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Absorption and Metabolism of γ-Oryzanol in Rats

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The metabolism and lymphatic transport of γ-oryzanol in rats were investigated following oral administration of the ^{14}C-labeled compound (triterpenyl esters of ferulic acid-3-^{14}C). Redioactivity excreted in the urine during 72 h after oral administration was 9.8 % of the dose (50 mg/kg). Unchanged γ-oryzanol was not detected at all in the urine, but an in situ intestinal absorption experiment suggested that more than 80% of the absorbed radioactivity was transferred into the mesenteric vein as an intact form, though a part was metabolized in the small intestine during absorption.

Ferulic acid, dihydroferulic acid, *m*-hydroxyphenylpropionic acid, *m*-coumaric acid, *m*-hydroxyhippuric acid and hippuric acid were identified as the major urinary metabolites. These metabolites, with the exception of hippuric acid, were also excreted in the form of glucuronide or sulfate.

The radioactivity transported into the thoracic duct was only 0.3% of the dose, and of this, more than 80% was found to be γ-oryzanol by thin-layer chromatography. From these results, it is considered that γ-oryzanol administered orally is absorbed mainly into the blood *via* the portal vein system, not into the lymph *via* the thoracic duct.

Keywords——γ-oryzanol; rat; metabolism; *in situ* absorption experiment; lymphatic transport; ferulic acid; *m*-hydroxyphenylpropionic acid; hippuric acid

In the previous paper,[1] we reported on the metabolism of γ-oryzanol in rabbits. Meanwhile, the metabolism of γ-oryzanol in rats was studied by Noda *et al.*[2] who identified only two metabolites, ferulic acid and *m*-hydroxyphenylpropionic acid, in urine. However, detailed investigation, including quantitative analysis of the urinary metabolites, has not been done.

The present study was undertaken in order to identify and quantitate the urinary metabolites, and also to investigate the metabolism in the intestine by the use of γ-oryzanol-^{14}C labeled at the C-3 position of ferulic acid. In addition, lymphatic transport was studied to clarify the main absorption route of γ-oryzanol.

Experimental

Materials——γ-Oryzanol-^{14}C (specific activity, 0.75 μCi/mg) synthesized by Sato and Awata[3] was used. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), *m*-hydroxyphenylpropionic acid, *m*-coumaric acid (*m*-hydroxycinnamic acid), dihydroferulic acid (4-hydroxy-3-methoxyphenylpropionic acid), *m*-hydroxyhippuric acid and hippuric acid were obtained from Tokyo Kasei Kogyo Co. Ltd. Triolein and polysorbate 80 were supplied by Wako Pure Chemicals Ltd., and sodium taurocholate was supplied by Sigma Chemical Co. β-Glucuronidase (EC 3.2.1.31, from *E. coli*) and arylsulfatase (EC 3.1.6.1. from *Helix pomatia*) were purchased from Boehringer Mannheim Yamanouchi Co. Other chemicals used were of analytical reagent grade and were obtained commercially.

The triolein solution and emulsion were prepared as follows; (1) triolein solution: γ-oryzanol-^{14}C was dissolved in triolein (10 mg/ml), (2) emulsion: 50 mg of γ-oryzanol-^{14}C was dissolved in 2.5 g of triolein containing 0.5 g of polysorbate 80, then 8 ml of 10 mM sodium taurocholate was added and the mixture was shaken vigorously and sonicated.

Animals——Male Sprague-Dawley rats weighing around 250 g were fasted for 20 h prior to the experiments, but water was given freely.

Urinary and Fecal Excretions——The triolein solution of γ-oryzanol-^{14}C was administered (50 mg/kg) orally to rats, then the urine and feces were collected daily for five days. The urine was diluted with water to a certain volume, then 0.5 ml of each sample was taken into a counting vial and 10 ml of a scintillation medium (PPO 16.5 g, POPOP 0.3 g, Toluene 2 l, Triton X-100 1 l) was added. The radioactivity was measured with a liquid scintillation spectrometer (Packard, Tri-carb 3385 or 2450). The feces were weighed and homogenized in a certain amount of added water. Aliquots of the homogenates were dried and subjected to combustion in an oxidizer (Packard, Tri-carb 306), and the radioactivity was measured.

Measurement of the Blood Level——An accurately measured 0.2 ml of bood was collected in a heparinized capillary tube from the tail vein prior to the administration and at appropriate times after oral administration of the triolein solution of γ-oryzanol-^{14}C (50 mg/kg). The blood samples were dried, and subjected to combustion, then the radioactivity was measured.

Separation, Identification and Quantitation of the Urinary Metabolites——Ten ml portions of the 24 h urine were extracted by the method shown in Chart 1, and the radioactivity recovered in each fraction was measured. The extracts of each fraction were evaporated to dryness under a vacuum, and appropriate portions of the methanol soluble fraction were spotted on a thin-layer cellulose plate (Merck, Cellulose F, No. 5718, 20×20 cm), and subjected to two-dimensional thin-layer chromatography (TLC) developed with 5% acetic acid in the first direction, followed by the lower organic layer of a mixture of chloroform, acetic acid and water (2:1:1, v/v) in the second direction. The radioactive metabolites on the plate were detected by contact with ^{3}H film (LKB, Ultrofilm ^{3}H). For the quantitative analysis of the urinary metabolites, the radioactive areas and all the remaining area on the plate were separately scraped from the plate, and taken into vials. Then 10 ml of the scintillation medium was added and the radioactivity was measured.

The structures of the metabolites were deduced by comparison of the locations of authentic samples with those of the radioactive spots obtained in autoradiography. The identities of the metabolites were confirmed by a reverse isotope dilution analysis. The locations of authentic samples on the plate were confirmed by spraying with diazotized *p*-nitroaniline[4] or by irradiation with ultraviolet (UV) light (254 nm).

Procedure for *in Situ* Intestinal Absorption Experiment——Rats were anesthetized with sodium pentobarbital. The jugular vein was cannulated and the mid-ileal portion of the intestine was exposed by a midline abdominal incision. An ileal segment (about 5 cm in length) was isolated, the lumen was washed with

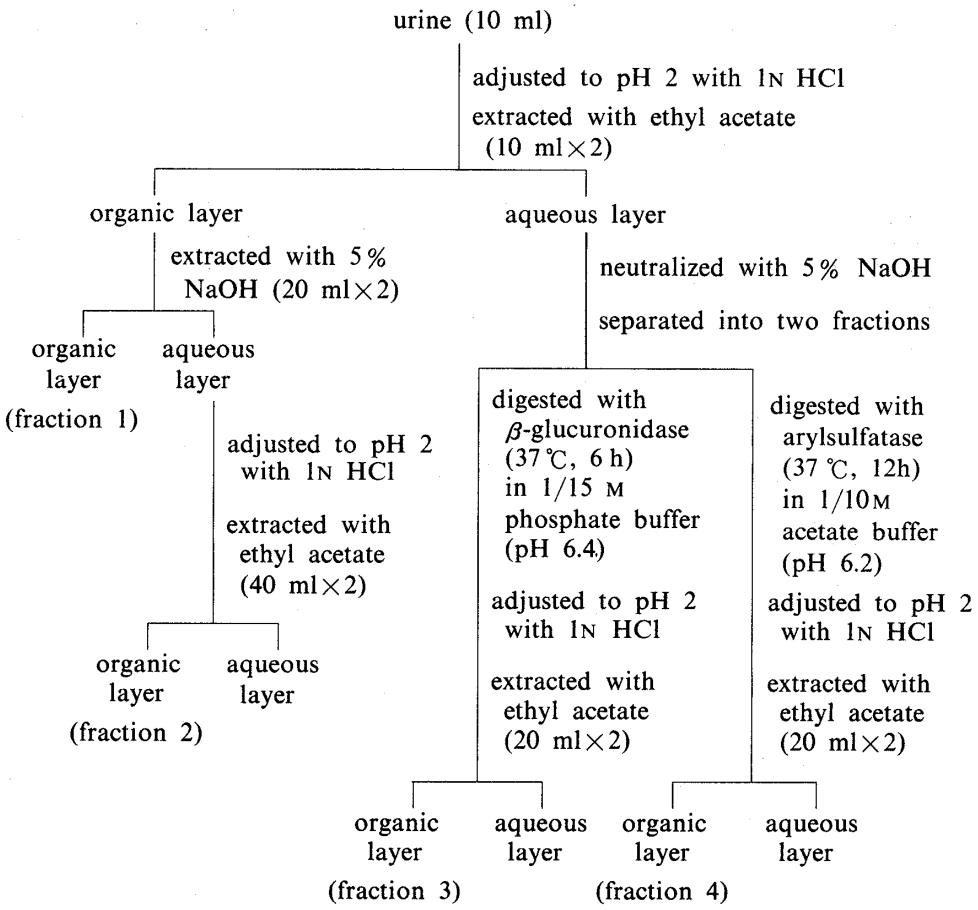


Chart 1. Procedure for the Separation of the Metabolites in Rat Urine

isotonic saline, one end of the segment was ligated, and the mesenteric vein was cannulated with polyethylene tubing (PE 50; i.d., 0.023 inch; o.d., 0.038 inch). An aliquot (0.5 ml) of the emulsion of γ-oryzanol-^{14}C was injected into the lumen, and the other end of the segment was ligated. All venous blood draining the segment was collected in heparinized, calibrated tubes for five 10 min periods. The blood lost from the mesenteric vein was continuously replaced by jugular infusion with an equal volume of heparinized whole blood previously collected from other rats. Rats were placed in a warm (37°C) box, and the isolated intestine was kept warm and moist by frequent application of warm (37°C) saline to a gauze covering the intestine. At the end of the experiment, the intestine was cut off, and the luminal fluid was withdrawn as completely as possible and washed with physiological saline. The washings were combined with the luminal fluid and made up to 10 ml with water. The intestine was homogenized in 5 ml of 10% trichloroacetic acid. Aliquots (0.5 ml) of the luminal fluid and the intestinal homogenates were used for the measurement of radioactivity. One ml of each blood sample was extracted by the method of Fujiwara *et al.*[5] for the quantitative analysis of γ-oryzanol and ferulic acid. The luminal fluid and the intestinal homogenates were acidified with 1 N HCl and extracted with ethyl acetate. These extracts were analyzed by TLC according to the method of Belfrage *et al.*[6] Thin-layer plates (Merck, Silica gel 60 F_{254}, 5×20 cm) were immersed in an ether solution containing 5% (v/v) acetic acid for 5 min. The ether was allowed to evaporate, and an appropriate amount of the extract was applied. Solutions of nonradioactive γ-oryzanol and ferulic acid (reference standards) were also applied on the same plate. Then the plate was developed with a mixture of ether, heptane, acetic acid and methanol (15:85:2:3, v/v). After development, the plate was removed and air-dried. The silica gel at the same positions as the reference standards detected under UV light (254 nm) on the plate was scraped off and taken into vials, and the radioactivity was measured as described previously.

Procedure for Lymphatic Transport Experiment——Rats were anesthetized with sodium pentobarbital given intraperitoneally (50 mg/kg body weight), and the thoracic duct in the abdomen was cannulated with a polyethylene catheter (PE 50) according to the method of Bollman *et al.*[7] The rats were kept in restraining cages and had free access to water containing 10% glucose and 0.9% NaCl. Three animals that had satisfactory lymph flow (more than 2 ml/h) were used for the absorption study, which was begun 16-24 h after the surgery. The triolein solution of γ-oryzanol-^{14}C was administered orally (50 mg/kg) by gastric intubation, and the lymph was collected in a heparinized tube for 32 h after the administration. Aliquots (0.5 ml each) of the lymph were taken into counting vials and the radioactivity was counted in a scintillation medium. Analysis of the lymph was performed by the following procedure. Ten ml of the lymph was acidified with 2 ml of 1 N HCl, and extracted twice with 30 ml of a mixture of ethanol and ether (1:3, v/v). After the removal of the solvent under a vacuum, the residue was analyzed by TLC as described above (in "Procedure for *in situ* Intestinal Absorption Experiment").

Results

Urinary and Fecal Excretions

Table I shows the urinary and fecal excretions of radioactivity following oral administration of γ-oryzanol-^{14}C. During 72 h, 9.8% of the dose was excreted in the urine, and most of the radioactivity was recovered within the first 24 h. Fecal excretion was 84.5% of the dose during 72 h, and thereafter no radioactivity was detected in feces.

Blood Levels

Figure 1 illustrates the time course of the radioactivity in blood. The radioactivity attained a maximum 4 h after administration, and decreased gradually after that time. At 24 h, a little radioactivity was found, but the radioactivity had disappeared from the blood at 48 h.

Urinary Metabolites

The percentages of the radioactivity extracted into each fraction of 24 h urine are

TABLE I. Urinary and Fecal Excretions of Radioactivity after Oral Administration of γ-Oryzanol-^{14}C to Rats

	% of dose[a]					
	24 h	48 h	72 h	96 h	120 h	Total
Urine	9.2±3.2	0.5±0.2	0.1±0.1	0.0	0.0	9.8±2.9
Feces	78.4±9.9	6.0±6.5	0.0±0.1	0.0	0.0	84.5±4.1

a) Each value represents the mean±S.D. of 3 rats.

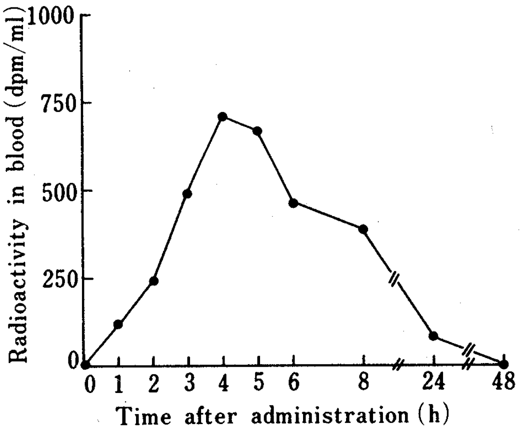


Fig. 1. Blood Levels of Radioactivity after Oral Administration of γ-Oryzanol-^{14}C to Rats

Each point represents the mean of 3 rats.

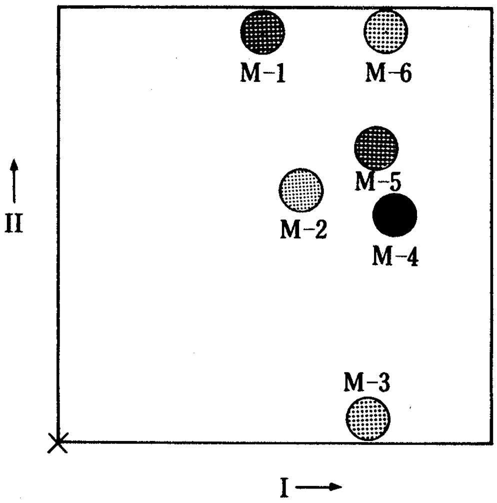


Fig. 2. Two-Dimensional TLC of Fraction 2 separated from Rat Urine by the Method shown in Chart 1

Solvent: I, 5% acetic acid; II, lower layer of a mixture of chloroform, acetic acid and water (2:1:1, v/v).

TABLE II. Recovery of Radioactivity in the Urine excreted for 24 h

Fraction	Recovery[a] (%) Rat No. 1	2	3	Mean
1	N. D.[b]	N. D.	N. D.	N. D.
2	45.8	48.3	54.1	49.4
3	16.1	12.3	12.6	13.7
4	37.8	36.8	31.4	35.5
Total	99.7	97.4	98.1	98.4

a) Each value represents % of the urinary excretion.
b) Not detectable.

summarized in Table II. Almost 100% of the radioactivity present in the urine was recovered. Fraction 1, which should contain γ-oryzanol, showed no radioactivity. This result indicated that γ-oryzanol was completely metabolized in the body. The recovery of radioactivity was the largest in fraction 2, and decreased in fraction 4 and fraction 3, in that order.

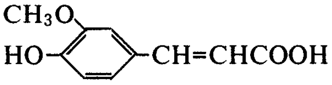
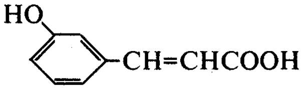
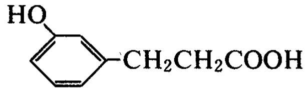
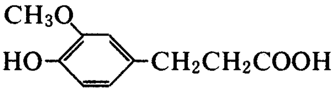
In order to identify the metabolites, fraction 2 was analyzed by two-dimensional TLC. As shown in Figure 2, six radioactive metabolites from M-1 to M-6 were detected chromatographically. Five metabolites, with the exception of M-4, were also detected from fractions 3 and 4. Two metabolites, M-1 and M-5, gave *Rf* values corresponding to those of authentic ferulic acid and *m*-hydroxyphenylpropionic acid, which were identified as metabolites by Noda *et al.*[2] On the other hand, Booth *et al.*[8] reported that nine metabolites including ferulic acid were excreted in rat urine after oral administration of unlabeled caffeic acid (3,4-dihydroxycinnamic acid). In co-TLC with authentic samples, M-2, M-3 and M-6 gave the same *Rf* values as *m*-coumaric acid, *m*-hydroxyhippuric acid and dihydroferulic acid, respectively. To identify the metabolite M-4, unlabeled γ-oryzanol was administered orally

TABLE III. Reverse Isotope Dilution Analysis of Urinary Metabolites

Metabolites	Carrier compound added	Specific activity (dpm/mg)		
		1 st.	2 nd.	3 rd.
M-1	Ferulic acid	463	465	463
M-2	*m*-Coumaric acid	52	48	49
M-3	*m*-Hydroxyhippuric acid	73	70	69
M-4	Hippuric acid	294	290	288
M-5	*m*-Hydroxyphenylpropionic acid	200	197	195
M-6	Dihydroferulic acid	153	154	151

The recrystallization solvent was water.

TABLE IV. Urinary Excretions of Metabolites in Rats following Oral Administration of γ-Oryzanol-^{14}C

Metabolites	% of urinary excretion[a]			
	Unconjugated	Glucuronide	Sulfate	Total
	11.3	8.1	27.1	46.5
	0.3	0.3	0.8	1.4
	1.9	< 0.1	< 0.1	2.0
	20.7	N.D.[b]	N.D.	20.7
	9.6	0.8	2.8	13.2
	4.6	4.4	3.5	12.5
Others	1.1	0.1	1.1	2.3

a) Each value represents the mean of 3 rats.
b) Not detectable.

to rats and the 24 h urine was extracted to obtain fraction 2, which contained a larger amount of M-4, according to the method described in Chart 1. Metabolite M-4 was isolated by two-dimensional TLC of the extract and recrystallized from water to yield colorless needles.

The nuclear magnetic resonance (NMR) spectrum[9] of this compound showed the signals of a secondary amino group at 8.74 ppm and methylene protons at 4.01 ppm as a doublet. Also, the signals of aromatic protons (5H) appeared at 7.30—7.92 ppm in the NMR spectrum, and the mass spectrum[9] showed a molecular ion peak at m/z 179 with fragment ions at m/z 134 (M^+-COOH), 105 (M^+-NHCH_2COOH) and 77 ($M^+-CONHCH_2COOH$). On the basis of these results, M-4 was assigned as hippuric acid. Moreover, as shown in Table III, a reverse isotope dilution analysis showed that every metabolite gave a uniform specific activity, confirming the presumed structures of the metabolites.

Table IV summarizes the urinary excretions of the metabolites during 24 h. The total amount of ferulic acid excreted as unconjugated form, sulfate and glucuronide was about 50% of the urinary excretions, and was in the order sulfate>unconjugated>glucuronide. Other metabolites such as hippuric acid, *m*-hydroxyphenylpropionic acid and dihydroferulic acid were also excreted in relatively large quantities.

Metabolism in the Intestine

Table V summarizes the percentages of radioactivity remaining in the luminal fluid and the intestinal tissue, and transferred into the mesenteric venous blood at 50 min after the infusion of γ-oryzanol emulsion. The amount transferred into the blood was only 0.2% of the dose and the major portion of the radioactivity remained in the infused emulsion, suggesting that γ-oryzanol is poorly absorbed.

The cumulative amounts of γ-oryzanol and ferulic acid recovered from the blood are shown in Fig. 3. The amount of γ-oryzanol or ferulic acid increased with time almost linearly. It was found that 10—20% of the administered γ-oryzanol was metabolized in the intestinal tissue to yield ferulic acid, but the remainder was absorbed into the portal vein as an intact γ-oryzanol form. Furthermore, it was confirmed by TLC that more than 95% of the radioactivity that remained in the luminal fluid or the intestinal tissue was in the form of γ-oryzanol.

TABLE V. Percentages of Radioactivity in the Luminal Fluid, Intestinal Wall and Mesenteric Venous Blood

Rat No.	% of dose		
	Luminal fluid	Intestinal wall	Blood
1	92.0	7.8	0.2
2	90.1	9.8	0.1
3	86.1	13.6	0.2
Mean	89.4	10.4	0.2

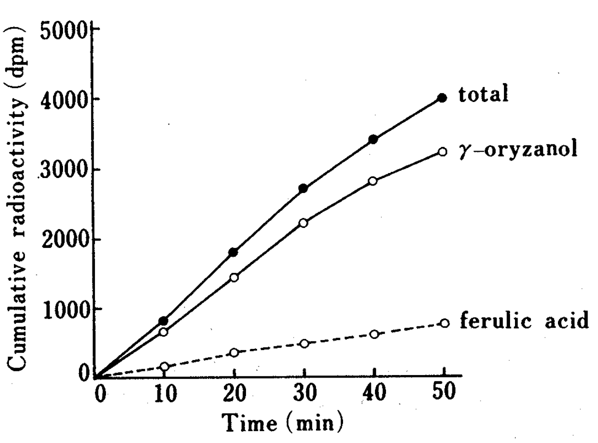


Fig. 3. Cumulative Radioactivity of γ-Oryzanol and Ferulic Acid transferred into the Mesenteric Vein

Each point represents the mean of 3 experiments.

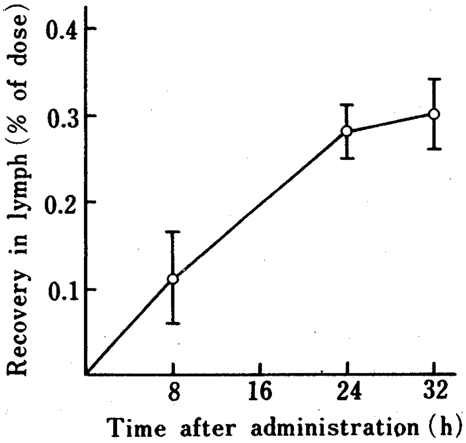


Fig. 4. Cumulative Recovery of Radioactivity in Thoracic Duct Lymph after Oral Administration of γ-Oryzanol-^{14}C

Each point represents the mean±S.D. of 3 rats.

TABLE VI. Composition of Radioactive Compounds in Lymph

Rat No.	Lymph used (h)	Fraction (%[a])		
		γ-Oryzanol	Ferulic acid	Others
1	8—24	80	6	14
2	0—8	81	13	6
3	0—8	92	7	1

a) % of the total radioactivity applied to the TLC plate.

Lymphatic Transport

Figure 4 shows the lymphatic transport after oral administration of γ-oryzanol-^{14}C. During 32 h, only 0.30% of the dose was transported into the thoracic duct lymph, and most of the radioactivity absorbed was recovered within the first 24 h. The form of the radioactivity in the lymph was analyzed by TLC, and the results are given in Table VI. In the lymph, more than 80% of the radioactivity was found to exist as γ-oryzanol, but a little ferulic acid, presumably produced by ester hydrolysis in the intestine, was also found in the lymph.

Discussion

When γ-oryzanol-^{14}C was administered orally to rats at the dose of 50 mg/kg, the radioactivity in blood reached a maximum 4 h after administration, and even at 24 h a little radioactivity was found. On the other hand, in rabbits,$^{1)}$ the radioactivity in blood reached a maximum 3 h after oral administration and no radioactivity was detected in blood at 24 h. This indicated that the blood level in rats was better sustained than that in rabbits. During 72 h, 9.8% of the dose was excreted in the urine, while Noda *et al.* reported that the urinary excretion was only 1.7% of the dose for 48 h after oral administration of γ-oryzanol-^{14}C (50 mg/kg). It is considered that the difference of the urinary excretion is due to an increase of the radioactive urinary metabolites resulting from the difference of the ^{14}C-labeling site, and/or due to an increase of the intestinal absorption induced by the administration of triglyceride.

Investigation of the urinary metabolites was performed with reference to the study of Booth *et al.*$^{8)}$ They reported that the amounts of nine phenolic acids in rat urine increased following oral administration of unlabeled caffeic acid (3,4-dihydroxycinnamic acid), and that ferulic acid was an intermediate metabolite. In the present study, among the metabolites which Booth *et al.* reported, ferulic acid, dihydroferulic acid, *m*-hydroxyphenylpropionic acid, *m*-coumaric acid and *m*-hydroxyhippuric acid were identified as urinary metabolites of γ-oryzanol by the reverse isotope dilution analysis, and hippuric acid was newly confirmed to be a metabolite. Further, quantitative analysis showed that the main metabolites were ferulic acid and its conjugates, accounting for about 50% of the urinary excretions.

γ-Oryzanol was not detected at all in the urine, as was the case in rabbits.$^{1)}$ The tissue distribution of the radioactivity after oral administration of γ-oryzanol-^{14}C was examined by Noda *et al.*, and they found that the radioactivity in the liver was the highest. It can be assumed from their result that γ-oryzanol is metabolized mainly in the liver, but it is well known that the drugs undergo various kinds of metabolism such as ester-hydrolysis$^{10)}$ and glucuronidation$^{11)}$ in the small intestine during absorption. Thus, the intestinal metabolism of γ-oryzanol was investigated by analyzing the mesenteric venous blood in an *in situ* intestinal absorption experiment. In the present investigation, it was found that the ester linkage of γ-oryzanol was partly hydrolyzed in the intestine during absorption, and the extent of hydrolysis was less than 20% of the radioactivity transferred into the mesenteric vein. In the previous work,$^{1)}$ we performed a similar experiment in rabbits and found that γ-oryzanol was absorbed mostly as the intact form. It has been reported by Fukuda *et al.*[12] that the intestinal esterase activity of the rat is twice that of the rabbit on the basis of biochemical assay of non-specific esterase in the small intestine (duodenum) of nine different mammalian species. The segment of the small intestine which we used is different from that used by Fukuda *et al.*, but the hydrolysis of the ester linkage of γ-oryzanol occurring in rat intestine, but not in rabbit intestine, is presumed to originate from the difference in esterase activity between the two.

On the other hand, many studies on the lymphatic transport of sterols have been reported, and it has been shown that the amounts of cholesterol and β-sitosterol absorbed into the lymph are 30—50[13] and 3—6%[14] of the dose in rats, respectively. Accordingly, in order to clarify the absorption route of γ-oryzanol, we examined the lymphatic transport of γ-oryzanol in rats. It was found that the amount of radioactivity transported into the lymph was extremely low, only 0.3% of the dose, and most of the radioactivity in the lymph was in the form of γ-oryzanol.

Therefore, the present results obtained from the lymphatic transport and *in situ* intestinal absorption experiments suggest that γ-oryzanol is absorbed mainly into the portal venous blood, mostly as the intact form, although it is partly metabolized to yield ferulic acid in the intestine during absorption.

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