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Stimulation of Serum Protein Synthesis in Ginsenoside Treated Rat

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Ginsenosides Rb_1 and Rc purified from the ethanol extract of a root of Panax ginseng were administered into rats at the dose of 5 mg/100 g of body weight. After 3 hours of the administration, biosynthesis of rat serum protein was stimulated at 2 times and more than the control, in 1 hour ^{14}C-amino acid incorporation experiment. From the analyses of serum proteins by salting-out, electrophoresis and NaBr flotation, apoproteins of chylomicrons and very low density lipoprotein showed mostly stimulated synthesis by the treatment with ginsenosides. This coincided with the stimulation of cholesterol synthesis reported previously, and a possible relationship between the synthesis of lipids and that of apo-lipoproteins was discussed.

The herbal, a sort of the oriental drugs has been used for thousands years in Asian countries empirically as analeptic, stomachic and erythropoietic. In this century, chemical analysis has been conducted to identify effective substances in these drugs. Particularly, a root of *Panax ginseng* C.A. Mayer has attracted much attentions and has been one of the most extensively studied.

To elucidate the effectivity of Panax ginseng, biochemical techniques have been applied. Oura found that an extract of the root (surely containing saponin) enhanced the synthesis of nucleic acid and protein in rat liver,[2] and also reported a stimulating activity of some purified saponins on serum protein synthesis in mouse.[3] Yamamoto described an increased cholesterol metabolism in rat by the administration of crude saponins.[4]

We selected three saponins, namely Ginsenoside Rb_1, Rc and Rg_1 by their high content in dried Panax root (0.47, 0.26 and 0.17% respectively),[5] and studied their biochemical activities. When administered into rats, these saponins showed a significant activity to stimulate biosynthesis of cholesterol.[6] Here, the effects of these purified saponins on serum protein metabolism are presented.

Materials and Methods

Ginsenoside Rb_1, Rc, and Rg_1: Purified by thin-layer chromatography from a hot ethanol extract of the powder of *Panax ginseng* C.A. Mayer.[5] Dissolved in physiological saline at the concentration of 10 mg/ml.

^{14}C-leucine, ^{14}C-amino acids mixture and ^{14}C-acetate were purchased from Daiichi Pure Chemicals.

Albino male rats (*ca.* 150 g) were given 5 mg of ginsenoside per 100 g of body weight intraperitoneally. After 3 hours, 10μCi of ^{14}C-leucine per 100 g of body weight was administered intraperitoneally and then after 1 hour rats were sacrificed by bleeding from aorta abdominalis under ether anesthesia. Serum was separated from the blood and proceeded for protein determination and radioactivity measurement after 5% trichloroacetic acid (TCA) precipitation.

1) Location: *1–5–8, Hatanodai, Shinagawa-ku, Tokyo.*
2) H. Oura, *J. Japan. Biochem. Soc.*, **45**, 804 (1973).
3) H. Oura, S. Hiai, Y. Odaka, and T. Yokozawa, *J. Biochem.*, **77**, 1057 (1975).
4) M. Yamamoto, *Metabolism*, **10**, 646 (1973).
5) S. Sanada, N. Kondo, J. Shoji, O. Tanaka, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **22**, 421, 2407 (1974).
6) K. Sakakibara, Y. Shibata, T. Higashi, S. Sanada, and J. Shoji, *Chem. Pharm. Bull.* (Tokyo), **23**, 1009 (1975).

Serum proteins were analyzed by salting-out in ammonium sulfate solution···a sequential precipitation at 1/3- and 1/2-saturation of the salt. The total volume never exceeded 2 folds of the starting serum volume. Protein amount was determined on each supernatant and whole serum, radioactivity was measured on each precipitate and 5% TCA precipitate of the supernatant of 1/2-salt saturation.

Agar gel plate ($10 \times 7 \times 0.2$ cm) was used as a bed in the electrophoretic analysis of serum proteins. The serum was dialyzed and an aliquot of 0.15 ml was applied. After electrophoresis (6 mA/cm 3 hours in pH 8.6 Veronal buffer) 3 mm-width slot was cut out, whose 1/3 was proceeded for protein determination and the rest 2/3 for radioactivity measurement. Disc gel (0.5×7 cm) was prepared with 7% polyacrylamide, and after electrophoresis (1.5 mA/tube, 1.5 hours in pH 8.9 Tris-buffer) the gel was stained with amido black 10 B and scanned by Atago densitometer for protein analysis, and radioactivity was measured with 1.5 mm-width of slice after direct combustion.

One ml of serum was applied on Sephadex G-100 column (2×25 cm, in pH 8.0 phosphate buffer), and after the gel filtration 4 ml each of the effluent was assayed for OD_{280} absorbance and radioactivity.

The analysis of serum lipoproteins was carried out by a modified method of NaBr density gradient flotation[7] (40000 rpm, 3.5 or 18 hours in Hitachi RPS-50 ultracentrifuge rotor) on the treated and the normal sera. For 3.5-hr centrifugation to analyze lower density lipoproteins, 400 mg of NaBr was dissolved into 1.0 ml of the serum to make a bottom layer in the tube, and then each 0.9 ml of 27.0, 17.5 and 6.6% NaBr solution and physiological saline was layered on succesively. For 18-hr centrifugation to analyze higher density lipoproteins, 700 mg of NaBr was dissolved into 0.8 ml of the serum to make a bottom layer, and then each 0.6 ml of 60 and 50% NaBr solution and physiological saline was layered on. After centrifugation, 0.1 ml each of the content was collected from the top of the tube and was proceeded for the measurements.

Protein content was determined by biuret reaction in the salting out fractionation and by Folin method in the agar gel electrophoresis. Radioactivity was measured by a liquid scintillation spectrometer (Beckman LC-100) after combustion by Aloka ASC 111 aparatus.

Results

Since in a preliminary study ^{14}C-leucine incorporation into serum proteins of normal rats showed a maximum at 30 min after injection, we labeled the animals for 60 min to compare the incorporation rate between normal and ginsenoside-treated rats. Ginsenoside Rb_1, Rc or Rg_1 was given to rats at a dose of 5 mg per 100 g of body weight, and after 3 hr ^{14}C-leucine followed to label for 60 min. Table I presents protein content, incorporated radioactivity and the specific activity of total serum proteins from normal and the treated rats. Judging from the averaged specific radioactivity, ginsenoside Rb_1 and Rc increased the leucine incorporation by a factor of 2.2 and 1.9 respectively, over the control. However, Rg_1 failed to stimulate the synthesis of serum proteins.

In other experiments using ^{14}C-amino acid mixture instead of ^{14}C-leucine for labeling, a parallel results were obtained. Therefore, an effect of the ginsenosides to delete leucine pool in protein-producing cells was excluded. Furthermore, the amount of whole serum proteins were not significantly affected by the treatment, so the increase in specific radioactivity was not derived from decreased protein concentrations. Two-three fold increase of de Novo synthesis of proteins for 1 hr does not seem to elevate the total protein level.

Table I. Protein Content and Incorporated Radioactivity of Sera in Ginsenoside Treated Rats

		Protein content (mg/ml)	Radioactivity (cpm/ml)	Specific activity (cpm/mg)
Control		56.0±1.4	31400±2470	560± 42
Treated with Ginsenoside	Rb_1	56.9±1.2	68900±2680	1210±123
	Rc	52.1±1.5	54100±9800	1040±170
	Rg_1	49.9	27300	545

Ginsenoside (5 mg/100 g)-treated rats were labeled with 10 uCi of ^{14}C-leucine for 60 min. TCA insoluble portions of each 1.0 ml of serum were assayed by biuret reaction and liquid scintillation counting. Figures are the averaged values from 16 normal, 8 Rb_1–, 8 Rc– and 2 Rg_1-treated rats, and the standard errors of the means.

7) H.G. Wilcox, D.C. Davis, and M. Heimberg, *J. Lipid Research*, **12**, 160 (1971).

To analyze what kind of serum proteins was affected in biosynthesis, at first, salting out fractionation with ammonium sulfate was employed as described in "Methods." In the precipitates of 1/3 and 1/2 saturation of the salt, and in the supernatant of 1/2 saturation, protein amounts after the treatment were not significantly changed, whereas radioactivities incorporated into protein were generally higher in Rb_1- and Rc-treated rats than the control. However, it was difficult to find any significant difference in the increases of specific radioactivity among these fractions.

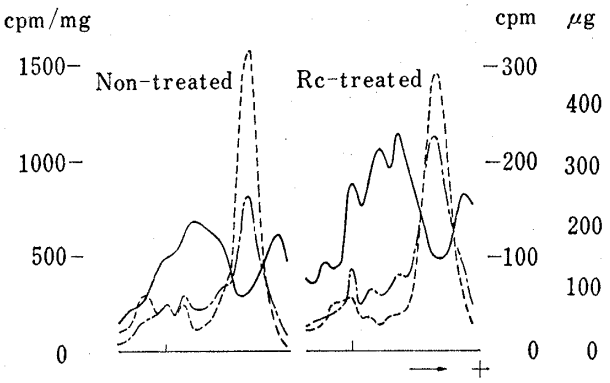

Fig. 1. Electrophoretic Profile of Serum Proteins Labeled with ^{14}C-Leucine

Each 0.15 ml of serum of a normal and an Rc-treated rat both injected with ^{14}C-leucine, was analyzed on agar-gel plate (10×7×0.2 cm). Values for each 3mm-width slot were traced.
— — —: amount of protein
—·—·—: radioactivity
———: specific radioactivity

Next, analysis of the labeled protein by electrophoresis was carried out in an agar-gel plate. Electrophoretic profiles of sera from a normal and, for example, an Rc-treated rats are illustrated in Fig. 1. In the control serum, the specific radioactivities around α- and β-globulins were higher than those of albumin, so these globulins should turn over faster. A high specific radioactivity seen in prealbumin region, is not meaningful, because of its small protein content. In Rc-treated rats the increase in specific activity among α- and β-globulins was remarkable. In γ-globulin an elevation in the specific activity was observed, but again the amount of protein was so small that it was not able to obtain any significant informations.

The summarized results in this kind of experiments including the ones with Rb_1 and Rg_1, are presented in Table II, which shows protein content, radioactivity and the specific activity in each of protein fractions.

No significant effect of the ginsenoside treatment was detected on the protein concentration in each fraction. However, in the specific radioactivity, 2—3 times increase was observed

Table II. Protein Amount and Radioactivity of Serum Proteins Fractionated by Agar-Gel Electrophoresis

		Control	Treated with Ginsenoside		
			Rb_1	Rc	Rg_1
Albumin	protein (mg)	5.6	5.7	5.3	4.1
	radioactivity (cpm)	2720	5410	4720	1880
	specific activity (cpm/mg)	485	950	890	460
α-Globulin	protein (mg)	0.89	0.91	0.90	1.29
	radioactivity (cpm)	710	2120	1770	930
	specific activity (cpm/mg)	795	2340	1970	725
β-Globulin	protein (mg)	0.98	0.98	1.00	0.44
	radioactivity (cpm)	535	1130	1145	270
	specific activity (cpm/mg)	545	1155	1145	600
γ-Globulin	protein (mg)	0.84	1.05	0.60	1.05
	radioactivity (cpm)	160	790	345	340
	specific activity (cpm/mg)	190	750	575	325

The sera (0.15 ml) were fractionated by electrophoresis described as the legend to Fig. 1. Then the gel was sliced in 3 mm-width slots. Each 1/3 was proceeded for Folin determination and 2/3 for radioactivity measurement. Next, these data were collected in each protein fraction which was devided accord with the profile as shown in Fig. 1.

in the treated groups. Both α- and β-globulins, which are fast in turn over in the normal serum received a higher stimulation in synthesis after Rb_1 and Rc treatment. The stimulation ratio (a ratio in the specific radioactivity of the treated to the control) was around 2.5 in α-globulin, and over 2.0 in β-globulin. Although 3 times increase was observed on γ-globulin, it hardly conclude a significant difference because of its low protein content. Rg_1 gave little effect on the biosynthesis of proteins in every fractions.

The finding shown in Fig. 1 and Table II were confirmed by a further experiment in which serum proteins of the normal and Rc-treated rats were labeled with ^{14}C-amino acid mixture and analyzed by disc electrophoresis. As shown in Fig. 2, the specific activity around α-globulin was high in both normal and the treated rats. However, the stimulation ratio did not exceed 2.0 in α-globulin, and rather higher in β_1 (2.5) and β_2 (3.0 regions.

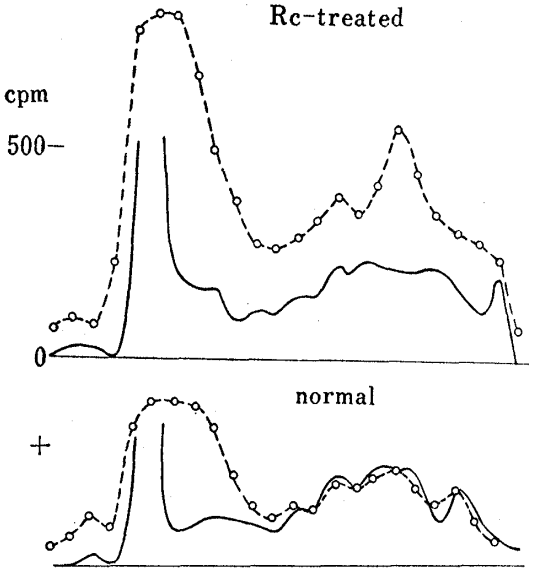

Fig. 2. Disc Electrophoretic Profile of Serum Proteins Labeled with ^{14}C-Amino Acid Mixture

Serum proteins were labeled with 10 μCi of ^{14}C-amino acid mixture per 100 g of body weight for 60 min. Each 0.12 ml of sera was analyzed in polyacrylamide gel (0.5×7 cm) as described in "Materials and Methods". Radioactivity profile (——) tracing each 2 mm-width slice data is arranged to densitometrical profile (——) of the stained gel.

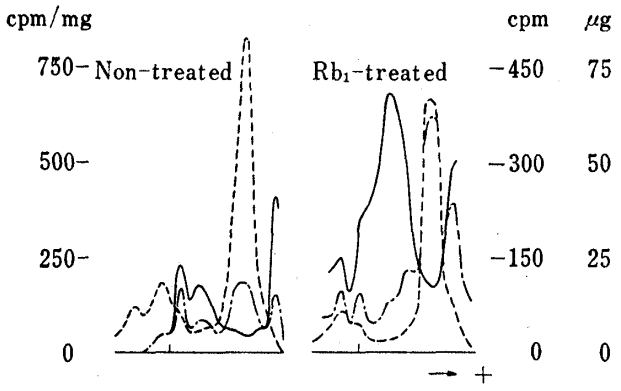

Fig. 3. Electrophoretic Profile of Serum Proteins Labeled with ^{14}C-Acetic Acid

Ginsenoside (5 mg/100 g)-treated rats were labeled with 10 μCi of ^{14}C-acetic acid for 30 min. Each 0.15 ml of dialyzed sera was analyzed by agar-gel electrophoresis.

– – – –: amount of protein
—·—·—: radioactivity
————: specific activity

On a discrepancy between the stimulation ratios in α-globulin region of the agar- and the disc-electrophoresis, the authors convince the latter, since more precise separation is considered to render.

In another experiments in which the labeling of cholesterol by injected ^{14}C-acetic acid was studied, simultaneously the incorporation of the ^{14}C into serum proteins were analyzed by agar electrophoresis. Both in normal and in Rb_1-treated rats (Fig. 3) a similar radioactivity profile was obtained with markedly higher peaks in α- and β-globulin areas in the latter, as was the case in the labeling experiments with ^{14}C-leucine (Fig. 1). The evidence would suggest that a stimulated synthesis of lipid is associated with that of apo-lipoproteins as well, since α-globulin contains high density lipoprotein (HDL) and β-globulin contains very low density lipoprotein (VLDL) and low density lipoprotein (LDL). But again, α-globulin failed to show a higher stimulation ratio in amino acid labeling on disc-electrophoresis (Fig. 2).

Judging from these different stimulation ratios in α- and β-globulin regions (Fig. 2), one may postulate a variable extent of stimulation on the biosynthesis of lipoproteins by the ginsenosides. To investigate further the increased synthesis of serum lipoproteins, a conven-

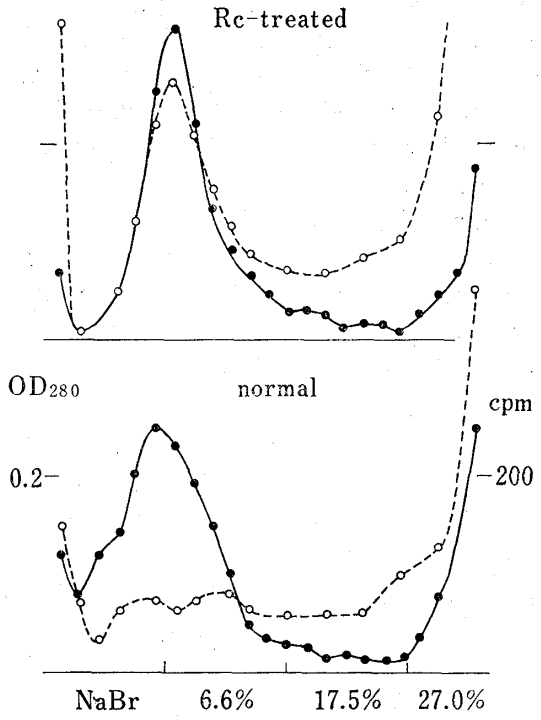

Fig. 4. NaBr Flotation Analysis (3.5-hr Centrifugation) of Serum Proteins Labeled with ^{14}C-Amino Acid Mixture

Serum (1.0 ml) was analyzed by 3.5-hr centrifugation as described in "Materials and Methods". Each 0.1 ml of effluent was diluted with physiological saline to 2.0 ml for measurement on OD_{280} (——), and TCA insoluble portion of a diluted solution was assayed for the radioactivity (----). Only an upper half is shown.

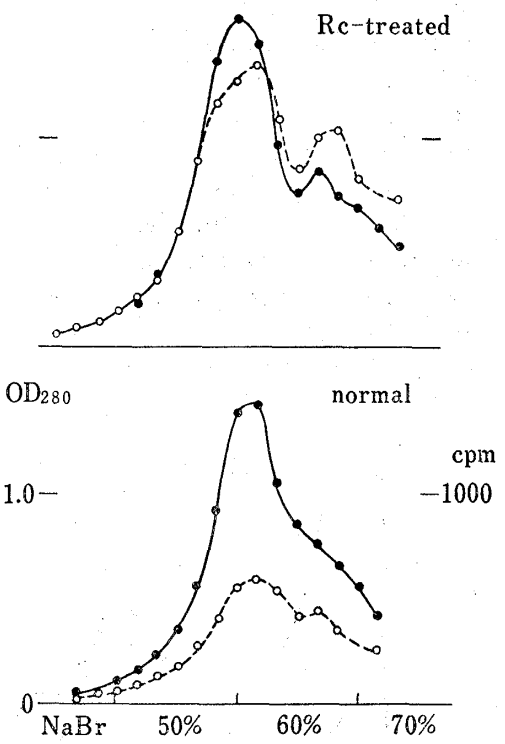

Fig. 5. NaBr Flotation Analysis (18-hr Centrifugation) of Serum Proteins Labeled with ^{14}C-Amino Acid Mixture

Serum (0.8ml) was analyzed by 18-hr centrifugation as described in "Materials and Methods". Other experimental conditions were the same as described in the legend to Fig. 4. ——: OD_{280}; ----: radioactivity. Only a lower part is shown.

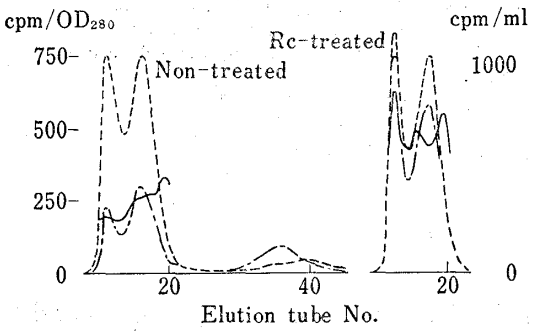

Fig. 6. Gel Filtration of Serum Proteins Labeled with ^{14}C-Amino Acid Mixture

Serum (1.0 ml) was analyzed through a Sephadex G-100 column (2×25 cm). Each 4.0 ml of effluent was assayed for OD_{280} (--------) and radioactivity (—·—·—). Also specific activity is shown (———).

tional NaBr flotation analysis was carried out. On 3.5 hr centrifugation as described in "Methods," much difference of the incorporated radioactivity was observed in the phase of chylomicrons (top) and VLDL (on 6.6% NaBr layer) between normal and Rc-treated rats (Fig. 4). The stimulation ratio was 5 in chylomicrons and 4 in VLDL. No concrete information was obtained on LDL, the phase on 17.5% NaBr layer.

In 18 hr centrifugation, as shown in Fig. 5, the difference of the specific radioactivity was greater rather in non-lipoprotein region (infranate) than HDL, and the stimulation ratios in these regions were not so high. These results are considered to support the result of the disc electrophoresis, that is, the low stimulation ratio in α-globulin.

The labeled serum proteins were also analyzed by gel filtration through a Sephadex G-100 column. Fig. 6 demonstrates a change in the labeling rate after Rc-treatment. In normal serum, proteins of lower molecular weight were rather higher in specific radioactivity than those of higher molecular weight, whereas in the serum of treated rats the tendency was reversed. The stimulation ratio was 2.5 in the larger proteins including chylomicrons and VLDL, and 2.0 in the smaller proteins.

Discussion

A crude extract of the root of Panax ginseng was found to have a stimulating activity on protein synthesis in rats, as reported by Oura.[2] He and his colleagues concluded that the active principle stimulating protein synthesis is saponin based on the results of their experiments with mice administered pure saponins isolated by thin-layer chromatography.[3] In the previous study, we injected into rats a large dose of purified ginsenoside Rb_1, Rc and others, observing a striking enhancement of cholesterol synthesis[6]. Here, a stimulation in serum protein synthesis by purified ginsenoside was demonstrated by the labeling experiments with ^{14}C-amino acid.

Ginsenoside Rb_1 as well as Rc stimulated ^{14}C-leucine incorporation into serum proteins more than 2 times compared with normal rats, and Rg_1 did not show any effect. However, Oura, *et al.*[3] reported that ginsenoside Rc and Rg_1 increased ^{3}H-leucine incorporation into mouse serum proteins by 20% over the control, whereas Rb_1 was not effective. Discrepancies between their results and ours are, at present, difficult to explain since experimental conditions, such as animal species, amount of ginsenosides administered, length of time of the treatment, isotopes injected, were absolutely different.

Although the incorporation was elevated, the protein concentration in serum remained unchanged after the ginsenoside treatment. Therefore, it is assumed that the turn-over rate of some of the serum proteins was increased by the treatment.

Our interest was excited in the fact that the treatment with ginsenosides enhanced the incorporation of ^{14}C-acetate into the lipids of lipoproteins and coincidently that of ^{14}C-amino acids into the protein moiety of lipoproteins, which is shown in the similarity of profiles between acetate-labeled and amino acid-labeled sera on the agar-electrophoresis and subsequent experiments. If the synthesis of lipids is associated with the synthesis of apo-lipoproteins, the next question would be which is the trigger. Frnka and Reiser[8] described that administration of cholesterol stimulated the synthesis of a certain kind of lipoproteins. However, in our previous experiment with ginsenoside-treated rats,[6] the level of cholesterol either in serum or in liver was found to be unchanged in spite of a remarkable increase in the incorporation of ^{14}C-acetate into cholesterol. On the other hand, there has been no report evidencing that an increased synthesis of apo-lipoproteins enhances the turn-over of lipids. In connection to this, Cooper and Margolis[9] reported a suppression of acetate incorporation into various lipids in isolated hepatocytes by some lipoproteins, the effect depending on the lipoprotein species.

Concerning to the lipoprotein species, in our experiment, de Novo protein was analyzed by the methods of salting-out, electrophoresis, NaBr-flotation and gel-filtration. The characteristics, slow in electrophoresis, light at flotation and large size on gel-filtration, indicated that the synthesis of the apoproteins for VLDL and chylomicrons were mostly stimulated by ginsenosides.

Considering our previous findings[6] on the stimulated synthesis of cholesterol in ginsenoside-treated rats, we expected an elevation in the synthesis of LDL, which was known to carry more cholesterol than other lipoproteins. The present results showed an increase in the synthesis of apo-LDL by ginsenoside-Rb_1 and -Rc, but the stimulation ratio was rather lower than that for chylomicrons and VLDL.

Recently, cholesterol-rich VLDL was reported to be found at considerable amount in the cholesteremic rabbit serum.[10] Although it is necessary to investigate on lipid composition of the increased VLDL in our experiment, the ginsenoside may be considered to stimulate

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this kind of apoprotein synthesis. An alternative interpretation is also possible. According to the current theories, the subunits of apo-lipoproteins are partly common to each lipoprotein in rat[11] and human,[12-14] and VLDL changes easily to LDL after releasing triglycerides,[15] *via* the form of "remnant" or by some modifications.[16] Therefore, the increased synthesis of VLDL apoproteins by the ginsenosides would result in a large supply of apo-LDL, which might take up cholesterol from the liver. As the concentration of LDL in rat serum is reported to be very low, the turn over of this lipoprotein should be very fast, and this seems to be a reason by which the detection of highly labeled LDL apoprotein is difficult. If the subunits of VLDL could be involved in apoprotein for LDL, they also could enter apoprotein for HDL. However no stimulation in HDL synthesis was observed in this experiment.

In any way, ginsenosides Rb_1 and Rc were proved to have the effect on lipid metabolism, and on protein synthesis. However, Rg_1 not only failed to exhibit a stimulating effect on protein synthesis in the present study, but also showed little enhancement on cholesterol metabolism in the previous experiment.[6] These three ginsenosides differ in their structures: Rg_1 has a triol sapogenin, but Rb_1 and Rc a diol. The more experiments with other kinds of ginsenosides are carried out, the more clearly will be demonstrated the relationship between the structure of ginsenosides and their biological functions.

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