Plasma Lipoproteins as Drug Carriers: Pharmacological Activity and Disposition of the Complex of β-Sitosteryl-β-D-glucopyranoside with Plasma Lipoproteins

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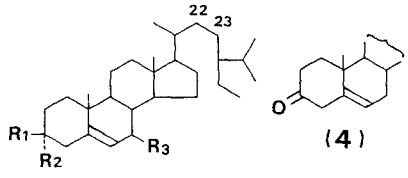
Abstract □ The ability of plasma lipoproteins to act as carriers in site-specific drug delivery systems was evaluated by determining the disposition and pharmacological effects of β-sitosteryl-β-D-glucopyranoside (SG, **3**). In the disposition studies, [^{3}H]SG was absorbed from the intestinal tract by the formation of chylomicrons and was specifically associated with lipoproteins in vivo. [^{3}H]SG was incorporated into various rat plasma lipoproteins in vitro. [^{3}H]SG complexed with the lower density lipoproteins ($d < 1.063$ g/mL), especially with the intermediate density lipoproteins ($1.006 \leqq d < 1.019$ g/mL) which disappeared more rapidly from the circulatory system than the [^{3}H]SG complexed with the higher density lipoproteins ($d \geqq 1.063$ g/mL) following intravenous administration to rats. In pharmacological studies, the hemostatic effect of SG in mice and the inhibitory effect of SG on vascular permeability in rats were only observed after intravenous administration of the complexes of SG with the lower density lipoproteins. The same results were obtained after the intravenous administration of the complexes of SG with human and mouse lipoproteins.

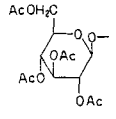
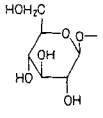
In the circulatory system, plasma lipoproteins transport not only water-insoluble lipids but also lipophilic compounds such as certain vitamins, hormones, toxins, pesticides, and drugs.[1-6] Plasma lipoproteins are classified into chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Each has a different metabolic fate.[7,8] Chylomicrons are responsible for the transport of dietary or exogenous lipids in the circulatory system and are rapidly metabolized by lipoprotein lipase to CM remnants which are rapidly cleared from the circulatory system by the liver. In contrast to CM, VLDL are responsible for the transport and delivery of endogenous lipids to extrahepatic tissues. They are metabolized to IDL by lipoprotein lipase more slowly than chylomicrons. The IDL are rapidly taken up by the liver in rats. In humans, IDL undergo a final conversion to LDL, the major carrier of plasma cholesterol to the extrahepatic tissues. Specific receptors on cultured human fibroblasts, smooth muscle, and other cells bind LDL with high affinity; the entire lipoprotein enters the cell by endocytosis.[9] An additional pathway for LDL metabolism involves a low affinity process associated with scavenger cells or macrophages of the reticuloendothelial system. HDL have the longest half-life in the circulatory system and the most complex metabolism of all the lipoproteins. HDL have been shown to bind cells, such as fibroblasts, at specific sites different from those that bind LDL.[10] In rats, HDL appear to play a role similar to that of LDL in humans. Namely, if a lipophilic compound partitions into the plasma lipoproteins, the amount of the compound entering cells may vary widely among tissues. Therefore, lipoproteins can be considered not only as possible transport vehicles for lipophilic drugs in the circulatory system but also as drug carriers for improved drug delivery.

β-Sitosteryl-β-D-glucopyranoside (SG, 3), an abundant plant sterylglucoside, exhibits an inhibitory effect on vascular permeability, antiulcerogenic and hemostatic effects, etc.[11,12] It was also reported that a mixture containing SG as the major constituent showed antitumor activity against P-388 leukemia.[13,14]

In the present paper, in order to examine whether lipoproteins are useful drug carriers, we determined the pharmacological potencies of SG after intravenous administration in various vehicles including plasma lipoproteins.



(4)

	R_1	R_2	R_3
1,	OH	H	H_2
2,		H	H_2
3, SG		H	H_2
5,	OAc	^{2}H	H_2
6,	OAc	^{2}H	=O
7,	OH	^{2}H	$^2H_2$

Results and Discussion

Water-insoluble lipids, such as cholesterol and triglycerides, are absorbed from the intestine by forming chylomicrons. Because SG is also a water-insoluble compound, the lymphatic absorption of SG was evaluated after the oral administration of [^{3}H]SG in thoracic duct cannulated rats.

As shown in Table I, a few percentages of the orally administered [^{3}H]SG was recovered in the lymph. The recoveries increased when 1 mL of condensed milk was administered orally 30 min before the administration of [^{3}H]SG. This lymphatic absorption was almost the same as the absorption efficiency (2.1%) of SG, which was estimated by comparing the urinary excretion ratios of the radioactivity after oral

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Table I—Lymphatic Absorption of [^{3}H]SG after Oral Administration[a]

Condensed Milk	Percent of Dose	
	[^{3}H]	[^{3}H]SG
With[b]	2.78 ± 0.05[c]	1.29 ± 0.05
Without	1.59 ± 0.29	0.61 ± 0.11

[a] The lymph was collected for 48 h following oral administration of [^{3}H]SG (5 mg/kg) in the thoracic-duct-cannulated rats. [b] Condensed milk (1 mL) was administered orally at 30 min before the administration of [^{3}H]SG. [c] The radioactivity in the lymph was expressed as cumulative absorption (mean ± SEM, n = 3).

and intravenous administration of [^{3}H]SG to intact animals. The recoveries of total radioactivity and [^{3}H]SG in the feces after the oral administration of [^{3}H]SG to rats were 83% and 76% of the dose, respectively. In addition, it was found that most of [^{3}H]SG in the lymph associated with CM when assessed by the centrifugal flotation technique. Thus, it was concluded that SG is barely absorbed from the GI tract and that SG associated with CM in the lymph enters into the circulatory system.

Because SG cannot be present in plasma as a free form, it was considered to be associated with a constituent of the plasma. Thus, the gel filtration of plasma was carried out to identify the binding constituents for SG in the plasma. Figure 1 illustrates the elution pattern of the plasma which had been incubated with [^{3}H]SG for 60 min in vitro. More than 88% of the applied [^{3}H]SG was recovered in the eluate. Most of the [^{3}H]SG eluted in the VLDL/LDL fraction (26%) and the HDL fraction (68%), whereas a small amount eluted in the albumin fraction. In addition, >95% of SG in plasma samples was recovered in the plasma lipoproteins when assessed by ultracentrifugal flotation. These results indicate that SG associates with the lipoproteins in plasma.

Figure 2 shows the relationship between the time courses of hemostatic time and that of the concentration of [^{3}H]SG in serum and its subfractions after the oral administration. The

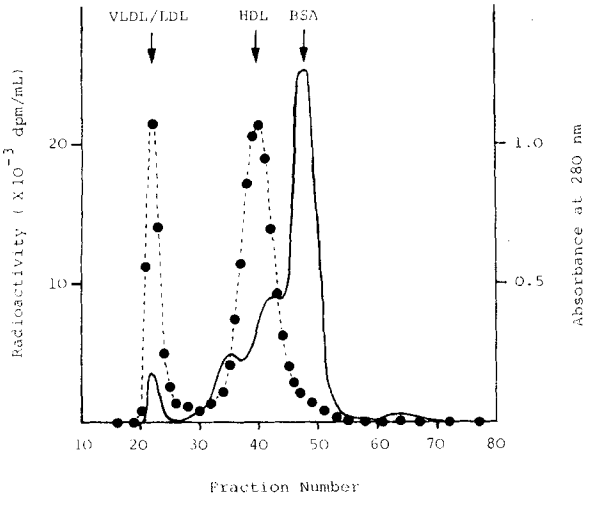

Figure 1—*Gel filtration of plasma containing [^{3}H]SG. Rat plasma (0.5 mL) which had been incubated with [^{3}H]SG (5μg) at 37°C for 60 min was chromatographed on the Sepharose CL-6B column (1.6 × 90 cm), equilibrated with 0.15 M NaCl, 0.2 mM Tris-HCl, pH 7.1. Three-milliliter fractions were collected and the absorbance at 280 nm (——) was monitored. One-milliliter aliquots were taken for scintillation counting (---●---). The arrows indicate the elution peaks of each lipoprotein isolated from rat plasma and bovine serum albumin (BSA) chromatographed in the same conditions.*

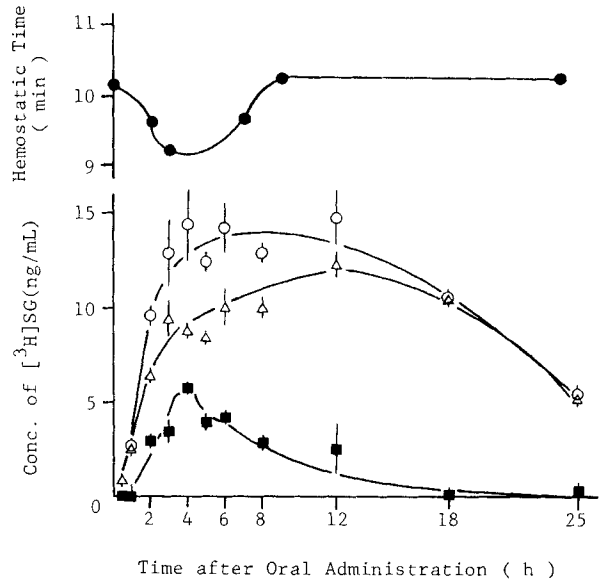

Figure 2—*Time courses of the hemostatic time and the concentration of [^{3}H]SG in serum and its subfractions after oral administration of [^{3}H]SG. The hemostatic time (●) was determined by the spectrophotometric measurement of the hemorrhage from the tail after oral administration of SG to mice (2 mg/kg, n = 5). Serum was obtained from the rats after the oral administration of [^{3}H]SG (5 mg/kg) and 1 mL of the serum obtained was treated with heparin–manganese to fractionate the lipoproteins. Each symbol shows the concentration of [^{3}H]SG in the serum (○), in the S (supernatant) fraction (△), and in the P (precipitate) fraction (■) (mean ± SEM, n = 3).*

amount of [^{3}H]SG in serum and the S (supernatant) fraction remained high level at 18 h following oral administration. However, the concentration of [^{3}H]SG in the P (precipitate) fraction decreased rapidly and this time profile was closely related to the time course of the hemostatic effect which appeared at 2 h and disappeared at 10 h following the oral administration. Thus, the hemostatic effect depends on the concentration of SG only in the P fraction consisting of the lower density lipoproteins (CM, VLDL, IDL and LDL). This observation suggests that SG needs to associate with the lower density lipoproteins in order to exhibit pharmacological effects.

Pharmacological potency was determined after intravenous administration of SG complexed with each lipoprotein. Incorporation of [^{3}H]SG into the lipoproteins isolated from rat plasma was carried out on the surface of Celite. The determination of radioactivity in the treated lipoproteins revealed that [^{3}H]SG was incorporated, to some extent, into each lipoprotein (Table II). There were no significant differences between the treated and the nontreated lipoproteins as demonstrated by disc electrophoresis and gel filtration. In addition, negligible radioactivity was found in the solution

Table II—Incorporation of [^{3}H]SG into Rat Lipoproteins In Vitro[a]

Vehicle	[^{3}H]SG, μg/mg of Protein
CM	300.9[b]
VLDL	151.9
IDL	126.1
LDL	116.4
HDL	12.1
BF[c]	0.8

[a] [^{3}H]SG (100 μg) was immobilized on the surface of Celite (50 mg) and then incubated with each lipoprotein solution (2 mL) at 37°C for 20 h. [b] Each value represents the mean concentration of [^{3}H]SG in the supernatant after triplicate incubations. [c] The final 9 mL of the last centrifugation was the bottom fraction (BF); d ≥ 1.21 g/mL.

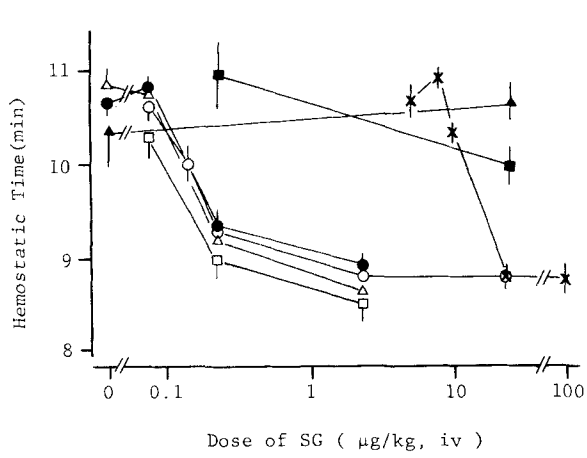

Figure 3—*Hemostatic activity of SG after administration of the SG–lipoprotein complex. The complex prepared in vitro was administered intravenously to mice and the hemostatic time was determined at 1 h following the injection of SG complexed with CM (○), VLDL (□), IDL (△), LDL (●), HDL (■), and BF (▲), and determined at 2 h after the injection of SG solubilized with 2% HCO-60 (×) (mean ± SEM, n = 5).*

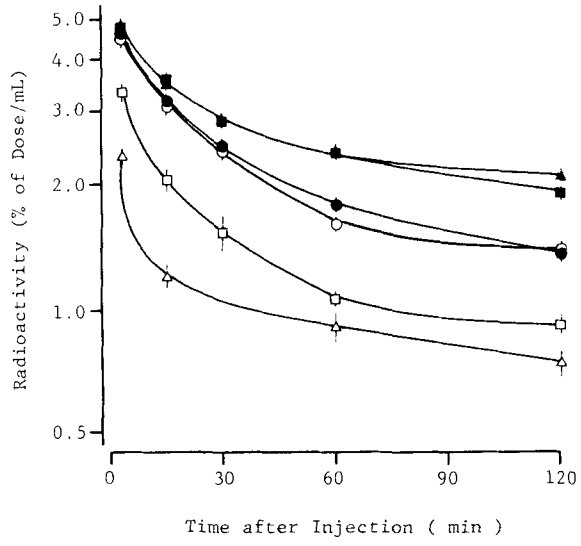

Figure 4—*Time courses of the concentration of the radioactivity in blood after the intravenous administration of [^{3}H]SG-lipoprotein complex in rats (dose of [^{3}H]SG = 9 μg/kg). Each symbol shows the total radioactivity in the blood after the injection of the [^{3}H]SG complexed with CM (○), VLDL (□), IDL (△), LDL (●), HDL (■) and BF (▲) (mean ± SEM, n = 3).*

without protein and no metabolite of [^{3}H]SG was formed during these incorporations.

The SG–lipoprotein complexes thus obtained were administered intravenously into mice and the hemostatic time was determined. As shown in Fig. 3, SG in the conventional vehicle, 1.5% HCO-60 (hydrogenated castor oil polyethylene glycol ether) exhibited the hemostatic effect at 2 h following administration at the dose of 25 μg/kg, whereas SG complexed with HDL and with HDL and the bottom fraction (BF; the final 9 mL of the last centrifugation was the bottom fraction, d ≥ 1.21 g/mL) had no hemostatic effect. The SG complexed with the lower density lipoproteins, such as CM, VLDL, IDL, and LDL, elicited the same hemostatic time as the conventional vehicle even at the dose of 0.23 μg/kg. There were no significant differences between the effect of the lower density lipoproteins. Specifically, CM, VLDL, IDL, and LDL used as drug carriers decreased significantly the dose of SG needed to exhibit the hemostatic effect. It was also found that SG complexed with the lower density lipoproteins showed the maximal effect at 30 min following administration.

SG inhibits vascular permeability; plasma protein leakage from vas capillare, determined by a method of Evans blue leakage, is inhibited by the treatment of SG. The vascular permeability, or the amount of the leakage, after the injection of histamine was inhibited by the pre-administration of SG complexed with the lower density lipoproteins (d < 1.063 g/mL) as shown in Table III. However, SG incorporated into the higher density lipoproteins (d ≧ 1.063 g/mL) failed to affect vascular permeability. These results show that SG complexed with the lower density lipoproteins exhibits not only a hemostatic effect but also an inhibitory effect on vascular permeability.

Figure 4 shows the time course of the radioactivity in the blood after intravenous administration of [^{3}H]SG complexed with the various lipoproteins. [^{3}H]SG complexed with VLDL and IDL disappeared rapidly from the circulatory system. But, [^{3}H]SG complexed with HDL and BF was retained in the circulatory system in relatively higher concentrations. Therefore, the SG–lower density lipoprotein complexes are rapidly and efficiently carried from the circulatory system into target cells which are the action site of SG. These properties must be essential for a drug carrier in site-specific drug delivery systems. The plasma half-lives of the SG–lipoprotein complexes did not agree with that of the various lipoproteins (CM, −5 min; VLDL, 1–3 h; LDL, 3–4 d; HDL, 5–6 d). It was reported that drug-induced alteration in lipoprotein structure in and of themselves might alter the metabolism of lipoproteins.[27] There may be also an interchange of [^{3}H]SG between the various lipoprotein classes.[28,29] This problem was complicated by the metabolism of [^{3}H]SG; considerable interchange or transport of [^{3}H]SG between the various lipoprotein classes is suggested in Table IV. At 30 min following the injection of the [^{3}H]SG–lipopro-

Table III—Inhibitory Effect of the SG–Lipoprotein Complexes on Vascular Permeability[a]

Vehicle	Dose μg/kg	Vascular permeability
Control		1.00 ± 0.18[b]
d < 1.063[c]	42	0.49 ± 0.15[d]
d ≧ 1.063	50	0.85 ± 0.16

[a] The SG–lipoprotein complex, prepared in vitro, was administered intravenously into rats. The vascular permeability was evaluated at 60 min following injection of SG by the determination of Evans blue. [b] Each value shows the relative permeability to that of control rats (mean ± SEM, n = 5). [c] The plasma proteins were fractionated into two classes at the density of 1.063 g/mL. [d] Significantly different from the control ($p < 0.05$).

Table IV—Distribution of Radioactivity in the Serum Lipoprotein Fractions after Intravenous Administration of the [^{3}H]SG–Lipoprotein Complex[a]

Vehicle	d < 1.006, %	1.006 ≦ d < 1.063, %	d ≧ 1.063, %
CM	2.7 ± 2.1[b]	68.4 ± 3.4	28.9 ± 1.3
VLDL	6.4 ± 3.4	64.6 ± 2.8	29.0 ± 6.8
IDL	18.3 ± 1.6	39.7 ± 1.0	42.0 ± 0.8
LDL	—	68.1 ± 6.4	33.1 ± 2.8
HDL	6.5 ± 4.1	42.7 ± 3.9	50.8 ± 0.9
BF	—	40.7 ± 1.5	60.2 ± 1.2

[a] The [^{3}H]SG–lipoprotein complex prepared in vitro was administered intravenously to rats. At 30 min following the injection, serum was obtained from the rats and to determine the ultracentrifuged distribution of radioactivity in the lipoprotein classes. [b] Each value represents the mean ± SEM of 3 animals.

tein complex, the distribution of radioactivity in the lipoprotein classes was determined by ultracentrifugation. At 30 min following administration, the radioactivity in the lipoprotein fractions was different from the radioactivity in the injected lipoproteins. When the [^{3}H]SG–CM complex was administered, the radioactivity in the $d < 1.006$ g/mL fraction was only 2.7%. This result is apparently coincident with the fact that CM has the fastest clearance rate. Distributions of radioactivity in the $1.006 < d < 1.063$ g/mL fraction were higher after injection of [^{3}H]SG complexed with the lower density lipoproteins than in the fractions complexed with HDL or BF. However, the distribution of radioactivity in this fraction after administration of the [^{3}H]SG–IDL complex was relatively low. This result corresponds with the fact that IDL are rapidly eliminated from the circulatory system in rats. The interchange and/or transport of SG was also confirmed in vitro. The [^{3}H]SG–CM complex (0.1 mL) was incubated with normal rat serum (0.4 mL) at 37°C, and then the lipoproteins were fractionated by the heparin–manganese method. The recoveries of [^{3}H]SG in the S fraction were 14.6%, 19.8%, and 24.6% at 30 min, 60 min, and 120 min, respectively.

The adrenal gland uptakes of [^{3}H]SG complexed with the various lipoproteins after injection differed markedly as shown in Table V. The lowest and highest uptakes by the adrenal gland were observed with IDL and HDL, respectively. The variety in the amount of uptake by the liver was less than that observed by the adrenal gland. It was confirmed that these results were caused by the nature of plasma lipoproteins: each lipoprotein has a different metabolic fate which has been the subject of a number of excellent reviews.[7,8] For example, rat HDL is internalized into the adrenal gland via a specific receptor mediated process[30] in a manner similar to that observed for LDL in the human fetal adrenal glands.

Each of lower density lipoprotein is a precursor or a product in the lipoprotein metabolism. As lipolysis occurs, VLDL becomes smaller, leading to formation of IDL. IDL serves as the precursor of LDL which is the major carrier of cholesterol to the extrahepatic tissue in humans. The metabolic alteration of lipoproteins occurs rapidly in vivo. In the present paper, it remains unclear whether or not all of the lower density lipoproteins have a carrier potential to elicit the pharmacological effects of SG.

The major finding in the present study is that the pharmacological effects of SG were closely related with lipoprotein metabolism. It was confirmed that the pharmacological effects of SG are mediated only by the uptake process of the lower density lipoproteins. It will be useful to the pharmacological study on the action site of SG. For example, as only a small amount of the SG–lower density lipoprotein complex was taken up by the adrenal gland, the adrenal gland was not considered as the site of action of SG. In fact, SG exhibits the hemostatic effect even in mice that had their adrenal glands surgically removed.

Table V—Concentration of [^{3}H]SG in the Liver and the Adrenal Gland after the Intravenous Administration of the [^{3}H]SG-Lipoprotein Complex[a]

Vehicle	Liver		Adrenal Gland	
	[^{3}H]	[^{3}H]SG	[^{3}H]	[^{3}H]SG
CM	1.26 ± 0.51[b]	0.88 ± 0.25	0.81 ± 0.08	0.61 ± 0.06
VLDL	1.83 ± 0.06	1.31 ± 0.58	0.64 ± 0.03	0.41 ± 0.01
IDL	1.32 ± 0.03	0.91 ± 0.02	0.20 ± 0.02	0.13 ± 0.01
LDL	1.31 ± 0.03	0.93 ± 0.03	0.64 ± 0.03	0.49 ± 0.02
HDL	1.36 ± 0.05	0.92 ± 0.03	4.03 ± 0.42	3.25 ± 0.38
BF	1.25 ± 0.02	0.97 ± 0.01	1.26 ± 0.12	1.64 ± 0.10

[a] The [^{3}H]SG-lipoprotein complex prepared in vitro was administered intravenously to rats. At 30 min after injection, the liver and the adrenal gland were isolated from the rats and homogenized to determine the concentration of the total radioactivity and [^{3}H]SG, and expressed as percent of dose per gram. Each value represents the mean ± SEM of 3 animals.

Lipoproteins obtained from healthy humans and mice were used as drug carriers to examine the species differences between the lipoproteins. Plasma from various species including humans contained a small amount of SG, and it was associated specifically with the lipoproteins in the plasma. The endogenous SG obviously comes from the diet because the negligible amount of SG found in the plasma of rats and mice fed on an SG-free diet for 1 week. Plasma used in this experiment was obtained from mice which had been fed on a diet containing 0.015% SG for 1 week, and the plasma concentration of SG was 507 ng/mL. Human plasma contained SG at a concentration of 61.4 ng/mL.

Figure 5 shows the hemostatic effect of SG which was associated endogenously with these plasma lipoproteins. The hemostatic effect was observed following the administration of SG which was associated not only with the mouse lower density lipoproteins ($d < 1.063$ g/mL) but also with the human lower density lipoproteins (VLDL and LDL). Although SG associated with the human HDL had some hemostatic potency, it may be due to contamination of the HDL fraction with LDL, because human plasma contains more LDL than HDL.[31]

It was concluded that any lower density lipoprotein had a carrier potential regardless of their origin. The incorporation procedures of SG into the lipoproteins, in vitro or in vivo, had no relation to their efficacy. This may be the result of partitioning into the same thermodynamically stable region for SG in the lipoproteins.

Recently, Counsell and Pohland reviewed current efforts to utilize the lipoproteins as site-specific delivery systems for drugs.[32] A few of the studies analyzed the possible role of plasma lipoproteins in the transport and the disposition of drugs[33–36] and in the delivery of various anticancer agents to cultured cancer cells.[37–41] However, the usefulness of lipoproteins as drug carriers has been investigated in in vitro drug–cell interactions, and it has not been demonstrated in vivo so far. In this paper, the pharmacological effectiveness and usefulness were shown in vivo not only by the study of drug

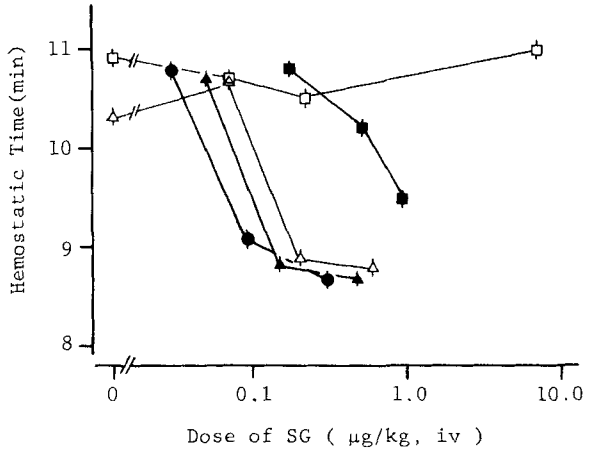

Figure 5—*Hemostatic activity following the intravenous administration of SG complexed with mouse and human lipoproteins. The complexes were obtained from mouse and human plasma which contained SG endogenously. Each symbol shows the hemostatic time at 1 h following the injection of SG complexed with the $d < 1.063$ g/mL (□) and $d \geqq 1.063$ g/mL (△) fractions of mouse plasma and VLDL (●), LDL (▲), and HDL (■) fractions in human plasma (mean ± SEM, $n = 5$).*

disposition but also by pharmacological tests.

To achieve the site-specific delivery of drugs, researchers have employed a variety of approaches.[42] The lipoproteins have site-specific properties. Moreover, they may be able to carry not only lipophilic drugs but also lipo-solubilized drugs such as a phospholipid–nucleoside conjugate[43] and dexamethasone palmitate.[44] Lipoproteins also show a stabilizing effect on the degradation of chloroethylnitrosoureas,[45] and appear to have great therapeutic possibilities as drug carriers.

Experimental Section

Sterylglucosides used in this study were synthesized in our laboratories as follows:

β-Sitosteryl-β-D-glucopyranoside tetraacetate (2)—β-Sitosterol (1) was prepared from stigmasterol via *i*-stigmasteryl methyl ether according to the method of Steele and Mosetting.[15] A mixture of β-sitosterol (**1**) (2.90 g, 7 mmol), α-D-glucopyranosyl bromide tetraacetate[16] (11.51 g, 28 mmol), calcium hydride (2.36 g, 56 mmol), silver oxide (7.3 g, 31.5 mmol), and iodine (0.1 g, 0.79 mmol) in dry dichloromethane (100 mL) was stirred in the dark for 48 h, filtered, and the solvent was concentrated under reduced pressure. The residue was fractionated on a column of silica gel with a cyclohexane–ethyl acetate gradient. The solvent was removed under reduced pressure and the residue was recrystallized from ethanol to give 3.70 g of **2** (71% yield), mp 169–170°C (lit.[17] mp 167–168°C); ^{1}H NMR ($CDCl_3$): δ 0.67 (s, 3, 18-H), 0.81 (d, 3, J = 6.7 Hz, 27-H,), 0.83 (d, 3, J = 6.6 Hz, 26-H), 0.84 (t, 3, J = 7.1 Hz, 29-H), 0.92 (d, 3, J = 6.4 Hz, 21-H), 0.98 (s, 3, 19-H), 2.00 (s, 3, acetyl), 2.02 (s, 3, acetyl), 2.05 (s, 3, acetyl), 2.07 (s, 3, acetyl), 3.49 (m, 1, J = 5.4 and 10.5 Hz, 3-H), 3.67 (ddd, 1, J = 2.5, 4.8, and 9.5 Hz, 5′-H), 4.11 (dd, 1, J = 2.5 and 12.2 Hz, 6′-H), 4.25 (dd, 1, J = 4.8 and 12.2 Hz, 6′-H′), 4.59 (d, 1, J = 8.1 Hz, 1′-H), 4.95 (dd, 1, J = 8.1 and 9.4 Hz, 2′-H), 5.07 (dd, 1, J = 9.3 and 9.3 Hz, 4′-H), 5.20 (dd, 1, J = 9.3 and 9.3 Hz, 3′-H), and 5.35 ppm (m, 1, 6-H); ^{13}C NMR ($CDCl_3$): δ 11.86 (C-18), 12.00 (C-29), 18.79 (C-21), 19.06 (C-27), 19.82 (C-26), 19.36 (C-19), 20.60, 20.69, 20.72, and 20.74 (CH_3CO), 21.07 (C-11), 23.10 (t, C-28), 24.30 (t, C-15), 26.11 (t, C-23), 28.24 (t, C-16), 29.19 (C-25), 29.47 (t, C-2), 31.89 (C-7 and C-8), 33.97 (t, C-22), 36.13 (d, C-20), 36.72 (s, C-10), 37.22 (t, C-1), 38.94 (t, C-4), 39.76 (t, C-12), 42.35 (s, C-13), 45.87 (d, C-24), 50.19 (d, C-9), 56.07 (d, C-17), 56.77 (d, C-14), 62.13 (t, C-6′), 68.59 (d, C-4′), 71.53 (d, C-5′), 71.72 (d, C-2′), 72.95 (d, C-3′), 80.07 (d, C-3), 99.66 (d, C-1′), 122.15 (d, C-6), 140.36 (s, C-5), 169.29, 169.36, 170.30, and 170.62 ppm (CH_3CO); MS: *m/z* (%), 169 (45.3), 396 (100), and 744 (M^+, 0.1).

Anal.—Calc. for $C_{43}H_{68}O_{10}$: C, 69.32; H, 9.20. Found: C, 69.48; H, 9.31.

β-Sitosteryl-β-D-glucopyranoside (3)—A mixture of (**2**) (3.5 g, 4.7 mmol) and 5% methanolic KOH (60 mL) was stirred at room temperature for 4 h and cooled in a refrigerator for 2 h. The precipitate was collected by filtration and washed several times with cold methanol, water, and acetone. Recrystallization from dioxane gave 2.58 g of **3** (95% yield), mp 292–296°C (dec.). Mass spectra of tetramethylsilyl (TMS) and tetrafluoroacetyl (TFA) derivatives of **3** on GC–MS were identical with that reported by Laine and Elbein[18] and Knights,[19] respectively.

Anal.—Calc. for $C_{35}H_{60}O_6$: C, 72.87; H, 10.48. Found: C, 72.75; H, 10.50.

[22,23-^{3}H]β-Sitosteryl-β-D-glucopyranoside ([^{3}H]SG)—In the method of Steele and Mosettig,[15] *i*-stigmasteryl methyl ether (500 mg, 1.17 mmol) was hydrogenated using a mixture of tritium (5 Ci) and hydrogen (37.5 mL) in ethyl acetate (4 mL) containing palladium black (30 mg). [22,23-^{3}H]β-Sitosterol was prepared and converted to [^{3}H]SG as described above. The yield was 34% from *i*-stigmasteryl methyl ether and its specific radioactivity and radiochemical purity, which was determined by TLC (chloroform:methanol, 5:1) as described below, were 14 mCi/mg and >99%, respectively. It was stored in benzene:ethanol (9:1), and an adequate amount of unlabeled SG was added before use.

[3α,7,7-$^2H_3$]Campesteryl-β-D-glucopyranoside—[3α,7,7-$^2H_3$]Campesteryl-β-D-glucopyranoside, employed as an internal standard in quantitative GC–MS, was obtained as a minor product during the synthesis of [3α,7,7-$^2H_3$]β-sitosteryl-β-D-glucopyranoside. The commercially available β-sitosterol (**1**) (~80%) was converted into 3-keto-compound(s) (**4**) using dimethyl sulfoxide:oxalyl chloride in the presence of triethylamine,[20] then into [3α-^{2}H]sterylacetate(s) (**5**) using sodium boro[$^2H_4$]hydride in ether:methanol[21] and acetic anhydride in pyridine, then into 7-keto[3α-^{2}H]sterylacetate(s) (**6**) using acetic acid, acetic anhydride, and *tert*-butyl chromate in carbon tetrachloride,[22] and fourth to [3α,7,7-$^2H_3$]sterol(s) (**7**) using lithium aluminum [$^2H_4$]hydride and aluminum chloride in dry ether.[23] After coupling **7** with α-D-glucopyranosyl bromide tetraacetate as described above, [3α,7,7-$^2H_3$]campesteryl-β-D-glucopyranoside tetraacetate, isolated from other sterylglucopyranosides by preparative HPLC (column, Polygosyl 10 C_{18}, 10 × 30 mm; mobile phase, acetonitrile), was hydrolyzed as described above. The spectra of TMS derivative on GC–MS showed an intense peak at *m/z* 386 corresponding to the fragment ion of the deuterated campesteryl-β-D-glucopyranoside.

All other reagents were commercially available and of analytical grade.

Determination of β-Sitosteryl-β-D-glucopyranoside (SG)—To determine the total radioactivity of liquid samples, a 0.5 mL portion was taken into a counting vial containing 10 mL of scintillation cocktail (ACS-II, Amersham). The radioactivity was determined by a liquid scintillation counter (model 460, Packard Tri-Carb) with external standards.

To determine the concentration of [^{3}H]SG, 4 mL of methanol and 3 mL of 15% NaCl were added to 1.0 mL of liquid sample, and [^{3}H]SG together with the metabolites was extracted with 8 mL of chloroform. The tissue samples were homogenized in 5 mL of methanol. After 4 mL of 15% NaCl was added to 4 mL of the homogenate, the mixture was extracted with 8 mL of chloroform. The organic phase was evaporated under reduced pressure, and the residue was dissolved in chloroform:methanol (1:1). The [^{3}H]SG was separated from the metabolites by TLC (silica gel, Merck) in a solvent of chloroform:methanol (5:1). Four spots were observed on the plate; R_f = 0.36 (SG), 0.55 (unknown), 0.68 (sitosterol), and 0.90 (sitosterol ester). Silica gel corresponding to [^{3}H]SG and the metabolites was moved into respective vials containing 1 mL of methanol, and the radioactivity was counted. The percent of the radioactivity of [^{3}H]SG on the TLC plate was calculated.

Unlabeled SG was determined as follows. [3α,7,7-$^2H_3$]Campesteryl-β-D-glucopyranoside (100 ng) as an internal standard was added to the samples. The sterylglucosides were extracted and fractionated from the samples as described above. These materials were eluted from silica gel (TLC) with 5 mL of methanol. After the trimethylsilylation of the sterylglucosides, the selected ion monitoring analyses were performed at *m/z* 386 and 397 corresponding to fragment ions of the internal standard and SG with a Shimadzu Auto GC/MS model 6020.

Operational conditions were as follows: GC column, 2% Dexsil 300 GC on chromosorb W-HP (80/100 mesh), 0.5 m in length and 2 mm i.d.; GC column temperature, 305°C; GC injector temperature, 310°C; both interface line and source temperature, 340°C; helium carrier gas flow rate, 20 mL/min; ionizing energy, 25 eV; filament current, 4.2 A; gain, 80. Under these conditions the retention times were 6.7 min for the internal standard and 8.0 min for SG. The linear calibration curve was obtained by the addition of varying amounts of SG (5–300 ng) to samples.

Lymph Collection—The thoracic ducts of male Wistar rats weighing ~250 g were cannulated under pentobarbital anesthesia. After awaking from anesthesia, 1.25 mg of [^{3}H]SG dissolved in 3% hydrogenated castor oil polyethylene glycol ether (HCO-60, Nikko Chemical Co., Ltd.) and 2% monolaurate (SPAN-20, Nakarai Chemicals, Ltd.) was administered orally to rats by intubation, and the lymph was collected for 48 h.

Gel Filtration—The sample (0.5 mL) was applied on a Sepharose CL-6B column (1.6 × 90 cm) and eluted with 0.15 M NaCl and 0.2 mM Tris-HCl, pH 7.1, at a flow rate of 0.5 mL/min.

Fractionation and Preparation of Lipoproteins—The heparin–manganese method was used for conventional fractionation of the lipoproteins in serum. A tube containing a 1.0-mL aliquot of the serum was chilled on ice prior to the addition of 50 μL of heparin sodium (4,000 IU/mL). After 30 min, 50 μL of 1 M $MnCl_2$ was added to the solution. The tube was shaken gently, allowed to stand for 30 min, and centrifuged (3,000 rpm for 10 min) to precipitate the LDL. The precipitate (P fraction) consisted of CM, VLDL, IDL and LDL, and the supernatant (S fraction) consisted of primarily HDL. An Airfuge (Beckman) micro-ultracentrifugation system was used for fractionation of the lipoproteins.

Preparative isolation of the lipoproteins was conducted as follows. Blood was collected from the subjects, male Wistar rats and male

ddY mice, after an overnight fast using EDTA (1 mg/mL), as the anticoagulant. The plasma was separated and the plasma lipoproteins were isolated by sequential ultracentrifugal flotation of the plasma at 100,000×*g*. The increase in the density of the latter solutions was accomplished by adding appropriate amounts of solid sodium bromide. Ultracentrifugation was carried out at 4°C for the appropriate periods, *i.e.*, 20 h for VLDL ($d < 1.006$ g/mL), IDL ($1.006 \leq d < 1.019$ g/mL), and LDL ($1.019 \leq d < 1.063$ g/mL); 46 h for HDL ($1.063 \leq d < 1.21$ g/mL). After each of four centrifugations, the top 1 mL constituted the lipoprotein fraction. The final 9 mL of the last centrifugation was the bottom fraction (BF, $d \geq 1.21$ g/mL), which is considered to be primarily protein. In some experiments, plasma lipoproteins were fractionated into roughly two groups with higher and lower density than 1.063 g/mL. The purity of each of the lipoprotein fractions was checked by polyacrylamide gel electrophoresis[24] and gel filtration.

Chylomicrons were isolated from the thoracic lymph by centrifugal flotation (10,000×*g* for 10 min). The lymph was obtained from the cannulated rats after oral administration of 1 mL of condensed milk.

The individual fractions were diluted to their original plasma concentrations, to correct for condensation yielded in the isolation process, and dialyzed against 0.15 M NaCl and 1 mM EDTA before use.

Protein was determined by the method of Markwell et al.[25] using bovine serum albumin (Fraction V, Sigma) as the standard.

Incubation of [^{3}H]SG with Lipoproteins—[^{3}H]SG was incorporated into rat lipoproteins by the procedure of Avigan[26] with some modifications. Briefly, 50 mg of Celite 545 (Wako Pure Chemical Industries, Ltd.), 100 μg of [^{3}H]SG (0.5 mCi), and 0.5 μg of α-tocopherol as antioxidant were dispersed in chloroform in a test tube. The solvent was evaporated at reduced pressure and 2 mL of the lipoprotein solution was added. The mixture was incubated at 37°C for 20 h on a shaking water bath. Celite was pelleted from the solution by centrifugation (3,000 rpm for 10 min). The [^{3}H]SG-lipoprotein complex was obtained in the supernatant.

Hemostatic Effect—β-sitosteryl-β-D-glucopyranoside (SG) in various vehicles was administered intravenously or orally to male ddY mice weighing ~25 g. After their tails were cut at ~1 cm from the tail end, the tail was steeped in gently stirred water which was recirculated in a spectrophotometer by a pump. Increase in the absorbance at 413 nm indicated hemorrhage from the tail. The hemostatic time was defined as the time when the absorbance no longer increased.

Inhibitory Effect on Vascular Permeability—SG in various vehicles was administered into the femoral vein of male Wistar rats weighing ~200 g. At 60 min postinjection, Evans blue (10 mg) was administered to the tail vein, and then histamine dihydrochloride (1 μg/50 μL) was injected intracutaneously into the shaved abdominal skin. Thirty minutes after the administration of histamine dihydrochloride the skin (~3 cm^2) with Evans blue was isolated and solubilized in 3 mL of 35% HCl overnight. After 3 mL of 10% benzalkonium chloride was added to the solution, Evans blue was extracted with 5 mL of chloroform. Absorbance of the organic phase was determined at 620 nm with a spectrophotometer.

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