Studies on Drug Metabolism by Use of Isotopes XXVII: Urinary Metabolites of Rutin in Rats and the Role of Intestinal Microflora in the Metabolism of Rutin

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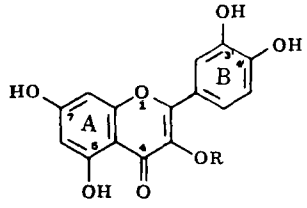
Abstract □ Analysis of urinary metabolites of orally administered rutin (I) labeled with deuterium ([2′,5′,6′-^{2}H]rutin, rutin-*d*) was carried out by GLC–MS. In rat urine, 3-hydroxyphenylacetic acid (III), 3-methoxy-4-hydroxyphenylacetic acid (IV), 3,4-dihydroxyphenylacetic acid (V), 3,4-dihydroxytoluene (VI), and 3-(*m*-hydroxyphenyl)propionic acid (VIII) were identified as rutin metabolites and were differentiated from the corresponding endogeneous compounds. Unchanged I and quercetin (II) were not present in the urine. Rutin-*d* was injected intraperitoneally in rats, administered orally to neomycin-treated rats, and incubated *in vitro* with the intestinal contents of rats. The experiments suggested the involvement of intestinal microflora in the metabolism of orally administered I.

Keyphrases □ Rutin—metabolites in rat urine, GLC–MS analysis, role of intestinal microflora □ Metabolites—rutin, analysis in rat urine, role of intestinal microflora □ Intestinal microflora—role in rutin metabolism, *in vitro* analysis with rat intestinal contents, GLC–MS detection

The metabolic fate of orally administered [2′,5′,6′-^{2}H]rutin (rutin-*d*) in humans was elucidated using GLC–MS (1, 2). 3-Hydroxyphenylacetic acid (III), 3-methoxy-4-hydroxyphenylacetic acid (IV), 3,4-dihydroxyphenylacetic acid (V), 3,4-dihydroxytoluene (VI), and β-*m*-hydroxyphenylhydracrylic acid (VII) were identified as urinary metabolites of rutin (I) and were differentiated from the corresponding endogenous compounds. All of these urinary metabolites were formed *via* reduction of the double bond of the γ-pyranone ring; III and VII were formed *via* dehydroxylation at the 4′-position of the B-ring.

The metabolism of I has been studied extensively in animals. In rat urine, III, IV, and V were identified as rutin metabolites after oral administration of I (3). Evidence for the involvement of the intestinal microflora in the metabolism of I was suggested by some investigators (4–8). These authors reported that anaerobic incubation of I with extracts of rat intestinal contents resulted in the formation of ring-fission metabolites (III and VIII). The intestinal origin of these metabolites was also demonstrated indirectly by the fact that excretion of the phenolic acids in the urine was suppressed when I was administered orally to neomycin-treated or germ-free rats (8, 9). However, an accurate determination of III–V derived from administered I is not possible, because the phenolic acids in question are also endogenous metabolites excreted in the ordinary urine and feces.

A radioactive isotope tracer technique is useful for the metabolic studies. Several radioactive metabolites were found in the urine after oral administration of [G-^{3}H]I in rats, but the structures were not elucidated (10). A mass chromatographic method using I labeled with stable isotopes is superior to a radioactive tracer technique in the structural elucidation of I metabolites. This report identifies the urinary metabolites of I in rats and elucidates the role of the intestinal microflora in the metabolism of I using a stable isotope tracer technique.



I: R = rutinose
II: R = H

EXPERIMENTAL[1]

Materials—Rutin[2] (I), quercetin[2] (II), 3-hydroxyphenylacetic acid[2] (III), 3-methoxy-4-hydroxyphenylacetic acid[2] (IV), 3,4-dihydroxyphenylacetic acid[2] (V), 3,4-dihydroxytoluene[3] (VI), 3-(3,4-dihydroxyphenyl)propionic acid[4] (IX), *p*-hydroxyphenyllactic acid[5] (X), neomycin sulfate[6], yeast extract[7], and peptone[7] were obtained commercially.

[2′,5′,6′-^{2}H]Rutin (Rutin-*d*)—A solution of NaOH (0.58 g) and I (6.0 g) in D_2O[8] (60.0 g) was heated at 95° for 8 hr in a sealed tube in a nitrogen atmosphere. The mixture was lyophilized, and D_2O (60.0 g) was added. The solution was heated for 8 hr in a similar manner and was acidified with 10% AcOH (200 ml). The resultant yellow solid was collected, washed with H_2O, dried, and then chromatographed using cross-linked dextran gel[9] with CH_3OH as eluant. Recrystallization from CH_3OH–H_2O (1:1) gave [2′,5′,6′,6,8-^{2}H]rutin as yellow needles (4.80 g). To a solution of NaOH (0.64 g) and H_2O (50.0 g) was added [2′,5′,6′,6,8-^{2}H]rutin (4.80 g). The mixture was stirred for 1 hr at 25° and then was acidified with 10% AcOH (150 ml). After repeating this procedure, the crude solid was purified as above for [2′,5′,6′,6,8-^{2}H]rutin to give [2′,5′,6′-^{2}H]rutin (rutin-*d*), 3.60 g (60.0% yield), mp 193°; ^{1}H-NMR(CD_3OD): δ 1.15 (d, 3, J = 6.0 Hz, rhamnosyl-CH_3), 6.16 (d, 1, J = 2.5 Hz, 6-CH), and 6.38 ppm (d, 1, J = 2.5 Hz, 8-CH). The ^{1}H-NMR spectrum showed that the isotopic composition (d_1, d_2, and d_3) was 1:6:3.

β-*m*-Hydroxyphenylhydracrylic Acid (VII)—To a solution of *m*-benzyloxyphenylhydracrylic acid (11) (1.0 g) in EtOH (70 ml) was added 5% Pd-C (0.25 g). The solution was stirred under a hydrogen atmosphere for 3 hr. The uptake of hydrogen was ~90 ml (theoretical 82 ml). The catalyst was removed by filtration. The filtrate was concentrated to dryness under reduced pressure, and the residue was crystallized from *n*-hexane–Et_2O (1:1) to give 0.65 g (97.1% yield). Recrystallization from *n*-hexane–EtOAc (1:1) gave V, 0.50 g (75% yield), mp 158–159° dec. [lit. (11) 159°]; ^{1}H-NMR (DMSO-d_6): δ 6.50–7.20 (m, 4, ArH), 4.84 (t, 1, J = 6.0 Hz, 3-CH), 2.48 ppm (d, 2, J = 6.0 Hz, 2-CH_2), and OH exchangeable; MS: m/z 182 (M^+).

[1] Melting points were determined in open-glass capillaries and are uncorrected. ^{1}H-NMR spectra were determined on a JEOL JM-MH-100 spectrometer using deuterated methanol for rutin-*d* and deuterated dimethyl sulfoxide for VII and VIII as solvents, with tetramethylsilane as the internal standard. Elemental analyses were performed by Analytical Center, Tokyo College of Pharmacy, Tokyo, Japan.

[2] Nakarai Chemicals Ltd., Kyoto, Japan.

[3] Wako Pure Chemical Industries, Tokyo, Japan.

[4] Aldrich Chemical Co., Milwaukee, Wis.

[5] Tokyo Chemical Industry, Tokyo, Japan.

[6] P-L Biochemicals, Inc., Milwaukee, Wis.

[7] Difco Laboratories, Detroit, Mich.

[8] Deuterium content ≥99.95%; E. Merck, Darmstadt, West Germany.

[9] Sephadex LH-20; Pharmacia Fine Chemicals, Uppsala, Sweden.

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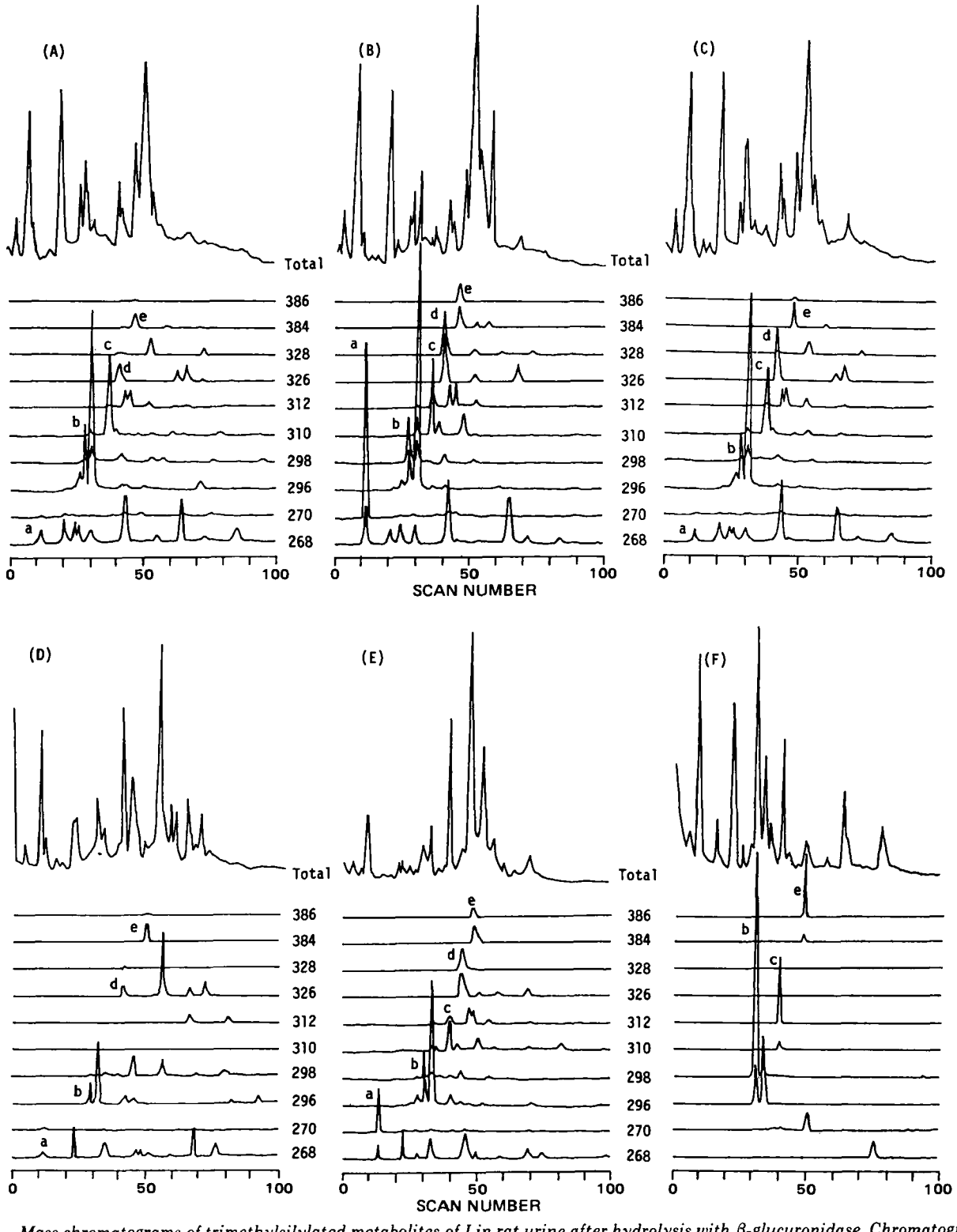

Figure 1—*Mass chromatograms of trimethylsilylated metabolites of I in rat urine after hydrolysis with β-glucuronidase. Chromatograms were obtained from the 24-hr urine of rats prior to (A) and after (B) oral administration of rutin-d and after intraperitoneal injection of rutin-d (C) before neomycin treatment. Chromatograms also were obtained from the 24-hr urine after oral administration of rutin-d in rats on the 2nd (D) and 14th (E) days after neomycin treatment. Chromatogram F was obtained from the incubation mixture of rutin-d and rat intestinal contents. Key: (a) VI [M^+:268, $(M+2)^+$:270]; (b) III [M^+:296, $(M+2)^+$:298]; (c) VIII [M^+:310, $(M+2)^+$:312]; (d) IV [M^+:326, $(M+2)^+$:328]; (e) V [M^+:384, $(M+2)^+$:386].*

Anal.—Calc. for $C_9H_{10}O_4$: C, 59.34; H, 5.53. Found: C, 59.38; H, 5.43.

3-(*m*-Hydroxyphenyl)propionic Acid (VIII)—To a solution of *trans*-*m*-hydroxycinnamic acid[5] (1.0 g) in EtOH (70 ml) was added 5% Pd-C (0.30 g). The solution was stirred under a hydrogen atmosphere for 2 hr. The uptake of hydrogen was ~150 ml (theoretical 137 ml). The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure to give 1.0 g (100% yield) of VIII which was recrystallized from Et_2O–benzene (1:1) to give white needles (VIII), 0.94 g (94% yield), mp 111°; ^{1}H-NMR (DMSO-d_6): δ 6.50–7.15 (m, 4, ArH), 2.40–2.84 ppm (A_2B_2 4, CH_2CH_2), and exchangeable OH; MS: *m/z* 166 (M^+).

Anal.—Calc. for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 65.15; H, 6.02.

GLC–MS—A GLC–mass spectrometer[10] and a data processing system[11] connected to a minicomputer[12] were used. The GLC system employed a 2 m × 3-mm i.d. glass column packed with 2% OV-105 on 60–80 mesh Chromosorb W[13]; it was packed with 1.5% OV-1 on 80–100 mesh Shimalite[3] for analysis of II. The columns were preconditioned with a helium flow of 30 ml/min at 230° (OV-105) and 280° (OV-1) for 24 hr, or until the baseline was stable. The column, flash heater, and separator temperatures were 160°, 180°, and 280°, respectively. The helium flow rate was 20 ml/min. The mass spectometer employed an ionization-source temperature of 310° and ionization energy of 20 eV.

One minute following injection of the sample, automatic magnet scanning was initiated, covering an m/z range of 50–500 every 8 sec. All scanning data were stored in the data processing system. Mass chromatograms were obtained on a digital plotter by monitoring the molecular ions of the $d_0(M^+)$ and $d_2[(M + 2)^+]$ forms of the trimethylsilylated derivatives of possible metabolites of I.

Drug Administration and Urine Collection—Three male Wistar albino rats, weighing 180–220 g, orally received 100 mg of rutin-*d*/kg. Urine was collected for 24 hr prior to and 24 hr after oral administration of rutin-*d*. Seven days later each animal was injected intraperitoneally with rutin-*d* (20 mg/kg). Urine was collected for 24 hr after the administration.

To inhibit the action of intestinal microflora, neomycin sulfate (100 mg/kg) was orally administered twice a day for 4 days to an additional three rats. Rutin-*d* (100 mg/kg) was administered orally to the rats on the 2nd and 14th day after treatment with neomycin ended. Urine was collected for 24 hr after each administration of rutin-*d*. Urine samples were diluted with distilled water to 30 ml and were kept frozen until analysis.

***In Vitro* Experiments**—The incubation medium was composed of 0.5% (w/v) yeast extract and 0.5% (w/v) peptone in 0.1 *M* phosphate buffer (pH 7.4). The rat intestinal contents were well mixed with 50 ml of the medium. The resulting suspension was centrifuged at low speed (500× *g*) for 1 min. To 5 ml of the supernatant thus obtained was added 3.0 mg of rutin-*d*. The mixture was incubated for 12 hr at 37° under anaerobic conditions.

Identification of I Metabolites by TLC—Urine samples (10 ml) collected prior to and after rutin-*d* administration were incubated at 37° for 24 hr with 10 ml of 0.1 *M* acetate buffer (pH 5.0) containing 3000 U of β-glucuronidase[14]. The incubation mixture was lyophilized and suspended in CH_3OH (10 ml). The suspension was centrifuged (1500× *g*) and the supernatant layer mixed with Et_2O (10 ml). The resulting suspension was centrifuged, and the precipitate was dissolved in CH_3OH (0.5 ml). A 0.1-ml volume of this solution was subjected to TLC[15]. Control urine (10 ml), collected prior to the rutin-*d* administration, was mixed with 200 μg of I and a sample (0.1 ml) was subjected to the TLC procedure. The TLC plate was developed with 1-butanol–AcOH–H_2O (4:1:2) and the spots were visualized by aluminum chloride (12), R_f 0.45 (I).

Identification of I Metabolites by GLC–MS—Urine samples (10 ml) collected prior to and after rutin-*d* administration were incubated at 37° for 24 hr in 10 ml of 0.1 *M* acetate buffer (pH 5.0) containing 3000 U of β-glucuronidase. Portions (10 ml) of urine obtained prior to and after the rutin-*d* administration and the incubation samples were subjected to the separation and extraction procedures without β-glucuronidase hydrolysis. Urine and incubation samples were acidified with 10% HCl (pH 1.0) and extracted with ether. The ether layer was concentrated to dryness under reduced pressure. The residue was dissolved in dry pyridine (0.1 ml), and *N,O*-bis(trimethylsilyl)acetamide[5] (0.05 ml) was added. The silylated derivatives were injected in the GLC–MS.

RESULTS AND DISCUSSION

The urine samples (10 ml) collected prior to and after I administration were subjected to TLC after extraction and separation procedures. The TLC results revealed that I was not present in the urine. To a 10-ml portion of the urine collected prior to the administration was added 200 μg of I; this urine sample was also subjected to TLC as above and I was detected. The results showed that the excretion of I in the urine after the I administration to rats was >20 μg/ml and suggested that orally administered I might be completely metabolized, as in humans (1).

[10] LKB-9000; Shimadzu Seisakusho Ltd., Kyoto, Japan.
[11] GCMSPAC-300; Shimadzu Seisakusho Ltd., Kyoto, Japan.
[12] OKITAC-4300; Oki Electric Industry Co., Tokyo, Japan.
[13] Nishio Industry Co., Ltd., Tokyo, Japan.
[14] Tokyo Zoki Kagaku Co., Ltd., Tokyo, Japan.
[15] Kieselgel $60F_{254}$; E. Merck, Darmstadt, West Germany.

Table I—Presence of Urinary Metabolites of Rutin in Rats and *In Vitro* with Rat Intestinal Contents [a]

Metabolite	Untreated Rats[b] po	Untreated Rats[b] ip	Neomycin-Treated Rats[b] After 2 Days	Neomycin-Treated Rats[b] After 14 Days	*In Vitro*[c]
III	+	—	—	+	+
IV	+	—	—	+	—
V	+	—	—	+	+
VI	+	—	—	+	—
VIII	+	—	—	+	+
I	—	—	—	—	recovered
II	—	—	—	—	—

[a] Urine and incubation samples obtained after the extraction and separation procedures were analyzed by GLC–MS or TLC. Key: (+) metabolite was detected; (—) metabolite was not detected. [b] Rutin-*d* was administered orally (po) or intraperitoneally (ip) to untreated rats and orally to neomycin-treated rats on the 2nd and 14th day after neomycin treatment ended. [c] Rutin-*d* was incubated with rat intestinal contents under anaerobic conditions.

The isotopic composition (d_1, d_2, and d_3) in rutin-*d* was 1:6:3. Therefore, particular attention was paid to the d_2 form for the determination of I metabolites by GLC–MS. The urine samples collected prior to and after the rutin-*d* administration were subjected to silylation with *N,O*-bis(trimethylsilyl)acetamide and analyzed by the GLC–MS computer system. Mass chromatograms were obtained by monitoring the molecular ions of the $d_0(M^+)$ and $d_2[(M + 2)^+]$ forms of the silylated derivatives of possible I metabolites. A metabolite was identified by examining the presence of duplicate peaks at the same retention time in the mass chromatogram.

Typical mass chromatograms obtained from the urine after hydrolysis with β-glucuronidase are given in Fig. 1A and B. The ratios of the peak intensities $[(M + 2)^+/M^+]$ shown in Fig. 1A were practically the same as those estimated from control urine. On the other hand, the ratios of the peak intensities $[(M + 2)^+/M^+]$ were larger in peaks a–e in Fig. 1B than those in the peaks shown in Fig. 1A, indicating the increment of the peak intensity in each $(M + 2)^+$ peak. These findings made it evident that orally administered rutin-*d* was metabolized to give five metabolites which were also present in the urine as endogenous materials.

These metabolites were identified as III (peak b), IV (peak d), V (peak e), VI (peak a), and VII (peak c). Identification was made by comparing the mass spectra and GLC retention times of the respective mass chromatogram peaks with the corresponding silylated authentic compounds. The five metabolites identified in the urine after hydrolysis with β-glucuronidase were also found in the urine without β-glucuronidase treatment. Four of these metabolites, *i.e.*, III–VI, were the same as those found in human urine. β-*m*-Hydroxyphenylhydracrylic acid (VII), detected in human urine, 3-(3,4-dihydroxyphenyl)propionic acid (IX), *p*-hydroxyphenyllactic acid (X), and β-*p*-hydroxyphenylhydracrylic acid (XI) were excluded from the metabolites of rutin-*d* by the mass spectra of the corresponding authentic compounds or the results of one investigation (13). Quercetin, an aglycone of I, was not found in the urine. It was impossible to follow the metabolic change occurring in the A-ring on which no atoms were deuterium labeled.

Rutin-*d* was injected intraperitoneally in rats on the 7th day after oral administration of rutin-*d*. The urine samples from the rats injected with rutin-*d* were subjected to the GLC–MS analysis. The mass chromatogram (Fig. 1C) thus obtained was similar to that obtained prior to oral administration of rutin-*d* (Fig. 1A). The five metabolites detected in urine after the oral administration of rutin-*d* were not excreted in the urine after the intraperitoneal injection of rutin-*d* (Fig. 1B and C). The results indicated that orally administered I must be metabolized by intestinal microflora or by enzymes in the intestinal walls. Petrakis *et al.* (14) injected [G-^{14}C]quercetin into rats intraperitoneally and found radioactive vanillic acid only in the urine. In the case of the rutin-*d* injection, this metabolite, though endogenously present, could not to be found in the urine.

The absorption of I in the GI tract has been investigated (10, 15–19). It has been reported that [G-^{3}H]I (10) and [G-^{14}C]II (14) are absorbed very slowly from the GI tract when administered orally to rats. Because of the slow absorption of I and II by the intestines, the intestinal microflora play a significant role in the metabolism of orally administered I.

Rutin-*d* was administered to another group of rats orally. The mass chromatogram thus obtained was similar to that shown in Fig. 1B and showed the presence of five metabolites derived from rutin-*d*. The rats then received neomycin orally twice a day for 4 days to inhibit the action of the intestinal microflora. Rutin-*d* was administered to the rats on the

2nd day after treatment with neomycin ended. The mass chromatogram is given in Fig. 1D. In peaks a, b, d, and e in Fig. 1D, the ratios of the peak intensities $[(M + 2)^+/M^+]$ were much smaller than those of the corresponding peaks in the mass chromatogram obtained when rutin-*d* was administered to untreated rats (Fig. 1B). In addition, peak c did not appear in Fig. 1D. Rutin-*d* was also administered to rats on the 14th day after neomycin treatment when the action of the intestinal microflora had been restored. In the mass chromatogram thus obtained (Fig. 1E), the $[(M + 2)^+/M^+]$ ratios were larger than those in the mass chromatogram obtained from neomycin-treated rats (Fig. 1D). The five metabolites were detected in the untreated rats. They were reduced or disappeared in the neomycin-treated rats and were increased on the 14th day after neomycin treatment. The results obtained from the intraperitoneal injection and the subsequent neomycin-treatment experiments showed that intestinal microflora greatly influence the metabolism of orally administered I.

Rutin-*d* was incubated with extracts of rat intestinal contents under anaerobic conditions. As shown in the mass chromatogram (Fig. 1F) thus obtained, three metabolites, *i.e.*, III (peak b), V (peak e), and VIII (peak c), appeared. Compound II and unchanged I were also found in the incubation mixture. The *in vitro* experiments suggested direct involvement of the intestinal microflora in the metabolism of I, even though all of the metabolites detected in the urine after oral administration of rutin-*d* were not found. The lack of formation of metabolites IV and VI in the mixture indicated that they were excreted in the urine *via* methylation or decarboxylation by enzymes in the intestinal walls or in other tissues after the absorption of orally administered I from the GI tract.

It has been reported that I was metabolized to give phenolic acids such as III, IV, VIII, *etc.* by the isolated perfused rat liver (19). These phenolic acids should be excreted in the urine *via* liver metabolism if I is absorbed as such from the GI tract after oral administration of rutin-*d* in neomycin-treated rats. However, these metabolites were not found in the urine from neomycin-treated rats (Fig. 1D), and neither I nor II was present. It is then reasonable to assume that orally administered I could not be absorbed from the GI tract *per se*. 3,4-Dihydroxyphenyl[carboxy-^{14}C]acetic acid (V), when administered to rats orally, was almost completely excreted in the urine in the form of III, IV, and V (20). Thus, I must be metabolized to the phenolic acids by intestinal microflora, with subsequent absorption from the GI tract.

In this study, five metabolites (III–VI and VIII) derived from orally administered rutin-*d* were differentiated from these compounds endogenously present in the urine and successfully identified by the mass chromatographic method. In addition, the involvement of intestinal microflora in the metabolism of I was investigated by this method (Table I). It is possible that the human intestinal microflora may also play a significant role in the formation of urinary metabolites of I (III–VII) in humans.

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Antitumor Agents LXII: Synthesis and Biological Evaluation of Podophyllotoxin Esters and Related Derivatives

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Abstract □ Synthetic esters of the C-4 hydroxyl group of podophyllotoxin (I) were prepared. In addition, esters were synthesized using the diol system of tetrahydropyranyl podophyllol (XV), produced by reducing the lactone ring of tetrahydropyranyl podophyllotoxin with lithium aluminum hydride. Six compounds, the acrylate (IV), 3,3-dimethyl acrylate (V), phenoxyacetate (IX), and ethyl adipate (XI) of I as well as podophyllol (XIV) and tetrahydropyranyl podophyllol dimesylate (XVIII), showed significant activity when tested using the P-388 lymphocytic leukemia screen at 3 mg/kg/day. None of the esters showed higher activity than that shown by the parent molecule I when tested at the same dosage level.

Keyphrases □ Podophyllotoxin—esters, synthesis, antileukemic activity in mice □ Synthesis—podophyllotoxin esters, antileukemic activity in mice □ Antileukemic agents—potential, podophyllotoxin esters, synthesis

The development of teniposide (VM-26) and etoposide (VP-16-213), two glucopyranosyl derivatives related to the lignan podophyllotoxin (I), as clinically effective anticancer drugs has been reviewed recently (1). An examination of the structural features of teniposide and etoposide indicated that a free hydroxyl group at C-4 in epipodophyllotoxin is not essential for potent activity. Thus, it was considered that further modification of the C-4 hydroxyl group of I or epipodophyllotoxin could yield additional potent antitumor agents. In view of the importance of an

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