Accumulation of (−)-Epicatechin Metabolites in Rat Plasma after Oral Administration and Distribution of Conjugation Enzymes in Rat Tissues1,2,3

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ABSTRACT Absorption of orally administered (−)-epicatechin (EC) in rats was studied to obtain plasma pharmacokinetic profiles of EC metabolites. Rats were administered 172 μmol/kg body weight of EC, and blood was collected from the tail for 8 h after administration. Seven groups of compounds possessing the basic structure of EC were identified by using a combination of enzymatic hydrolysis, HPLC and electron impact mass spectrometry. Metabolites were quantified with a new, simple and sensitive method using HPLC with electrochemical detection. Ingested EC was absorbed from the alimentary tract and was present in the rat common blood circulation in the form of glucuronide and/or sulfate conjugates. The activity of conjugative enzymes in rat tissues was studied. The highest activity of glucuronosyltransferase was found in the intestinal mucosa of both the small and large intestine; the highest activity of phenolsulfotransferase occurred in the liver, and that of catechol-O-methyl transferase was found in the liver and kidney. It has been proposed that the first detoxification step of dietary EC, namely, glucuronidation, occurs at the level of the intestinal mucosa in rats, and EC enters the common blood circulation exclusively in the glucuronized form. The compound is then sulfated in the liver and methylated in the liver and kidney. Because ingested EC undergoes extensive conjugation, its biological activities previously demonstrated in vitro may not be occurring in vivo systems. J. Nutr. 128: 1172–1178, 1998.

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Flavonoids, which have attracted much attention recently, are widespread in the plant kingdom, mainly as glycosides. Humans consume substantial amounts of flavonoids in fruits, vegetables, herbs and beverages (Herrmann 1976, Hertog et al. 1992). The average intake of flavonoids by humans was estimated to range from 25 mg/d (Hertog et al. 1993) to 1 g/d (Kühnau 1976). However, the former value includes only five aglycones (myricetin, quercetin, kaempferol, apigenin and luteolin). In vitro, flavonoids act as antioxidants by inhibiting lipid peroxidation, oxygen radical scavenging and metal ion chelating (Afnan et al. 1989, Cotelle et al. 1992, Terao et al. 1994); this activity is related to the unique flavonoid structure (Rice-Evans et al. 1995, van Acker et al. 1996). Potential physiologic activity of dietary flavonoids has often been the center of interest (Kinsella et al. 1993, Nakagami et al. 1995). Epidemiologic evidence has emerged, showing an inverse association between the intake of dietary flavonoids and coronary heart disease and cancer (Hertog et al. 1995, Knekt et al. 1996). The so-called French paradox, a lack of positive correlation between a high intake of saturated fat and occurrence of coronary heart disease, is related at least in part to the consumption of red wine, which is rich in phenolic compounds including flavonoids (Renaud and de Lorgeril 1992). The site and mechanism of flavonoid action are yet to be assessed; they might act directly, interacting with other dietary components in the digestive tract, or after absorption and metabolic alteration in the body.

The metabolism of flavonoids has not yet been well characterized. Absorbed flavonoids are present in the common blood circulation in the form of glucuronide, sulfate and methylate conjugates and are excreted via urine or bile. In addition, several metabolites of ingested flavonoids are formed by the intestinal microflora. Microorganisms are responsible for hydrolysis of flavonoid conjugates as well as for the formation of easily absorbed flavonoid ring fission products. A number of new reports on flavonoid absorption have been published recently. There are studies on absorption of quercetin (Hollman et al. 1996, Manach et al. 1997, Paganga and Rice-Evans 1997), naringin (Fuhr and Kummert 1995), (−)-epigallocatechin gallate (Unno et al. 1996) and tea catechins (Okushio et al. 1996). The authors usually measured the total amounts of absorbed or excreted compound, rarely as a function of time. In this study, our goals were to provide information on the quantitative changes of particular metabolites in the blood plasma after flavonoid ingestion and to identify the sites of metabolite formation. We used (−)-epicatechin (EC), a fla-
Monitored with an amperometric electrochemical detector (ICA-3062, TOA, Tokyo, Japan) with working potential set at +800 mV. Experiments with EC-spiked plasma showed that this procedure ensured a minimum recovery of 95% EC. Quantitative EC determination was performed with an external standard method. It was verified that the detector response was linear with standards up to 20 μmol/L plasma. When necessary, samples were diluted with mobile phase before HPLC analysis.

Because the detector was calibrated for EC, and methyl-EC standard was not available, a correction factor was established for quantitative recalculation of EC into methyl-EC. For that purpose, a known EC amount was methylated in a system similar to that for the determination of catechol-O-methyltransferase activity (see below), with the use of an enzyme preparation from rat liver. EC and methyl-EC were determined in the same HPLC run, with retention times of 10 and 21 min, respectively. After complete EC conversion into methyl-EC, the areas of corresponding peaks were compared, and a correction factor of 1.2 was calculated, allowing us to quantify plasma methyl-EC.

Enzymatic hydrolysis and determination of EC conjugates. Glucuronides. Plasma (50 μL) was mixed with 50 μL of β-glucuronidase solution in 0.1 mol/L sodium acetate buffer, pH 5, containing 50 units of enzyme. The mixture was incubated at 37°C in a shaking water bath for 2 h. Released compounds were extracted and analyzed by HPLC according to the EC determination procedure as described above. The difference in EC and methyl-EC content after and before incubation was assumed to be the amount of respective glucuronide conjugates in the sample.

Sulfates. Plasma (50 μL) was mixed with 50 μL of sulfatase type VIII solution in 0.1 mol/L acetic acid buffer, pH 5, containing 25 units of enzyme. To prevent the hydrolysis by β-glucuronidase present in the sulfatase preparation (<3.0 units), 21 mmol/L of d-saccharic acid 1,4-lactone was added to the enzymatic solution as a β-glucuronidase inhibitor (Hackett and Griffiths 1982). The mixture was incubated at 37°C in a shaking water bath for 4 h. The difference in EC and methyl-EC content before and after incubation was assumed to be the amount of respective sulfate conjugates in the sample.

The tissue enzymecentrifugation at 800 g for 5 min at 4°C. Homogenates from the liver, kidney, lung and mucosa (Lundth 1990) from the upper half of the small intestine, cecum and upper half of the large intestine were prepared in a Potter-Elvehjem tissue grinder with teflon pestle. Tissues were homogenized with 0.15 mol/L Tris-HCl buffer, pH 7.6, (1,9, wt/v) and centrifuged at 1000 x g for 15 min at 4°C. The protein content of the supernatant was determined by using bovine serum albumin as a protein standard (Bradford 1976). Tissue preparations were stored at −80°C until the assay of enzymatic activities.

Determination of EC metabolic enzyme activities. Uridine 5′-diphosphate glucuronosyltransferase (UGT; EC 2.4.1.17). Tissue enzyme preparation was diluted 1:1 (v/v) with 0.15 mol/L Tris-HCl buffer, pH 7.6, containing 21 mmol/L of d-saccharic acid 1,4-lactone, 20 mmol/L MgCl₂, and 2 mmol/L di-thiothreitol. The mixture (total volume 0.5 mL), containing 50 μL of diluted enzymatic preparation in 0.15 mol/L Tris-HCl buffer, pH 7.6, with final concentration of 1.25 mmol/L uridine 5′-diphosphogluconic acid, 10 μmol/L EC, 10 mmol/L of d-saccharic acid 1,4-lactone, 10 mmol/L MgCl₂, and 1 mmol/L di-thiothreitol, was incubated at 37°C in a shaking water bath for 30 min. The difference in EC content between the control sample incubated without uridine 5′-diphosphogluconic acid and the sample incubated with uridine 5′-diphosphogluconic acid was assumed to be the amount conjugated with glucuronic acid. UGT
activity was expressed as pmol EC conjugated/(min·mg of enzyme preparation protein).

Phenolsulfotransferase (PST; EC 2.8.2.1). Tissue enzyme preparation was diluted 1:1 (v/v) with 0.1 mol/L Tris-HCl buffer, pH 7.9, containing 2 mmol/L MgCl₂ and 20 mmol/L of dL-dithiothreitol. The mixture (total volume 0.5 mL), containing 50 μL diluted enzyme preparation in 0.1 mol/L Tris-HCl buffer, pH 7.9, with final concentration of 0.5 mmol/L adenosine-3'-phosphate-5'-phosphosulfate, 10 μmol/L EC, 10 mmol/L MgCl₂, and 1 mmol/L dL-dithiothreitol, was incubated at 37°C in a shaking water bath for 30 min. The difference in EC content between the control sample incubated without adenosine-3'-phosphate-5'-phosphosulfate and the sample incubated with adenosine-3'-phosphate-5'-phosphosulfate was assumed to be the amount conjugated with sulfate. PST activity was expressed as pmol EC conjugated/(min·mg of enzyme preparation protein).

Catechol-O-methyltransferase (COMT; EC 2.1.1.6). Before the assay, enzyme preparations from liver and kidney were diluted with 0.1 mol/L Tris-HCl buffer, pH 7.9, containing 20 mmol/L MgCl₂ and 2 mmol/L dL-dithiothreitol, at a ratio of 1:1 and 1:4 (v/v), respectively. Other tissue enzyme preparations were used without dilution. The mixture (total volume 0.5 mL), containing 50 μL of appropriate enzyme preparation in 0.1 mol/L Tris-HCl buffer, pH 7.9, with final concentration of 5 mmol/L S-adenosyl-L-methionine, 20 μmol/L EC, 10 mmol/L MgCl₂, and 1 mmol/L dL-dithiothreitol, was incubated at 37°C in a shaking water bath for 30 min. COMT activity was expressed as pmol methyl-EC formed/(min·mg of enzyme preparation protein).

In all enzymatic activity determinations, EC and methyl-EC were determined with the HPLC method as described above.

Isolation and mass spectral analysis of EC metabolites. Four rats were orally administered 860 μmol EC/kg body weight in 2 mL of 3.4 mol/L propylene glycol by direct stomach intubation; 2 h later, blood was taken from the abdominal aorta under diethyl ether anesthesia into heparinized tubes, and plasma was prepared by centrifugation at 800×g for 20 min at 4°C. Plasma EC metabolites were hydrolyzed with sulfatase type H-5 as described above. EC and methyl-EC released during incubation were extracted three times in a final volume of 100 mL methanol. Methanolic extracts were combined and centrifuged at 5000×g for 10 min at 4°C. The supernatant was evaporated completely at 40°C on a rotary evaporator under partial vacuum. The residue was extracted with 3×10 mL portions of methanol, and combined extracts were concentrated to ~5 mL. EC and methyl-EC were isolated from concentrated methanolic extracts on a C18 LiChroprep RP18 column (Merck, Darmstadt, Germany) with methanol/water (30:70, v/v) as the mobile phase at a flow rate of 0.8 mL/min. The elute was monitored with a UV detector at 280 nm (SPD-6A, Shimadzu, Kyoto, Japan). Two fractions, containing EC or methyl-EC, were collected during one run. Combined relevant fractions from several separations were evaporated completely and, after being dissolved in methanol, were analyzed by mass spectrometry. An electron impact mass spectrometer was obtained in the total ion monitoring mode on JMS-SX102 apparatus (JEOL, Tokyo, Japan). The operating conditions were as follows: source temperature 800°C, filament emission current 300 μA, ionizing voltage 70 eV, and scan range from 0 to 500 m/z.

Data analysis. Reported values represent means ± SEM (n = 3). Statistical analysis was evaluated by one-way ANOVA followed by Tukey’s honestly significant difference test to identify significantly different means; SPSS for Windows software, Release 6.0 (SPSS, Chicago, IL) was used. Significance was set at P < 0.05.

RESULTS

Quantitative changes of EC and its metabolites in rat plasma. A typical chromatographic profile of rat plasma is shown in Figure 2 (control plasma in Fig. 2A, plasma after EC administration in Fig. 2B, plasma after EC administration and hydrolysis of conjugates in Fig. 2C). Enzymatic treatment released two compounds that eluted at 10 and 21 min. The first compound had the same chromatographic properties as the EC standard in HPLC analysis. The electron impact mass spectrometry (EI-MS) showed an M⁺ at m/z 290, correspond-

FIGURE 2 The HPLC chromatograms of rat plasma extracts of blood taken before (--)epicatechin administration (panel A), 2 h after oral administration of 172 μmol/kg (--)epicatechin (panel B), 2 h after oral administration of 172 μmol/kg (--)epicatechin and hydrolyzed with sulfatase/b-glucuronidase (panel C). Peaks with retention times of 10 and 21 min were identified as (--)epicatechin and methyl(--)-epicatechin, respectively.
olites of EC and free EC were quantified. This method did not allow us to distinguish the number and position of conjugated moieties; thus metabolites were grouped according to their conjugated moieties (Fig. 3). All metabolites reached their maximum concentrations within the first 2 h after administration; then their plasma levels started to decrease. Free EC (Fig. 3A) was at the lowest maximum concentration of all metabolites (1.2 ± 0.1 μmol/L) and was cleared from the plasma within 6 h. It must be emphasized that nonconjugated methyl-EC was not detected after administration to rats of 172 μmol EC/kg of body weight. Plasma EC-glucuronide and EC-sulfate/glucuronide were the metabolites that started to decrease at the highest rates (Fig. 3C, D), indicating that they may be intermediates in further EC metabolism or may be excreted via bile or urine. EC-sulfates were present in rat plasma at relatively low levels (Fig. 3B) compared with glucuronides (Fig. 3C) or with mixed sulfate/glucuronide conjugates (Fig. 3D). Methyl-EC-sulfate/glucuronide and EC-glucuronide, in particular, became the main plasma metabolites 2 h after administration, with concentrations of 11.5 ± 1.6 and 10.7 ± 0.8 μmol/L, respectively, each comprising ~30% of all of the metabolites determined at this time (Fig. 3D). Moreover, although the plasma concentration of methyl-EC-sulfate/glucuronide decreased to 5 ± 0.9 μmol/L after 8 h, its proportion of total metabolites rose to 50%. As was observed 8 h after EC administration, its methylated derivative was the dominant form.

The relative distribution of metabolites that underwent one of three metabolic processes, glucuronidation, sulfation or methylation, is shown in Figure 4. The metabolites of EC that possessed glucuronide, sulfate or methyl moieties were grouped separately, and the percentage contribution of each group to total metabolites was calculated. The sum of proportions at each time point is not 1.0 because the majority of plasma EC metabolites were conjugated with two or three moieties. Because the discrimination was based on the conjugated moiety, some of the metabolites, such as the methyl-EC-sulfate/glucuronide conjugates (Fig. 3D), were included simultaneously in the glucuronized, sulfated and methylated groups. Thirty minutes after EC administration, glucuronized metabolites comprised nearly 90% of the total present in plasma, and this high share continued throughout the experimental period. The second rapid process appeared to be sulfation because within 30 min, ~50% of the total metabolites present in plasma were sulfated; within 8 h, this proportion slowly increased to 70%. Finally, ~40% of the plasma EC metabolites were methylated during the first 30 min and continued to increase in proportion to reach 75% after 8 h.

**Distribution of enzymatic activities in rat tissues.** The highest UGT activity was found for the preparations from the mucosa of the upper half of the small intestine, cecum and the upper half of the large intestine (Fig. 5A). UGT activity in the lower half of the small intestine was on a similar level (data not shown). The differences in activity between intestinal samples were not significant. The only organ possessing

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**Figure 3** (-)-Epicatechin metabolite concentrations in rat plasma after oral administration of 172 μmol/kg (-)-epicatechin. Panels represent rat plasma concentration of (-)-epicatechin (EC) and methyl(-)-epicatechin (methyl-EC) conjugates: (A) nonconjugated, (B) sulfates, (C) glucuronides and (D) sulfates/glucuronides. Metabolites were extracted from plasma obtained from rat tail blood taken before and for 8 h after EC administration. After hydrolysis with sulfatase and/or β-glucuronidase, metabolites were quantified by HPLC with electrochemical detection. Values are means ± SEM, n = 3. Means with no lower-case (EC) or upper-case (metyl-EC) letters in common are significantly different, P < 0.05.
phenolic compounds in the intestinal mucosa was proposed earlier (Powell et al. 1974) when simple phenol was the substrate. In another report (Mizuma et al. 1994), it was demonstrated that substantial amounts of naphthyl glucosides were changed to respective glucuronides after passing through the intestinal mucosa. Conjugation with glucuronic acid on the intestinal level was also proposed in a study on the conjugation of plant estrogens (Lundh 1990, Sfakianos et al. 1997). This study (with EC as substrate) demonstrated the highest UGT activity in the intestinal mucosa (Fig. 5A) and the simultaneous presence, from the very beginning, of the glucuronide moiety in almost 90% of plasma EC metabolites (Fig. 4); this strongly implies that absorbed EC is immediately conjugated with glucuronic acid in the intestinal mucosa. The idea that the first step of detoxification of some xenobiotics occurs in the ability to conjugate EC with sulfate (PST) was the liver (Fig. 5B). The liver had the highest COMT activity (P < 0.05) and is the main organ engaged in methylation of the EC catechol group (Fig. 5C). The COMT activity of the kidney was significantly lower than that in the liver, but was significantly higher than in the other tissues studied.

DISCUSSION

Although a number of papers have reported absorption of flavonoids in rats (Hackett and Griffiths 1982, Hackett et al. 1982, Manach et al. 1997, Okushio et al. 1996), they do not provide pharmacokinetic profiles of particular metabolites. The objective of our research was to study EC absorption and identify the metabolites formed. We monitored EC plasma metabolites after oral EC administration to rats and attempted to determine the sites of EC metabolite formation. It must be emphasized that a dose of 172 μmol/kg of body weight is much greater than normal dietary intake. Nevertheless, we selected such a dose as the one often used previously in studies on flavonoid absorption. The results presented here indicate that EC absorption takes place exclusively before glucuronidation. Okushio et al. (1996) reported the presence of nonconjugated EC in rat plasma; however, in that study, a huge dose (1720 μmol/kg of body weight) was administered orally to rats. In this study, after 172 μmol/kg of body weight EC administration to rats, maximum concentrations of 1.2 ± 0.1 μmol/L of unchanged EC and 10.7 ± 0.8 μmol/L of EC-glucuronides were found in plasma. However, a fivefold dose increase (860 μmol/kg of body weight) resulted in about an eightfold increase in intact EC and only a 2.5-fold increase in EC-glucuronides (Piskula, M. and Terao, J., unpublished results). This suggests the following: first, absorption of high EC doses is not dose dependent and, second, rats possess high but limited ability to conjugate EC with glucuronic acid. The glucuronidation of

![FIGURE 4](https://academic.oup.com/jn/article-abstract/128/7/1172/4722413)

**FIGURE 5** Tissue distribution of conjugative enzyme activities in rats. Panels represent enzyme activities measured in rat tissue preparation, of uridine 5′-diphosphate glucuronosyltransferase (UGT) (panel A), phenolsulfotransferase (PST) (panel B) and catechol-O-methyltransferase (COMT) (panel C). Activities were determined using (−)-epicatechin as substrate. Values are the means ± SEM, n = 3. Means with different letters are significantly different, P < 0.05.
the alimentary tract appears likely, especially in light of some evolutionary and adaptive considerations (Singleton 1981). The next conjugative reaction studied was the enzymatic sulfation of EC. Phenolsulfotransferase activity was found only in the rat liver (Fig. 5B). Although other tissues were also reported to exhibit this activity (Shirkey et al. 1979), EC was never used as a substrate. The possibility of flavonoid sulfation by the intestinal bacterium was also reported (Koizumi et al. 1990); however, in this case, the sulfate group is transferred from sources other than adenosine-3'-phosphate-5'-phosphosulfate. There is a balance between sulfation and glucuronidation of various phenolic substances, which is affected by the dose administered (Koster et al. 1981, Mehta et al. 1978). Generally, the capacity to conjugate with sulfate is very limited compared with glucuronidation, and at a large dose, there is a shift from sulfation toward glucuronidation. Nevertheless, the high level of mixed sulfate/glucuronide conjugates in blood plasma suggests that conjugation with glucuronic acid is not an obstacle for further conjugation with sulfate. The metabolic process is completed after elimination of its products, principally via bile and urine. Capel et al. (1974) showed that the composition of urine metabolites varied, depending on the route of administration. After intravenous phenol administration to hens, by-passing the intestine, only 4% of metabolites had a glucuronic moiety but as much as 93% were sulfated. In another study, Zhu et al. (1994) proved that after intraperitoneal administration of quercetin to hamsters, 35% of urinary metabolites were not conjugated with glucuronide or sulfate, but 87% were methylated. It should be noted here that interspecies differences in metabolism are also very important (Mehta et al. 1978, Hackett and Griffiths 1981). Two points seem to follow clearly from the already published research on flavonoid absorption: first, the level of unmetabolized compounds found in urine was always very low and probably caused by overdosing the experimental animals; and second, the route of administration influenced the proportions among the kinds of conjugates excreted.

The third and the last metabolic path explored was methylation. This process seems to proceed less rapidly than glucuronidation (Fig. 4), but its products remain in the common blood circulation for the greatest amount of time (Fig. 3B, C, D), indicating that they are likely the final products formed from the absorbed EC. MacGregor and Jurd (1978) showed that some of flavonoids were mutagenic in the Salmonella typhimurium Ames test, with mutagenicity depending on flavonoid structure. Moreover, mutagenicity of catechol-type flavonoids decreased substantially when one of hydroxyl groups in the catechol moiety was methylated (Czeckot et al. 1990). There was also a proposal that the polarity of the compound to be methylated by COMT is of importance, and catechols with polar substituents are preferentially methylated in position 3'; however, without this substitution, they are methylated randomly in position 3' or 4' (Creveling et al. 1970). As demonstrated in this report, almost all of the absorbed EC is present in the common blood circulation as polar EC-glucuronide and/or EC-sulfate conjugates, and therefore methylation in position 3' can be expected. In a recently published study on rats fed a diet supplemented with quercetin, Manach et al. (1997) reported different methylation patterns in two groups. The first group of rats was not adapted to high quercetin doses [745 μmol/(kg body wt · d)], and plasma contained quercetin metabolites methylated at position 3' or 4'. In contrast, in plasma of rats fed a high quercetin diet [745 μmol/(kg body wt · d)] for 10 d, the authors could not detect quercetin metabolites methylated at position 4'. This suggests that before rats become adapted to high quercetin doses, some of the ingested quercetin could escape the intestinal conjugation process and appear in the common blood circulation as a compound without polar substitution and consequently could be methylated at position 3' or 4'.

Summing up all of the results presented here, we propose a metabolic pathway of orally administered EC in rats (Fig. 6). We postulate that EC is absorbed throughout the digestive tract, beginning at least from the small intestine. After glucuronidation, the first metabolic step occurring in the intestinal mucosa, EC enters the portal vein exclusively in the conjugated form. Next, following sulfation in the liver and methylation in the liver and kidney, metabolized EC is excreted via bile or urine.

After absorption, EC is present in the common blood circulation in the form of various metabolites for a period of time sufficiently long to affect the rats. The previously mentioned biological activity of flavonoids was often associated with their in vitro antioxidative potential. They were considered to be protective agents against degenerative changes induced by oxidative damage. Because antioxidative activity is associated in part with the number of hydroxyl groups and their structural relationship in the molecule, it is very likely that during metabolic conjugation, at least some of this activity is lost. In this case, the beneficial actions of flavonoids, shown in epidemiologic studies, could be related to their other properties. It was demonstrated that flavonoids possess the ability to induce metabolizing enzymes in vivo (Siess et al. 1996) or recently, through the use of computer modeling, to inhibit urokinase, an enzyme crucial for cancer growth (Jankun et al. 1997). However, in the latter case, the authors did not consider the metabolic alteration of the flavonoid.

Finally, the results presented here show that ingested EC is absorbed from the alimentary tract and is present in the rat common blood circulation in the form of various conjugates. Because of this rapid and effective metabolic alteration, in vitro findings may not be applicable to in vivo systems. It is therefore possible that the various biological activities of EC already demonstrated in vitro may not be occurring in vivo systems.

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LITERATURE CITED