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Mechanism of the Blood Urea Nitrogen-decreasing Activity of Rhatannin from Rhei Rhizoma in the Rat. I

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The effects of rhatannin (condensed tannin) purified from Rhei Rhizoma on urea nitrogen and amino acid concentrations and on the activities of various enzymes concerned with the urea cycle and amino acid metabolism in the rat were investigated. Rhatannin significantly reduced the blood urea nitrogen (BUN) concentration by 16—31% at 4—8 h after intraperitoneal administration. The reduction of BUN concentration was proportional to that of urea nitrogen concentration in the liver. The activities of five urea cycle enzymes were not affected by the administration of rhatannin. On the other hand, the concentrations of eleven amino acids (Gln, Ala, Gly, Ser, Glu, Lys, Met, Pro, Orn, Cit, Arg) in the plasma were decreased. In addition, the concentrations of three amino acids (Gln, Glu, Asp) in the liver were decreased and the Gly concentration was increased. The glutamine synthetase activity in the liver was significantly increased by 52—57% at 2—4 h, while glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, glutamate dehydrogenase, and glutaminase activities were not altered. Ammonia concentration in the hepatic vein was reduced at 4—8 h prior to the reduction of ammonia concentration in the portal vein.

Keywords——Rhei Rhizoma; rhatannin; condensed tannin; blood urea nitrogen; urea cycle; amino acid; ammonia; glutamine synthetase

In an attempt to examine the individual effects of Kanpo prescriptions (Oriental medicines) on rat serum constituents after intraperitoneal administration, we found that the administration of 6 Kanpo prescriptions resulted in a decrease of blood urea nitrogen (BUN) concentration.[1] In addition, from among the crude drugs which constituted the 6 Kanpo prescriptions, 7 crude drugs (Rhei Rhizoma, Paeoniae Radix, Ephedra Herba, Coptidis Rhizoma, Bupleuri Radix, Glycyrrhizae Radix, and Scutellariae Radix) reduced BUN concentration.[2]

The aqueous extract from Rhei Rhizoma was the most effective in decreasing BUN activity in the rat. After administration of the extract, urea nitrogen concentration in the liver was maximally decreased at 8 h, and the concentrations of seven amino acids in plasma and three amino acids in the liver were reduced at 2 h. In addition, with respect to the net balance of plasma amino acids across various organs, release of Gln from the liver and uptake of Gln by non-hepatic visceral organs were decreased at 2 h after the administration.[3]

After fractionation of the extract from Rhei Rhizoma by dialysis and Sephadex LH-20 column chromatography, the BUN-decreasing activity of Rhei Rhizoma was found in the water-soluble, non-dialyzable fraction which presumably contained the high molecular-weight phenolic compounds, so-called "tannin."[4] A new component, rhatannin (condensed tannin), was purified from this fraction.[5] Another BUN-decreasing component purified from Paeoniae Radix was 1,2,3,4,6-penta-*O*-galloyl glucose (gallotannin), which was one of the hydrolyzable tannins.[6]

In this work, it was found that rhatannin was responsible for decreasing the BUN concentration. Therefore, in an attempt to clarify the biological action mechanism of rhatannin, we have studied the effects of rhatannin on urea nitrogen and amino acid concentrations and on the activities of various enzymes concerned with the urea cycle and amino acid metabolism.

Materials and Methods

Animals and Treatments——Male Wistar rats weighing *ca.* 140 g were used throughout the experiments. The animals were maintained in an air-conditioned room with lighting from 6 a.m. to 6 p.m. The room was kept at 25 ± 1 °C and 60% relative humidity. Laboratory pellet chow, CE-2, purchased from CLEA Japan Inc., Tokyo, and tap water were given freely. Rhatannin (2.5 mg/rat) made soluble in saline was administered intraperitoneally to the rats, while control rats were treated with an equal volume of saline. In all experiments, the pellet chow was removed from the rat cage at 9 a.m. and rats were killed at 6 p.m. after the administration of rhatannin.

Reagents——Carbamyl phosphate dilithium salt was obtained from Wako Pure Chemicals Industries, Ltd., Japan. Nicotineamide adenine dinucleotide (NADH) was obtained from P-L Biochemicals, Inc., U.S.A. Adenosine 5′-triphosphate (ATP), phosphoenol pyruvate, argininosuccinic acid barium salt, γ-glutamylhydroxamate, pyruvate kinase type III, ornithine carbamyltransferase, arginase, malic dehydrogenase, L-lactic dehydrogenase type XI, and argininosuccinate lyase type II were purchased from Sigma Chemical Co., U.S.A. All other reagents were of the highest grade commercially available.

Determination of Serum, Plasma, and Liver Constituents——a) Urea Nitrogen Assay: The rats were killed by decapitation, and blood samples were collected and allowed to stand for several hours in a cold room at 4 °C. Serum was separated by centrifugation ($1000 \times g$, 10 min, 4 °C). BUN concentration was measured by the urease-indophenol method,[7] using a DSA-560 discrete sample analyzer (Beckman Instrument Inc., U.S.A.) according to the previous paper.[1] The liver was quickly removed and then frozen in dry ice-acetone. One gram of the liver was homogenized in 6 volumes of ice-cold distilled water with a motor driven Teflon homogenizer. The homogenate was filtered through 4 layers of gauze. Urea nitrogen concentration in the liver was determined by the method of Archibald.[8]

b) Ammonia Assay: Under pentobarbital (Abbott Laboratories, U.S.A.) anesthesia, blood was taken from the hepatic vein and the portal vein as described previously.[3] Estimation of ammonia concentration in blood was carried out by a modification of the method of Okuda and Fujii,[9] with the commercial reagent, Ammonia-Test Wako (Wako Pure Chemical Industries, Ltd.).

c) Free Amino Acid Assay: Plasma and liver samples were taken as described in the previous paper.[3] Equal volume of plasma from 3 rats were pooled, and then one ml of the plasma was deproteinized with 25 mg of solid sulfosalicylic acid and left to stand for 2 h at 4 °C. The supernatant obtained by centrifugation at $15000 \times g$ for 15 min was applied directly to a Hitachi 835 amino acid analyzer (Hitachi, Ltd., Japan). Equal weights of liver from 3 rats were pooled and homogenized in 4 volumes of 3% ice-cold sulfosalicylic acid with a Teflon homogenizer. The tissues extracts obtained by centrifugation were subjected to amino acid analysis.

Determination of Enzyme Activities——One gram of the liver excised after decapitation of the rat was homogenized in 9 ml of ice-cold distilled water. A part of the homogenate was diluted with ice-cold distilled water in order to make the most convenient preparation for assay of each urea cycle enzyme. The other part of the homogenate was sonicated (7 kHz, 30 s, 0 °C) by using a cell disruption sonicator, model W220F (Heat Systems-Ultrasonics, Inc.), for the assay of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), glutamate dehydrogenase (GDH), and glutamine synthetase. Protein was determined by the procedure of Lowry *et al.*,[10] with bovine serum albumin as the standard.

a) Urea Cycle Enzymes Assay: The activities of urea cycle enzymes were assayed by the method of Shimbayashi and Yonemura,[11] which is a modification of the method of Brown and Cohen[12] for the assay of urea cycle enzymes in tadpole liver.

b) GOT and GPT Assay: GOT was determined by the method of Karmen[13] as modified by Boyd,[14] except that the reaction was carried out at 37 °C. GPT was assayed by the method of Segal *et al.*[15]

c) GDH Assay: GDH was measured by the method of Olsen and Anfinsen.[16] One unit of the enzyme was defined in terms of an absorbance change, ΔOD_{340}, of 0.001.

d) Glutamine Synthetase Assay: Glutamine synthetase was determined by the colorimetric method of Pamiljans *et al.*[17] One unit of the enzyme was defined as the activity forming one μmol of γ-glutamylhydroxamate per hour at 37 °C.

e) Glutaminase Assay: The liver was homogenized in 4 volumes of chilled 0.25 M sucrose–TMK buffer (50 mM Tris-HCl, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.5) with a Teflon homogenizer. The homogenate was centrifuged at $2500 \times g$ for 10 min, at 0 °C. The precipitate obtained by centrifugation was taken as the mitochondrial fraction,

suspended in 0.1 M Tris-HCl buffer, pH 7.5, and sonicated at 7 kHz for 30 s, at 0 °C. Phosphate-independent glutaminase (PIG) and phosphate-dependent glutaminase (PDG) were assayed according to the method of Katsunuma *et al.*[18] with slight modifications. Ammonia liberated during incubation was measured colorimetrically by the method of Henry[19] with slight modifications. One unit of the enzyme was defined as the activity forming one μmol of ammonia per hour at 37 °C.

Results

Effect of Rhatannin on BUN Concentration and Urea Nitrogen Concentration in the Liver

Figure 1 shows the effects of rhatannin on BUN concentration and urea nitrogen concentration in the rat liver after intraperitoneal administration. Rhatannin significantly reduced the BUN concentration by 16—31% at 4—8 h as compared with the control value. Similarly, urea nitrogen concentration in the liver was decreased by 16—32% at 4—8 h. Thus,

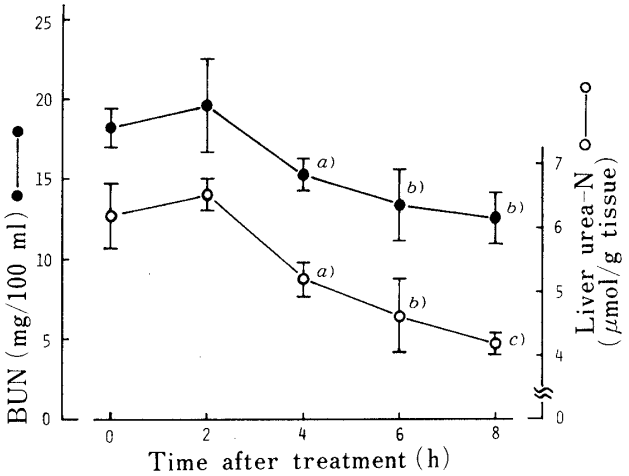

Fig. 1. Time Course of the Effect of Rhatannin on Urea Nitrogen Concentrations in Serum and Liver

Control at 0 h is expressed as the mean ± S.D. of values obtained at each period after saline treatment.
a) $p<0.05$, student's t test.
b) $p<0.01$.
c) $p<0.001$.

TABLE I. Effect of Rhatannin on Urea Cycle Enzyme Activities of Rat Liver

Time after treatment (h)	No. of rats	Carbamyl phosphate synthetase	Ornithine carbamyl-transferase	Arginine synthetase	Argininosuccinate cleavage enzyme	Arginase
		units/mg protein[a] (%)				
0[b]	6	3.29±0.26 (100)	56.6±3.7 (100)	0.41±0.03 (100)	0.91±0.08 (100)	151± 9 (100)
2	4	3.68±0.24 (112)	53.2±1.6 (94)	0.40±0.02 (98)	0.84±0.04 (92)	150±14 (99)
4	4	3.68±0.36 (112)	55.8±2.9 (99)	0.40±0.02 (98)	0.92±0.07 (101)	161± 6 (107)
6	4	3.21±0.48 (98)	53.6±3.1 (95)	0.44±0.03 (107)	0.97±0.07 (107)	175±11 (116)
8	4	3.84±0.38 (117)	54.8±3.1 (97)	0.43±0.01 (105)	0.95±0.03 (104)	174±36 (115)

a) Data are expressed as means ± S.D.
b) Control at 0 h is expressed as the mean ± S.D. of values obtained at each period after saline treatment.

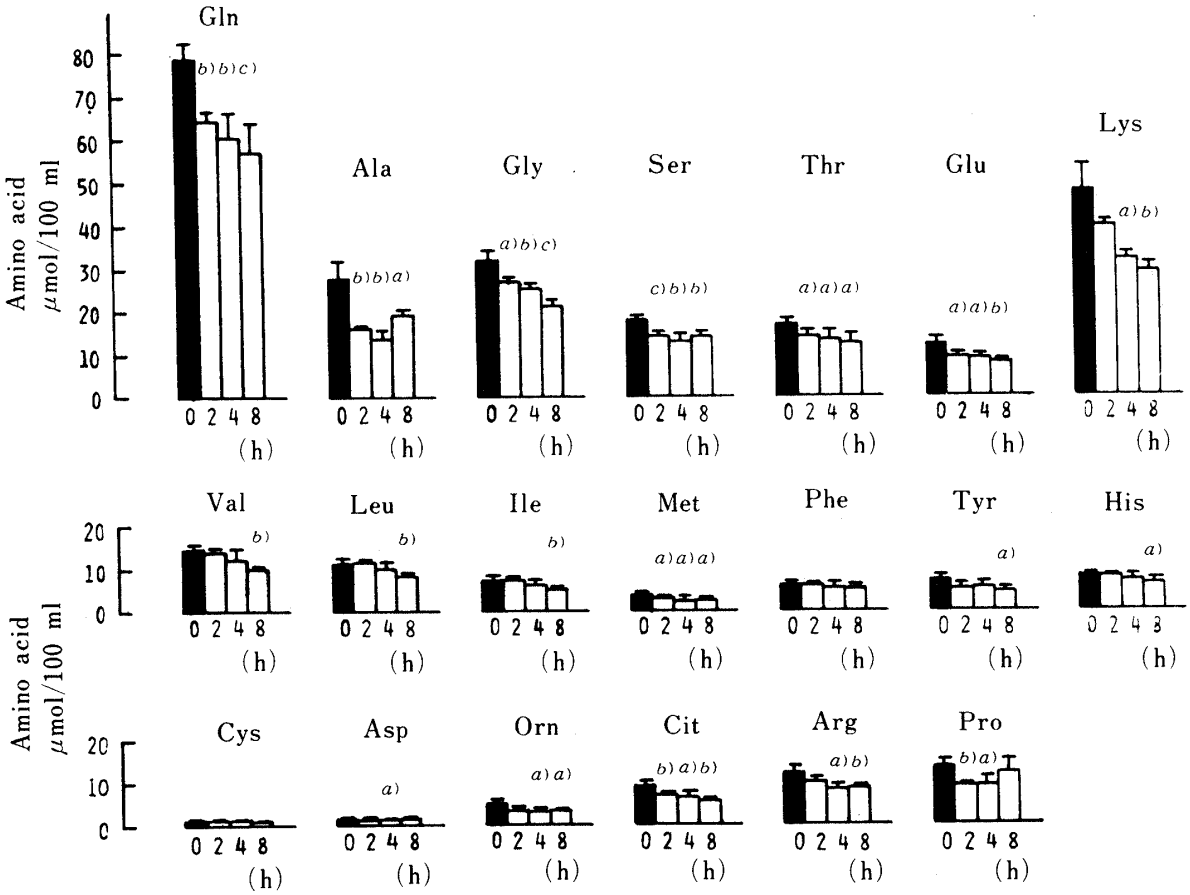

Fig. 2. Time Course of the Effect of Rhatannin on Plasma Free Amino Acid Concentrations

Control at 0 h is expressed as the mean ± S.D. of values obtained at each period after saline treatment.
a) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$, student's *t* test.

it was indicated that the reduction of BUN concentration was proportional to that of urea nitrogen concentration in the liver.

Effect of Rhatannin on Urea Cycle Enzyme Activities

In an attempt to examine the effects of rhatannin on the activities of five urea cycle enzymes (Table I), it was found that the activity of argininosuccinate synthetase, which is the rate-limiting enzyme, was not affected, and the activities of other urea cycle enzymes were also not affected.

Effects of Rhatannin on Free Amino Acid Concentrations in Plasma and the Liver

α-Amino nitrogen of amino acids is one of the nitrogen sources for urea synthesis. Therefore, the effect of rhatannin on α-amino acid nitrogen concentration in serum was examined. The α-amino acid concentration was decreased at 2—8 h, as was reported previously[3] with the aqueous extract from Rhei Rhizoma. Then, we examined the effect of rhatannin on free amino acid concentrations in plasma of peripheral blood.

As shown in Fig. 2, the concentrations of amino acids in plasma were significantly decreased at 2—8 h after administration as compared with the control values, as follows: Gln (18—28%); Ala (31—41%), Gly (16—33%); Ser (22—28%); Glu (26—35%); Lys (33—39%); Met (18—29%); and Pro (30—32%). The concentrations of Orn, Cit, and Arg were reduced by 33—36, 23—37, and 29—33%, respectively. The maximal reduction of total amino acid

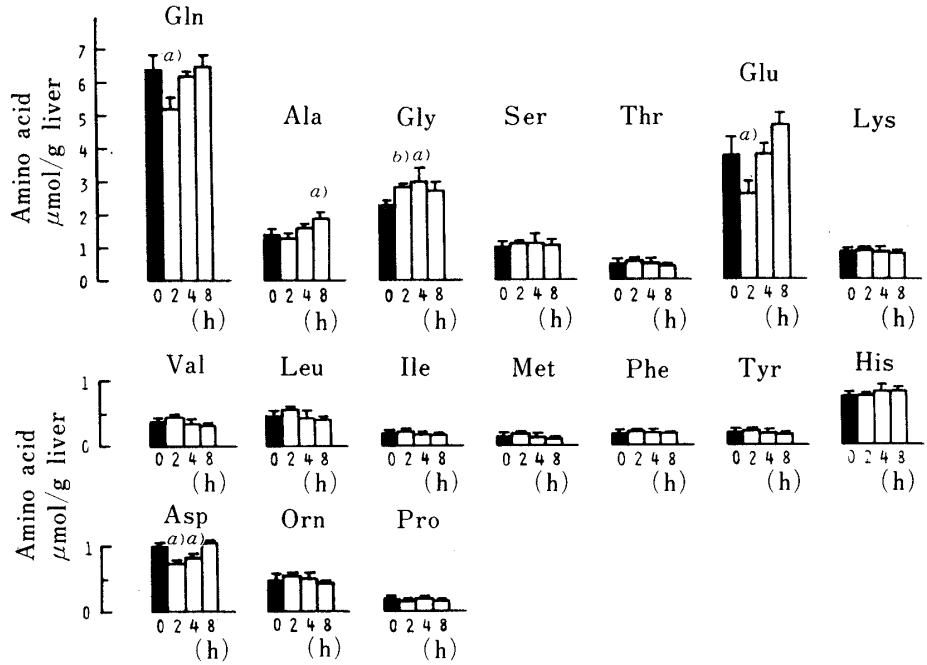

Fig. 3. Time Course of the Effect of Rhatannin on Hepatic Amino Acid Concentrations

Control at 0 h is expressed as the mean ± S.D. of values obtained at each period after saline treatment.
a) $p<0.05$, *b*) $p<0.01$, student's *t* test.

TABLE II. Effect of Rhatannin on Enzyme Activities in Rat Liver Homogenate

Time after treatment (h)	No. of rats	GOT $\Delta OD_{340} \cdot min^{-1} \cdot mg^{-1}$ protein[a] (%)	GPT $\Delta OD_{340} \cdot min^{-1} \cdot mg^{-1}$ protein[a] (%)	GDH $\Delta OD_{340} \cdot min^{-1} \cdot mg^{-1}$ protein[a] (%)	Glutamine synthetase μmol γ-glutamylhydroxamate formed $\cdot h^{-1} \cdot mg^{-1}$ protein[a] (%)
Control (Saline)	6	1690±160 (100)	52±19 (100)	486±39 (100)	0.23±0.05 (100)
1	4	1700±150 (101)	53±16 (102)	519±11 (107)	0.27±0.05 (117)
2	4	1680± 40 (99)	56±28 (108)	506±33 (104)	0.35±0.05[b] (152)
4	4	1740±140 (103)	45±17 (87)	510±54 (105)	0.36±0.03[b] (157)
6	4	1690± 90 (100)	59± 8 (113)	537±38 (110)	0.27±0.07 (117)

a) Data are expressed as means ± S.D.
b) $p<0.01$, student's *t* test.

concentration was observed at 8 h. Thus, rhatannin had a prolonged decreasing effect on the amino acid concentration in plasma as compared with the effect of the aqueous extract from Rhei Rhizoma described in the previous paper.[3]

Furthermore, Fig. 3 shows the effect of rhatannin on free amino acid concentrations in the liver. The concentrations of Gln, Glu, and Asp were decreased by 20 (at 2 h), 28 (at 2 h), and 16—26% (at 2—4 h), respectively. The concentrations of Gly and Ala were increased by 25—32 (at 2—4 h) and 34% (at 8 h), respectively.

TABLE III. Effect of Rhatannin on Glutaminase Activities in Rat Liver

Time after treatment (h)	No. of samples	Glutaminase[a] PIG	PDG
		μmol NH_3 formed·h^{-1}·mg^{-1} protein[b] (%)	
Control (Saline)	4	0.45±0.04 (100)	4.90±0.60 (100)
2	3	0.51±0.04 (113)	4.84±0.63 (99)
4	4	0.44±0.07 (98)	5.43±0.73 (111)
6	3	0.43±0.11 (96)	4.44±0.41 (91)

a) Mitochondrial fraction.
b) Data are expressed as means±S.D.

TABLE IV. Effect of Rhatannin on Blood Ammonia Nitrogen Concentrations in Portal and Hepatic Vein

Time after treatment (h)	No. of rats	Portal vein NH_3–N (μg/100 ml)[a] (%)	Hepatic vein	Portal-hepatic vein difference
Control (Saline)	16	479±35 (100)	193±24 (100)	286±47 (100)
2	7	505±46 (105)	193±34 (100)	312±47 (109)
4	7	471±57 (98)	152±17[b] (79)	319±42 (112)
6	7	394±43[b] (82)	159±15[b] (82)	235±33[c] (82)
8	8	385±24[b] (80)	168±22[c] (87)	217±41[d] (76)

a) Data are expressed as means±S.D.
b) $p<0.001$, student's t test.
c) $p<0.05$.
d) $p<0.01$.

Effects of Rhatannin on the Enzyme Activities of GOT, GPT, GDH, Glutamine synthetase, and Glutaminase in the Liver

With respect to the reductions of Gln and Glu in plasma and the liver, the effects of rhatannin on the activities of various enzyme reactions concerned with Gln and Glu were investigated.

As shown in Table II, glutamine synthetase activity was significantly increased by 52—57% at 2—4 h, while GOT, GPT, and GDH activities were not altered. Furthermore, Table III shows the effect of rhatannin on glutaminase activity. Neither PIG nor PDG activity was altered at any time after the administration.

Effect of Rhatannin on Free Ammonia Concentrations in the Portal and Hepatic Veins

Table IV shows the effects of rhatannin of free ammonia concentrations in the portal and hepatic veins. Ammonia concentration in the portal vein was decreased at 6—8 h as compared with the control value. On the other hand, ammonia concentration in the hepatic vein was

reduced at 4—8 h prior to the reduction of ammonia concentration in the portal vein. Furthermore, the portal-hepatic difference of ammonia concentration was decreased at 6—8 h.

Discussion

Urea synthesis is changed by protein intake[20] and is subject to hormonal regulation, *e.g.* by growth hormone, cortisone,[21] and glucagon.[22] *N*-Acetyl-L-glutamate acts as a allosteric activator of carbamyl phosphate synthetase in the regulation of urea synthesis.[23] In this paper, the effects of rhatannin on the activities of all five enzymes of the urea cycle were examined, and none of the enzyme activities was altered after administration (Table I). Furthermore, we reported that the excretion of urea in the urine was not different from the control value after the administration of rhubarb extract.[3] These findings suggested that the reduction of urea nitrogen concentration was not due to depression of the enzyme activities of the urea cycle or to increase of the excretion of urea.

One mole of urea was formed from one mole each of ammonia, carbon dioxide, and α-amino nitrogen of aspartate. Aikawa *et al.*[24] suggested that glutamine and alanine were the "end product" of amino acid metabolism in many tissues and acted as nitrogen carriers from organ to organ. Aspartate is a substrate for argininosuccinate synthesis by argininosuccinate synthetase on the pathway of the urea cycle. As shown in Figs. 2 and 3, the concentrations of eleven amino acids, Gln, Ala, Glu, *etc.*, in plasma and of three amino acids, Gln, Glu, and Asp, in the liver were decreased after the administration of rhatannin. Furthermore, free ammonia concentrations in the portal and hepatic veins were reduced (Table IV), and the portal-hepatic difference of ammonia concentration was decreased. Thus, it is suggested that the reduction of urea synthesis was caused by the decreases of its substrates, such as ammonia, aspartate, and the "end product" amino acids.

It was reported that the ammonia concentration in the portal vein was reduced after the oral administration of an antibiotics mixture to conventional rats.[25] Kobashi *et al.*[26] reported that the oral administration of nicotino-hydroxamic acid (H·X·A), a specific urease inhibitor, or antibiotics depressed ureolysis in rat intestine. The effects of the antibiotics and urease inhibitors such as thiophenycol and caprylo H·X·A on BUN concentration at 6 h after a single intraperitoneal or oral administration were examined, and it was found that the administration of thiophenycol or capryro H·X·A showed no effect, while that of rhatannin reduced BUN concentration.[27] Consequently, the administration of rhatannin may not affect the enterohepatic circulation of urea nitrogen.

Next, the reduction of amino acid concentrations in plasma and the liver was of interest. We noticed decreases of Gln and Glu concentrations and investigated the effect of rhatannin on the various enzyme reactions in which Gln or Glu acts as a substrate or a product in the liver. It was observed that glutamine synthetase activity was increased at 2—4 h after the administration of rhatannin, while GOT, GPT, GDH, and glutaminase activities were not altered (Tables II and III). In addition, Gln concentration in the liver was not increased at 2—8 h after administration. Furthermore, in an attempt to examine the effect of rhatannin on the portal-hepatic difference of plasma amino acid concentrations across the liver, it was observed that the release of Gln from the liver was decreased, as described in the case of the administration of the aqueous extract from Rhei Rhizoma.[3] Thus, it was supposed that glutamine was produced from glutamic acid through glutamine synthetase and was immediately converted into some metabolites in the liver.

It is widely known that glutamine not only serves as a required amino acid in the biosynthesis of protein, but is also a necessary nitrogen source in other biosynthetic pathways. The amide nitrogen of glutamine is used for the synthesis of purines, pyrimidines, gluco-

samine, and carbamyl phosphate. The α-amino nitrogen of glutamine is used for the synthesis of amino acids from the corresponding α-keto acids by transamination.[28] One possible explanation of the reduction of amino acid concentrations may be that glycine is produced by glutamine transaminase, because of the observed increase of glycine in the liver at 2—4 h (Fig. 3). In the future, we expect to examine what metabolites glutamine was converted into after the administration of rhatannin.

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