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The Effects of Digitonin and Glycyrrhizin on Liposomes

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Abstract □ Digitonin is a strong hemolysin and glycyrrhizin has protective activity against the deterring effect of other hemolytic saponins. The interaction of these saponins with liposomes was studied as a function of cholesterol in membrane. In the case of multilamellar vesicles, which act as ideal osmometers, digitonin disrupted the barrier function of liposomes composed of phosphatidyl choline, dicetyl phosphate and cholesterol, however, did not influence on cholesterol-lacking liposomes. Glycyrrhizin had similar effect on liposomes irrespective of cholesterol in membrane. In the test with large unilamellar vesicles, digitonin increased the lysis with increasing cholesterol content in membrane, but glycyrrhizin showed no detectable change in cholesterol-containing liposomes. These results suggest that incorporation of cholesterol into liposomes increases the susceptibility to digitonin, resulting in lysis of liposomes, and that the inhibitory effect of glycyrrhizin against other hemolytic saponins is cholesterol-independent.

Keywords □ Digitonin, Glycyrrhizin, Liposome, Cholesterol, Barrier function, Lysis.

In recent years, model membrane system (liposomes) has been developed as one of the tools for clarifying the interaction of the substances with the natural membrane(1). They are useful in studying the permeability properties of the lipid barrier of biological membrane (2).

Saponins are known to be of potential medical value. Their therapeutic applications are, however, very limited, since when taken orally they are poorly absorbed at the intestines and when injected, especially intraveneously, most of them are very toxic and induce marked hemolysis(3). Saponins are classified into several categories according to their reactivities. Recently, some purified nonhemolytic saponins were reported to protect washed erythrocytes against the deterring effect of other hemolytic saponins. Glycyrrhizin which is used as sweetening agent, antiulcer agent and demulsant was found to possess this capacity (3). Digitonin has been known to be strong hemolysin (4, 5). Many studies have been described in the literature about saponin hemolysis (4, 6-7), however, the mode of action is much less clear. Therefore, elucidation of the saponin interaction in model membrane system might be very useful in understanding the lytic or protective action in the plasma membrane. This report was carried out to obtain a better understanding of the saponin effects on membrane, and for comparison of lytic and protective action of saponins, the role of cholesterol in membrane was noted by using liposomes with or without cholesterol. The barrier function is one of the essential properties of a biomembrane, and the barrier property of liposomes would be changed (or destroyed) as a result of the interaction with saponin.

It has been already demonstrated that liposome acts as an ideal osometer (8-10) and the reciprocal of turbidity of the dispersion is linearly proportional to the osmotically induced volume change of the liposomes (11). If a saponin interacts with membrane and has an influence on its barrier funtion, the liposomes will no longer exhibit an ideal osmotic behavior. This result appears disruption of linearity between $1/A$ and C_{in}/C_{out} of liposomes. Following is applied to the test of saponin effects on large unilamellar vesicles. Hidden enzyme activity sequestered in liposomal internal volume from the substrate in the medium becomes apparent by the entrance of substrate through the mediation of saponin-induced lysis of membrane. The degree of lysis can be measured by monitoring the absorbance of light.

EXPERIMENTAL METHODS

Materials

Digitonin was purchased from Sigma Co. and glycyrrhizin from Tokyo Chemical, Ltd..Chemical formulas of these saponins are shown in Fig.1. Cholesterol was obtained from Nakarai Chemical, Ltd. in Japan and used without further purification. Egg phosphatidylcholine was purchased from Merck Co. and purified by alumina column chromatography. Dicetylphosphate was purchased from Sigma Co. *p*-nitrophenylphosphate and alkaline phosphatase were obtained from Sigma Co. and all other reagents were commercial of analytical grade.

Preparation of Liposome

(1) **Multilamellar vesicle(MLV)**

Liposome were made from phospholipids, with or without cholesterol. Appropriate amounts of egg phosphatidylcholine (PC), dicetyl phosphate (DCP) and/or cholesterol (Ch) in $CHCl_3$ were mixed in a round bottomed flask in a ratio of 96 : 4 : 16 by weight. The $CHCl_3$ was removed in a rotary evaporator and further dried in vacuum for 2 hours. To this thin dry lipid film, aqeous solution of 60mM glucose (2mM EDTA, 10mM Tris-HCl buffer, pH7.5) was added and lipid was suspended by vortex mixer. Lipid concentration of this liposome stock dispersion was 12.5mg phospholipid/m*l*.

(2) **Large unilamellar vesicle(LUV)**

LUV was prepared by the reverse-phase evaporation method. The $CHCl_3$ with desired lipid constituents was evaporated. Resulting lipid film was dissolved in diethylether and then 1ml of a solution of 5mg/ml alkaline phosphatse in Borate -HCl buffer (pH9.2) was added to this phospholipid-ether mixture. The preparation is mixed for a brief period, forming a homogeneous emulsion. While the organic solvent was removed in rotary evaporator, the material first formed a viscous gel-like intermediate, and then it became a liposome dispersion by continued evaporation. Untrapped enzymes in prepared LUV dispersion were inhibited by CNBr saturated solution. Liposomes containing enzymes were separated by centrifugation at 100,000g for 20 minutes on Beckman Ultracentrifuge. The pellet containing liposomes is resuspended in 25mM Borate-HCl buffer (pH9.2). Further purification was obtained by repeated centrifugation and resuspension. Lipid concentration of liposome stock dispersion was 12.5mg phospholipid/ml.

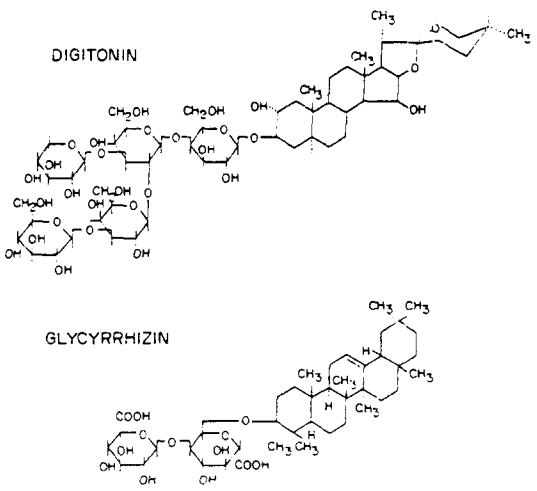

Fig.1. Chemical structures of saponins.

Determination of the CMC of Saponin

The critical micelle concentration (CMC) values in Phosphate buffer were determined by the enhancement of 8-anilino-1-naphthalene sulfonic acid (ANS) fluorescence (12) performed with a Biard Atomic Fluoricord Spectrofluorimeter. ANS concentration in diluted saponin solutions was 2.0×10^{-5}M.

Effects of Saponin on Osmotic Behavior of MLV

Two sets of experiments were run in parallel. a) The aliquots (0.067ml) of stock dispersion were mixed with glucose solutions at various concentrations to give a final volume of 3ml. It was incubated for 1 hour to achieve equilibrium before measurment. b) To the liposome-glucose solution of concentration gradients, appropriate amounts of saponin were added to give a final volume of 3ml. The reaction mixture was incubated for 1 hour, and absorbance was measured at 450nm on a LKB Ultrospec 4050.

Lytic Effects of Saponin on LUV

Lysis of LUV was studied by following: Aliquots of 0.067ml liposomes dispersion were incubated with the series of saponin solutions. After 1.5 hour, 0.2m*l* of 18N-PNPP solution was added. During enzyme substrate reaction, the time and temperature were controlled. The enzyme reaction was terminaced by addition of 0.2m*l* 16N-NaOH solution and the absorbance at 410 nm was measured on LKB Ultrospec 4050.

RESULTS

CMC of Saponin

In Fig.2, ANS fluorescence vs. saponin concentration was plotted. ANS is almost nonfluorescent in water, whereas it becomes strongly fluorescent when located in regions of lower polarity such as the micelle/water surface (13). There is virtu-

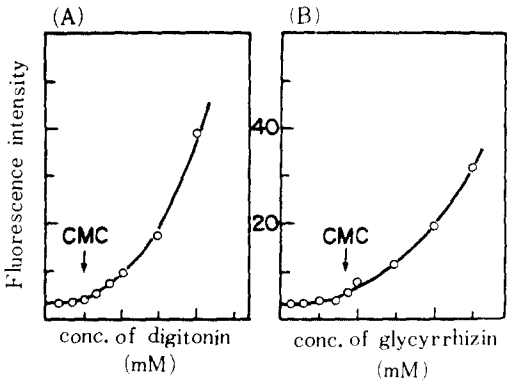

Fig.2. Critical micelle concentrations of digitonin(A) and glycyrrhizin(B).

CMC values were determined by the fluorescence of ANS as a function of concentration for solutions of saponins. Fluorescence was excited at 387nm and the emmision detected at 474nm at room temperature.

ally no detectable fluorescence below CMC. The CMC values of digitonin and glycyrrhizin were about 0.1mM and 1.6mM, respectively.

Effects of Saponin on Osmotic Behavior of MLV

Multilamellar liposomes of phospholipids including a small amount of charged lipids were shown to be osmotically active and the osmotic behaviors can be investigated using turbidimetry (8-11). Phospholipid-liposome suspension responds to the osmotic gradient and the total average volume of liposomes changes to the Boyle-van't Hoff's law,

$$V_{total} = V_{act} \cdot (C_{in}/C_{out}) + V_{dead}$$

where V_{act} and V_{dead} are the volumes of osmotically active and non-active part. C_{in}/C_{out} is the ratio of glucose concentration of inner part to that of outer part of lipsomes. The volume change of liposome can be easily detected by optical measurement using the equation derived by Yoshikawa *et. al.* (11),

$$V = K \cdot (1/A)^{3/2}$$

where A and K are the absorbance and a constant, respectively. From these two equations following would be derived,

$$(1/A)^{3/2} = 1/K \cdot (V_{act} \cdot (C_{in}/C_{out}) + V_{dead})$$

There is a linear relationship between $(1/A)^{3/2}$ and C_{in}/C_{out}. The slope of the straight line may represent the relative volume change of liposomes due to the effect of osmotic pressure.

Fig.3-Fig.6 show the osmotic behavior of liposomes composed of PC, DCP and/or Ch (96 : : 16). It can be seen that the good linear relationships between $(1/A)^{3/2}$ and C_{in}/C_{out} exist for liposomes prepared in the absence of saponins.

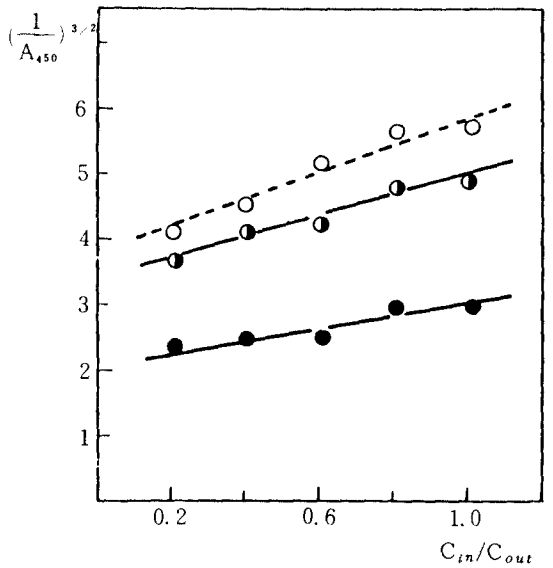

Fig.3. Effects of digitonin on osmotic behavior of PC, DCP liposomes.

Liposomes were treated with 0.1mM (◐) and 0.3mM (●) of digitonin. Dashed line (○) shows osmotic behavior of digitonin-untreated liposomes.

Therefore it is regarded that liposome acted as an ideal osmometer in this study. When liposomes without cholesterol were treated with high concen-

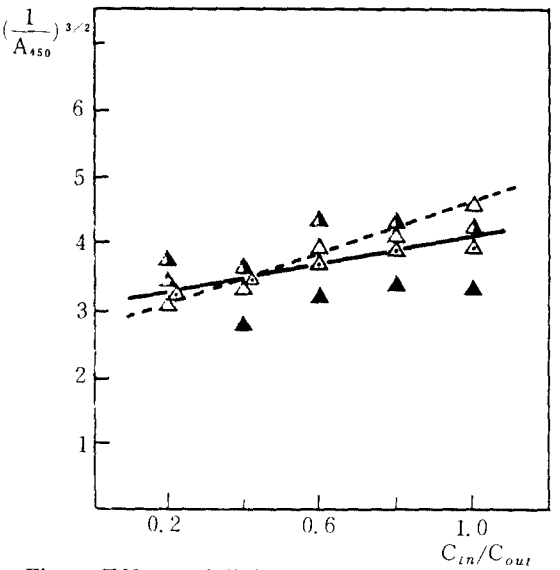

Fig.4. Effects of digitonin on osmotic behavior of PC, DCP, Ch liposomes.

Digitonin concentrations are 0mM (△), 0.08mM (◬), 0.1mM (▲) and 0.3mM (▲).

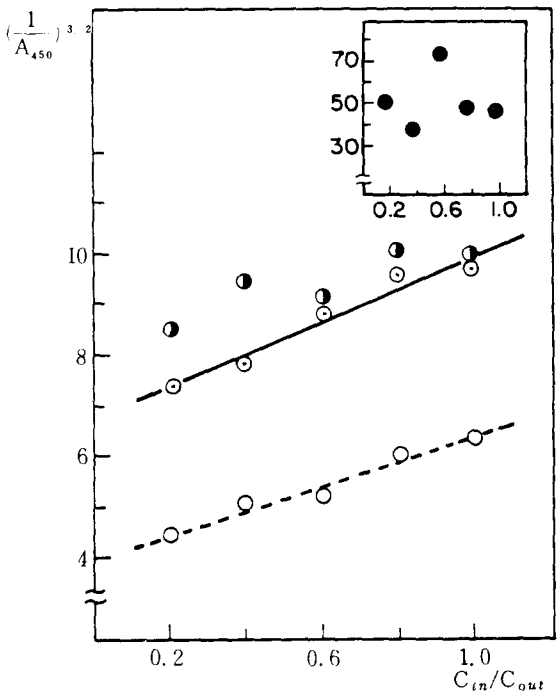

Fig.5. Effects of glycyrrhizin on osmotic behavior of PC, DCP liposomes.
Liposomes were treated with 0.4mM (⊙), 0.5mM (◐) and 1.5mM (●). Dashed line (○) shows osmotic behavior of glycyrrhizin-untreated liposomes.

trations of digitonin (Fig.3), the linearity was not disturbed. It suggests that digitonin does not damage the osmotical barrier function of cholesterol-lacking liposomes up to 0.3mM of digitonin. On the other hand, 0.1mM of digitonin had influence on cholesterol-containing liposomes (Fig.4). Resulting disturbance of linearity is indicative of fragility of basic membrane structure by the interaction between digitonin and membrane.

The effect of glycyrrhizin on the osmotic behavior of multilamellar liposomes is shown in Fig.5 and Fig.6. At concentrations above 0.6mM, glycyrrhizin influenced on all liposomes irrespective of cholesterol in membrane, however, the susceptibility to glycyrrhizin was somewhat higher for cholesterol-free liposomes than for cholesterol-containing liposomes. Cholesterol-free liposomes were influenced at concentrations above 0.5mM, and cholesterol-containing liposomes were at concentrations above 0.6mM. At enough concentrations of glycyrrhizin (above 1mM), $(1/A)^{3/2}$ was increased very much, and the dispersion became eye-detectably clear.

Lytic Effects of Saponin on LUV

In order to test the extent of the lytic effect of saponins on membrane, large unilamellar vesicles (LUV) were used. LUVs containing enzymes in their internal volume were preincubated with saponin solution. By addition of substrates to this mixutre, saponin-induced lysis would permit the substrate to enter liposome bilayer and it reacted with enzyme, producing yellow color. The greater the extent of saponin-induced lysis is, the greater absorbance would be. The degree of membrane lysis was measured by the increasing absorbance of light at 410 nm. LUV is sufficiently large to encapsulate a high percentage of the initial aqueous enzyme phase, and centrifugation has proved a desirable procedure for concentrating liposomes after inhibition of non-entrapped enzymes. *p*-Nitrophenyl-phosphate (PNPP) is a substrate for the enzyme, alkaline phosphatase (AP). Under alkaline conditions (pH greater than 7) AP will snip off the phosphate group from PNPP (colorless) producing *p*-nitro-phenol (yellow). AP and PNPP are conveninent for this study bacause the color reaction is easily detectable and the time required for the reaction is fast.

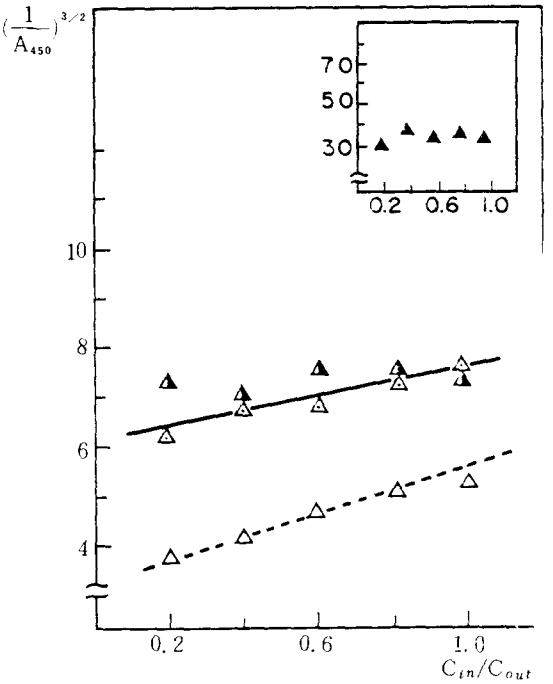

Fig.6. Effects of glycyrrhizin on osomtic behavior of PC, DCP, Ch liposomes.
Glycyrrizin concentrations are 0mM (△), 0.5mM (◬), 0.6mM (▲), 0. and 1.5mM (▲).

Fig.7 and Fig.8 show the extent of liposome lysis estimated from the absorbance after addition of saponins. In Fig.7 the presence of the cholesterol in membrane caused a lysis by addition of digitonin, and the increase of lysis was especially pronounced with increasing amount of cholesterol content in the liposome. But no difference occured upon addition of glycyrrhizin (Fig.8) whether cholesterol is present in membrane or not. Even liposomes with PC:DCP:Ch ratios 96 : 4 : 24 showed no detectable increase in absorbance. It can be deduced from the result that interaction of glycyrrhizin with membrane is not related with cholesterol. The maximal range of liposomal lysis by saponin was determined by comparing the enzyme activity in the case of saponin lysis with that in the case of complete lysis by triton X-100.

DISCUSSION

The hemolytic activity is one of the characteristic properties of most saponins, and each saponin shows this activity in different degrees. Recently, saponins which show a slight or no hemolytic activity were isolated, and non-hemolytic saponins which protect against hemolysis might be effective inhibitors to the toxic effects of saponins.

Results show that digitonin changed the permeability of the cholesterol-containing liposomes, but did not in the case of cholesterol-free liposomes.

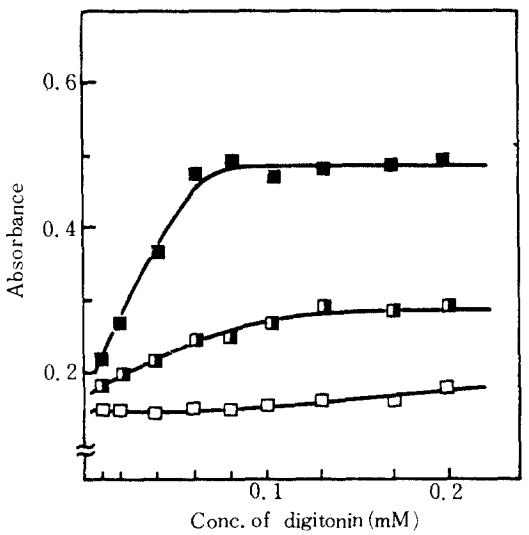

Fig.7. Lytic effects of digitonin on large unilamellar vesicles estimated from the absorbance at 410nm.
Liposome composion, □ PC, DCP (96 : 4) ◨ PC, DCP, Ch (96 : 4 : 8) ■ PC, DCP, Ch (96 : 4 : 24).

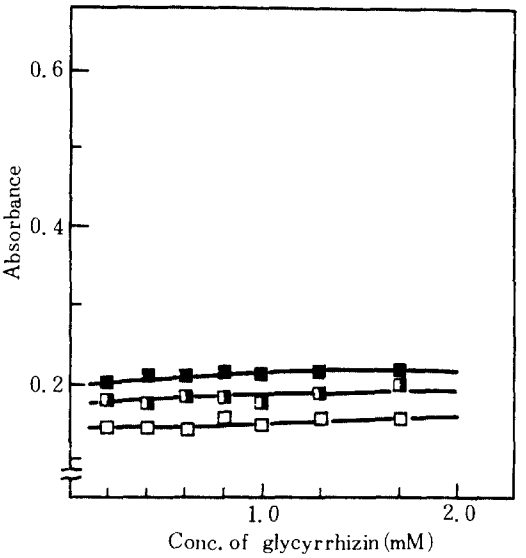

Fig.8. Effects of glycyrrhizin on large unilamellar vesicles estimated from the absorbance at 410nm.
Liposome composion, □ PC, DCP (96 : 4) ◨ PC, DCP, Ch (96 : 4 : 8) ■ PC, DCP, Ch (96 : 4 : 24).

Incorporation of cholesterol into liposome increased the susceptibilty to digitonin. The fact that specific interaction between digitonin and cholesterol occurs in membrane is reported (14, 15). It is worth noting that when the liposomes with cholesterol were treated with relatively high concentrations of digitonin (0.3mM), though linearity was disturbed, average value of $(1/A)^{3/2}$ was similar to that of saponin-untreated liposomes. Since $(1/A)^{3/2}$ is representative of liposomal volume, it can be assumed that the volume of digitonin-treated liposomes still remains when compared with saponin-untreated liposomes. The reason of disruption of barrier function which is represented as dis turbance of linearity is formation of pores in membrane rather than fragmentation of liposomes, and this view is supported by the observation of the irreversible change in the ^{2}H NMR spectra studied by T. Akiyama *et. al.* (14). There is another result that digitonin could make plasma membranes permeable to various substances, without impairing the function of the intracellular organelles (7). Though it was not possible to obtain a more precise mechanism of saponin action by this method, one possibility is that lipids surrounded by stable complexes between digitonin and cholesterol might be easily removed, resulting in lysis (14).

The lytic susceptibility to digitonin was clearly higher for cholesterol-containing liposomes than

for cholesterol-lacking liposomes, which indicates that the lytic effect of digitonin was highly cholesterol-dependent. For digitonin lysis, no threshold value for cholesterol concentration was observed. There was a small increase of absorbance even in liposomes without cholesterol at large enough concentrations of digitonin (above CMC). From this, it can be assumed that digitonin has an unspecific lytic effect on lipids membrane at above CMC, in addition to the specific cholesterol-dependent lysis (16).

There was a slight difference in linearity-disturbing concentrations of glycyrrhizin whether cholesterol was present in membrane or not. But the reason why liposomes with cholesterol needed somewhat smaller amount of glycyrrhizin to disrupt the linearity than those without cholesterol might be not due to the interaction of glycyrrhizin with membrane but due to the effect of cholesterol in membrane. Incorporation of cholesterol into membrane enhances the mechanical stability of lipid bilayer (17). At room temperature, reduced membrane fluidity and permeability by cholesterol influences the sensitivity to glycyrrhizin. Hence it can be deduced that cholesterol does not serve as the specific binding site for glycyrrhizin, but acts only as depressor for water permeability during adsorption of glycyrrhizin by the liposomes. It seems likely that the great increase of reciprocal absorbance induced by glycyrrhizin above 1mM is due to solubilization of liposomes into mixed micelle structure by the detergent-like action of saponin which decreases average size of liposomes markedly (18). Practically, the turbidity of the reaction dispersion was reduced eye-detectably at higher concentration.

In the effect of glycyrrhizin on large unilamellar vesicles, there was not any increase of absorbance with increasing cholesterol content in membrane even at 24% of cholesterol, which might indicate that there is no specific interaction between glycyrrhizin and cholesterol. It was reported that preincubation of erythrocytes with glycyrrhizin decreased the degree of lysis by hemolysin, and the inhibition was found to be reversible (3). However, the protective property was not investigated in detail. On the basis of the results from this experiment, the cholesterol in membrane is not responsible for the protective effect of glycyrrhizin against other saponin-induced hemolysis and the factors rather than direct glycyrrhizin-cholesterol interactions are involved in inhibitory action of glycyrrhizin. The possible explanation about the protective effect would be that glycyrrhizin is nonspecifically adsorbed by the membrane and prevents the access of the hemolysin to its receptor(3). The results from these model systems are useful for the understanding of phenomena occuring in the more complex biological membranes.

Being amphiphiles, saponin has its CMC. At concentrations below its CMC, saponin interacts with liposomes as monomers. To characterize the effects of saponins as monomers or micelles, CMC values of saponins were determind. But, the CMC appears to provide poor explanation of lytic activity (19), since the data show that there is no relationship between membrane-disturbing (or lytic) activity and is CMC. The CMC may be considered a measure of the compound solubility under the given conditions reflecting the relative hydrophobicity. It was pointed out that the relative hydrophobicity of the surfactant appeared to affect its affinity for the membrane but not its lytic activity (20). Further study is necessary to investigate the more detailed mechanism of digitonin and glycyrrhizin on the hemolytic action and protective action in molecular level.

ACKNOWLEDGEMENT

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