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Effect of Rhatannin on the Incorporation of Precursors into Proteins and Ribonucleic Acids of Rat Liver

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The mechanism by which rhatannin (condensed tannin purified from Rhei Rhizoma) produces a prolonged decrease in plasma amino acids in the postabsorptive state was investigated *in vivo* by measuring the incorporation of [^{14}C]phenylalanine (Phe) into proteins in serum, liver, kidney and muscle. Enhanced incorporation into serum proteins was observed 4 to 8 h after the intraperitoneal administration (12.5 mg/kg body weight), and the maximal enhancement was observed 6 h after the treatment. Incorporations into other proteins did not change. Further, a fluorogram of the polyacrylamide gel electrophoretic pattern of serum obtained from rats after rhatannin treatment revealed incorporation of the labeled amino acid into protein(s) which corresponded to albumin in terms of electrophoretic mobility. Incorporation of [^{3}H]phenylalanine after a 20 min *in vivo* labeling 6 h after rhatannin treatment was enhanced in hepatic microsomal and mitochondrial fractions. In addition, stimulated incorporation of [^{3}H]orotic acid into rat hepatic nuclear and cytoplasmic ribonucleic acids (RNA) was observed after rhatannin treatment and the maximal enhancements were achieved at 6 h. Thus, the decrease of plasma amino acid level 4 to 8 h after rhatannin treatment may be due in part to the increased removal of amino acids by the liver, although the underlying mechanisms of enhancement of the syntheses of hepatic protein and RNA in rhatannin-treated rats remain obscure.

Keywords——rhatannin; tannin; rhubarb; hepatic protein synthesis; hepatic RNA synthesis; plasma protein

It was shown previously that the concentrations of rat plasma amino acids were decreased 2 to 8 h after an intraperitoneal injection of rhatannin (condensed tannin purified from Rhei Rhizoma)[1,2] and that a part of the effects of the tannin on amino acids within 2 h is mediated through its action on the glucagon regulatory pathway without changes in the levels of glucagon and insulin in blood.[3] However, the reasons for the prolonged decreasing effect of rhatannin on amino acids have not been established. Therefore, a question has remained as to whether the alteration observed in plasma amino acids following rhatannin treatment was a result of decreased release from endogeneous protein stores, or increased utilizations by various tissues, or a combination of these changes.

In the present work, protein and ribonucleic acids (RNA) syntheses were determined by measuring the incorporation of labeled precursors. The results suggested that the mechanism by which rhatannin causes a prolonged decrease in the levels of plasma free amino acids after treatment might involve a stimulation of protein synthesis in the liver.

Materials and Methods

Materials——Rhatannin was isolated from rhubarb (Rhei Rhizoma) by the method described previously.[2] The following compounds were purchased: [L-^{14}C(U)]phenylalanine, [L-2,3-^{3}H]phenylalanine and [5-^{3}H]orotic acid (New

England Nuclear, U.S.A.), scintillation cocktail ACS II (Amersham Corp. U.S.A.) and rat albumin (Cappel Laboratories, U.S.A.).

Animals and Treatment——Male Wistar rats were fed on laboratory pellet chow (CE-2, CLEA Japan Inc., Tokyo) and tap water freely. Rats were fasted for 24 h and animals weighing *ca.* 100 g were employed in all experiments. Rhatannin (12.5 mg/kg body weight) was administered intraperitoneally to rats, and control animals were treated with an equal volume (0.5 ml/rat) of saline.

Determination of Radioactivity Incorporated into Proteins——The liver and kidney after a 1 h [^{14}C]phenylalanine (4 μCi/rat) *in vivo* labeling were each homogenized with 10 volumes of ice-cold distilled water in a Potter-Elvehjem device with 10 strokes of a motor-driven Teflon pestle, and the gastrocnemius muscle was homogenized with 9 volumes of 0.02 M $NaHCO_3$ in a Polytron homogenizer at full speed for 30 s 5 times. The homogenates were filtered through four layers of gauze. Blood samples were collected and allowed to stand for several hours in a cold room at 4 °C. Serum was prepared by centrifugation (1000 × *g*, 10 min, 4 °C). Sera and the filtered fractions of the homogenized livers and kidneys were each treated with an equal volume of 10% trichloroacetic acid (TCA). The TCA-insoluble residue was washed three times with 5% TCA. After extraction of nucleic acid with 5% TCA for 20 min at 90 °C, residues were washed once with 5% TCA, twice with ethanol, and once with ether. The prepared fractions were taken as individual proteins. The homogenized muscle was extracted with two volumes of 0.1 N NaOH for 1 h at 38 °C. The alkali-soluble fraction was treated with an equal volume of 10% TCA. The TCA-insoluble residue was treated according to the same procedure as described above. The prepared fraction was taken as the noncollagen protein (NCP). The individual proteins and NCP were each dissolved in 0.1 N NaOH, and the solution was subjected to the determination of protein by the Lowry method,[4] with bovine serum albumin as a standard. The radioactivity was determined in a liquid scintillation cocktail ACS II with an Aloka liquid scintillation spectrometer, model LSC-900. The specific radioactivity was estimated as dpm/mg protein from the radioassay and colorimetric determination.

Determination of Radioactivity in Subcellular Fractions from the Liver——Subcellular fractions were prepared by a modification of the method of Fleisher and Kervina[5] as follows. The liver, after a 20 min [^{3}H]phenylalanine (5 μCi/rat) *in vivo* labeling, was homogenized with 4 volumes of chilled 0.25 M sucrose in TKM buffer (50 mM Tris–HCl, 25 mM KCl, 5 mM Mg_2Cl, pH 7.5) in a Potter-Elvehjem device with 10 strokes of a motor-driven Teflon pestle. The homogenate was filtered through 4 layers of gauze. The filtered fraction was taken as liver homogenate fraction. This fraction was centrifuged successively at 1000 × *g* for 10 min, at 9500 × *g* for 10 min, and at 105000 × *g* for 60 min. The resulting precipitates from individual centrifugations were taken as nuclear fraction, mitochondrial fraction, and microsomal fraction, respectively. These fractions were each dissolved in chilled distilled water. The supernatant fraction resulting from the centrifugation at 105000 × *g* for 60 min was taken as soluble protein fraction. Specific radioactivities in these fractions were determined by the same procedure as described above.

Determination of Radioactivity Incorporated into Hepatic Nuclear RNA——The livers from three rats after a 20 min [^{3}H]orotic acid (5 μgCi/rat) *in vivo* labeling were pooled in each experimental or control group. The purified hepatic nuclei were prepared by the same procedure as described previously.[6] The nuclear RNA was prepared by a modification of the Schmidt-Thannhauser method.[7] The residue of extracted RNA was mixed with 0.3 N KOH and the sample was hydrolyzed at 37 °C for 18 h. The hydrolysate was neutralized with chilled $HClO_4$ and the precipitate was removed by centrifugation. The supernatant fluid was used for the determination of RNA by means of the orcinol reaction as modified by Mejbaum.[8] The radioactivity was determined with the aliquots of this supernatant in liquid scintillation cocktail ACS II with an Aloka liquid scintillation spectrometer, model LSC-900. Specific radioactivity was estimated as dpm/mg RNA from the radioassay and colorimetric determination.

Determination of Radioactivity Incorporated into Hepatic Cytoplasmic RNA——The purified cytoplasmic ribosome fraction from the liver after a 90 min [^{3}H]orotic acid (5 μCi/rat) *in vivo* labeling was prepared by the method of Blobel and Potter.[9] The specific radioactivity was determined by the same procedure as used for nuclear RNA.

Analysis of Serum Proteins——Electrophoresis was performed on samples of serum according to the method of Ogita and Markert,[10] using 7% polyacrylamide gels for 2 h at a constant current of 20 mA. The running gel buffer was 187 mM Tris–HCl (pH 8.8), the stacking gel buffer was 15 mM Tris–HCl (pH 6.8), and the electrode buffer was 12.5 mM Tris–glycine (pH 8.3). The gels were then stained for 2 h with 0.1% Coomassie brilliant blue R-250 (dissolved in 40% methanol, 12.5% TCA). The gels were destained in 7% acetic acid. To prepare fluorograms, the gels were treated for 1 h with a solution containing 10% TCA, 10% acetic acid, and 30% methanol, and were then impregnated with EN^{3}HANCE (New England Nuclear). The gels were pressed with filter paper, dried under a vacuum, and exposed to Fuji X-ray film at −70 °C for 4 weeks.

Results

Effect of Rhatannin on Incorporation of [^{14}C]Phenylalanine into Proteins of Serum, Liver, Kidney, and Muscle

We first examined whether or not rhatannin caused a stimulation of protein synthesis. Changes in plasma free amino acids after rhatannin treatment were reported previously.[1] The

TABLE I. Time Course of the Effect of Rhatannin on Incorporation of [^{14}C]Phenylalanine into Proteins Contained in Serum, Liver, and Kidney and into Noncollagen Proteins in Gastrocnemius Muscle after a 60-min *in Vivo* Labeling

Time after treatment (h)	No. of rats	Incorporated radioactivity (dpm/mg protein) Serum (%)	Liver (%)	Kidney (%)	Muscle (%)
Control	6	5444±1010 (100)	2139±460 (100)	1478±170 (100)	348±12 (100)
2	3	6701± 750 (123)	2510±260 (117)	1508±304 (102)	290 (83)
4	3	7814± 785[a] (143)	2263±295 (106)	1421±177 (96)	334 (96)
6	3	10124±1613[b] (186)	2538±231 (119)	1447±144 (98)	315 (98)
8	3	8753± 800[a] (161)	2134±150 (100)	1423± 73 (96)	260 (75)

a) $p<0.05$. *b*) $p<0.001$. The values are expressed as means±S.D.

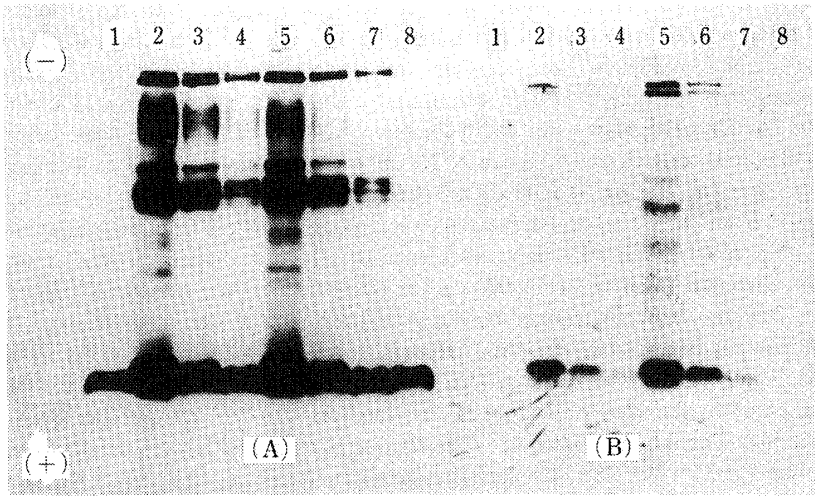

Fig. 1. Polyacrylamide Gel Electrophoretic Patterns of Serum Samples obtained from Control and Rhatannin-Treated Rats

Electrophoresis was carried out in a thin layer polyacrylamide gel slab. The spectrotypes and fluorotypes were developed as described in Methods and Materials.
(A) spectrogram, (B) fluorogram.
Lanes 1 and 8, rat albumin; 2, 3, and 4 (2 times, 4 times, and 8 times diluted, respectively), control rat; 5, 6, and 7 (2 times, 4 times, and 8 times diluted, respectively), rhatannin-treated rat.

concentrations of phenylalanine in plasma and liver did not change during 2 to 8 h after the treatment. Control and rhatannin-treated rats were given [^{14}C]phenylalanine (4 μCi/rat) intraperitoneally 1 h before killing them by decapitation. Table I shows the time course of the effects of rhatannin on the incorporation of labeled amino acid into proteins in serum, liver, kidney, and gastrocnemius muscle. The rate of incorporation of labeled precursor into serum proteins was enhanced 4, 6 and 8 h after treatment. The increments were 43%, 86%, and 61%, respectively, as compared with the control. However, the incorporations into the other examined proteins did not change significantly as compared with the individual control values.

Distribution of [^{14}C]Phenylalanine in Serum Proteins

A fluorogram of the electrophoretic pattern was prepared to examine the incorporation

of radioactive precursor into serum proteins obtained from rats after a 1 h *in vivo* labeling. Figure 1 shows the spectrogram and fluorogram patterns for individual serum samples obtained from the control rat (lanes 2—4) and the rat 6 h after rhatannin treatment (lanes 5—7), and for rat albumin (lanes 1 and 8). The fluorogram demonstrates that the samples obtained from the control and rhatannin-treated rats both exhibit an incorporation of [^{14}C]phenylalanine into protein(s) which corresponds in terms of electrophoretic mobility to albumin. Further, the fluorogram of the serum obtained from the rhatannin-treated rat indicates that the labeled precursor was also incorporated into other proteins (lane 5). Although the proteins other than albumin which exhibit fluorescence were not identified, they might include transferrin and γ-globulin, judging from the observed pattern.

Effect of Rhatannin on the Incorporation of [^{3}H]Phenylalanine into Hepatic Subcellular Fractions

The effect of rhatannin on the distribution of radioactivity in the liver was determined by measuring the incorporation of labeled precursor into subcellular fractions prepared from the liver after a 20 min [^{3}H]phenylalanine (5 μCi/rat) *in vivo* labeling. Table II shows the distribution of radioactivity in the proteins extracted from the liver and serum. Increased incorporation of labeled amino acid was found in every fraction of the liver extract from rhatannin-treated rats as compared with the corresponding control. In particular, considerable increases of incorporation into the microsomal and mitochondrial fractions were observed 63% and 75%, respectively.

Stimulatory Effect of Rhatannin on Hepatic Nuclear and Cytoplasmic RNA Syntheses

In order to determine the contribution of transcription to the effect of rhatannin on protein synthesis, the incorporation of a labeled precursor into rat hepatic nuclear and cytoplasmic RNA was examined. Table III shows the time course of the effect of rhatannin on the incorporation of radioactive precursor into rat hepatic heterogeneous nuclear RNA after a 20 min [^{3}H]orotic acid (5 μCi/rat) *in vivo* labeling. The result showed that the incorporation of radioactive precursor into nuclear RNA was increased 4, 6, and 8 h after rhatannin treatment. The increments were 22%, 50%, and 43%, respectively. The maximal stimulation was observed 6 h after treatment. To see whether this stimulation of nuclear RNA synthesis induced by rhatannin resulted in an increase of cytoplasmic RNA synthesis, the effect of rhatannin on the cytoplasmic RNA synthesis was determined by measuring the incorporation of labeled precursor into cytoplasmic RNA after a 90 min [^{3}H]orotic acid (5 μCi/rat) *in vivo* labeling. As shown in Table IV, the incorporations of radioactive precursor into membrane-bound ribosomal fraction and free ribosomal fraction were increased 6 and 8 h after rhatannin

TABLE II. Effect of Rhatannin on the Distribution of [^{3}H]Phenylalanine in Fractions of the Liver Extract and Serum *in Vivo* during a 20-min Pulse 6 h after Treatment

Fraction	Incorporated radioactivity (dpm/mg protein)			
	Control	(%)	Rhatannin-treated	(%)
Homogenate	2248 ± 359	(100)	2806 ± 119[a]	(125)
Nuclear fr.	1993 ± 369	(100)	2791 ± 87[b]	(140)
Mitochondrial fr.	3905 ± 556	(100)	6850 ± 596[c]	(175)
Microsomal fr.	6501 ± 820	(100)	10595 ± 1632[b]	(163)
Soluble fr.	1777 ± 350	(100)	2295 ± 171[a]	(129)
Serum	270 ± 62	(100)	813 ± 94[c]	(301)

Data are expressed as means ± S.D. *a*) $p<0.05$. *b*) $p<0.01$. *c*) $p<0.001$.

Table III. Time Course of the Effect of Rhatannin on Incorporation of [^{3}H]Orotic Acid into Rat Hepatic Nuclear RNA after a 20-min *in Vivo* Labeling

Time after treatment (h)	No. of rats	Incorporated radiactivity dpm/mg RNA	(%)
Control	6	164859±14829	(100)
2	3	171554	(104)
4	3	201845	(122)
6	3	247189	(150)
8	3	235094	(143)

Table IV. Effect of Rhatannin on Incorporation of [^{3}H]Orotic Acid into Cytoplasmic RNA in Rat Liver *in Vivo* during a 90-min Pulse 6 and 8 h after Treatment

Time after treatment (hr)	No. of rats	Incorporated radioactivity dpm/mg RNA	
		Membrane-bound ribosomal fr. (%)	Free ribosomal fr. (%)
Control	6	7822±2577 (100)	9711±2814 (100)
6	6	22282±4808 (285)	17694± 376 (182)
8	6	16503±4637 (211)	16258±1363 (167)

Data are expressed as means±S.D.

treatment. The increments of the former were 185% and 111%, respectively, and those of the latter were 82%, and 67%, respectively. The maximal stimulation of incorporation was observed in both fractions 6 h after treatment.

Discussion

It is widely known that in the postabsorptive state, the steady-state concentrations of free amino acids in blood are maintained by the net balance between release from endogeneous protein stores and utilization by various tissues. In the present *in vivo* investigation, the incorporation of [^{14}C]phenylalanine into proteins of the serum, liver, kidney, and muscle was measured in order to analyze the mechanism by which rhatannin causes a prolonged decrease in the levels of plasma amino acids.

It was shown that incorporation of the labeled amino acid was increased in serum proteins, while that into the other proteins did not change as compared with the control (Table I). Further, a fluorogram of the polyacrylamide gel electrophoretic pattern of the serum obtained from rhatannin-treated rats demonstrated that radioactivity was incorporated into albumin (Fig. 1), which is synthesized in and secreted from the liver.

It has been demonstrated that the pathway of albumin secretion is first the rough endoplasmic reticulum, then the smooth endoplasmic reticulum and the Golgi apparatus, and finally the blood.[11] Newly synthesized secretory proteins such as albumin[12] and transferrin,[13] are not secreted in less than 15 and 20 min, respectively; these two proteins are the most abundant plasma protein and another major one, respectively. It was shown that the incorporation of labeled precursor into microsomal and mitochondrial fractions was in-

creased 6 h after rhatannin treatment (Table II). This result suggested that the increased radioactivity in the microsomal fraction might be derived from unfinished and unreleased peptides or proteins of the endoplasmic reticulum synthesized by membrane-bound ribosomes, since under the present experimental conditions, newly synthesized plasma protein molecules were present in the liver predominantly (Table II). This observation could not be ascribed to a change in the specific activity of the intracellular amino acid pool, since the level of amino acid used as the tracer in the liver and blood did not change after rhatannin treatment.[1] It seems probable that the distribution and increased incorporation of labeled amino acid in hepatic subcellular fraction might reflect a stimulatory effect of rhatannin on the process of plasma protein synthesis.

It is well known that hormones such as insulin,[14] growth hormone,[15] and thyroid hormone[16] stimulate plasma protein production by enhancing messenger RNA (mRNA) production in the liver. In the present case, the underlying mechanism of enhancement of the incorporation of labeled precursors into hepatic RNA in rhatannin-treated rats (Tables III and IV) is obscure, since the effects of rhatannin on these hormone levels were not determined. However, it is likely that the increased incorporation of labeled precursor into hepatic nuclear and cytoplasmic RNA in rhatannin-treated rats might reflect an increase of the RNA content. Therefore, the observed effects of rhatannin indicate that the stimulated hepatic protein synthesis may be due in part to enhancement of the hepatic RNA synthesis.

Thus, the present work showed that the increase of the incorporation of [^{14}C]phenylalanine into serum proteins might have resulted from the stimulation of hepatic protein synthesis. This might be closely related to the prolonged decrease of plasma free amino acids observed after rhatannin treatment.

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