JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Urinary Excretion of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone, a Ring-Fission Metabolite of (−)-Epicatechin, in Rats and Its in Vitro Antioxidant Activity

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There is great interest in the nutritional potential of (−)-epicatechin, a common polyphenolic constituent of many foods and beverages, because of its potent antioxidant capacity. To better evaluate the biological role of (−)-epicatechin, we studied the urinary excretion of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, a ring-fission metabolite of (−)-epicatechin by intestinal microflora, in rats as well as its antioxidant activity in vitro. The method for measuring the urinary levels of (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was based on the enzymatic hydrolysis of β-glucuronidase and sulfatase, and was subsequently determined by HPLC coupled to an electrochemical detector. Following administration of (−)-epicatechin at doses of 0, 20, 40, and 80 μmol per rat, (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone were excreted into the urine within 24 h in a dose-dependent manner. Urinary 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was mostly in the conjugated form, with a higher ratio of conjugation than (−)-epicatechin. We assessed the relative antioxidant potentials for scavenging radicals in the aqueous phase as expressed in the Trolox equivalent antioxidant capacity (TEAC). The results demonstrated that the degradation of (−)-epicatechin into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone attenuated the antioxidant ability of the former. However, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone showed stronger antioxidant activity than L-ascorbic acid. These results led us to suppose that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, a microbial metabolite of (−)-epicatechin, circulating in the body may also at least be biologically active in terms of contributing to its combined antioxidant effect.

KEYWORDS: (−)-Epicatechin; 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; urinary excretion; antioxidant

INTRODUCTION

There is increasing interest in the beneficial health effects of polyphenols distributed in the human diet (*1*, *2*). Recent work has been focused on the association of polyphenol-rich foods with a reduced risk of many cancers and coronary heart disease (*3*−*8*). Tea (green, oolong, and black), cocoa, chocolate, and red wine are abundant sources of polyphenols (flavan-3-ols), of which (−)-epicatechin is one of the antioxidant components (*9*, *10*). Current interest in the antioxidant properties of (−)-epicatechin is linked to a reversal of the pathogenesis of many degenerative diseases.

Due to the increasing importance of the beneficial role of polyphenols, there is a growing demand for research on their absorption, distribution, metabolism, and excretion. Over the past few years, a number of studies have been performed on the intestinal absorption and metabolic fate of (−)-epicatechin by animals (*11*−*16*) and humans (*17*−*21*). After ingesting green tea, cocoa, or chocolate, the plasma level of (−)-epicatechin in human volunteers reached its peak level in from 1 to 2 h (*18*−*21*). Ingested (−)-epicatechin undergoes extensive conjugation such as glucuronidation, sulfation, and/or methylation (*13*). These conjugation reactions seem to be the most common type of metabolic pathways for (−)-epicatechin. One comparative study is of interest in that it shows that a stereoisomeric significance could govern the bioavailability at equimolar doses of pure (−)-epicatechin and (+)-catechin (*22*).

It has also been reported that flavan-3-ols could degrade into low molecular weight phenolic acids. The first report on the degradation of (+)-catechin in rats was published in the late 1950s by Watanabe (*23*−*25*), who found 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone in rabbit urine collected after (+)-catechin administration. A subsequent paper by Griffiths (*26*) identified *m*-hydroxyphenylpropionic acid as a major ring-fission metabolite of (+)-catechin by intestinal microflora. Moreover, additional metabolites of (+)-catechin such as *m*-hydroxyhippuric acid, *m*-hydroxybenzoic acid, *p*-hydroxyphenylpropionic acid, 5-(4′-

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10.1021/jf034578e CCC: $25.00 © 2003 American Chemical Society
Published on Web 10/10/2003

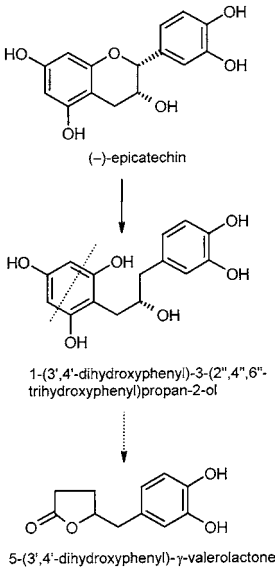

Figure 1. Pathway of the microbial degradation of (−)-epicatechin.

hydroxy-3′-methoxyphenyl)-γ-valerolactone, and 5-(3′-hydroxyphenyl)valeric acid were found (*27*−*31*). These phenolic compounds were excreted into human urine in both free and conjugated forms, including their glucuronides and, to a lesser degree, their sulfate esters (*32*). In the past decade, the microbial metabolites of (−)-epicatechin have been extensively characterized. An in vitro study by Meselhy et al. using a human fecal suspension confirmed that (−)-epicatechin first undergoes reductive cleavage to yield 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, which is further lactonized to give 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**Figure 1**) (*33*). Moreover, some studies showed that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone appeared in the plasma and urine of human subjects after ingesting green tea or pure (−)-epicatechin solution (*34*, *35*). However, the quantitative importance and biological activities of the microbial metabolites have seldom been assessed through a comparison with intact polyphenolic precursors. Therefore, our study is intended as an investigation into and an evaluation of the relative abundance and possible antioxidant importance of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone compared with those of (−)-epicatechin.

MATERIALS AND METHODS

Chemicals. (−)-Epicatechin, β-glucuronidase type X-A (G 7896), and sulfatase type VIII (S 9754) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Ethyl gallate, L-ascorbic acid, *o*-methyleugenol, *m*-chloroperbenzoic acid, ethyl malonate, and hydrobromic acid were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan).

Chemical Synthesis of 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone. The procedure for the synthesis of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was that reported previously by Watanabe (*36*) and Grosse Düweler and Rohdewald (*37*). First, 1,2-epoxy-3-(3′,4′-dimethoxyphenyl)propan was prepared from *o*-methyleugenol and *m*-chloroperbenzoic acid in chloroform for 3 days at room temperature. The product was then mixed for 3 days with ethyl sodiomalonate prepared from sodium and ethyl malonate in ethanol. This mixture was continuously refluxed with 10% sodium hydroxide solution for 30 min at 100 °C to yield 5-(3′,4′-dimethoxyphenyl)-γ-valerolactone. For the preparation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, a mixture of 5-(3′,4′-dimethoxyphenyl)-γ-valerolactone, acetic acid, and 48% hydrobromic acid was refluxed for 1 h at 100−110 °C. After acetic acid and hydrobromic acid were removed under reduced pressure, the residue was dissolved in distilled water, neutralized with sodium carbonate, extracted with ethyl acetate, and dried with anhydrous sodium sulfate. The resulting residue was finally purified by a preparative HPLC with a 250 mm × 20 mm i.d., 5 μm, Wakosil-II $5C_{18}$HG column (Wako Pure Chemical Industry, Ltd., Osaka, Japan) eluted with 22% (v/v) aqueous methanol containing 0.1% (v/v) phosphoric acid at 10 mL/min. Further optical separation was undertaken. The compound was subjected to JEOL JNM-LA500 ^{1}H NMR and ^{13}C NMR spectroscopic measurements (JEOL, Ltd., Tokyo, Japan), and characterization of chemically synthetic 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was identical with the previously reported values (*33*, *37*).

Animal Study. Eight-week-old male Wistar rats weighing 180−200 g were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and were acclimatized for 1 day in a room with controlled temperature (23 ± 2 °C), and a 12-h cycle of light and dark (7:00 a.m.−7:00 p.m.). All rats were fed a commercial diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) ad libitum with free access to tap water during the experimental period, and then fasted for 16 h before being orally administered (−)-epicatechin. They were divided into 4 groups to orally receive (−)-epicatechin at doses of 0, 20, 40, and 80 μmol dissolved in 2 mL of distilled water ($n = 7$ in each dosage group). Urine samples at time intervals of 0−24 and 24−48 h after dosing were mixed with 0.1% (v/v) phosphoric acid and adjusted to 50 mL with distilled water. The diet was resumed 5 h after (−)-epicatechin administration. Fifty microliters of the resultant urine sample was mixed with 10 μL of a preservative solution (10% (w/v) L-ascorbic acid and 0.1% (w/v) Na_2-EDTA), and the mixture was stored at −70 °C until analysis. This study was conducted in conformity with the guidelines for the care and use of laboratory animals.

HPLC Analysis of (−)-Epicatechin and 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone in Urine. The enzymatic method for liberating (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone from their conjugated forms by β-glucuronidase and sulfatase was that reported by Lee et al. (*17*). The thawed urine was mixed with 100 μL of 0.1 M sodium phosphate buffer (pH 6.8) and 10 μL of a mixture of β-glucuronidase (250 units) and sulfatase (20 units). The reaction mixture was incubated at 37 °C for 45 min and stopped by adding 100 μL of 20% (w/v) metaphosphoric acid. Next, 300 μL of acetonitrile and 10 μL of 100 μM ethyl gallate were added to the reaction mixture, which was vigorously vortexed for 1 min. After centrifuging at 10000*g*, 4 °C for 15 min, the resulting supernatant (500 μL) was transferred to another tube and diluted with 4.5 mL of distilled water. This sample was passed through an Oasis HLB size 30 mg/1 mL solid-phase extraction cartridge (Waters Co., Milford, MA) that had been preconditioned by rinsing with 1 mL of methanol followed by 1 mL of distilled water. After being washed with 1 mL of 30% (v/v) methanol, the polyphenolic samples were collected in another tube by eluting with 1 mL of methanol. The resulting eluate was concentrated under reduced pressure, and the residue was redissolved in 1 mL of the mobile phase of HPLC. After being filtered through a 0.45-μm filter, 10 μL of the resulting solution was applied to an HPLC system (Tosoh, Co., Tokyo, Japan) consisting of a controlled dual pump (model PX-8010), an autoinjector (model AS-8010), a column oven (model CO-8010), an electrochemical detector (model EC-8011), and an integrator (model SIC Chromatocorder 12). The mobile phase was comprised of 20 mM of sodium phosphate buffer containing 1 mM Na_2EDTA (pH 3.1) and 10% acetonitrile at the flow rate of 0.5 mL/min. A 150 mm × 4.6 mm i.d., 3 μm, WAKOPAC $3C_{18}$ column (Wako Pure Chemical Industries Ltd., Osaka, Japan) with a 10 mm × 4.6 mm i.d. guard column was operated at 40 °C. The applied potential of the electrochemical detector was set at +500 mV versus Ag/AgCl.

Assay of Antioxidant Activity. The antioxidant activity of (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was measured by using the methods of Miller et al. (*38*) with a Total Antioxidant Status kit (Randox Laboratories Ltd., San Francisco, CA). The chemical basis of the assay is the generation of a long-lived specific radical cation chromophore based on the peroxidative activity of metmyoglobin and its interaction with the phenothiazine compound, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), in the presence of hydrogen peroxide to form the ABTS radical cation with an absorption maximum at 734 nm. This reflects the ability of a putative hydrogen-donating antioxidant to scavenge the ABTS radical cation. Absorbance was read with a Shimadzu UV-1400 spectrometer 3 min after the addition of

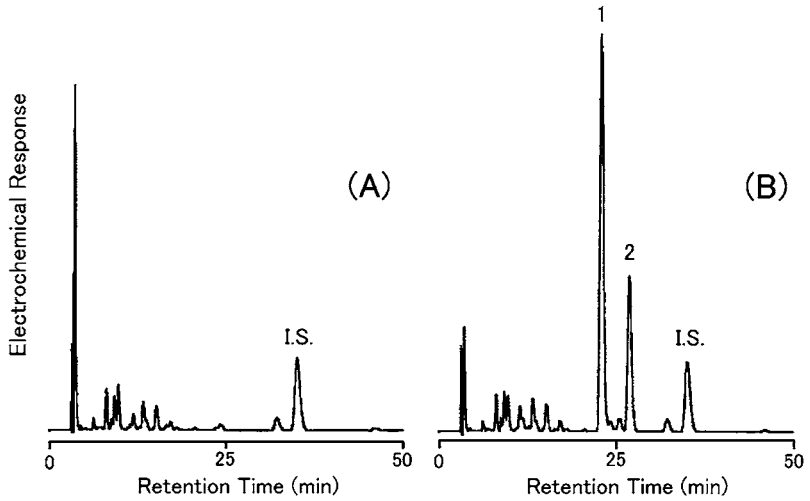

Figure 2. Chromatograms of rat urine after enzymatic treatment of β-glucuronidase and sulfatase as analyzed by HPLC with electrochemical detection: (A) urine collected 24 h after administration of distilled water; (B) urine collected 24 h after oral administration of 80 μmol of (–)-epicatechin. Peaks: 1 = (–)-epicatechin; 2 = 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; IS = internal standard.

Table 1. Urinary Excretions of (–)-Epicatechin and 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone after Oral Administration of (–)-Epicatechin in Rats[a]

	(–)-epicatechin dose (/rat)			
	0 μmol	20 μmol	40 μmol	80 μmol
(–)-epicatechin				
free, μmol/rat	ND[b]	0.5 ± 0.1[c]	0.9 ± 0.3	1.9 ± 0.4
total (free + conjugate), μmol/rat	ND	1.4 ± 0.1	3.2 ± 0.2	5.7 ± 0.6
free vs total, %		39 ± 9	29 ± 9	33 ± 6
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone				
free, μmol/rat	ND	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
total (free + conjugate), μmol/rat	ND	0.7 ± 0.1	1.3 ± 0.3	1.7 ± 0.4
free vs total, %		12 ± 2	12 ± 5	14 ± 3

[a] Urine was collected 24 h after a single administration. [b] ND, not detected. [c] Values are represented as mean ± SEM (n = 7).

hydrogen peroxide. The concentration needed to inhibit the control by 50% (IC_{50}) was calculated from the dose−response curve by three different concentrations of the compound under investigation. By using an IC_{50} value of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble vitamin E analogue, the Trolox equivalent antioxidant capacity (TEAC) was defined as the concentration of Trolox solution with antioxidant potential equivalent to a 1 mM concentration of the investigated compound.

RESULTS

HPLC Chromatograms. Typical chromatographic profiles of rat urine with enzymatic hydrolysis of conjugates are shown in **Figure 2** (control urine in **Figure 2A**, and urine after (−)-epicatechin administration in **Figure 2B**). As can be seen in **Figure 2B**, two distinct peaks were indicated in the HPLC chromatogram of urine, identified by the retention time of each compound as (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, in that order. These were not detected in the chromatogram of the control sample (**Figure 2A**).

Dose-Dependent Excretion of (−)-Epicatechin and 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone into Urine. Table 1 shows the 24-h urinary excretions of (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone after oral administration of (−)-epicatechin to rats. When distilled water was orally given, neither compound was detected in the urine. In the 24-h urine collected after single doses of 20, 40, and 80 μmol of (−)-epicatechin, the dose-dependent excretions amounted to 1.4, 3.2, and 5.7 μmol of total (−)-epicatechin, and 0.7, 1.3, and 1.7 μmol of total 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, respectively. They were also completely absent from the second sample (24−48 h).

Free versus Conjugated Forms in Urine. In the present study, the free versus total (free plus conjugated form) levels of each compound in the 24-h urine were evaluated (**Table 1**). For the analysis of free forms of (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, the enzymatic treatment of urine samples with β-glucuronidase and sulfatase was omitted. In the samples collected after doses of 20, 40, and 80 μmol of (−)-epicatechin, 39%, 29%, and 33% of the total (−)-epicatechin were present in the free form, whereas 12%, 12%, and 14% of the total 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone were found in the free form. Thus, a substantial amount of urinary 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone accounted for the conjugated form, and at a higher level of conjugation than (−)-epicatechin.

Antioxidant Activity. The antioxidant activity of (−)-epicatechin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was evaluated by the TEAC method, with the results shown in **Table 2**. L-Ascorbic acid was used as the reference. TEAC values of (−)-epicatechin and L-ascorbic acid were 2.7 and 1.0, respectively. These values were almost the same as those in a previous study (*39*). The antioxidant power of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was about half that of (−)-epicatechin (TEAC value of 1.4).

DISCUSSION

Sufficient evidence has accumulated since the late 1950s to demonstrate the catabolism of flavan-3-ols into low molecular

Table 2. Relative Antioxidant Potentials of (–)-Epicatechin, 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone in Comparison with L-Ascorbic Acid[a]

	Trolox equivalent antioxidant capacity; TEAC (mM)
(–)-epicatechin	2.7 ± 0.1
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	1.4 ± 0.1
L-ascorbic acid	1.0 ± 0.1

[a] Value are represented as mean ± SEM from quadruplicate determinations.

weight phenolic compounds by intestinal microflora. The fact that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone is the most abundant ring-fission metabolite of (−)-epicatechin has become widely accepted in recent years (*6*). It is recognized that the main site for the formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone is the large intestine, in which the ring cleavage of (−)-epicatechin appears to be due to intestinal microflora. However, there is room for debate about the quantitative information on its biological role in the body. Therefore, this study sought to provide supporting evidence for the presence of microbial metabolites in rats, and to determine their possible role in antioxidant activity.

The most important finding in this study is the clear demonstration of the microbial degradation products of (−)-epicatechin in rats. The present study proved that the administration of (−)-epicatechin to rats induced a dose−response excretion of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone into only the 24-h urine. The result that the microbial metabolism of (−)-epicatechin in the intestine could be accomplished within 24 h is in agreement with the account given by Takizawa et al. (*40*), who reported that the cumulative urinary excretion of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone reached the plateau level 24 h after oral administration to rats of an (−)-epicatechin derivative, (−)-epicatechin gallate. This present study also demonstrated that the urinary amounts of total (−)-epicatechin (free + conjugated) collected after an oral administration of 20, 40, and 80 μmol of (−)-epicatechin were respectively 7.2%, 8.0%, and 7.2% of the dose, while the amounts of its ring-fission metabolite excreted were 3.6%, 3.1%, and 2.1%. The urinary amounts of free (−)-epicatechin were 2.6%, 2.3%, and 2.4%, against 0.36%, 0.30%, and 0.26% of free 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. This shows that the free-versus-total ratio of (−)-epicatechin in the urine was higher than that of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, which is thus indicated to be primarily present in the conjugated form. It is well documented that the enzymatic conjugation should be accomplished by using uridine-5′-diphosphoglucuronosyl transferase for glucuronidation, and phenolsulfotransferase for sulfation. Piskula et al. (*13*) showed that a high level of uridine-5′-diphosphoglucuronosyl transferase activity existed not only in the small intestine but also in the cecum and large intestine, while the only organ possessing the phenolsulfotransferase was the liver. Such results may well explain the assumption that portions of orally administered (−)-epicatechin are degraded into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in the colon where the glucuronidized conjugation begins immediately due to the activity of mucosal uridine-5′-diphosphoglucuronosyl transferase, followed by further conjugations of glucuronidation, sulfation, and/or methylation in the liver. Conjugated flavonoids may pass into the bile through enterohepatic circulation, and thereby reach the luminal side of the colon as glucuronides or other metabolites (*41*). Considering that the fecal microflora showed a high level of deglucuronidation activity (*42*), it makes sense to assume that bacterial β-glucuronidase hydrolyzes the glucuronides, enabling reabsorption of the free aglycone. Because of low absorption of (−)-epicatechin through the small intestine (*43*), the role of the large intestine in the metabolism of (−)-epicatechin prior to subsequent absorption is increasingly considered to be important. In this regard, the fecal excretions of (−)-epicatechin and its degradation products provide support for the argument on the metabolic fate of dietary (−)-epicatechin, an issue requiring further substantiation.

Provided that the beneficial health effects of (−)-epicatechin are linked to its antioxidant potential, the next step must be the evaluation of the antioxidant impact of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. Accordingly, its antioxidant activity was compared with the parent compound by the TEAC method, which is widely used for evaluating the ability of putative antioxidants in the aqueous phase. The result of the experiment confirmed that the degradation of (−)-epicatechin into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone diminished the TEAC value, yet this ring-fission metabolite showed stronger activity than L-ascorbic acid. In the limited sense that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone may at least partly retain its antioxidant activity, the role of this intermediate product in the biological system is a relatively important one. There is fairly general agreement that the *o*-dihydroxyl structure in the B-ring of the flavan-3-ol skeleton exhibits antioxidant activity (*44*). It is quite possible that such antioxidant activity would be attenuated if the *o*-dihydroxyl structure were to be conjugated with glucuronic acid and/or sulfate. Our conclusion that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone predominantly exists in the conjugated form takes on importance in connection with the comparative contribution of the ring-fission metabolite in relation to the total antioxidant effect of dietary (−)-epicatechin on the biological system. Baba et al. (*22*) showed that the ratio of free versus conjugated forms in the urine obtained after oral administration of (−)-epicatechin differed from that in the plasma, suggesting that deconjugation activity occurred in rat kidney. The biological significance of the conjugated form of plasma and urinary levels may offer the key to understanding the total antioxidant activity of (−)-epicatechin and its ring-fission metabolites. The microbial metabolite, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, can also be further metabolized into simple phenolic acids such as 3-hydroxyphenylpropionic acid, 3-hydroxyhippuric acid, or 3-hydroxybenzoic acid, which may be among the minor compounds present in animal urine after a catechin dose (*45*). This fact has further complicated the overall assessment of the antioxidant effect of (−)-epicatechin on the biological system.

In conclusion, although the use of dietary polyphenols may be increasing because of their known beneficial properties, there is a lack of detailed information on their metabolic fate in the body system. This study thus provides a basis to assess both the urinary appearance of the microbial metabolite of (−)-epicatechin and its antioxidant activity. Further investigation is needed to produce direct evidence for the activity of microbial metabolites in relation to their biological and pharmacological effects in vivo.

ABBREVIATIONS USED

Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TEAC, Trolox equivalent antioxidant capacity; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate).

ACKNOWLEDGMENT

Thanks are due to Professor N. Watanabe of the Department of Applied Biological Chemistry, Faculty of Agriculture at Shi-

zuoka University for his technical suggestions on synthesizing 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.

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Received for review June 2, 2003. Revised manuscript received September 2, 2003. Accepted September 3, 2003.

JF034578E