Metabolic Fate of (−)-[4-^{3}H]Epigallocatechin Gallate in Rats after Oral Administration

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After oral administration of [4-^{3}H]EGCg to rats, the radioactivity in blood, major tissues, urine, and feces was measured over time. The radioactivity in blood and most tissues remained low for 4 h postdose, began to increase after 8 h, peaked at 24 h, and then decreased. Major urinary excretion of radioactivity occurred in the 8−24 h period, and the cumulative radioactivity excreted by 72 h was 32.1% of the dose. The radioactivity in the feces was 35.2% of the dose within 72 h postdose. In the case of rats pretreated with antibiotics (antibiotic-pretreated rats), the radioactivity levels of the blood and urine were definitely lower than those in rats not pretreated with antibiotics (normal rats). The radioactivity recovered in the antibiotic-pretreated rat urine was estimated to be only $^1/_{100}$ of that in the normal rat urine. These results clearly demonstrated that the radioactivity detected in the blood and urine of normal rats mostly originated from degradation products of EGCg produced by intestinal bacteria. Furthermore, a main metabolite in the normal rats was purified and identified as 5-(5′-hydroxyphenyl)-γ-valerolactone 3′-*O*-β-glucuronide (M-2). In feces of the normal rats, EGC (40.8% of the fecal radioactivity) and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (M-1, 16.8%) were detected. These results suggested that M-1 was absorbed in the body after degradation of EGCg by intestinal bacteria, yielding M-1 with EGC as an intermediate. Furthermore, M-2 was thought to be formed from M-1 in the intestinal mucosa and/or liver, then to enter the systemic circulation, and finally to be excreted in the urine. Taking into account all of the above findings, a possible metabolic route of EGCg orally administered to rats is proposed.

Keywords: *(−)-Epigallocatechin gallate; catechin; tea; metabolism; flavonoids*

INTRODUCTION

The physiological effects of green tea catechins, both in vitro and in vivo, have been reported extensively in the past. They include antioxidative (*1*), anticancer (*2*, *3*), blood cholesterol lowering (*4*, *5*), blood sugar level lowering (*6*), antibacterial (*7*), and antiviral actions (*8*). In particular, because (−)-epigallocatechin gallate (EGCg) is the most abundant component of green tea catechins and has stronger physiological activities than the other catechin compounds, there are high expectations that it will exert beneficial effects on human health. Accordingly there has been a growing interest in determining the metabolic profiles of EGCg to clarify the mechanisms of the physiological activities in vivo.

Our previous studies have shown that a part of EGCg administered orally to rats appears in the portal vein (*9*, *10*) and that conjugates of EGCg, 3′-*O*-methyl-EGCg, 4′-*O*-methyl-EGCg, 3″-*O*-methyl-EGCg, 4″-*O*-methyl-EGCg, and 4′,4″-di-*O*-methyl-EGCg, are excreted in the bile (*11*). It has also been reported that when EGCg was orally administered, EGCg and/or its conjugates were detected and peaked 1−2 h postdose in rat systemic circulation (*12*−*15*). Thus, it is now known that a part of the orally administered EGCg is absorbed from the intestine and that EGCg and/or its conjugates are present in the blood and bile after dosing. Recently, Suganuma et al. (*16*) traced the radioactivity of tritium labeled in the B ring and/or galloyl moiety of EGCg after its oral administration to mice. They reported that the radioactivity was widely distributed in the blood and various organs. In addition, they found that 6% of the dosed radioactivity was excreted in the urine by 24 h postdose. On the other hand, EGCg and its conjugates were not detected in the urine of the rat (*11*, *14*) or of humans (*17*, *18*) after oral administration of EGCg. From the above observations, it is thought there is a possibility that when EGCg is orally administered, metabolites other than EGCg and its conjugates are excreted in the urine. Thus, although research on the metabolism of EGCg after oral administration has made advances, the metabolic profiles of EGCg have not been clarified.

This study was conducted with the aim of revealing the whole picture of the metabolic fate of EGCg as fully as possible. After oral administration of [4-^{3}H]EGCg to rats, we quantitatively examined the absorption, distribution, and excretion of EGCg and its metabolites by tracing the radioactivity. Next, to examine the influence of intestinal bacteria on the metabolism of EGCg,

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radioactivity levels of blood and urine in rats pretreated with antibiotics were compared with those in rats not pretreated with antibiotics (normal rats). Furthermore, major radioactive metabolites excreted in the urine and feces of the normal rats were identified. On the basis of all the research on this topic, we discuss the possible metabolic fate of EGCg orally administered to rats.

MATERIALS AND METHODS

Materials. [4-^{3}H]EGCg (13 Ci/mmol) was prepared according to the method previously described (*19*). EGCg, (−)-epigallocatechin (EGC), β-glucuronidase type H-1 from helix pomatia (G 0751), and D-saccharic acid 1,4-lactone were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were available products of analytical or HPLC grade.

Animals and Treatments. Male Wistar rats (6 weeks of age, 180−210 g) purchased from Charles River Japan, Inc., were given a polyphenol-free diet (*10*) for a week. Water was provided ad libitum throughout this experiment. The rats were fasted overnight before dosing with [4-^{3}H]EGCg, and the diet was resumed 4 h after dosing. In the first study, absorption, distribution, and excretion of ingested EGCg were examined. [4-^{3}H]EGCg (200 μCi) was added to 2 mL of physiological saline containing cold EGCg (4 mg). The solution was administered to rats (220−250 g) by intragastric gavage at a dose of 2 mL/kg. The rats were then placed in stainless steel metabolism cages. Samples of the blood and tissue were taken at the time points of 0.5, 1, 2, 4, 8, 24, 48, and 72 h after the dose according to the following methods. At each time point rats ($n = 4$) were anesthetized with ether, and blood was taken from inferior vena cava with a syringe containing a small amount of heparin. The rats were perfused with 50 mL of physiological saline, which was injected from inferior vena cava to remove the remaining blood in the tissues. Then the following tissues were first removed and frozen in liquid nitrogen: eyes, brain, heart, lungs, thymus, liver, spleen, pancreas, adrenals, kidneys, testes, and prostate. Next the alimentary tract was divided into stomach, small intestine, cecum, and large intestine, with respective contents intact, and frozen in liquid nitrogen. These tissues were transferred to a −20 °C freezer before use. For analysis of radioactivity in blood, aliquots (200 μL in duplicate) of the blood were taken. The remainder of the blood was centrifuged at 2000*g* for 15 min at 4 °C to obtain plasma and blood cells. The blood cells were washed three times with physiological saline. Each aliquot (200 μL in duplicate) of the plasma and the blood cells was taken for analysis of radioactivity. Urine and feces from the rats sacrificed at 72 h were separately collected at 0−4, 4−8, 8−12, 12−24, 24−48, and 48−72 h after dosing in chilled vessels with dry ice and were then stored in a −20 °C freezer until use.

In the second study, to examine dose dependency, [4-^{3}H]-EGCg preparation in physiological saline (4 mg, 200 μCi/kg; 20 mg, 1 mCi/kg; or 100 mg, 1 mCi/kg) was administered to rats ($n = 4$) by intragastric gavage. The rats were placed in stainless steel metabolism cages and urine samples at time intervals of 0−4, 4−8, 8−12, 12−16, 16−20, and 20−24 h after dosing were collected in chilled vessels with dry ice. Blood (50 μL) was taken from the caudal vein at 0.5, 1, 2, 4, 8, 12, 16, 20, and 24 h postdose. The samples collected were stored at −20 °C until use.

In the third study, the influence of intestinal bacteria on the metabolic fate of EGCg was examined. The polyphenol-free diet and previously autoclaved water were given to rats. To prepare rats with a suppressed intestinal bacterial activity, rats ($n = 4$) were given antibiotic saline solution containing bacitracin, neomycin sulfate, and streptomycin sulfate (200 mg of each antibiotic/kg) by intragastric gavage every 12 h for 2 days before [4-^{3}H]EGCg administration according to the methods of Uejima et al. (*20*). With this antibiotic treatment, bacterial counts in the rat cecum were confirmed to decrease sharply as shown in Table 1. The rats were fasted overnight and were then given [4-^{3}H]EGCg (4 mg, 200 μCi/kg) 4 h after the dose of the antibiotic solution, to minimize the contact of [4-^{3}H]EGCg with the antibiotics in the stomach. The diet was resumed 4 h after [4-^{3}H]EGCg dosing, and administration of the antibiotic solution to the rats was continued every 12 h throughout this experiment. Urine and blood samples were collected in a manner similar to that described for the second study.

Table 1. Number of Bacteria in Cecum Contents of Rats Pretreated with or without Antibiotics

		no. of bacteria (CFU/g)	
	n	aerobic[a]	anaerobic[b]
no treatment	4	$(1.95 \pm 0.36) \times 10^8$	$(10.10 \pm 3.03) \times 10^8$
antibiotics	4	$<10^2$	$<10^2$

[a] Cecum contents were cultured aerobically with GAM agar medium at 35 °C for 3 days. [b] Cecum contents were cultured anaerobically with GAM agar medium at 35 °C for 3 days.

Determination of Total Radioactivity. Total radioactivity in blood, plasma, blood cells, and urine samples was determined according to the methods as described previously (*19*). The fecal samples were weighed and, while cooling with ice, were homogenized with 4 volumes of 0.2 M sodium acetate buffer (pH 3.0) containing 1% ascorbic acid and 0.15 mM ethylenediaminetetraacetic acid (EDTA). Aliquots (100 μg in duplicate) of the fecal homogenate were taken, followed by the addition of Soluene-350 (1 mL) (Packard Bio Science B.V.). The tissue samples were weighed and homogenized with 3−10 volumes of phosphate-buffered saline (pH 7.2). Each aliquot (200−300 μL in duplicate) of the tissue homogenate was taken, and 1 mL of Solvable (Packard Bio Science B.V.) was added. Total radioactivity in each fecal and tissue homogenate preparation was measured according to the methods described previously (*19*).

HPLC Analysis of Radioactive Metabolites in Urine and Feces. Each urine sample collected in the first study was adjusted to pH 5.0 with 0.2 M sodium acetate buffer (pH 3.0) containing 1% ascorbic acid and 0.15 mM EDTA. Each sample of feces collected in the first study was homogenized as described under Determination of Total Radioactivity, and the fecal homogenate (2 g) was extracted with 2 mL of methanol, followed by centrifugation at 2000*g* for 10 min at 4 °C. The precipitate was further extracted three times with 1 mL of 80% aqueous methanol. The supernatant was pooled and evaporated to dryness. The resultant residue was dissolved in 2 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 1% ascorbic acid and 0.15 mM EDTA. Then each of these urinary and fecal preparations was divided into three portions. Each portion was mixed separately with 20 μL of β-glucuronidase type H-1 (750 units of β-glucuronidase and 40 units of sulfatase) dissolved in 0.1 M sodium acetate buffer (pH 5.0), with 20 μL of β-glucuronidase type H-1 solution containing 250 mM D-saccharic acid 1,4-lactone (β-glucuronidase inhibitor) and with 20 μL of 0.1 M sodium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 6 h. The reaction mixture was cooled and centrifuged at 10000*g* for 5 min at 4 °C, and then the supernatant was subjected to analytical HPLC. The analytical HPLC was carried out on a Capcell Pak C18 UG120 (Shiseido Co., Ltd., 4.6 mm i.d. × 250 mm) in a Jasco liquid chromatograph apparatus equipped with a Jasco UV-870 detector (280 nm) and a β-radioactivity detector (Beckman) as previously described (*19*).

Isolation of Metabolite from Urine. Male Wistar rats (15 rats) were administered cold EGCg (100 mg/kg) in saline by intragastric gavage. Urine (200 mL) was collected at 8−24 h postdose and adjusted to pH 5.0 with 0.2 M sodium acetate buffer (pH 3.0) containing 1% ascorbic acid and 0.15 mM EDTA. The urine sample was washed three times with the same volume of ethyl acetate. The organic phase was discarded, and the aqueous phase was concentrated to remove ethyl acetate. To the concentrated solution was added β-glucuronidase type H-1 (400 mg), which is a mixture of β-glucuronidase (140000 units) and sulfatase (7000 units). The reaction mixture was incubated at 37 °C for 6 h with gentle shaking, followed by extraction three times with the same

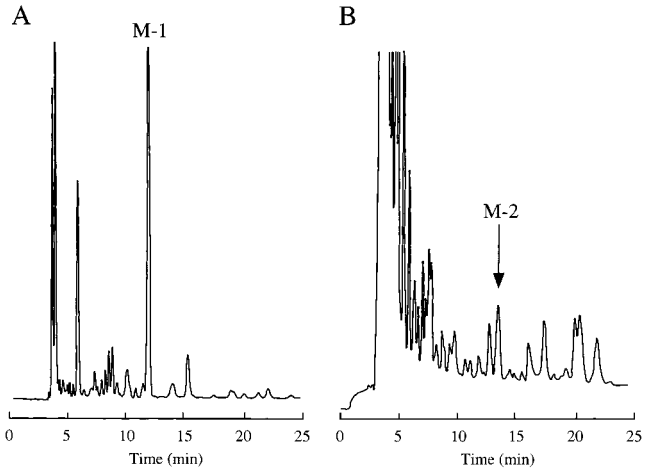


Figure 1. HPLC profile of rat urine sample after an oral dose of EGCg. (A) Prior to HPLC analysis, the urine sample was treated with a β-glucuronidase/sulfatase mixture, and then the reaction mixture was extracted with ethyl acetate. The analytical HPLC was carried out as described under Materials and Methods. (B) Prior to HPLC analysis, the urine sample was washed with ethyl acetate. The analytical HPLC was carried out as described under Materials and Methods except for using a mobile phase of acetonitrile/ethyl acetate/0.05% TFA (12:0.6:215, by volume).

volume of ethyl acetate. The organic phase was concentrated to dryness. The resultant residue was dissolved in acetonitrile/ethyl acetate/water (12:0.6:90, by volume) and was subjected to preparative HPLC. The preparative HPLC was performed with a Capcell Pak C18 UG120 (Shiseido Co., Ltd., 20 mm i.d. × 250 mm) in a Jasco liquid chromatograph apparatus equipped with a Jasco UV-870 detector. Elution was done with acetonitrile/ethyl acetate/water (12:0.6:90, by volume) at a flow rate of 10 mL/min at room temperature (24 °C). The elution pattern was monitored by measuring the absorbance at 280 nm. A peak (Figure 1A, M-1) was collected, and the fraction was concentrated in vacuo to remove the organic solvents and then freeze-dried (12 mg). HPLC analysis showed that the retention time of the purified compound (M-1) coincided with that of a major radioactive compound, which was detected in rat urine treated with the β-glucuronidase after oral administration of [4-^{3}H]EGCg. The chemical structure of the purified compound, M-1, was analyzed by MS, NMR, polarimetry, and circular dichroism (CD) measurements.

Second, the conjugated form of M-1 (M-2) was isolated. Urine samples (70 mL) collected from five rats in a manner similar to that described above were adjusted to pH 5.0 with 0.2 M sodium acetate buffer (pH 3.0) containing 1% ascorbic acid and 0.15 mM EDTA. The solution was washed three times with the same volume of ethyl acetate. The aqueous phase was concentrated to remove ethyl acetate. The concentrated aqueous phase was freeze-dried and reconstituted in 20 mL of distilled water. The solution was centrifuged at 10000*g* for 10 min at 4 °C, and the resultant supernatant was subjected to preparative HPLC. The preparative HPLC was carried out in the same JASCO HPLC system and under the same conditions as described above, except for using a mobile phase of acetonitrile/ethyl acetate/0.05% TFA (12:0.6:215, by volume). The elution pattern was monitored by measuring the absorbance at 280 nm, and the UV spectra were obtained by using wavelengths in the range of 200−400 nm. A peak (Figure 1B, M-2) having a UV spectrum very similar to that of M-1, was collected, and the fraction was concentrated in vacuo to remove the organic solvents. The resulting solution was applied to an HPLC column of Capcell Pak C18 UG120 (Shiseido Co., Ltd., 20 mm i.d. × 250 mm), which had been equilibrated with distilled water. The column was washed with distilled water to remove TFA and then was eluted with 50% aqueous methanol. The methanol fraction was collected and concentrated to remove methanol and then freeze-dried (1 mg). HPLC analysis of the purified metabolite (M-2) showed that its

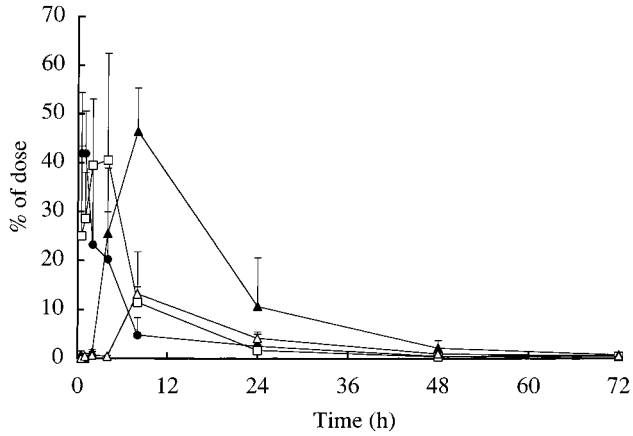


Figure 2. Time course of radioactivity in alimentary tract after an oral dose of [4-^{3}H]EGCg to rats. The alimentary tract was divided into stomach (●), small intestine (□), cecum (▲), and large intestine (△) with respective contents intact. The radioactivity was expressed as a percentage of the administered dose. Values are the mean ± SD of four rats.

retention time was in good agreement with that of a major radioactive compound which was detected in rat urine after administration of [4-^{3}H]EGCg. The metabolite, M-2, was analyzed by MS and NMR. Furthermore, enzymatic analysis of isolated M-2 was performed. An aliquot of M-2 was dissolved in 0.1 M sodium acetate buffer (pH 5.0) containing 1% ascorbic acid and 0.15 mM EDTA. The mixture was incubated with the β-glucuronidase type H-1 or with β-glucuronidase type H-1 containing D-saccharic acid 1,4-lactone (β-glucuronidase inhibitor) at 37 °C for 6 h, followed by extraction and concentration in a similar manner as described above. Each resultant residue was dissolved in acetonitrile/ethyl acetate/water (12:0.6:90, by volume) and was subjected to analytical HPLC as described under HPLC Analysis of Radioactive Metabolites in Urine and Feces.

Analytical Methods. Mass spectra were recorded on a JEOL DX-303 mass spectrometer. NMR spectra were obtained on a JEOL Lambda-500 system. The NMR chemical shifts were given in parts per million (δ) with tetramethylsilane (TMS) as an internal standard. Specific rotation data were determined by using a Jasco DIP-1000 polarimeter. CD measurements were performed by a Jasco J-600 apparatus.

RESULTS

Absorption, Distribution, and Excretion of Radioactivity in Rats after Oral Administration of [4-^{3}H]EGCg. In the first study, [4-^{3}H]EGCg (4 mg, 200 μCi/kg) was administered to rats by intragastric gavage, and absorption, distribution, and excretion of the radioactivity were determined. The radioactivity (percent of dose) in the alimentary tract (stomach, small intestine, cecum, and large intestine with respective contents intact) is shown in Figure 2. In the stomach the radioactivity had mostly disappeared by 8 h after dosing. The radioactivity moved into the small intestine, cecum, and large intestine, with peak levels at 4 h (40.5% of dose), 8 h (46.4% of dose), and 8 h (13.2% of dose), respectively, and was greatly reduced in all three tracts by 24 h, disappearing mostly by 72 h after dosing.

The time course of the radioactivity in the blood is depicted in Figure 3A. The level of radioactivity remained low for 4 h postdose. The radioactivity increased after 8 h, peaked [523 ± 439 ng equiv/mL (mean ± SD of four rats)] at 24 h postdose, and then decreased. Similar time course patterns were also observed in the plasma (Figure 3B) and blood cells (Figure 3C). The ratio of the radioactivity in plasma and blood cells at

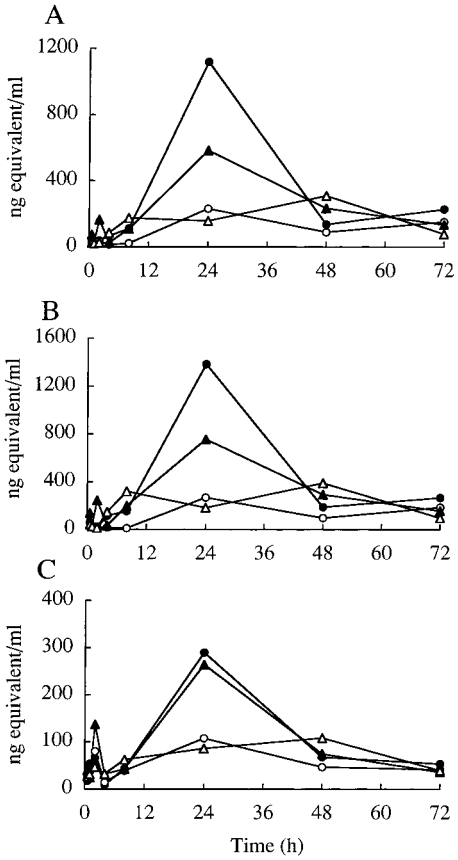


Figure 3. Concentration−time profiles of radioactivity in blood (A), plasma (B), and blood cells (C) after an oral dose of [4-^{3}H]EGCg to rats. Each nanogram equivalent of EGCg was calculated from the level of radioactivity in blood, plasma, and blood cells. The symbols (●, ○, ▲, △) represent four individual rats.

24 h after dosing was about 3:1 when calculated by taking into account the hematocrit (53.1%) of the rats.

Time course patterns of the radioactivity in the tissues examined were similar to those in the blood, except that in the liver a peak of the radioactivity was observed at 2 h postdose (0.5% of dose) in addition to the peak at 24 h postdose (0.7% of dose) (Figure 4B). The maximal level of radioactivity in all of the tissues, except the liver, was <0.2% of the dose. These observations indicated that the radioactivity was not preserved in the tissues. Accordingly, it is thought that no metabolites derived from EGCg are accumulated in the body. An increase of the radioactivity observed in the liver at 2 h postdose did not influence the blood radioactivity, implying that metabolites derived from EGCg may undergo first-pass effect and hence be excreted in the bile before blood circulation.

Excretion of the radioactivity in urine and feces is shown in Table 2. The cumulative radioactivity excreted in the urine by 72 h was 32.1% of dose; that is, at least 30% of the radioactivity derived from [4-^{3}H]EGCg was regarded as being absorbed. High levels of the radioactivity were excreted in urine at 8−12 h (12.7% of dose) and 12−24 h (11.2% of dose) after dosing. The radioactivity in the feces was detected after 8 h, and 35.2% of the dosed radioactivity was excreted in the feces within 72 h after administration.

Influence of Different EGCg Doses on Radioactivity in Blood and Urine. Three different doses of [4-^{3}H]EGCg preparations (4 mg, 200 μCi/kg; 20 mg, 1 mCi/kg; or 100 mg, 1 mCi/kg) were separately administered to rats ($n = 4$) by intragastric gavage, and the radioactivity in the blood and urine was measured. Figure 5 shows the time course of the radioactivity in the blood and cumulative urinary excretion of radioactivity. The blood level of the radioactivity in rats given 20 mg/kg of EGCg was similar to that in rats given 4 mg/kg. In rats given 100 mg/kg of EGCg, the radioactivity in the blood was higher than that in rats given 4 and 20 mg/kg of EGCg but was not parallel with its dosage. Cumulative urinary excretion (0.33 mg equiv/rat) of the radioactivity for 24 h postdose in rats given 20 mg/kg of EGCg was similar to that (0.27 mg equiv/rat) in rats given 4 mg/kg of EGCg. The cumulative excretion (3.5 mg equiv/rat) in rats given 100 mg/kg of EGCg was higher than that in rats given 4 and 20 mg/kg of EGCg but was not parallel with its dosage in a manner similar to their blood levels. The cumulative radioactivities (percent of dose) in urine were 26.8, 6.6, and 13.9% in rats given 4, 20, and 100 mg/kg of EGCg, respectively. Thus, clear dose dependency was not

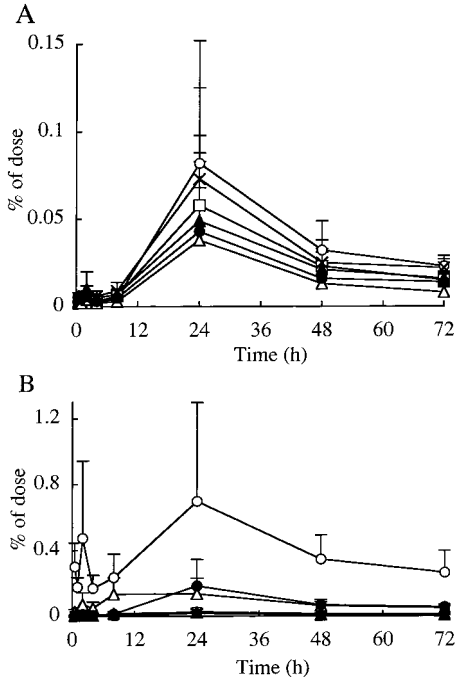


Figure 4. Time course of radioactivity in tissues after an oral dose of [4-^{3}H]EGCg to rats. The radioactivity was expressed as a percentage of the administered dose. Values are the mean ± SD of four rats. (A) (○) brain, (□) eye, (△) thymus, (×) lung, (▲) heart, and (●) spleen. (B) (○) liver, (□) pancreas, (△) kidney, (×) adrenal gland, (▲) prostate, and (●) testis.

Table 2. Excretion of Radioactivity in the Urine and Feces of Rats Following Oral Administration of [4-^{3}H]EGCg (4 mg, 200 μCi/kg)[a]

	urinary excretion (% of dose)		fecal excretion (% of dose)	
time (h)	each time interval	cumulative	each time interval	cumulative
0−4	0.4 ± 0.9	0.4 ± 0.9	0	0
4−8	2.6 ± 2.6	3.0 ± 3.0	0	0
8−12	12.7 ± 7.7	15.7 ± 10.1	3.4 ± 4.8	3.4 ± 4.8
12−24	11.2 ± 4.7	26.8 ± 10.6	12.1 ± 7.8	15.5 ± 7.4
24−36	2.2 ± 0.7	29.0 ± 11.2	7.6 ± 3.8	23.1 ± 5.6
24−48	1.2 ± 0.4	30.3 ± 10.3	8.1 ± 4.2	31.2 ± 5.2
48−72	1.8 ± 0.7	32.1 ± 11.1	4.0 ± 2.5	35.2 ± 4.2

[a] Values are the mean ± SD of four rats.

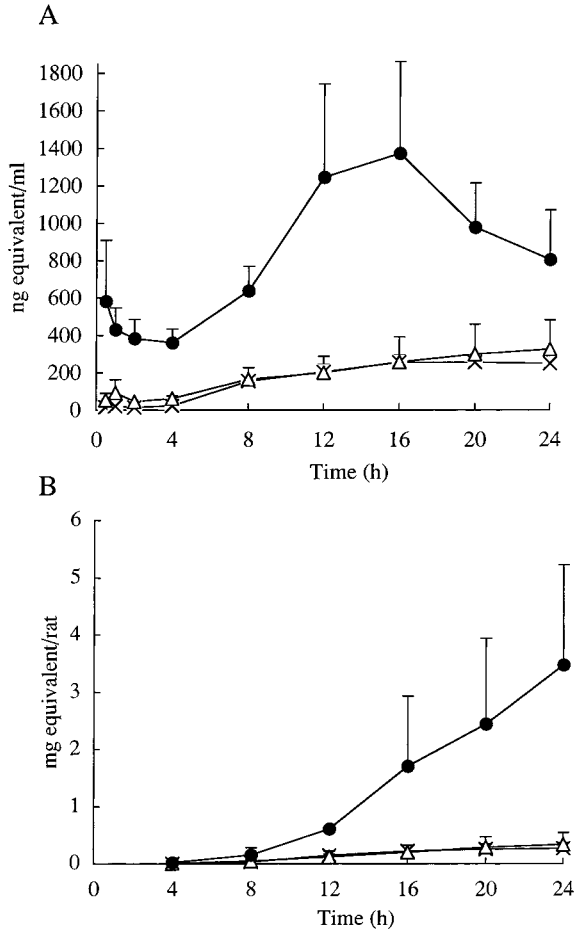


Figure 5. Influence of different EGCg doses on blood concentration (A) and cumulative urinary excretion of radioactivity (B) in rats. Three different doses of [4-^{3}H]EGCg [(×) 4 mg, 200 μCi/kg; (△) 20 mg, 1 μCi/kg; (●) 100 mg, 1 μCi/kg] were separately administered to rats by gavage. Each nanogram or milligram equivalent of EGCg was calculated from the level of radioactivity in the blood and urine. Values are the mean ± SD of four rats.

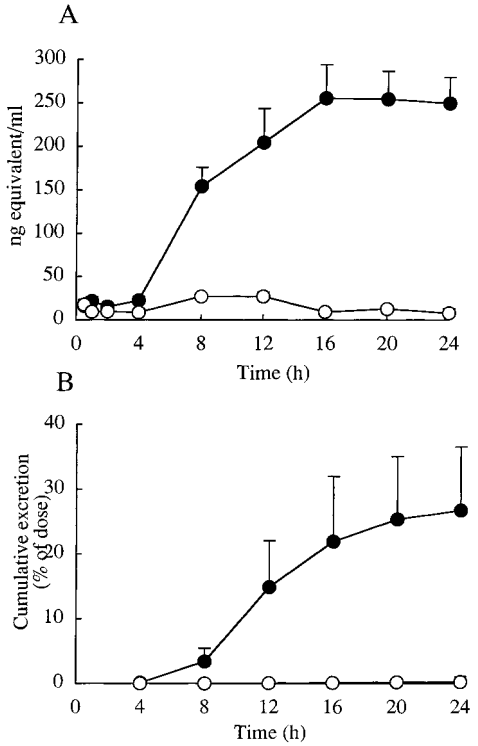


Figure 6. Blood concentration−time profiles (A) and cumulative urinary excretion (B) of radioactivity in rats pretreated with (○) or without antibiotics (●). (A) Each nanogram equivalent of EGCg was calculated from the level of radioactivity in the blood. Values are the mean ± SD of four rats. (B) Cumulative excretion of radioactivity was expressed as a percentage of the administered dose. Values are the mean ± SD of four rats.

observed in rats after oral administration of EGCg at the different doses.

Influence of Antibiotic Pretreatment on Radioactivity in Blood and Urine. Some studies (*11*, *14*, *17*, *18*) have demonstrated that EGCg and its conjugates were detected in plasma with their maximal concentration at 1−2 h after oral administration and that they were hardly detected in urine. However, in this study the radioactivity in the blood peaked at ∼24 h, and ∼30% of the dosed radioactivity was excreted in urine as described above. We considered that the above discrepancy may be caused by the degradation of EGCg by rat intestinal bacteria. Then, to examine the influence of intestinal bacteria, radioactivity levels of blood and urine in rats pretreated with antibiotics (antibiotic-pretreated rats) were compared with those in rats not pretreated with antibiotics (normal rats) after oral dose of [4-^{3}H]EGCg (Figure 6). The blood level of the radioactivity in the antibiotic-pretreated rats was definitely lower than that in the normal rats as shown in Figure 6A. Cumulative urinary excretion of the radioactivity in the normal rats accounted for 26.8% of the dose within 24 h after dosing, whereas only 0.3% of dose was recovered in the urine of the antibiotic-pretreated rats within the same period (Figure 6B). These results clearly demonstrated that the radioactivity detected in the blood and urine of normal rats mostly originated from degradation products of EGCg which were formed by intestinal bacteria.

Radioactive Metabolites in Urine and Feces. Radioactive metabolites in urine and feces collected over a period of 48 h after the ingestion of [4-^{3}H]EGCg in the first study were analyzed by HPLC. Representative HPLC profiles of urine samples are illustrated in Figure 7. A major radioactive metabolite (M-2) was detected in the urine (Figure 7B). The radioactivity of M-2 accounted for 68% of total radioactivity excreted in the urine. When the urine sample was treated with β-glucuronidase type H-1 (β-glucuronidase/sulfatase mixture), the radioactive M-2 disappeared and a new major radioactive peak (M-1) emerged (Figure 7C). However, treatment of the urine sample with β-glucuronidase type H-1 in the presence of β-glucuronidase inhibitor (D-saccharic acid 1,4-lactone) caused no change in its retention time (Figure 7D). These results suggested that the radioactive urinary metabolite (M-2) was a glucuronide of M-1.

Figure 8 shows a representative HPLC profile of a fecal sample. The retention times of radioactive peaks P-1 and P-3 were in good agreement with those of EGC and EGCg, respectively. In addition, the retention time of radioactive peak P-2 coincided with that of radioactive M-1 found in the urine sample after the β-glucuronidase treatment. The radioactivity of P-1, P-2, and P-3 accounted for 40.8, 16.8, and 5.4% of the total radioactivity excreted in the feces, respectively. No changes in their retention times were observed by the treatment of fecal sample with β-glucuronidase type H-1 in the absence or presence of β-glucuronidase inhibitor (data not

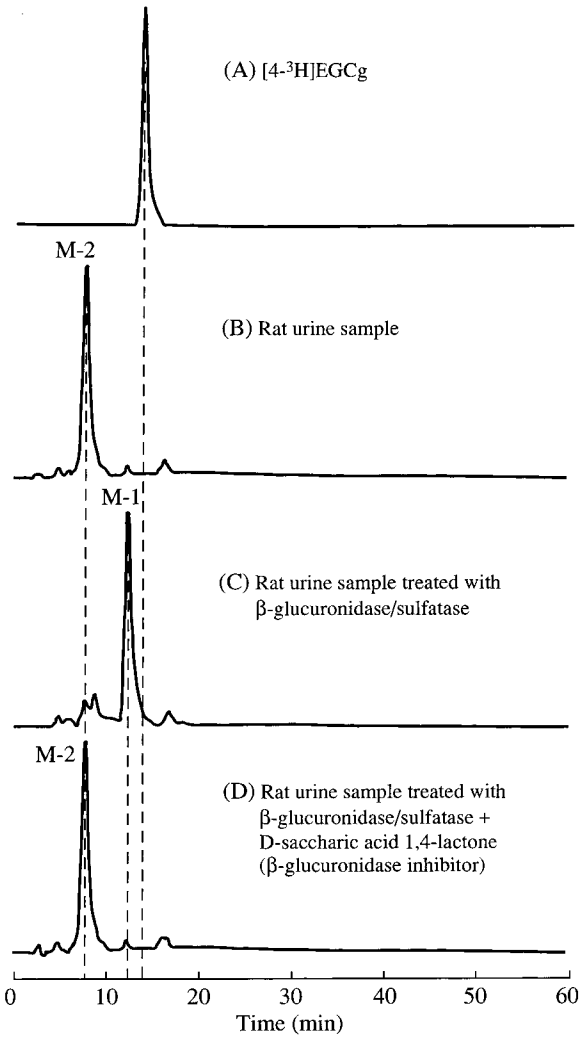


Figure 7. HPLC chromatograms of rat urine sample before and after β-glucuronidase/sulfatase treatment. The HPLC conditions and β-glucuronidase/sulfatase treatment were described under Materials and Methods. Detection was performed by β-radioactivity detector.

shown). From these results, it was concluded that the radioactive peaks, P-1, P-2, and P-3, were EGC, M-1, and EGCg, respectively.

Structural Identification of M-1 and M-2. We elucidated the chemical structures of the urinary radioactive metabolite, M-2, found in rats administered [4-^{3}H]EGCg in the following way. After oral administration of cold EGCg (100 mg/kg), urine was collected and metabolites M-1 and M-2 were purified as described under Materials and Methods.

The ^{1}H NMR spectrum of M-1 was similar to those of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (*21, 22*) and 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone (*22*). Positive FAB-MS data of M-1 showed a protonated molecular ion peak at *m/z* 209 $[M + H]^+$, in good agreement with the molecular weight of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. However, in the ^{1}H NMR data ABC-type aromatic protons in 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone changed to AB_2-type aromatic protons at δ 6.14 (1H, dd, J = 2.2, 2.2 Hz) and δ 6.19 (2H, d, J = 2.2 Hz) in M-1. From this observation, the proton signals at δ 6.14 and 6.19 in M-1 were assigned to H-4′ and H-2′ and 6′ aromatic protons, respectively. Further confirmation was substantiated by H,H-correlation spectroscopy (H,H-COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments. Full assignments of the ^{1}H NMR and ^{13}C NMR signals of M-1 are shown in Table 3. As a result, M-1 was identified as 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone. The optical rotation value, $[\alpha]_D$ −12.9° (c = 0.4, methanol at 27 °C), of this compound was very similar to that of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone [$[\alpha]_D$ −8.6° (c = 0.5, methanol)]. Hattori et al. (1997) showed that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone had an *R* configuration at the 4-position (personal communication). Accordingly, it was concluded that M-1, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone had an *R* configuration. The compound, M-1, showed (+) Cotton effect at ∼280 nm in CD measurement.

M-2 was a major metabolite found in rat urine in this study. The purified compound was hydrolyzed to yield M-1, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, by β-glucuronidase/sulfatase mixture. However, M-1 was not formed from M-2 by the enzymatic treatment in the presence of β-glucuronidase inhibitor. These results

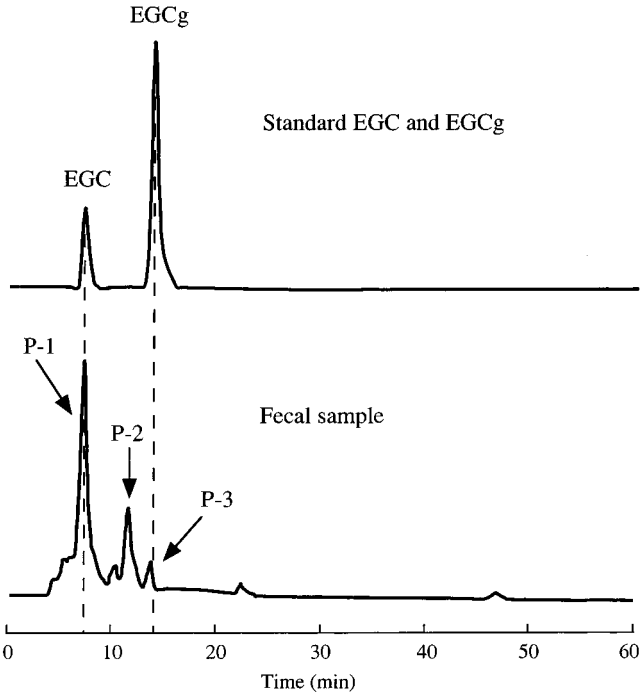


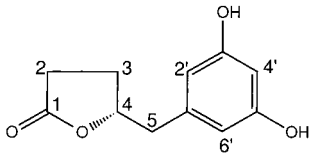
Figure 8. HPLC chromatogram of rat fecal sample. The HPLC conditions were described under Materials and Methods. Detections of standards and fecal sample were performed by UV (280 nm) and β-radioactivity detector, respectively.

Table 3. ^{1}H and ^{13}C NMR Chemical Shifts of 5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone[a]

^{1}H NMR		^{13}C NMR	
H-3β	1.95 dddd (0.5, 7.0, 9.5, 17.5)[b]	C-3	28.1
H-3α	2.25 dddd (4.6, 7.0, 8.9, 17.5)	C-2	29.5
H-2β	2.38 ddd (4.6, 9.5, 17.7)	C-5	42.2
H-2α	2.50 ddd (0.5, 8.9, 17.7)	C-4	82.9
H-5b[c]	2.76 dd (6.3, 13.9)	C-4′	102.1
H-5a[c]	2.87 dd (6.3, 13.9)	C-2′	109.0
H-4	4.73 dddd (6.3, 6.3, 7.0, 7.0)	C-6′	109.0
H-4′	6.14 dd (2.2, 2.2)	C-1′	139.9
H-2′	6.19 d (2.2)	C-3′	159.7
H-6′	6.19 d (2.2)	C-5′	159.7
		C-1	180.1

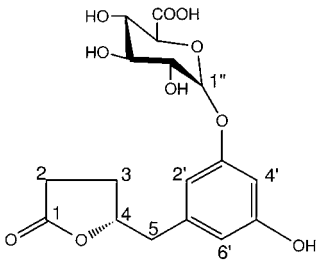
[a] Chemical shifts are expressed in ppm downfield from the signal of TMS in CD_3OD. [b] Coupling constants in Hz are in parentheses. [c] H-5a and 5b were provisionally determined according to literature (*21*).

M-1



(-)-5-(3', 5'-dihydroxyphenyl)-(4R)-γ-valerolactone

M-2



(-)-5-(5'-hydroxyphenyl)-(4R)-γ-valerolactone 3'-O-β-glucuronide

Figure 9. Structures of major urinary metabolite (M-2) and its deconjugated form (M-1).

indicated that M-2 was a glucuronide of M-1. In addition, HR-FAB-MS data ($[M - H]^-$ *m/z* 383.1050, calculated for $C_{17}H_{19}O_{10}$ 383.0978) showed that M-2 is a monoglucuronide of M-1. In ^{1}H NMR, the two signals at δ 6.14 (H-4′) and δ 6.19 (H-2′, 6′) in M-1 changed to three signals at δ 6.31, 6.33, and 6.36 in M-2, suggesting that glucuronic acid is attached at the 3′ (or 5′)- position of M-1. The anomeric proton of the glucuronide moiety was also observed at δ 4.81. All of these observations enabled M-2 to be identified as 5-(5′-hydroxyphenyl)-γ-valerolactone 3′-*O*-β-glucuronide. The assignment of the ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) was as follows: δ 1.86 (m, H-3β), 2.19 (m, H-3α), 2.38 (ddd, J = 4.6, 9.2, 17.7 Hz, H-2β), 2.76 (dd, J = 6.1, 13.9 Hz, H-5b), 2.84 (dd, J = 6.1, 13.9 Hz, H-5a), 4.68 (m, H-4), 4.81 (d, J = 6.9 Hz, H-1″), 6.31 (s, H-6′), 6.33 (s, H-4′), 6.36 (s, H-2′). The H-2α proton and ring protons of glucuronic acid were obscured by overlapping with methyl protons derived from DMSO-d_6 and H_2O, respectively. The chemical structures of M-1 and M-2 are illustrated in Figure 9.

DISCUSSION

Many physiological functions of EGCg have been reported, but the mechanisms of these functions have yet to be clearly defined, and thus elucidation of the metabolic fate of EGCg after oral administration is indispensable to this purpose. In this study we administered the previously reported [4-^{3}H]EGCg (*19*) orally to rats and investigated its metabolism. Results showed that up until 4 h after administration of [4-^{3}H]EGCg, the level of radioactivity in the blood and various tissues (excluding the digestive tracts) remained low. After this time, the radioactivity began to increase, peaked at 24 h, and decreased thereafter. Radioactivity excretion in the urine peaked at 8−24 h, and the cumulative amount excreted up until 72 h was ∼35% of the dose. On the other hand, when [4-^{3}H]EGCg was orally administered to rats pretreated with antibiotics (antibiotic-pretreated rats, with suppressed intestinal bacterial activity), the radioactivity was hardly detected in the blood and urine 24 h after administration. Moreover, on analysis of urinary radioactive metabolites in rats not pretreated with antibiotics (normal rats), neither EGCg nor its conjugates were detected up until 48 h after the dose, and it was found that ∼68% of radioactivity in the urine was derived from 5-(5′-hydroxyphenyl)-γ-valerolactone 3′-*O*-β-glucuronide (M-2). These results strongly suggest that most of the EGCg taken into the body is not absorbed intact but first undergoes degradation by intestinal bacteria before being absorbed, distributed, and excreted. In a recent study by Suganuma et al. (*16*), the radioactivity in the blood, tissues, and excreta was measured in mice after oral administration of [^{3}H]EGCg (the tritium atoms of which are considered to be in the B ring and/or galloyl moiety of EGCg). They showed that the radioactivity in the blood and most of the tissues was at a low level 1 h after the administration but increased after 6 h and was at an even higher level 24 h postdose. Furthermore, up until 24 h postdose, 6% of the dosed radioactivity was detected in the urine. From these findings, they proposed that EGCg is well absorbed from the intestine and is widely distributed to the blood and various tissues. However, because the time course of radioactivity in the blood, various tissues, and urine in the mice was similar to that of our present study with rats, it is reasonable to conclude that the radioactivity detected in the blood, various tissues, and urine of the mice is not derived from intact [^{3}H]EGCg but originated mainly from degradation products formed by intestinal bacteria as described above.

In this study, a metabolite in the urine of normal rats was isolated by HPLC, and upon determination of its structure by NMR and MS it was identified as 5-(5′-hydroxyphenyl)-γ-valerolactone 3′-*O*-β-glucuronide (M-2). The radioactivity of this compound was found to comprise ∼68% of total radioactivity excreted in the urine by 48 h. Furthermore, upon analysis of the radioactive components in the feces, radioactivity levels of EGC and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (M-1) were 40.8 and 16.8% of total radioactivity excreted in the feces by 48 h, respectively. These compounds detected in the urine and feces were not detected in antibiotic-pretreated rats (data not shown), indicating that they were the degradation products of EGCg formed by intestinal bacteria. Meselhy et al. (*21*) identified degradation products including 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone by anaerobic cultivation of (−)-epicatechin gallate (ECg) with human feces in vitro and suggested a metabolic pathway for ECg by intestinal bacteria. On the basis of their research and the results of our present study we proposed the following metabolic pathway for EGCg by intestinal bacteria (Figure 10). First, the galloyl group of EGCg undergoes hydrolysis in the intestine, and (−)-epigallocatechin (EGC) and gallic acid are formed. Next, EGC undergoes reduction cleavage, forming 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol. Then, this compound undergoes dehydroxylation at the 4′-position of the B ring, the phloroglucinol part (A ring) is decomposed, and after lactonization of C_2−OH with the residual carbonyl, the M-1 detected in the feces is produced. Alternatively, 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol undergoes degradation in the A ring to form 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, after which dehydroxylation occurs at the 4′-position of the B ring, forming M-1. Other examples of such ring fission by intestinal bacteria have been reported on (+)-catechin (*23*), (−)-epicatechin (*22*), and (−)-epigallocatechin (*22*).

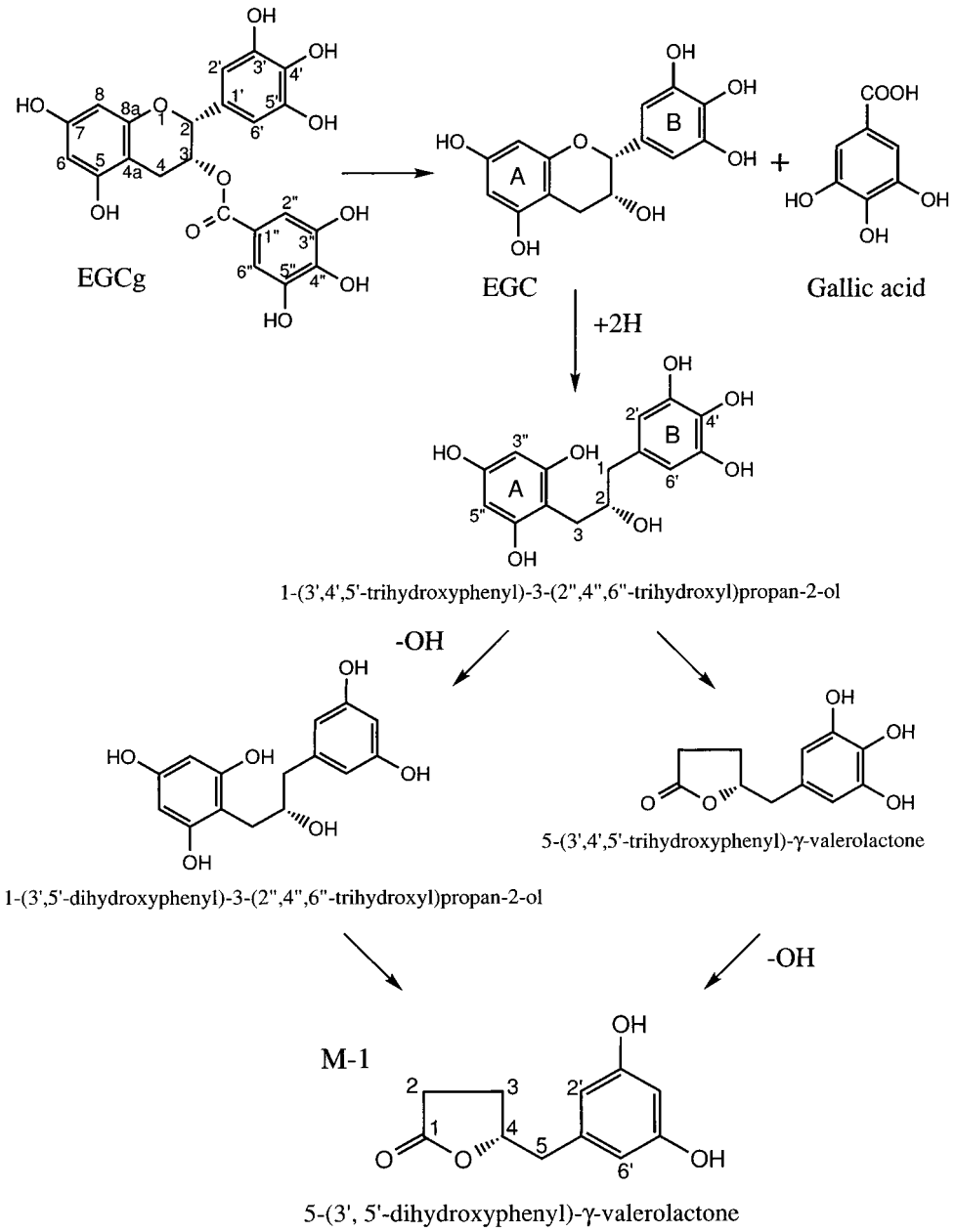


Figure 10. Proposed metabolic pathways of EGCg by rat intestinal bacteria.

On the basis of our results and past research on the metabolism of EGCg, we outlined a proposal for the metabolic route of EGCg as shown in Figure 11. Part of the orally administered EGCg is absorbed in the intestine and enters the liver via the portal vein (*9*, *10*). During this process most of the EGCg undergoes conjugation in the intestinal mucosa and/or the liver, whereas some is further methylated in the liver. Most of the EGCg metabolites formed are then excreted in the bile (*11*), but a part (including intact EGCg) enters the blood circulation, peaking at 1−2 h after administration (*12*−*14*). It has been reported that the bioavailability of EGCg (and its conjugates) in the blood circulation system is 0.1% (*14*). Moreover, calculation of the area under the curve (AUC) when [4-^{3}H]EGCg was intravenously administered to bile cannulated rats (AUC_{iv0-4h}) (*19*) and of the AUC of the present study when it was orally administered to antibiotic-pretreated rats ($AUC_{oral0-4h}$) showed the bioavailability ($AUC_{oral0-4h}/AUC_{iv0-4h}$) to be a very low 0.26%. Thus, it was considered that the amount of EGCg (and its conjugates) that enters the blood circulation and is distributed to the organs is nominal. This is probably because the greater part of EGCg absorbed undergoes first-pass effect due to hepatic elimination (*11*). On the other hand, most of the unabsorbed EGCg moves into the cecum and large intestine and then undergoes degradation by intestinal bacteria to 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (M-1) with EGC as an intermediate (Figure 10). A great part of M-1 formed is absorbed in the body, undergoing glucuronidation in the intestinal mucosa and/or liver, to form 5-(5′-hydroxyphenyl)-γ-valerolactone 3′-*O*-β-glucuronide (M-2), which enters the blood circulation, is distributed to various tissues, and is finally excreted in the urine. In addition, it is presumed that M-1 undergoes further degradation by intestinal bacteria before being absorbed in the body, because degradation products such as 3,4-dihydroxybenzoic acid and 3-methoxy-4-hydroxyhippuric acid are reported to be found in human urine after green tea intake (*24*).

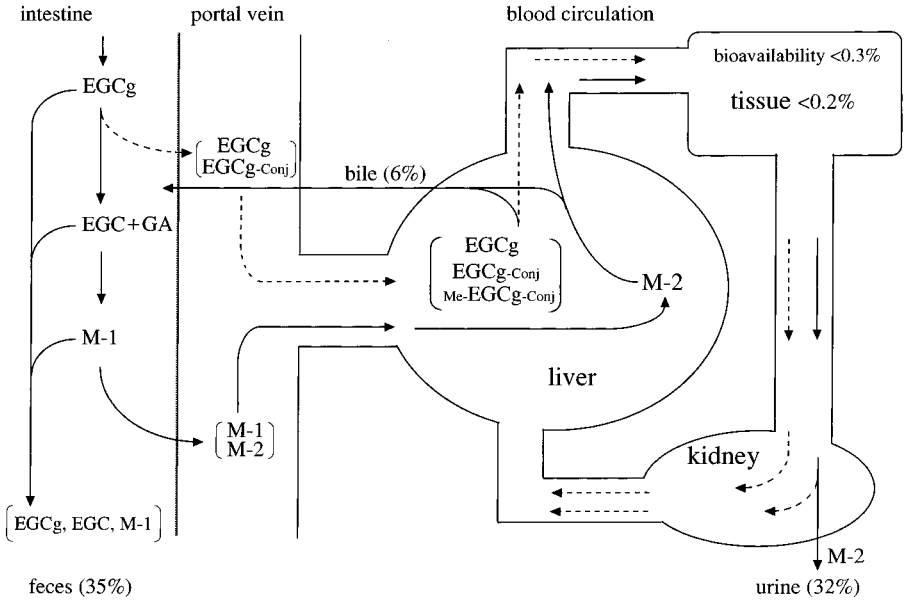


Figure 11. Possible metabolic route of EGCg orally administered to rats. GA, gallic acid; M-1, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; M-2, 5-(5′-hydroxyphenyl)-γ-valerolactone 3′-*O*-β-glucuronide; EGCg-Conj, EGCg conjugates; Me-EGCg-Conj, methylated EGCg conjugates; feces (35%), mean radioactivity excreted in feces by 72 h after a dose of [4-^{3}H]EGCg; urine (32%), mean radioactivity excreted in urine by 72 h after a dose of [4-^{3}H]EGCg; bile (6%), mean radioactivity excreted in bile by 48 h after an dose of [4-^{3}H]EGCg.

Although many studies on the urinary excretion of degradation products have been reported, information on biliary excretion is limited. However, from the fact that 5% of the dosed radioactivity was detected in the bile between 8 and 48 h postdose (data not shown) and results of a study (*25*) which reported that conjugates of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and its derivatives, with structures very similar to those of M-2, are excreted in rat bile, it is thought to be likely that degradation products including M-2 may be excreted in the bile at least in part.

Epidemiological studies conducted on tea consumption and the risk of certain cancers have been conflicting, with some reporting a reduction in risk (*26*, *27*) and others reporting a protective effect was not observed (*28*−*30*). In animal studies it is well recognized that tea catechins show an inhibitory activity against various cancers (*31*) such as skin, lung, liver, esophagus, and colon, although there have been a few studies that found no inhibitory effect (*32*). Thus, results concerning the relationship between tea or tea catechin consumption and cancer remain conflicting and inconclusive. In the case of human studies, these discrepancies may possibly be explained by taking into account factors such as drinking, smoking, and eating habits, but no such explanation exists for animal studies. On the basis of the results of this research we attempted to interpret the above situation. As described above, a minimal amount of EGCg is absorbed intact, but for the most part it is absorbed in the form of degradation products produced by intestinal bacteria. Accordingly, this leads to the logical inference that the amount of degradation products absorbed in the body is dependent on the degradation activity of intestinal bacteria and hence differs with each individual. Such a dependency may also imply that the amount of degradation products absorbed does not simply increase in proportion to the amount of EGCg dosed. These inferences are supported by the fact that in this study a change from 1 to 5 mg in the amount of [4-^{3}H]EGCg dosed hardly influenced the levels of radioactivity in the blood and urine. Thus, it may be considered that in the process of EGCg metabolism, degradation activity of intestinal bacteria is an influential factor in the formation of degradation products, thus greatly affecting the concentration of EGCg degradation products in the body. Furthermore, it is well recognized that there are differences in the degradation products according to each individual intestinal microflora. The following examples illustrate the above. Li et al. (*22*) found 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone in human urine after oral dosing of EGC, whereas we identified 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (M-1), which is a further dehydroxylation product of the compound they reported. Pietta et al. (*24*) detected even higher degradation products: 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxyhippuric acid, and 3-methoxy-4-hydroxybenzoic acid in human urine after green tea intake, which they regarded as being formed by intestinal bacteria from EGCg. Thus, administration of the same compound was found to yield various degradation products due possibly to differences in the intestinal bacteria, and these degradation products may have their own respective influences on physiological activities. For example, the antioxidative potency of 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (M-1) isolated in this study is predicted to show weaker antioxidative activity than the above degradation products having pyrogallol or catechol structure on the basis of the information (*33*−*36*) obtained so far. It is quite likely that the physiological activity following oral intake of EGCg is influenced appreciably both by the degradation activity of the bacteria and by the variety of degradation products formed. These influences may be important in determining whether the physiological activity in vivo is effectively exhibited or not, which in turn may throw light on the discrepancy between the results of the epidemiological and animal studies.

On the basis of our results and other research pertaining to the metabolism of EGCg, we proposed the outline of a possible metabolic route of EGCg orally administered to rats. However, to obtain a more detailed

picture it is necessary to identify other EGCg metabolites including the degradation products formed by intestinal bacteria. Furthermore, the individual physiological activities of the EGCg metabolites, including M-1 and M-2 identified in this study, must be determined so as to elucidate the physiological functions that result from the oral intake of EGCg.

ABBREVIATIONS USED

EGCg, (−)-epigallocatechin gallate; EGC, (−)-epigallocatechin; ECg, (−)-epicatechin gallate; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TMS, tetramethylsilane; HR-FAB-MS, high-resolution fast atom bombardment mass spectrometry; CD, circular dichroism; AUC, area under the blood concentration vs time curve.

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