloylated flavan-3-ols detected in ileal fluid after ingestion of the green tea (*12*). This in vivo figure is broadly in keeping with the 68% degalloylation of (−)-epigallocatechin-3-*O*-gallate observed with the in vitro fecal incubates (**Table 4**).

Other 0−24 h urinary catabolites, excreted in significantly higher amounts after green tea consumption, and probably derived from the flavan-3-ol skeleton, possibly via routes proposed in **Figure 2**, are 5-(3,4,5-trihydroxyphenyl)-γ-valeric acid, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, and 4-hydroxybenzoic acid (**Table 5**). Additional compounds excreted over the 24 h collection period in higher but not in statistically different amounts after the ingestion of green tea compared to water were low quantities of 3-methoxy-4-hydroxyphenylacetic acid and much more substantial amounts of 4-hydroxyphenylacetic acid and hippuric acid. Total excretion of these latter two catabolites,

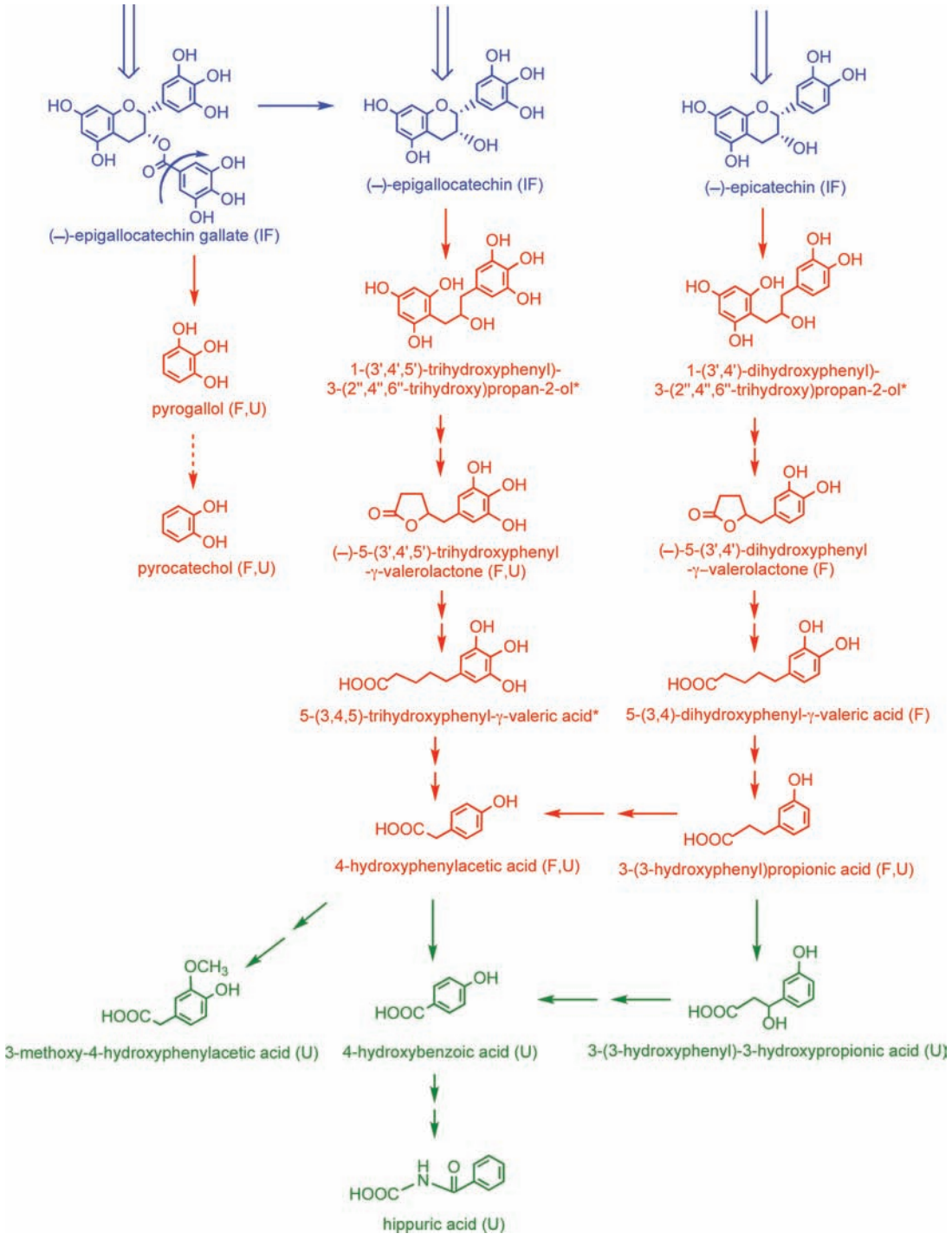

Figure 2. Proposed pathways involved in the colonic catabolism and urinary excretion of green tea flavan-3-ols. Following consumption of green tea >50% of the ingested (−)-epicatechin, (−)-epigallocatechin, and (−)-epigallocatechin-3-*O*-gallate (blue structures) pass into the large intestine. When incubated with fecal slurries, these compounds are catabolized by the colonic microflora probably via the pathways illustrated with red structures. Analysis of urine after green tea consumption indicates that some of the colonic catabolites enter the circulation and undergo further metabolism before being excreted in urine. Green structures indicate catabolites detected in urine but not produced by fecal fermentation of (−)-epicatechin, (−)-epigallocatechin, or (−)-epigallocatechin-3-*O*-gallate. The dotted arrow between pyrogallol and pyrocatechol indicate this is a minor conversion. Double arrows indicate conversions where the intermediate(s) did not accumulate and are unknown, although metabolism of 4-hydroxyphenylacetic acid to 3-methoxy-4-hydroxyphenylacetic acid probably proceeds via 3,4-dihydroxyphenylacetic acid. IF, compounds detected in ileal fluid after green tea consumption; F, U, catabolites detected in fecal slurries and in urine, respectively; (*) potential intermediates that did not accumulate in detectable quantities in fecal slurries.

at 503 and 873 μmol, respectively, was in excess of the 438 μmol of flavan-3-ols that entered the large bowel after green tea ingestion.

There was a relatively low excretion of 3-methoxy-4-hydroxyphenylacetic acid during the 0−2, 2−5, and 5−8 h collection periods after the consumption of either water or green tea, but there was a marked and significant increase in the quantity excreted 8−24 h after the consumption of green tea, although the total amounts excreted over the 0−24 h collection period were not significantly different (**Table 5**). A similar situation was apparent with the 8−24 h excretion of 4-hydroxyphenylacetic

acid (605 μmol) and hippuric acid (721 μmol). However, the quantities of these compounds that were excreted are both in excess of the 438 μmol of flavan-3-ols entering the large bowel after green tea ingestion. This indicates that they are produced by additional routes unrelated to colonic degradation of flavan-3-ols. It is, for instance, well-known that there are pathways to hippuric acid from compounds such as benzoic acid, quinic acids (*38*), tryptophan, tyrosine, and phenylalanine (*39*−*41*). Nonetheless, the elevated urinary excretion of hippuric acid and 4-hydroxyphenylacetic acid, occurring after green tea consumption, is likely to be partially derived from flavan-3-ol degradation. Earlier research showing statistically significant increases in urinary excretion of hippuric acid after the consumption of both green and black tea by human subjects (*38*, *42*) supports this supposition.

Four catabolites, 4-hydroxybenzoic acid, hippuric acid, 3-methoxy-4-hydroxyphenylacetic acid, and 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, were detected in urine (**Table 5**) but did not accumulate in the fecal slurries following incubation with flavan-3-ols (**Table 4**). Conversely, (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3,4-dihydroxyphenyl)-γ-valeric acid, which were major (−)-epicatechin catabolites in fecal slurries, were not excreted in urine. Other compounds, pyrogallol, pyrocatechol, 4-hydroxyphenylacetic acid, and 5-(3,4,5-trihydroxyphenyl)-γ-valeric acid, appeared in both fecal incubations and urine. This suggests that the disappearing compounds are products of microbial degradation in the large intestine and that, after absorption into the circulatory system, they are converted, presumably in the liver or kidneys, to at least some of the compounds that are excreted in urine but which did not accumulate in the fecal slurries. Potential routes for such conversions are summarized in **Figure 2**.

As discussed earlier, the excretion of pyrocatechol and pyrogallol indicates a ca. 47% cleavage of the gallate group from the 3-*O*-galloylated flavan-3-ols entering the colon from the small intestine. Quantitative estimates of the extent of ring fission of the flavan-3-ol skeleton are more difficult to assess because of the contribution of additional routes to the production of urinary catabolites, in particular, hippuric acid and 4-hydroxyphenylacetic acid. If these two compounds are excluded, along with pyrogallol and pyrocatechol, from the data in **Table 5**, 0−24 h excretion of the remaining five catabolites after the consumption of green tea was 210 μmol compared to 38 μmol after the consumption of water. The 172 μmol difference between these two figures corresponds to a 39% degradation of the flavan-3-ols entering the colon after the consumption of green tea. This figure is a minimum value as some urinary catabolites, such as valerolactone sulfates and glucuronide, may have escaped detection. In addition, there is a ca. 8% excretion of glucuronide, sulfate, and methylated metabolites of flavan-3-ols originating from absorption in the small intestine (*10*, *12*).

In summary, the current study establishes that, despite absorption in the small intestine, sizable quantities of flavan-3-ol monomers pass from the small intestine to the lower bowel where, as result of the action of the colonic microflora, they are degraded and phenolic acid catabolites, after being absorbed and passing through the circulatory system, are excreted in urine in quantities corresponding to ca. 40% of flavan-3-ol intake. Although most studies to date have focused on absorption in the small intestine, it is evident that the role of the colonic microflora is a key part of the overall bioavailability equation and may well be an important aspect of bioactivity of dietary flavonoids. In this regard, it is of interest that a recent study has demonstrated that 3,4-dihydroxyphenylacetic acid, dihydrocaffeic acid, and dihydroferulic acid, colonic catabolites found in high concentrations in plasma, urine, and fecal water after the consumption of fruit and vegetables and coffee (*43*, *44*), have anti-inflammatory activities (*45*).

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Supporting Information Available: Bioavailability of green tea flavan-3-ols and the role of the colon. This material is available free of charge via the Internet at http://pubs.acs.org.

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