Nutrient Metabolism

Polymeric Proanthocyanidins Are Catabolized by Human Colonic Microflora into Low-Molecular-Weight Phenolic Acids[1]

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ABSTRACT   Polymeric proanthocyanidins are common constituents of many foods and beverages. Their fate in the human body remains largely unknown. Their metabolism by human colonic microflora incubated in vitro in anoxic conditions has been investigated using nonlabeled and ^{14}C-labeled purified proanthocyanidin polymers. Polymers were almost totally degraded after 48 h of incubation. Phenylacetic, phenylpropionic and phenylvaleric acids, monohydroxylated mainly in the *meta* or *para* position, were identified as metabolites by gas chromatography coupled to mass spectrometry (GC-MS). Yields were similar to those previously reported for flavonoid monomers. These results provide the first evidence of degradation of dietary phenolic polymers into low-molecular-weight aromatic compounds. To understand the nutritional properties of proanthocyanidins, it is therefore essential to consider the biological properties of these metabolites.   J. Nutr. 130: 2733–2738, 2000.

KEY WORDS: • *proanthocyanidins* • *flavonoids* • *bioavailability* • *biodegradation* • *human colonic microflora*

Proanthocyanidins (PA,[3] or condensed tannins) are among the most abundant polyphenolic compounds in plants and are common constituents of many foods (various fruits, legume seeds, chocolate) and beverages (fruit juices, wine, beer, cider, tea) (Santos-Buelga and Scalbert 2000, Shahidi and Naczk 1995, Swain 1962). PA differ from all other natural polyphenols by their polymeric nature. They are made of flavan-3-ol units and their average degree of polymerization generally varies between 3 and 11 (**Fig. 1**) (Santos-Buelga and Scalbert 2000). Polymerization degree may reach values as high as 17 as was shown in an apple cider extract by liquid chromatographic-mass spectrometric (LC-MS) analysis (Guyot et al. 1997). Two main types of PA can be distinguished according to the substitution pattern of their B-ring, i.e., procyanidins have two vicinal hydroxy groups and prodelphinidins three. The phenolic and polymeric nature of PA makes them good complexants of proteins and explains the astringent character of foods and beverages rich in PA (e.g., grape skin and seeds, unripe fruits, wine or cider) (Haslam 1998). The daily intake of PA has been estimated at 0.1–0.5 g (Kühnau 1976, Santos-Buelga and Scalbert 2000, Scalbert and Williamson, 2000).

However, the complexity of their chemical structure and the lack of a suitable method for their estimation make these figures uncertain.

Various epidemiologic data on PA-rich wine and tea and experimental studies on both animals and cell cultures using either PA-rich food or pure PA have suggested that they prevent cancers and cardiovascular diseases in humans [for a review, see Santos-Buelga and Scalbert (2000)]. These effects suggest that some PA are absorbed through the gut barrier. Their absorption depends on their degree of polymerization. In some in vitro experiments, only PA dimers and trimers, but not polymers with an average polymerization degree of 7, were absorbed through an intestinal epithelium cell monolayer (Déprez 1999). PA polymers were not better absorbed in vivo in chicken and sheep (Jimenez-Ramsey et al. 1994, Terrill et al. 1994).

These PA polymers could be degraded by the colonic microflora into low-molecular-weight compounds, which would be subsequently absorbed. This has been demonstrated for other dietary polyphenols such as isoflavones by comparing normal and germfree animals or humans before and after antibiotic administration (Axelson and Setchell 1981, Axelson et al. 1982, Setchell et al. 1981). A procyanidin dimer (dimer B3) (Groenewoud and Hundt 1986) and (+)-catechin (Groenewoud and Hundt 1984, Scheline 1991) were shown to be degraded into phenolic acids and nonphenolic aromatic metabolites. Some of these metabolites were also found in the urine of rats fed a grape extract containing a mixture of catechin monomers and PA oligomers of low polymerization
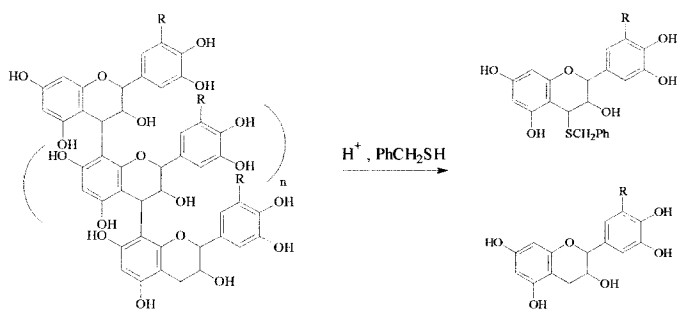
FIGURE 1 Proanthocyanidin depolymerization by thiolysis into benzylthio(gallo)catechins and (+)-catechin. R = H: procyanidins; R = OH: prodelphinidins.

degree (Harmand and Blanquet 1978). No study of this kind has been carried out to date with human microflora and high polymerization degree PA, the most abundant in the human diet.

We report here the in vitro degradation of ^{14}C-labeled PA polymers by human microflora. Labeled PA polymers were obtained by incorporation of a radiolabeled precursor to willow tree shoots. They were carefully purified and were free of any PA dimer and trimer or any other phenolic or nonphenolic contaminants (Déprez et al. 1999). PA in willow tree leaves are procyanidins, typical of the most common PA in food (Santos-Buelga and Scalbert 2000). The radiolabeled PA had an average polymerization degree of 6, close to that of other PA found in apples (Guyot et al. 1998) or grape seeds (Prieur et al. 1994).

MATERIALS AND METHODS

General. 5-(*m*-Hydroxyphenyl)valeric and 5-(*m,p*-dihydroxyphenyl)valeric acids were kindly provided by Ronald Scheline (University of Bergen, Norway). 3-(*m*-Hydroxyphenyl)propionic acid was a gift of Gerhard Groenewoud (Farmovs Research Center, Bloemfontein, South Africa). Benzoic acid was purchased from Prolabo (Nogent-sur-Marne, France) and all other aromatic acids from Sigma-Aldrich (Saint-Quentin Fallavier, France). Total radioactivity of samples was determined by liquid scintillation counting of aliquots (10 μL). Liquid scintillation radioactivity counting was controlled with a Packard Tri-Carb 1500 analyzer (Meriden, CT) (liquid scintillation cocktail: Ecolite, ICN Pharmaceuticals France, Orsay, France); counting efficiency was 95%.

Nonlabeled and ^{14}C-labeled proanthocyanidin polymers. PA polymers were purified from the leaves of an adult willow tree (*Salix caprea* L.) by chromatography on a Sephadex LH 20 (Pharmacia, Guyan-Court, France) (Déprez et al. 1999). They were free of any catechin monomers or PA dimer and trimer. ^{13}C nuclear magnetic resonance spectra indicated a procyanidin-type polymer, with a predominance of 2,3-*trans* (catechin) units. Polymers were analyzed by thiolysis (**Fig. 1** (Déprez 1999, Matthews et al. 1997) and gave two main thioadducts, 3,4-*cis*- and 3,4-*trans*-benzylthiocatechins, formed from inner catechin units (yield 45.0%), low amounts of 3,4-*trans*-benzylthioepicatechin derived from inner epicatechin units (yield 1.4%) and (+)-catechin derived from terminal units (yield 7.7%). The ratio of thioethers and (+)-catechin allowed the calculation of an average polymerization degree of 7.

^{14}C-PA polymers were prepared by administration of [1-^{14}C]-acetate to willow shoots and similar purification by chromatography on Sephadex LH 20 (Déprez et al. 1999). Specific activity was 7.0 MBq/g. The yield of thioadducts formed by thiolysis was 42.2%. Thioadducts 3,4-*cis*- and 3,4-*trans*-benzylenzylthiocatechins represented 95% of the thioethers. An average polymerization degree of 6 was calculated. All other characteristics were similar to those given above for nonlabeled PA.

Degradation of proanthocyanidin polymers by the microflora. The brain heart infusion (BHI) medium was made of calf brain and beef heart infusion (37 g/L; Difco, Becton Dickinson France SA, Le Pont de Claix, France), hemine (5 mg/L; Sigma-Aldrich) and yeast extract (5 g/L; Difco). The pH was adjusted to 7.4 with NaOH and the medium sterilized (120°C, 20 min). It was placed in anaerobic conditions (5% CO_2, 10% H_2, 85% N_2) 48 h before fermentation experiments. Fecal suspensions were prepared by mixing fecal samples (1 g) (freshly collected from a human subject habitually consuming a Western type diet) in BHI medium (100 mL).

Aqueous solutions of nonlabeled PA polymers (100 mmol/L expressed as catechin unit equivalents, i.e., 29 g/L) were sterilized by filtration on Millex-GS sterile units (25 mm, 0.22 μm, Millipore, Saint-Quentin en Yvelines, France); 150 μL was added to the fecal suspensions (2.85 mL; final PA concentration, 5 mmol/L) under anaerobic conditions (Freter glove box) at 37°C without shaking. Aliquots (500 μL) were sampled at 0, 6, 12, 24 and 48 h. All experiments were carried out in duplicate.

Similar experiments were carried out 1 mo later with aqueous solutions of radiolabeled PA polymers (50 mmol/L expressed as catechin unit equivalents). These solutions (200 μL) were added to fecal suspensions (1.80 mL; final concentration of polymers, 5 mmol/L; 18 kBq/flask). Samples (300 μL) were taken at 0, 6, 12, 24, 36 and 48 h. Four controls were performed, i.e., polymers incubated in water or in BHI medium without flora; flora in BHI medium without PA; flora heat-inactivated (120°C, 20 min) and in BHI medium with PA. All experiments were conducted in duplicate.

Samples were stored at −20°C before analysis. The raw extracts were centrifuged for 5 min at room temperature (15,000 × g) before analysis by cellulose TLC. Aliquots (40 μL) were also acidified with HCl (6 mol/L) saturated with NaCl (final pH ~1), extracted three times by ethyl acetate and concentrated under vacuum before silica TLC and gas chromatography coupled to mass spectrometry (GC-MS) analysis. Due to the identification of an arylsulfotransferase enzyme in a bacterial strain isolated from human feces (Koizumi et al. 1990 and 1991), some fermentation samples (48 h) were treated with a sulfatase of *Patella vulgata* (Sigma-Aldrich; 5 h at 37°C, 0.5 mg; 10 U/mg) before extraction by ethyl acetate (EtOAc); however, no change in GC chromatograms was observed.

Analysis of PA by thiolysis. Thiolysis was performed on the raw fermentation samples according to a procedure established on plant extracts (Matthews et al. 1997) except that the amount of added acetic acid (AcOH) was increased to take into account the buffering capacity of the growth medium. Toluene-α-thiol (90 μL), AcOH (300 μL) and the fermentation sample (200 μL) were added to ethanol/water 5:1 (2.5 mL) and the mixture acidified to pH 3.8 by further addition of AcOH, purged with nitrogen. The tubes were tightly closed (Teflon-lined screw cap), and the thiolysis reaction was conducted at 105°C in an oil bath over a period of 24 h under stirring. Samples were cooled and centrifuged (2 min, 20°C, 11,300 × g) and the supernatant dried under reduced pressure. Thiolysis products were solubilized in methanol (200 μL) and the solution filtered on Millex-GV units (Millipore, 4 mm, 0.22 μm) before HPLC analysis (Matthews et al. 1997). The chromatographic conditions were slightly modified to avoid an overlap between one of the thioadducts and a compound present in the samples but not derived from the PA polymers. Analyses were carried out on a Lichrospher 100 RP-18 column (5 μm, 25 cm × 4 mm i.d.; Merck, Darmstadt, Germany) with the following elution conditions: solvent A, H_3PO_4 0.1% in water; solvent B, methanol; linear gradient: 20–70% B in 30 min; flow rate, 1 mL/min; detection was performed at 280 nm. Yields are expressed as the percentage of the products obtained by thiolysis of the nonfermented PA polymer.

TLC analysis. Raw extracts were analyzed on high performance cellulose thin layer plates (aluminum foils, thin layer thickness 0.1 mm, Merck) with AcOH/water 6:94 (v/v) as eluent. EtOAc extracts were spotted on silica plates (aluminum foil, ~5 × 10 cm, 0.25 mm, Merck) and eluted with CHCl$_3$/AcOH/water 4:1:1 (v/v/v) on a length of 5–8 cm (Griffiths and Smith 1972). Electronic autoradiographies of thin layer chromatograms were obtained with a Storm 860 (Molecular Dynamics, Amersham Pharmacia Biotech, Orsay, France) electronic system. Dried cellulose plates were imaged for 3–24 h.
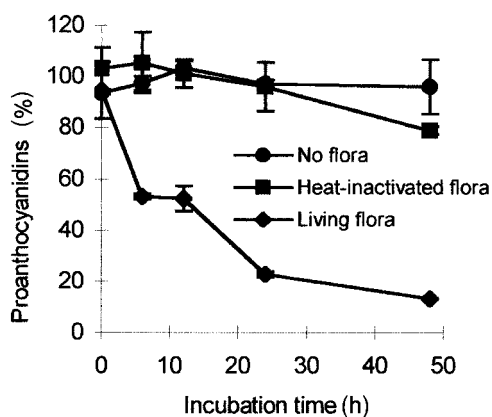
FIGURE 2 Anaerobic degradation of proanthocyanidin polymers by human colonic microflora assessed by thiolysis. Values are expressed as means ± SD, $n = 2$.

Calibration was performed with a standard solution of [U-^{14}C]-phenylalanine.

GC-MS analysis. EtOAc extracts were dried over Na_2SO_4 and aliquots (10 μL) silylated with 50 μL bis-trimethylsilyltrifluoroacetamide and 5 μL pyridine before GC-MS analysis. Silylated compounds were analyzed with a Varian Star 3400 chromatograph (Les Ulis, France), on a SPB5 polydimethylsiloxane capillary column (30 m × 0.2 mm i.d., 0.25 μm, Supelco, Sigma-Aldrich) with helium as carrier gas (0.5 bar inlet pressure). The column temperature was raised by 30°C/min from 40 to 110°C, then by 2.5°C/min from 110 to 280°C. MS of trimethylsilylated derivatives (70 eV electronic impact, 50–650 m/z range, positive mode) was performed with a Saturn 2000 ion trap instrument (Varian). Compounds were identified by comparison of their MS spectra to those of reference compounds. Standards of phenolic acids hydroxylated in the *ortho*, *meta* or *para* position or nonhydroxylated were well separated by GC; the elution order was as follows: nonhydroxylated < *ortho* < *meta* < *para* as previously reported (Groenewoud and Hundt 1986). Positional isomers all gave different mass spectra by GC-MS. The relative abundance of the phenolic acids was estimated from the relative surface area of their trimethylsilylated derivatives on the total ion chromatogram.

RESULTS

PA polymers were incubated in the presence of freshly isolated human colonic microflora in anaerobic conditions and their degradation followed by thiolysis; this method allows a reliable estimation of PA in complex samples (Fig. 1) (Matthews et al. 1997). A major drop of thiolysis yield was observed after 48 h of incubation (**Fig. 2**). This drop was not observed in the presence of a heat-inactivated flora or in the absence of flora.

To trace the newly formed metabolites, incubation was repeated with ^{14}C-labeled PA. Some labeled products that formed after 36 h of incubation were observed on autoradiograms of cellulose TLC chromatograms (**Fig. 3**). These products represented 9–22% of the initial PA radioactivity (according to the sample and duration of incubation). They were not observed in killed microflora controls. The total radioactivity measured in the liquid medium after incubation was close to the initial radioactivity introduced into the medium. The formation of gaseous metabolites such as CO_2 and CH_4 was therefore negligible.

Labeled metabolites were then extracted from the crude medium by EtOAc and analyzed by silica HPTLC. Four main metabolites (M1–M4) were detected (**Fig. 4**). After 48 h of incubation, these EtOAc-soluble metabolites represented 2.7% of the initial radioactivity. Their chemical structures were determined by GC-MS analysis. Six phenolic acids [2-(p-hydroxyphenyl)acetic acid (pHPAc), 2-(p-hydroxyphenyl)-propionic acid (pHPP) and their m-hydroxy isomers 2-(m-hydroxyphenyl)acetic acid (mHPAc) and 2-(m-hydroxyphenyl)propionic acid (mHPP), 5-(m-hydroxyphenyl)valeric acid (mHPV) and phenylpropionic acid (PP)] were specifically observed after incubation of PA polymers with the living flora and were absent in all controls (**Figs. 5** and **6**). No dihydroxylated phenolic acid could be detected. The nonphenolic aromatic compounds phenylacetic acid (PAc) and benzoic acid (BA) were also identified. However, they were not specific for PA and could also derive from the metabolism of other compounds such as protein aromatic acids present in the brain heart growth medium or could have been present initially in the fecal sample. Co-chromatography with authentic samples allowed us to identify the metabolites M2 and M3 as mHPP and mHPV, respectively. Radioactive compounds eluted in the solvent front (M4) are likely nonhydroxylated aromatic acids as suggested by the similar chromatographic behavior of BA, PAc and PP.

Relative abundances of aromatic acids were deduced from GC-MS chromatograms (**Fig. 7**). pHPAc and pHPP acids detected after 6 h-incubations disappeared progressively after a longer period of incubation. The proportions of their m-isomers and of PP increased in the first 10 h of incubation and then remained stable. mPP was the most abundant aromatic acid in all samples whatever the time of incubation. Some differences in the kinetics of accumulation of mHPP were
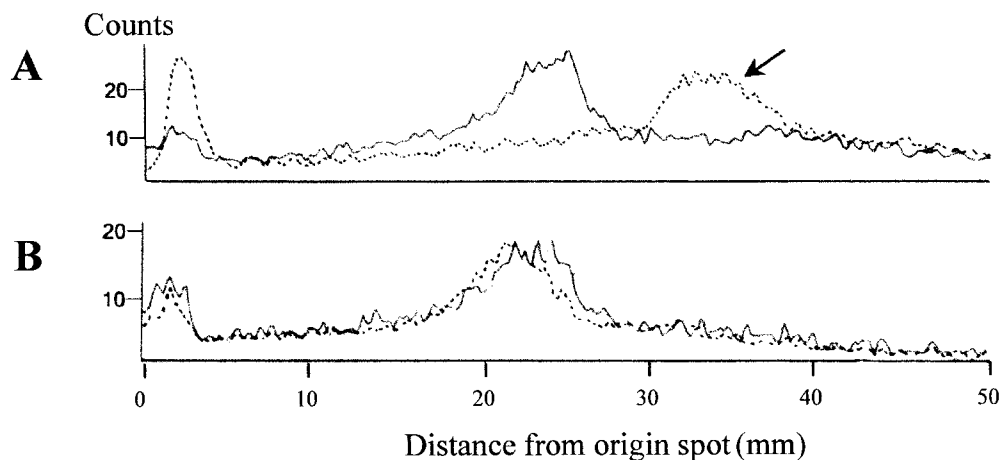

FIGURE 3 Autoradiograms of the crude incubation media of ^{14}C-labeled proanthocyanidin polymers with human colonic microflora. Proanthocyanidins and their degradation products were separated by cellulose TLC with acetic acid (AcOH)/water 6:94 as eluent. (*A*) Living microflora; (*B*) heat-inactivated microflora. Plain curve: medium sampled just after inoculation; dotted curve: medium sampled after 36 h incubation. The arrow shows the newly formed metabolites.
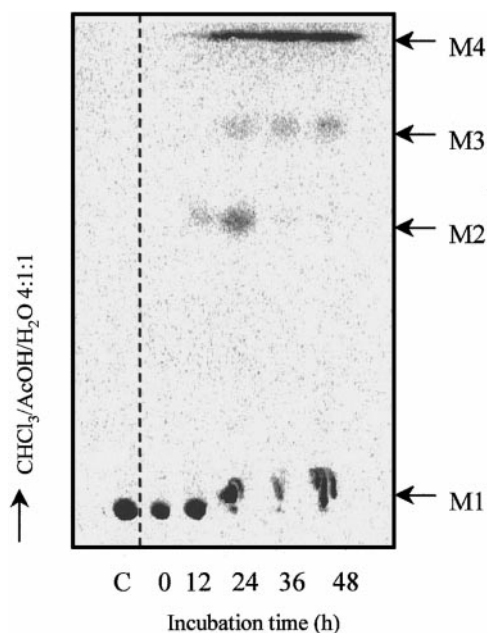
FIGURE 4 Autoradiogram of ethyl acetate extracts of proanthocyanidin polymers incubated with human colonic microflora over various time periods. The products were separated by silica TLC with CHCl₃/acetic acid (AcOH)/water 4:1:1 as eluent. C, control with a heat-inactivated microflora incubated for 36 h. M1–M4: proanthocyanidin metabolites.
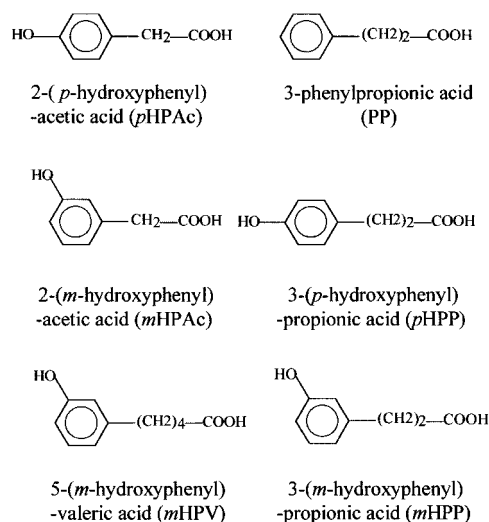

FIGURE 6 Proanthocyanidin metabolites formed by incubation with human colonic microflora.

observed in different experiments. In the first experiment, *m*HPP (Fig. 4, metabolite M2) disappeared after 36 h of incubation, whereas its concentration was found to be stable when the experiment was repeated 1 mo later. These differences may be explained by variations in the composition and activity of the microflora induced by diet modifications (Cummings et al. 1989).

DISCUSSION

These results show for the first time that PA polymers, which cannot be absorbed through the small intestinal barrier because of their high molecular weight (Déprez 1999), can be degraded by the colonic microflora into low-molecular-weight aromatic acids, which differ according to their hydroxylation profile and the length of the aliphatic side chain. Three types of hydroxylation pattern were observed for the phenolic acids. Phenolic acids with a *m*-hydroxyphenyl group and the non-
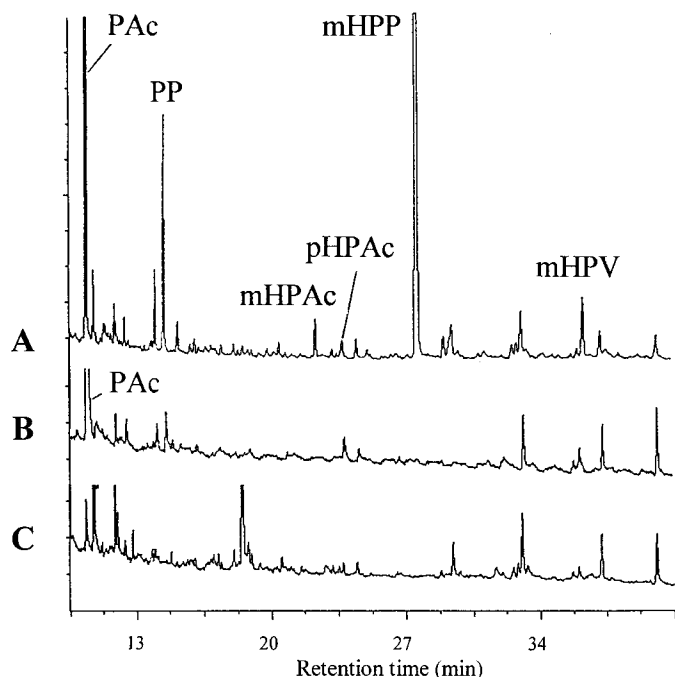

FIGURE 5 Gas chromatography-mass spectrometry (GC-MS) total ion chromatograms showing aromatic acids formed from proanthocyanidin polymers incubated for 48 h with living flora or under control conditions. Samples were extracted by ethyl acetate and silylated. (*A*) Living microflora; (*B*) control with no proanthocyanidins; (*C*) control with a heat-inactivated microflora. *m*HPAc, 2-(*m*-hydroxyphenyl)acetic acid; *m*HPP, 2-(*m*-hydroxyphenyl)propionic acid; *m*HPV, 5-(*m*-hydroxyphenyl)valeric acid; PAc, phenylacetic acid; *p*HPAc, 2-(*p*-hydroxyphenyl)-acetic acid; PP, phenylpropionic acid.
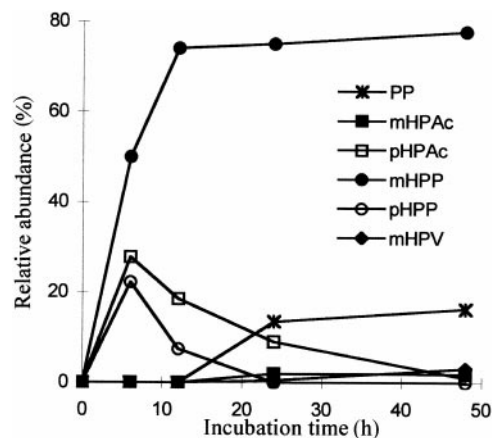

FIGURE 7 Relative abundances of aromatic acids formed by degradation of proanthocyanidin polymers by human colonic microflora after different incubation periods. Values were deduced from gas chromatography (GC) analysis of silylated ethyl acetate extracts. *m*HPAc, 2-(*m*-hydroxyphenyl)acetic acid; *m*HPP, 2-(*m*-hydroxyphenyl)propionic acid; *m*HPV, 5-(*m*-hydroxyphenyl)valeric acid; *p*HPAc, 2-(*p*-hydroxyphenyl)acetic acid; *p*HPP, 2-(*p*-hydroxyphenyl)propionic acid; PP, phenylpropionic acid.

hydroxylated aromatic acids both reached stable values after 24 h of incubation. In contrast, phenolic acids with a *p*-hydroxyphenyl group rose up to a maximum after 6 h of incubation to decrease to near-zero values after 24–48 h.

p-Hydroxy–substituted phenolic acids are likely produced by dehydroxylation in the 3-position. They may be further rearranged into their *m*-isomers by migration of either the hydroxyl group or the aliphatic side chain or dehydroxylated into their nonphenolic analogs as suggested by microflora degradation experiments of deuterated *p*HPP (Curtius et al. 1976). However, the major route leading to the *m*-hydroxy–substituted phenolic acids is likely to occur through the selective dehydroxylation in the 4-position of 3,4-dihydroxylated phenolic acid intermediates as previously shown in catechin metabolism studies by human and rat microflora (Meselhy et al. 1997). Nonhydroxylated phenolic acids are formed by dehydroxylation of the monohydroxylated phenolic acids. Only PP could be identified as a metabolite of PA polymers. PAc and BA are also likely formed because they are known metabolites of some aromatic acids identified here (Curtius et al. 1976, Drasar and Hill 1974, Scheline 1991). No definitive evidence of their formation could be obtained because of the simultaneous presence of other precursors in the growth medium.

Metabolites also differ by the length of the aliphatic side chain. Phenylvaleric acids are transformed into propionic acids and benzoic acids by a progressive loss of carbon atoms through β-oxidation (Das and Griffiths 1968, Harmand and Blanquet 1978, Meselhy et al. 1997, Scheline 1991). α-Oxidation, which would explain the formation of *m*HPAc from *m*HPP (Curtius et al. 1976), may also occur.

All aromatic acids presently identified as metabolites of PA polymers are similar to those produced by colonic microflora metabolism of either (+)-catechin (Meselhy et al. 1997) or procyanidin dimer B3 (Groenewoud and Hundt 1986). In agreement with the present work, *m*-HPP was the main metabolite produced from tea catechins by human or rat microflora (Meselhy et al. 1997) and also the main phenolic acid found in urine of volunteers who had ingested (+)-catechin (Das 1971). Other known metabolites of (+)-catechin and dimer B3 such as phenylvalerolactones (Groenewoud and Hundt 1986, Meselhy et al. 1997, Scheline 1991) and diaryl-propan-2-ol metabolites (Groenewoud and Hundt 1984 and 1986, Meselhy et al. 1997, Scheline 1991) could not be detected here, possibly because the duration of exposure to the microflora was too long. (+)-Catechin was not identified as an intermediate in contrast to previous results obtained with a rat microflora and the dimer B3 (Groenewoud and Hundt 1986). This contradiction may arise from the difference in the nature of the microflora in both rats and humans as suggested by the incapacity of the rat microflora to hydrolyze galloyl esters in tea catechins (Meselhy et al. 1997). It could also be explained by diet-mediated changes in the microflora.

The results obtained by in vitro anaerobic degradation by an isolated colonic microflora can be compared with animal and human in vivo data. Aromatic acid metabolites are formed exclusively by the colonic microflora and not by animal tissues as indicated by the comparison of the metabolites formed after oral and intraperitoneal administration of (+)-catechin and the suppression of their formation after administration of antibiotics (Das and Griffiths 1968, Griffiths 1964). The total yield of such aromatic acids recovered in 24-h urine from (+)-catechin or a mixture of (+)-catechin and PA oligomers fed to either rats or humans varies between 2 and 4% (Das and Griffiths 1969, Das 1971, Harmand and Blanquet 1978). These figures are close to those obtained after a 48-h in vitro

degradation by the microflora (see above the yields of ethylacetate-soluble metabolites). However, the similarity of these figures may be fortuitous because the amounts of aromatic acids excreted in urine depend not only on the catabolism of polyphenols in the colon but also on the amounts of substrate reaching the colon (which decrease with its absorption in the small intestine) and on the absorption of the products through the colon barrier. The absorption of aromatic acids may be quite high after oral administration. BA (Bridges et al. 1970) or PAc (James and Smith 1973) are absorbed very efficiently when ingested by humans, with 100% of the dose recovered in urine. The absorption of hydroxylated aromatic acids may be lower. When *m*HPP was administered orally to guinea pigs, 5% of the dose was recovered in urine as unchanged *m*HPP, *m*HBA or *m*-hydroxyhippuric acid (Das and Griffiths 1968). However, these data do not give any indication on their specific absorption at the colon level.

These results also suggest the formation of metabolites different from the aromatic acids described above. Indeed, radioactivity of EtOAc-soluble metabolites formed from PA polymers accounted for only 2.7% of the initial activity of PA. Their formation cannot compensate for the quasi-total disappearance of PA-derived thiolysis products. Some of the metabolites may still have a polymeric structure but may be modified in a way that would explain their loss of reactivity toward the thiolysis reagents. Some of the phenolic compounds may have been converted into microbial biomass and would therefore have escaped detection. A more detailed scrutiny of the distribution of the radioactivity in different soluble and insoluble fractions of the raw incubation media should give more insight into the nature of these metabolites.

PA polymers, which were usually considered to be relatively inert in the digestive tract and recovered unchanged in the feces, appear to be as easily degraded as other flavonoid monomers. More interest has been paid to their effects on microorganisms rather than to the effects of the microorganisms on PA polymers (Scalbert 1991). The only previous report clearly showing a chemical biodegradation of PA polymers was published more than 25 years ago and concerned fungi (Grant 1976). To understand the nutritional properties of dietary PA, it will be necessary to study not only their biological properties as such, but also those of their degradation products. Anticancer properties of aromatic acids such as PAc are well documented (Samid et al. 1997, Thibout et al. 1999). Such degradation products may be equally important as has been realized for fibers and their fatty acid metabolites.

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