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Phosphatidylcholine Liposomes Containing the Saponin Aglycone Diosgenin or Tigogenin in Place of Cholesterol—Their Properties and Sensitivities to Various Saponins

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1. Diosgenin and tigogenin, which are aglycones of saponins, formed liposomes with phosphatidylcholine, and these liposomes showed a rather stable barrier function. These aglycones both have the same 3β-hydroxyl group and flat steroid nucleus as cholesterol, but have different side chains.

2. The effects of the aglycones on the phase transition of phosphatidylcholine were examined by measuring their effects on the permeability increase due to perturbation of phase equilibrium and by differential scanning calorimetry. These analogs with spirostane structure have a fluidizing effect similar to that of cholesterol.

3. The permeability increase observed in egg yolk phosphatidylcholine liposomes at 42° was not suppressed by diosgenin, suggesting that the aglycone does not have a condensing effect on phospholipid bilayers.

4. Egg yolk phosphatidylcholine liposomes with diosgenin are less sensitive to digitonin and akebia saponins than those with cholesterol.

Keywords——phosphatidylcholine liposomes; diosgenin; tigogenin; aglycones of saponins; cholesterol

Introduction

Saponins are steroid or triterpene glycosides, most of which form an insoluble complex with cholesterol in aqueous ethanol and show hemolytic activity.[2] In previous work, we examined the hemolytic activities of various saponins and their interactions with liposomal membranes.[3] The results suggested that the permeability changes induced by some saponins might be caused by the formation of complexes with cholesterol in membranes. Recently Akiyama *et al.* obtained evidence by ^{2}H nuclear magnetic resonance (NMR) spectroscopy that digitonin interacts directly with cholesterol in liposomes.[4] Based on the structure of saponins, it is quite possible that the aglycone, the hydrophobic moiety of saponins, may be inserted into hydrophobic regions of the membrane when saponins are added exogenously to liposomes or cells. Incorporation of saponin derivatives that lack a sugar residue into liposomes may provide useful information about the structural change of the lipid membrane induced by saponins.

Cholesterol controls the fluidity of phospholipid in membrane bilayers. The condensing and fluidizing effects of cholesterol in model membrane systems have been studied by various techniques.[5] Earlier studies demonstrated that the 3β-hydroxyl group and the planar nucleus structure of the cholesterol molecule are essential for specific interaction between cholesterol and phospholipid. The side chain of cholesterol has also been shown to be important in steroid-phospholipid interaction.[5c,6] Studies on the permeability properties of liposomes with sterol analogs having side chains of various lengths indicate that the condensing effect of sterol on phospholipid requires a side chain, but does not require the full length of the side chain.[6]

Diosgenin and tigogenin, both of which are steroid-aglycones and cholesterol analogs, have a 3β-hydroxyl group and a flat steroid nucleus. Cholesterol has a side chain with eight

carbon atoms, but diosgenin and tigogenin each have a spirostane structure with eight carbon atoms and two oxygen atoms. It seemed interesting to see how the spirostane structure affects the interaction between sterol and phospholipid.

It is also known that cholesterol in membranes functions as a receptor for many reagents.[3,6,7] Studies of the sterol structural requirements necessary for the interaction between saponins and cholesterol have indicated that the interaction may be mainly through the planar nucleus structure and 3β-hydroxyl group of the sterol molecule.[3,7c] The side chain of cholesterol is also important for the interaction, since we found that for the maximum interaction of liposomes with digitonin, the side chain of the sterol should be longer than 6 carbon atoms.[6] It seemed interesting to examine whether further modification of the side chain would affect the mode of interaction between sterol and saponins.

In a previous paper, we reported the interaction of various saponins with human red blood cells and with liposomal membranes.[3] Saponins could be classified into several categories according to their reactivities. It is possible that different saponins have different structural requirements for interaction with sterols. Thus,, we examined the reactivities of several saponins with liposomes containing diosgenin or tigogenin instead of cholesterol.

Materials and Methods

Diosgenin, oleanolic acid, hederagenin and platicodigenin were obtained by published or known methods.[8] Akebia saponins B, C Pd and Pg[9] were kindly provided by Dr. T. Kawasaki (Department of Plant Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, The University of Kyushu). Tigogenin (Nakarai Chemicals, Ltd., Kyoto) was recrystallized from hot methanol solution. All analogs were shown to be pure by high performance thin-layer chromatography (Merck). Egg yolk phosphatidylcholine was prepared by the procedure described previously.[5e] The purities of dimyristoylglycerophosphocholine, dipalmitoylglycerophosphocholine and cholesterol (Sigma Chemical Co., St. Louis, Mo., U.S.A.) were confirmed by thin-layer chromatography on silica gel G (Merck). The structures of cholesterol and the aglycones used in the present experiments are shown in Fig. 1. Dicetylphosphate (K and K Laboratories, Inc., Plainview, N.Y., U.S.A.), hexokinase, glucose-6-phosphate dehydrogenase, NADP (Oriental Yeast Co., Tokyo) and ATP (Wako Pure Chemical Industries, Ltd., Osaka) were used without further purification.

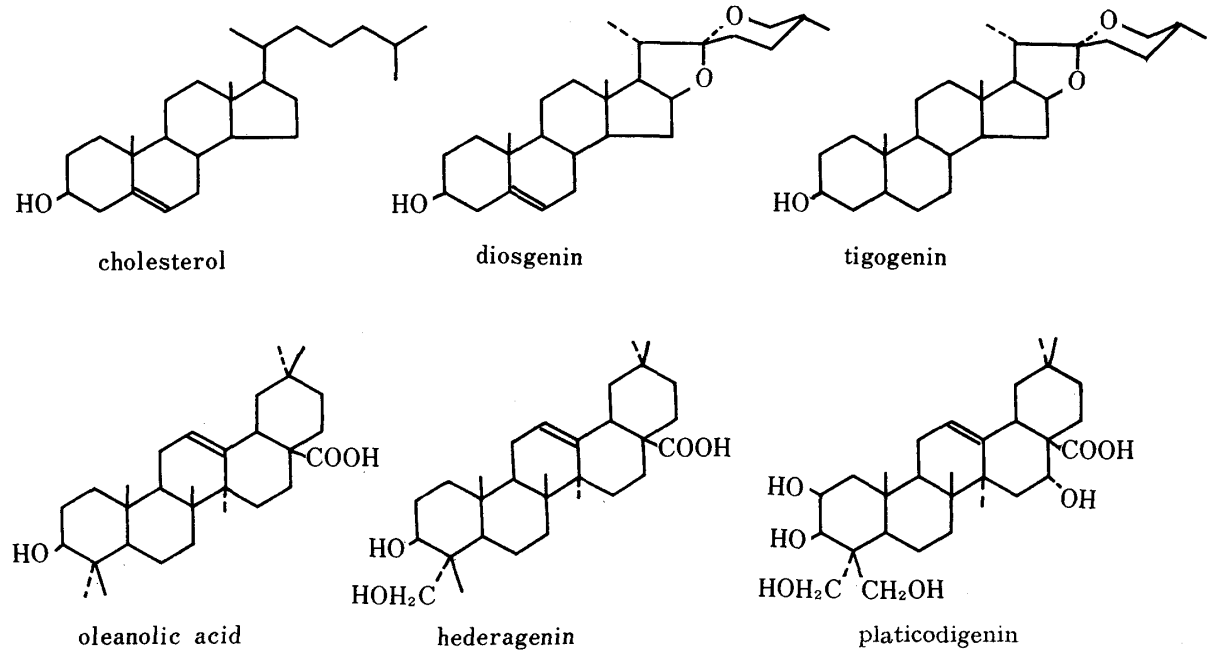

Fig. 1. Structures of Aglycones

Liposomes were prepared and assayed by the method of Kinsky *et al.*[10] A mixture of lipids in chloroform was placed in a 10-ml conical flask. The solvent was removed in a rotary evaporator at room temperature under reduced pressure, and then the flask was placed in a desiccator with anhydrous silica gel, and evacuated

for at least 1 h to ensure that the lipids were completely dry. The dried lipids were then dispersed with a vortex mixer in phosphate-buffered saline (pH 7.2). The final concentration of liposomes was 10 μmol as phospholipid per ml. Glucose release was assayed enzymatically with hexokinase and glucose-6-phosphate dehydrogenase. The amounts of NADH generated were determined photometrically. The effect of temperature on the permeability of liposomes to glucose was determined as described previously.[5e]

Calorimetric experiments were performed in a Daini Seikosha SSC-544 apparatus.

Results

Formation of Phosphatidylcholine Liposomes with Diosgenin

We tried to form liposomes using egg yolk phosphatidylcholine with various aglycones of saponins instead of cholesterol. As shown in Table I, spirostane aglycones, such as diosgenin and tigogenin, formed liposomes with phosphatidylcholine which showed the capacity to trap glucose, but triterpene aglycones, such as oleanolic acid, hederagenin and platicodigenin, could not replace cholesterol and form stable liposomes, since mixtures containing these aglycones did not trap appreciable amounts of glucose.

Table I. Effects of Aglycones on Glucose Trapping in Egg Yolk Phosphatidylcholine Liposomes (μmol Glucose Trapped/μmol Phospholipid)

	Liposomes with					
——	Cholesterol	Diosgenin	Tigogenin	Oleanolic acid	Hederagenin	Platycodigenin
	μmol glucose trapped/μmol phospholipid					
2.3	3.7	4.2	2.9	0.5	0.4	0.2

Liposomes were composed of egg yolk phosphatidylcholine and dicetylphosphate (molar ratio, 1: 0.1), without aglycone or with equimolar (with respect to phosphatidylcholine) cholesterol, diosgenin, tigogenin, oleanolic acid, hederagenin or platicodigenin. All liposomes were prepared at room temperature.

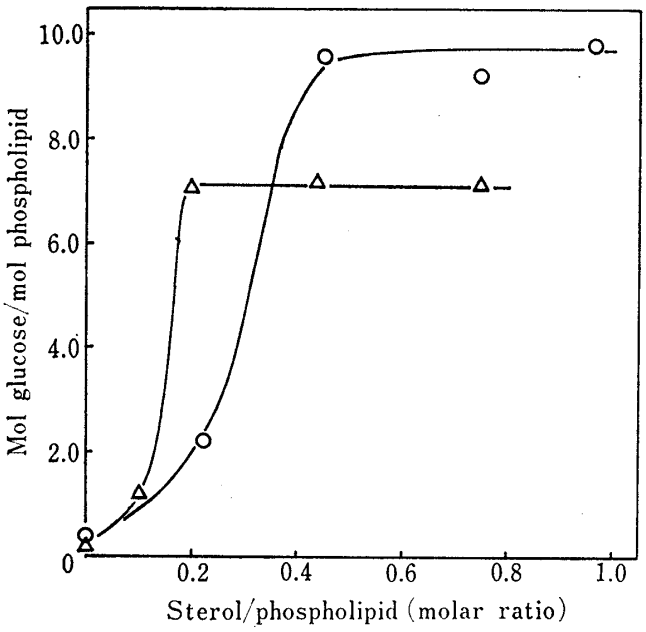

Fig. 2. Effect of Incorporation of Diosgenin (○) or Cholesterol (△) into Dipalmitoylglycerophosphocholine Liposomes on the Amount of Trapped Glucose

Liposomes composed of dipalmitoylglycerophosphocholine, dicetylphosphate (molar ratio, 1: 0.1) and various amounts of sterols were prepared at room temperature.

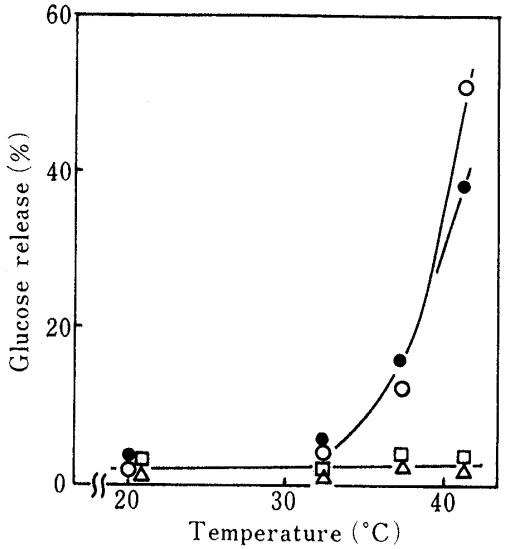

Fig. 3. Effect of Temperature on Glucose Release from Dipalmitoylglycerophosphocholine Liposomes

Liposomes were composed of dipalmitoylglycerophosphocholine and dicetylphosphate (molar ratio, 1: 0.1) without (○) or with molar ratios of diosgenin to phosphatidylcholine of 0.2 (●), 0.4 (□), 0.6 (△). Aliquots of liposome suspensions (2.5 μl) were incubated in cuvettes containing 500 μl of the assay buffer for 10 min at various temperatures.

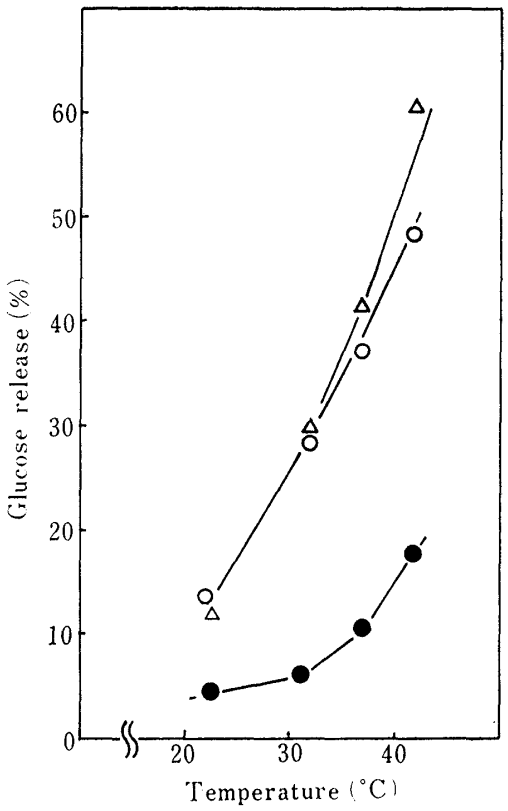

Fig. 4. Effect of Temperature on Glucose Release from Egg Yolk Phosphatidylcholine Liposomes

Liposomes were composed of egg yolk phosphatidylcholine and dicetylphosphate (molar ratio, 1: 0.1) without sterol (○) or with equimolar (with respect phosphatidylcholine) diosgenin (△) or cholesterol (●). Aliquots of liposomes (2.5 μl) were incubated at various temperatures for 30 min in cuvettes containing 490 μl of buffer without enzymes and cofactors. The solution of enzymes and cofactors (10 μl) was then added and mixtures were incubated for a further 3 min. The amount of glucose release was measured.

At room temperature (20—25°), dipalmitoylglycerophosphocholine forms liposomes spontaneously in the presence, but not in the absence of cholesterol.[5e] It is well known that dipalmitoylglycerophosphocholine, with a transition temperature (Tc) of 41°, cannot be dispersed well below the Tc. The observed effect of cholesterol incorporation is due to fluidization of phospholipid membranes. The amounts of cholesterol and diosgenin required for fluidizing the phospholipid in the gel state were examined by measuring the ability of dipalmitoylglycerophosphocholine liposomes to trap glucose when prepared at room temperature (Fig. 2). A molar ratio of cholesterol to phospholipid of more than 0.2 was required for preparing liposomes which could trap glucose, while a ratio of diosgenin to phospholipid of more than 0.4 was required for their formation (Fig. 2). Cholesterol can also fluidize distearoylglycerophosphocholine (Tc: 58°), since distearoylglycerophosphocholine liposomes with cholesterol trapped glucose even when prepared at room temperature. Unlike cholesterol, diosgenin cannot fluidize this phosphatidylcholine, since mixtures with diosgenin did not trap appreciable glucose when prepared at room temperature (data not shown).

Effects of Aglycones on the Release of Glucose from Liposomes upon Perturbation of the Bilayers by Temperature Change

Liposomes prepared with dipalmitoylglycerophosphocholine, dicetylphosphate (molar ratio, 1: 0.1) and various amounts of diosgenin were incubated for 10 min at different temperatures (Fig. 3). Liposomes prepared without diosgenin released about 50% of their trapped glucose when incubated at 41°. Incorporation of diosgenin in a molar ratio to phospholipid of less than 0.2 did not have any significant effect on the permeability increase. No glucose was released from liposomes containing diosgenin in a molar ratio of more than 0.4 to phospholipid.

The effect of incorporation of diosgenin on the leakage of glucose from egg yolk phosphatidylcholine was next examined. Liposomes prepared with egg yolk phosphatidylcholine and dicetylphosphate (1: 0.1, molar ratio) in the presence or absence of an equimolar amount of sterol to phospholipid were incubated for 30 min at various temperatures. Figure 4 shows that about 50% of the glucose was released from liposomes without sterols on incubation at 42°. Cholesterol suppressed glucose release at 42°, while diosgenin did not, suggesting that diosgenin does not have a "condensing effect" on phosphatidylcholine bilayers.

Scanning Calorimetric Study of Dipalmitoylglycerophosphocholine Liposomes Containing Diosgenin

The effect of diosgenin on the thermotropic phase transition (gel to liquid crystalline) of dipalmitoylglycerophosphocholine was studied by differential scanning calorimetry. The

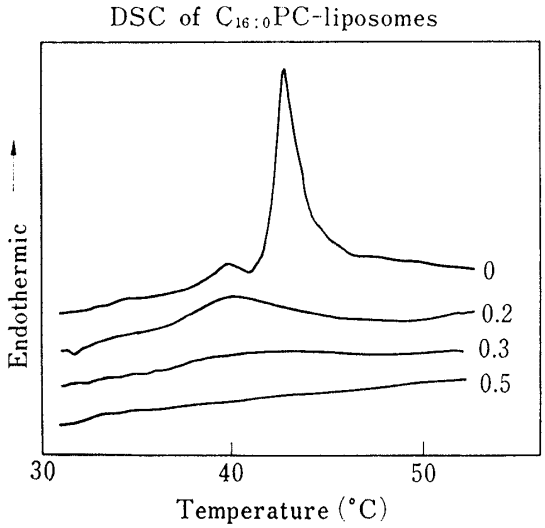

Fig. 5. Differential Calorimeter Scans of Dipalmitoylglycerophosphocholine Liposomes (50 mM Phospholipids) with or without Diosgenin

Ratios of diosgenin to phospholipid are indicated in the figure. Details are given in the text.

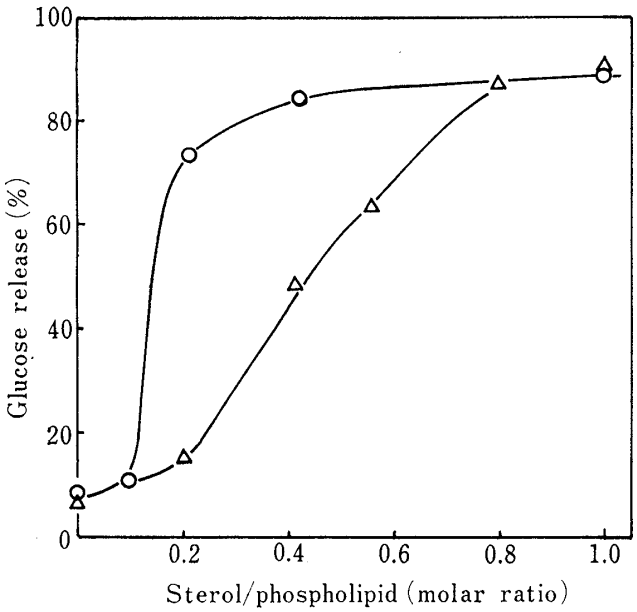

Fig. 6. Effect of Digitonin on Egg Yolk Phosphatidylcholine Liposomes

Liposomes were composed of egg yolk phosphatidylcholine and dicetylphosphate (molar ratio, 1: 0.1) with various amounts of cholesterol (○) or diosgenin (△). Liposomes (25 nmol phosphatidylcholine) were incubated with 100 nmol/ml of digitonin for 30 min at room temperature. The molar ratio of sterol/phospholipid in liposomes is indicated on the abscissa.

calorimetric curves of the liposomes without diosgenin showed a pre-transition endotherm at 39° and a main transition endotherm at 41° (Fig. 5). The transition endotherm bands became broader and eventually disappeared on increase in the amount of diosgenin in the liposomes.

Effects of Digitonin and Akebia Saponins on Liposomes with Diosgenin or Tigogenin

The effects of digitonin on liposomes with cholesterol or diosgenin were examined (Fig. 6). With increase in the amount of cholesterol or diosgenin, the liposomes became increasingly sensitive to digitonin. Maximal sensitivity of the liposomes to digitonin was observed at a molar ratio of diosgenin to phospholipid of more than 0.8 and at a much lower ratio of cholesterol to phospholipid (0.2).

We previously reported that akebia saponins change the glucose-permeability of liposomes containing cholesterol.[3] Their abilities to induce permeability change were affected by the chain length of phosphatidylcholine: with increase in the chain length, the sensitivity of liposomes to these saponins decreased (Table II).[3] It was also noteworthy that akebia saponins C, B, Pg and Pd showed different reactivities with liposomes with cholesterol. Studies on the effect of variation in the sterol structure on the reactivities of liposomes with akebia saponins should provide further information on the structural requirement for the maximum interaction of sterol with akebia saponins. Thus, we prepared various liposomes with diosgenin or tigogenin instead of cholesterol and examined their sensitivities to various akebia saponins. Liposomes containing diosgenin or tigogenin showed different sensitivities to akebia saponins from those of liposomes with cholesterol (Table II). Liposomes with diosgenin or tigogenin were not sensitive to akebia saponins C and B, irrespective of the acyl chain length of phosphatidylcholine. However, they were sensitive to P_G and P_D, but their sensitivities were greatly affected by the acyl chain length of phosphatidylcholine. Among the saponins, P_D was the most potent, since it could react with all the liposomes tested except those containing distearoylglycerophosphocholine.

Table II. Effects of Akebia Saponins on Liposomes

Liposomes with: Phosphatidylcholine	Sterol	Saponins Akebia saponin C	B	P_G	P_D
		% glucose release			
Egg	Cholesterol	91	100	100	100
	Diosgenin	6	5	74	89
	Tigogenin	3	7	86	74
$diC_{14:0}$	Chol.	8	41	99	100
	Dios.	7	12	80	86
	Tigo.	6	2	47	88
$diC_{16:0}$	Chol.	13	48	57	66
	Dios.	6	6	7	35
	Tigo.	3	2	3	33
$diC_{18:0}$	Chol.	5	5	71	63
	Dios.	3	7	8	7
	Tigo.	2	3	2	1

Liposomes were composed of egg yolk phosphatidylcholine, dimyristoyl-, dipalmitoyl or distearoylglycerophosphocholine and dicetylphosphate with cholesterol, diosgenin or tigogenin (1: 0.1: 1, molar ratio) as shown in the table. Liposomes (25 nmol phosphatidylcholine) were incubated with 100 μg/ml of akebia saponins for 30 min at room temperature. Values show percent releases of glucose from liposomes.

Discussion

In a previous paper[6] we examined the effect of the side chain length of cholesterol on its interaction with phospholipids by measuring the permeability properties of liposomes and the phase transition of phospholipid. The results showed that an analog (C22) having a short side chain, an isopropyl chain, had the maximum condensing and fluidizing effects on phospholipid bilayers. Although diosgenin is an analog having a longer side chain than C22, this compound did not have any appreciable condensing effect in the present work. Diosgenin may, however, have a "fluidizing effect," because it suppressed the permeability increase due to perturbation of phase equilibrium near the phase transition temperature and the transition endotherm of dipalmitoylglycerophosphocholine bilayers. The "fluidizing effect" of diosgenin was also suggested by the findings that the ability of dipalmitoylglycerophosphocholine liposomes to trap glucose when prepared at 20° (below its phase transition temperature) was dramatically improved by incorporation of diosgenin (Fig. 2). Epicholesterol also suppressed the permeability increase near Tc and reduced the transition endotherm band (data not shown). The observed effect of epicholesterol is not consistent with the observation of de Kruijff *et al.*[5d] that epicholesterol had no influence on the transition endotherm band of $C_{18:1}/_{18:0}$ phosphatidylcholine. This discrepancy may be due to a difference in the phosphatidylcholine used. The observed effect might be due to a "fluidizing effect," since, as judged from permeability and monolayer studies,[5a] it is clear that epicholesterol does not have any condensing effect. For the dual effect of cholesterol (condensing and fluidizing effects), a β-hydroxyl group and a side chain longer than isopropyl are required. Modification of the side chain, as seen in diosgenin, also has a marked influence on the condensing effect of the sterol.

The structure of the sterol molecule is important for maximum interaction with saponins. It was reported previously that a side chain of more than six carbon atoms was required for the maximum reactivity with digitonin.[6] Diosgenin showed rather weaker reactivity with digitonin than cholesterol, though it has a side chain of similar length to that of cholesterol, suggesting that the spirostane ring structure may interfere with the maximum interaction between sterol and digitonin. It is reasonable that tigogenin showed a similar effect to diosgenin,

since its structure is quite similar to that of diosgenin. Unlike spirostane aglycones, triterpene aglycones from unstable bilayers with phosphatidylcholine. Failure to form stable liposomes may be due to a steric effect of the carboxyl residue in these triterpenes.

Since many saponins have high affinities for cholesterol and form stable complexes with it in aqueous ethanol,[2] these properties are generally attributed to their interaction with membrane cholesterol to cause hemolysis.[3,6,7b,11] There is, however, some uncertainty about whether cholesterol is the actual membrane component responsible for hemolysis. In fact, Tschesche and Wulff[2b] found that there was no quantitative correlation between the hemolytic activities of saponins and the stabilities of their cholesterol complexes. Furthermore, Segal *et al.*[12] suggested that cholesterol does not serve as a specific binding site for several saponins, including digitonin, since these workers observed that intact saponins were not hemolytic, showing this property only after conversion to the corresponding aglycones by some hydrolytic process occurring on the erythrocyte membrane. Segal *et al.* further suggested that aglycones, such as diosgenin and digitogenin, might become inserted into the membrane bilayer, causing perturbation of the membrane. Judging from the present results, however, we consider that diosgenin incorporation seems to stabilize the liposomal membrane. Furthermore, in preliminary experiments, diosgenin did not cause hemolysis or permeability increase of liposomes at concentrations of up to 600 nmol/ml (more than 100 times the concentration of gracillin, dioscin or digitonin required for 100% hemolysis and glucose release from liposomes). Segal *et al.*[13] again insisted that cholesterol does not serve as a specific binding site for several saponins, including digitonin, since cholesterol depletion (40%) of cells did not affect hemolysis induced by these saponins. Our findings do not, however, support their view, since we found that egg yolk phosphatidylcholine liposomes with a molar ratio of cholesterol to phosphatidylcholine of 0.4 showed almost the same sensitivity as those with equimoler amounts of cholesterol and phospholipid.[3] It is noteworthy, however, that one of the saponins, chikusetsu saponin III is exceptional in not requiring cholesterol for interaction with lipid bilayers.[3]

Our findings suggest that the sugar residues of digitonin may play an important role in the formation of a complex with cholesterol in the membrane causing membrane damage.

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