Effect of Ginseng Saponins on Nuclear Ribonucleic Acid (RNA) Metabolism. II.[1] RNA Polymerase Activities in Rats treated with Ginsenoside

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The activities of ribonucleic acid (RNA) polymerases I, II and III in a sodium deoxycholate (DOC)-treated enzyme preparation have been differentially determined using three combinations of assay conditions (metal ion; Mg^{2+} or Mn^{2+}, $(NH_4)_2SO_4$ and α-amanitin).

Ginsenoside-Rb_1 enhanced, while -Rc repressed, the activities of RNA polymerases I and II, whereas both had no effect on that of RNA polymerase III.

Rb_1-treated rats showed different profiles of stimulation of RNA polymerases I and II; the maximum increase of RNA polymerase I activity was about $+70\%$ at 2 hr, and that of RNA polymerase II activity was $+40\%$ at 3 hr after the injection of Rb_1.

Actinomycin D and cycloheximide both blocked the increase in RNA polymerase I activity on treatment with ginsenoside-Rb_1. On the other hand, increased activity of RNA polymerase II was blocked by actinomycin D but not by cycloheximide. These results suggest transcriptional regulation in the enhancement by ginsenoside-Rb_1 of RNA polymerase activities I and II, though the mechanisms may differ in detail.

Keywords——ginseng saponins; RNA polymerase activity; rat liver; nuclear RNA; actinomycin D; cycloheximide

In the preceding paper,[1] we reported that ginsenoside-Rb_1 increased, while -Rc decreased, the incorporation of ^3H-orotic acid into the nuclear ribonucleic acid (RNA) of rat liver 4 hr after intraperitoneal injection. We also observed that ginsenoside-Rb_1 enhanced, while -Rc repressed, the RNA polymerase activity in liver 3 hr after intraperitoneal administration. The results suggest that the effects of the ginsenosides on the synthesis of nuclear RNA are due to changes in RNA polymerase activity. The present paper deals with the effects of these two purified saponins on the activities of three different RNA polymerases (I, II and III). In addition, the effect of cycloheximide or actinomycin D on the increased RNA polymerase activity in Rb_1-treated rats was studied, in order to investigate whether the stimulation occurred at the transcriptional or translational stage.

Experimental

Animal ——Male Wistar rats weighing 150—200 g were used.

Saponins——Ginsenoside-Rb_1 and -Rc, isolated and purified from Ginseng, were gifts from Dr. S. Sanada and Dr. J. Shoji, School of Pharmaceutical Sciences, Showa University.

Radiochemicals——[5-^3H]CTP (26.2 Ci/mmol) was a product of New England Nuclear Corp., U.S.A. [5-^3H]Orotic acid (17.9 Ci/mmol) was obtained from the Radiochemical Centre, England.

General Procedures for the Assay of RNA Polymerase Activity in Liver Nuclear Enzyme Preparation of Saponin-treated Rats——Each ginsenoside (5 mg/100 g body weight) was administered to rats intraperitoneally in saline solution. Control rats were treated with an equal volume of saline. The rats were killed at appropriate times after administration of the ginsenosides, and their livers were removed and the nuclei separated.

Separation of Nuclei of Rat Liver and Preparation of Lysed Nuclear Enzyme——The separation of nuclei and preparation of lysed nuclear enzyme were carried out as described in the preceding paper.[1]

Assay of RNA Polymerase Activity——RNA polymerase activity was expressed in terms of the radioactivity of ^3H-CMP incorporated into the RNA fraction. To assay each activity of RNA polymerase (I, II

1) Part I: M. Iijima, T. Higashi, S. Sanada, and J. Shoji, Chem. Pharm. Bull. (Tokyo), 24, 2400 (1976).
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and III) in a DOC-lysed enzyme preparation, three reaction mixtures were prepared as follows: Mixture A (250 µl) contained 25 µmol of Tris–HCl buffer, pH 8 0, 2 µmol of β-mercaptoethanol, 1.5 µmol of $MgCl_2$, 1.25 µmol of NaF, 0.1 µmol each of adenosine, guanosine and uridine triphosphates, 0.5 µCi of ³H-cytidine triphosphate, and α-amanitin (10 µg/ml). Mixture B (250 µl) contained 25 µmol of Tris–HCl buffer, pH 8.0, 2 µmol of β-mercaptoethanol, 1.25 µmol of $MnCl_2$, 30 µmol of ammonium sulfate, 0.1 µmol each of adenosine, guanosine and uridine triphosphates, and 0.5 µCi of ³H-cytidine triphosphate. Mixture C contained α-amanitin (1.0 µg/ml) in addition to the other components of mixture B. The reaction was initiated by the addition of enzyme solution and the mixture was incubated at 37° for 15 min. Fifty µl aliquots were withdrawn from each tube and spotted on DEAE-cellulose discs (Whatman DE-81 filters, 2.5 cm diameter). The filters were washed six times in 5% $Na_2HPO_4 \cdot 12H_2O$, twice in distilled water, twice in 90% ethanol and twice in ether successively, then dried according to Weil and Blatti.[3]

General Procedure for Assaying Radioactivity of Liver Nuclear RNA in Saponin-treated Rats——This was carried out as described in the preceding paper,[1] except that rats were killed at selected times after saponin treatment.

Radioactivity Measurement——Radioactivity was counted in a Beckman scintillation spectrometer.

Scintillator Fluid——Scintillator fluid A was used for the measurement of ³H-CMP incorporated into RNA, and fluid B was used for the measurement of ³H-orotic acid incorporated into liver nuclei RNA. The compositions of the scintillators were as follows. A; One liter of the solution contained 4 g of 2,5-diphenyloxazole, 100 mg of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene and toluene. B; One liter of the solution contained 50 ml of methanol, 10 ml of ethyleneglycol, 60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene and dioxane.

Protein Determination——Lowry's method[4] was employed.

RNA Determination——Schneider's method[5] was employed.

Results

Differential Determination of the Activities of RNA Polymerases I, II, and III in Enzyme Preparation of DOC-treated Nuclei

In the preceding paper[1] the effects of ginsenoside-Rb_1 and -Rc on RNA polymerase activity were studied, but the enzyme activity assayed under the conditions described there was that of all three RNA polymerases as a whole. In this study, we attempted to distinguish the effects of the ginsenosides on each of the RNA polymerases. Three different reaction mixtures (A, B and C) were used for this purpose. Mixture A contained 10 µg/ml of α-amanitin, which inhibits the activity of RNA polymerase II completely and that of RNA polymerase III by about 50%.[6] Mixture B contained a high concentration of ammonium sulfate; RNA polymerase I shows no activity in this case.[7] Addition of α-amanitin (1.0 µg/ml) to the reaction mixture B (i.e., mixture C) abolished the activity of RNA polymerase II.[6] Therefore, the activities in A, B and C correspond to I+III/2, II+III, and III, respectively. However, to calculate RNA polymerase I activity it was necessary to determine the relationship between the activities of RNA polymerase III assayed in A and in C.

TABLE I.   Incorporation of ³H–CMP into RNA under Various Conditions

Reaction mixture	α-Amanitin (µg/ml)	³H–CMP incorporated (cpm/mg protein)
A	10	3070
A + extra amanitin	200	2220
B	0	28850
C	1.0	3220

3) P.A. Weil and S.P. Blatti, *Biochemistry*, **14**, 1636 (1975).

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As shown in Table I, the enzyme activity was assayed in the presence of an amount of α-amanitin (200 μg/ml) sufficient to suppress the activity of RNA polymerase III completely. The decrease in the activity compared to that in reaction mixture A (3070—2220=850) was about 25% of the activity assayed in reaction mixture C (3220).

Thus, RNA polymerase I activity was calculated as the activity in A minus a quater of the activity in C.

Characterization of the RNA Polymerase Reaction

In order to determine the optimum conditions for the assay of RNA polymerases, the RNA polymerase reaction was studied. Fig. 1-a shows that there was a linear relationship between the amount of protein and the enzyme activities. A suitable amount of protein for the reaction mixture is 150 to 200/μg.

The kinetics of the reactions are shown in Fig. 1-b. The reactions of RNA polymerases I, II and III proceeded linearly with time for 5 min, then reached a plateau, using 250 μg of protein of DOC-lysed enzyme preparation.
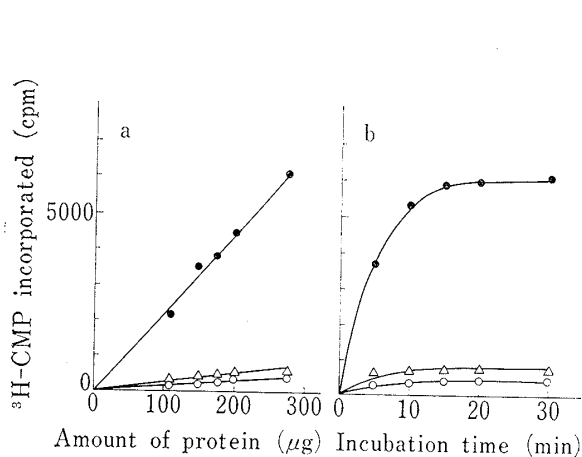

Fig. 1. Characterization of RNA Polymerase Reactions

Lysed nuclear enzyme was prepared from normal rats and the enzyme activity was assayed as described in the text. RNA polymerase activity is expressed as ^{3}H-CMP incorporated into RNA. a: Relationship between the amount of protein in lysed nuclear enzyme preparation and RNA polymerase activity. The incubation time was 15 min. b: Kinetics of RNA polymerase reactions. The amount of protein in the enzyme preparation used was 250 μg/250 μl of reaction mixture. —○—, RNA polymerase I activity; —●—, RNA polymerase II activity; —△—, RNA polymerase III activity.
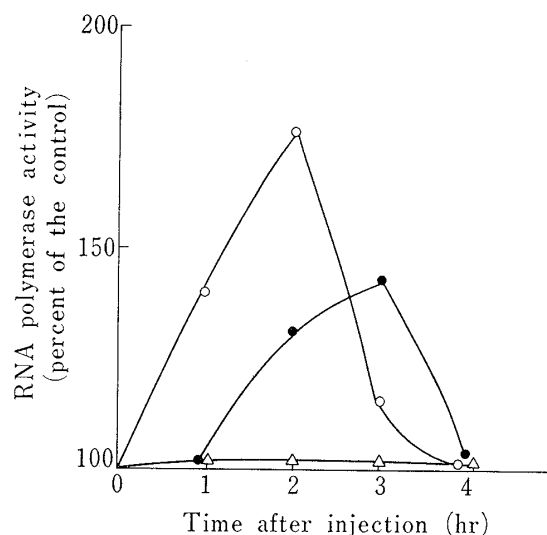

Fig. 2. Time Course of the Effect of a Single Dose of Ginsenoside-Rb$_1$ on the Activities of RNA Polymerases I, II and III

Rats received a single dose of ginsenoside-Rb$_1$ and were killed after the indicated times. Nuclear enzyme was prepared from four rats at each time, and the activities of RNA polymerase I, II, and III were differentially assayed as described in the text. RNA polymerase activity is expressed as percent of the control. —○—, RNA polymerase I activity; —●—, RNA polymerase II activity; —△—, RNA polymerase III activity.

Time Course of the Effect of a Single Dose of Ginsenoside-Rb$_1$ on RNA Polymerase Activity

In the preceding study,[1] we observed increased RNA polymerase activity at 3 hr after ginsenoside treatment. However, the increase in the activity was less than that obtained on treatment with a hormone such as estradiol.[8] It was expected that larger effects might be detected on the activity of individual polymerases at some stage after the treatment. Therefore, the time course of the effect of ginsenoside-Rb$_1$ on each of the RNA polymerase activities was studied. As shown in Fig. 2, ginsenoside-Rb$_1$ markedly stimulated RNA polymerase I activity. The increase in the enzyme activity was found to be 50% after 1 hr, reaching a maximum (70%) 2 hr after the injection of Rb$_1$. RNA polymerase II activity

8) T.H. Hamilton, C.C. Windel, and J.R. Tata, *J. Biol. Chem.*, **243**, 408 (1968).

was also stimulated, the maximum increase being 40% after 3 hr. Both activities showed little increase 4 hr after the injection of Rb_1. The ginsenoside had no effect on the activity of RNA polymerase III. The increase in RNA polymerase activity caused by ginsenoside-Rb_1 is probably not due to a decrease in RNase activity in the RNA polymerase preparations: no significant difference was observed between the control and Rb_1-treated preparation (data not shown).

Time Course of the Effect of Ginsenoside-Rb_1 on the Incorporation of ^3H-Orotic Acid into Liver Nuclei RNA

In the previous paper,[1] we reported an increased (17%) incorporation of ^3H-orotic acid into rat liver nuclear RNA 4 hr after treatment with ginsenoside-Rb_1. To obtain more detailed information the time course was studied; the results are shown in Fig. 3. The maximum increase in the incorporation of ^3H-orotic acid into the nuclear RNA was obtained at 4 hr after the treatment. Since the maximum increase in the RNA polymerase activities occurred shortly before that as regards RNA synthesis (cf. Fig. 2), it is suggested that the observed increase in the incorporation rate of nuclear RNA due to ginsenoside-Rb_1 may be caused by stimulation of the RNA polymerase activities (I and II).

Fig. 3. Time Course of the Effect of Ginsenoside-Rb_1 on the Incorporation of ^3H-Orotic Acid into Liver Nuclear RNA

Ginsenoside-Rb_1 was administered intraperitoneally to rats and ^3H-orotic acid was injected 20 min prior to sacrifice. The specific radioactivity of nuclear RNA isolated at each time was assayed as described in the text, and expressed as percent of the control.

Effect of Ginsenoside-Rc on RNA Polymerase Activities

Ginsenoside-Rc was reported in the preceding paper[1] to repress RNA polymerase activity, unlike Rb_1. In this study, the effect of ginsenoside-Rc on each of the RNA polymerases I, II and III was also examined. As shown in Table II, Rc repressed RNA polymerases I activity by 50% and II by 30%. It had no effect on RNA polymerase III activity, as was the case for Rb_1.

TABLE II.   Effects of Ginsenoside-Rc on the Activities of RNA Polymerases I, II and III

RNA Polymerase	^3H-CMP incorporated (cpm/mg protein)		Percent of control
	Control(4)[b]	Ginsenoside-Rc treated[a] (4)[b]	
	(Mean ± S.E.)		
RNA polymerase I	2260 ± 190	1150 ± 110	51
RNA polymerase II	24800 ± 1250	17190 ± 570	69
RNA polymerase III	3370 ± 200	3420 ± 230	101

a) Ginsenoside-Rc was injected into rats 3 hr before sacrifice.
b) Figures in parentheses indicate the number of animals.

Effects of Inhibitors of RNA and Protein Synthesis

The effects of actinomycin D and cycloheximide on the increased activity of RNA polymerase I and II due to ginsenoside-Rb_1 were investigated in order to study whether the effect is transcriptional or translational. The results are shown in Tables III and IV.
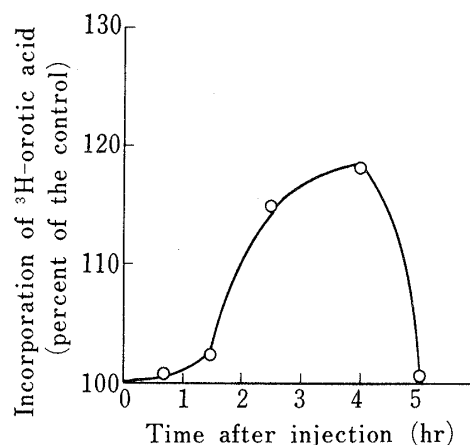
Administration of actinomycin D at a dose of 100 μg/100 g body weight markedly reduced the RNA polymerase I activity both in Rb_1-treated and in intact rats. When a smaller amount of this inhibitor (10 μg/100 g) was given, a slight decrease (14% in intact rats) of the enzyme activity was observed, and furthermore the effect of Rb_1 (+63%) was abolished. Cycloheximide at a dose of 500 μg/100 g body weight inhibited RNA polymerase I activity by 40% in intact animals and also suppressed the effect of Rb_1. A smaller amount of cycloheximide (250 μg/100 g), which had little effect on RNA polymerase I activity, could block the stimulating effect of Rb_1.

The effects of two inhibtors on the increased activity of RNA polymerase II were also investigated, and the results are shown in Table IV. Actinomycin D (100 μg/100 g body weight) blocked the increase of RNA polymerase II activity due to ginsenoside-Rb_1. On the other hand, cycloheximide (500 μg/100 g body weight) had no effect on the enhancement caused by Rb_1. The increase in RNA polymerase activity caused by Rb_1 may be regulated transcriptionally for both I and II, but the mechanisms may differ in detail.

TABLE III.   Effects of Actinomycin D and Cycloheximide on the Increase
of Activity of RNA Polymerase I due to Ginsenoside-Rb_1

Ginsenoside-Rb_1 treatment[a]	Inhibitor[b]	^{3}H-CMP incorporated (cpm/mg protein)	%
No treatment	None (6)[c]	2200± 50	100
	Actinomycin D		
	(100 μg)[b](4)	370± 10	17
	(10 μg)[b] (2)	1900	86
	Cycloheximide		
	(500 μg)[b] (5)	1230±140	60
	(250 μg)[b] (2)	2100	95
Treated	None (4)	3580±150	163
	Actinomycin D		
	(100 μg) (4)	340± 10	15
	(10 μg) (2)	1930	87
	Cycloheximide		
	(500 μg) (3)	1230	60
	(250 μg) (2)	2030	92

a) Ginsenoside-Rb_1 was injected into rats 2 hr before sacrifice.
b) Actinomycin D(100 μg or 10 μg/100 g body weight) or cycloheximide (500 μg or 250 μg/100 g body weight) was administered 1 hr before the Rb_1 injection.
c) Figures in parentheses indicate the number of animals.

TABLE IV.   Effects of Actinomycin D and Cycloheximide on the Increase
of Activity of RNA Polymerase II due to Ginsenoside-Rb_1

Ginsenoside-Rb_1 treatment[a]	Inhibitor[b]	^{3}H-CMP incorporated (cpm/mg protein) (Mean ±S.E.)	Percent of control
No Treatment	None (8)[c]	27760± 760	100
	Actinomycin D (4)	28090± 330	101
	Cycloheximide (4)	28210± 410	102
Treated	None (4)	41530±1500	150
	Actinomycin D (4)	27900± 410	101
	Cycloheximide (4)	42480± 980	153

a) Ginsenoside-Rb_1 was injected into rats 3 hr before sacrifice.
b) Actinomycin D(100 μg/100 g body weight) or cycloheximide (500 μg/100 g body weight) was administered 1 hr before the Rb_1 injection.
c) Figures in parentheses indicate the number of animals.

Discussion

In the present study, we did not aim to employ the optimum conditions for each of the RNA polymerases but we sought to find conditions adequate for distinguishing each enzyme activity in a crude preparation containing all three RNA polymerases, I, II and III. RNA polymerase I is known to be insensitive to α-amanitin,[3,6] whereas RNA polymerase II is sensitive (100% inhibition at a concentration of 0.15 µg/ml) in the presence (0.10 M)[3,6a] or absence[6b] of ammonium sulfate. RNA polymerase III shows intermediate sensitivity to α-amanitin (100% inhibition at a concentration of 200 µg/ml), independent of ammonium sulfate.[3,6a,6b] Furthermore, at a high concentration of ammonium sulfate such as that in reaction mixtures B and C in this study, RNA polymerase I exhibits no activity.[7] Based on these results and those shown in Table I, the conditions described here are considered to be satisfactory for assaying the activity of each RNA polymerase activity differentially.

Figure 2 shows that RNA polymerase I is affected by ginsenoside-Rb_1 more significantly and more rapidly than RNA polymerase II. Stimulation of RNA polymerase I and II activities by Rb_1 disappeared within 4 hr. These results are different from those obtained by Hiai et al.[9] with a crude saponin preparation. The discrepancy may be due to the difference in the purity of the saponin: our ginsenoside-Rb_1 was purified extensively, whereas the fraction 3 used by Hiai et al. was not.

The maximum increase in the incorporation of ^{3}H-orotic acid into nuclear RNA caused by Rb_1 occurred at 4 hr, which is later than the peak in RNA polymerase activity. Therefore, it is suggested that the latter is the primary response to the ginsenoside.

Both actinomycin D and cycloheximide blocked the increase in RNA polymerase I activity due to ginsenoside-Rb_1. On the other hand, increased activity of RNA polymerase II due to Rb_1 was not observed after treatment with actinomycin D, but cycloheximide did not suppress the increase in RNA polymerase II activity caused by Rb_1. In rat uterus, actinomycin D (200 µg/100 g body weight) was shown to inhibit RNA synthesis preferentially,[10] and the drug also inhibited the RNA synthesis in rat liver nuclei.[11] As for cycloheximide, it is known to inhibit protein synthesis in liver in vivo[12] but does not inhibit RNA synthesis drastically, even at a high dose (2 mg/100 g body weight).[13] Thus, it appears that regulation of the increase of RNA polymerase I activity may be transcriptional, and de novo protein synthesis precedes the increase of the enzyme activity. The increased activity of RNA polymerase II also seems to be regulated transcriptionally rather than translationally, and may not require prior protein synthesis. The mechanisms operating in the cases of polymerases I and II may differ in detail, however.

Chromatin RNA,[14] protein factors[15] from rat liver or Ehrlich ascites tumor cells, and phosphorylation[16] of RNA polymerases by protein kinase, have been reported to stimulate RNA polymerase activity. However, it is not yet possible to correlate the results in this study with these other factors.

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Some hormones, such as growth hormone,[17] estrogen[8] and hydrocortisone,[18] have been reported to stimulate RNA polymerase activity. Most of them have been suggested to increase RNA polymerase I activity preferentially for as long as 24 hr. The effect of ginsenoside-Rb$_1$ reported in the present parer is similar to that of these hormones, except that the effect disappeared within 4 hr. Hamilton *et al.*[8] reported that stimulation of RNA polymerase I activity by estrogen was prevented by actinomycin D or cycloheximide, and that the synthesis of RNA preceded the increase in the enzyme activity. Such evidence has not been obtained in the case of ginsenoside-Rb$_1$.

An increase in the template activity of chromatin has also been reported in hormone-treated animals.[19] Sajdel and Jacob[20] reported, on the other hand, that hydrocortisone induced an allosteric change in RNA polymerase which resulted in increased activity of the enzyme.

It is not clear whether the increased activity of RNA polymerase on treatment with ginsenoside-Rb$_1$ is due to a change in RNA polymerase itself, or to the change in the template activity of the chromatin, and it may be worth investigating the template activity after treatment with ginsenoside. In any case, studies on the effects of ginsenoside-Rb$_1$ and other compounds on RNA polymerase activities might provide useful information on the regulation of this enzyme activity.

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