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Saponin–Cholesterol Interaction in the Multibilayers of Egg Yolk Lecithin As Studied by Deuterium Nuclear Magnetic Resonance: Digitonin and Its Analogues†

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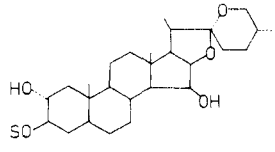
ABSTRACT: In order to gain an understanding of the hemolytic activity of digitonin, a ^2H NMR study was attempted with [26,26,26,27,27,27-$^2H_6$]cholesterol (Chol-d_6) and [18,18,18-$^2H_3$]stearic acid (SA-d_3) as probes for the terminal methyl portions of cholesterol and lipids, respectively, for the multibilayers of egg yolk lecithin containing digitonin or its analogues. It was found by ^2H NMR spectroscopy of the SA-d_3 probe that digitonin caused disordering of lipids noticeably, especially in the presence of cholesterol. The interaction between digitonin and cholesterol was characterized by the following three stages which depend upon the digitonin/cholesterol ratio: (1) "aggregated" species (the mole ratio being between 0 and 0.35), (2) the intermediate complex (between 0.35 and 0.9), and (3) the equimolecular (rigid) complex. The molecular ordering of Chol-d_6 was distorted by digitonin in the case of lower digitonin/cholesterol ratios, which is characterized as the aggregated species arising from cholesterol rapidly exchanging between free and complexed states with digitonin. At an elevated temperature (47 °C), however, these species were converted to the more rigid complex as in (2) or (3). ^2H NMR spectra of Chol-d_6 at the digitonin/cholesterol ratio 1.0 gave a quadrupole splitting as large as 14 kHz, which is very close to that of polycrystalline Chol-d_6. Cholesterol, in this case, is rather immobilized as a result of formation of the rigid complex with digitonin in the bilayers. In the case of the intermediate complex, the rigid complex was present together with the aggregated species. Saponins with a reduced number of terminal sugar moieties with lower hemolytic activity exhibited no distinct feature to form the rigid complex. Thus, the configuration of the terminal sugar moiety should play a specific role in forming the rigid complex which might be related to the hemolytic activity.

The saponins are steroid or triterpene glycosides that have the distinctive property of forming a soapy lather in water and have hemolytic activity (Tschesche & Wulff, 1973; Agarwal & Rastogi, 1974). Another characteristic is the formation of an insoluble equimolecular complex with cholesterol (Steiner & Holtzem, 1955). Digitonin (I) surpasses all other saponins in yielding complexes of great insolubility, called "digitonides" (Fieser & Fieser, 1959; Brooks, 1970). Digitonin has been used for the determination of cholesterol content in blood plasma, bile, and tissues and also is widely used to disperse membrane-bound proteins. In the early days, it was suggested that saponin hemolysis might be caused by the formation of a complex with cholesterol in the erythrocyte membrane. This postulate, however, is still controversial (Steiner & Holtzem, 1955). Therefore, elucidation of the specific interaction in model membrane systems might be very useful in understanding the hemolytic action in the plasma membrane.



I, S = Xyl-Glu-Gal-Glu-Gal
II, S = Xyl-Glu-Gal-Gal
III, S = Glu-Gal-

Recently, ^2H NMR[1] has proved to be a very powerful nonperturbing tool with which to examine molecular organization and molecular motion in model and biological membranes (Mantsch et al., 1977; Seelig, 1977; Stockton et al., 1977; Davis et al., 1979; Kang et al., 1979a). It is a natural extension to employ selectively deuterated cholesterol as a ^2H probe molecule since cholesterol is assumed to be the target for saponins. It should be mentioned that modification of even the side chain in cholesterol significantly alters the behavior

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[1] Abbreviations used: NMR, nuclear magnetic resonance; Chol-d_6, [26,26,26,27,27,27-$^2H_6$]cholesterol; SA-d_3, [18,18,18-$^2H_3$]stearic acid; EPR, electron paramagnetic resonance.

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of the interaction as manifested by digitonide formation in aqueous alcohol (Akiyama et al., unpublished experiments). Thus, we attempted here to use [26,26,26,27,27,27-$^2H_6$]-cholesterol (Chol-d_6) and [18,18,18-$^2H_3$]stearic acid (SA-d_3) probes, the latter of which was employed to examine the organization of lipids (Stockton et al., 1976) dispersed in the multibilayers of egg yolk lecithin. To clarify the role of the sugars in saponins, we used three glycosides with different sugars: digitonin (I), desglucodigitonin (II), and glucosylgalactosyldigitogenin (III). The former two are contained in commercial "digitonin".

Here we demonstrate that cholesterol complexed with digitonin (equimolar complex) exists as solid-state-like digitonides in the bilayers as manifested by their ^2H NMR spectra. At an initial stage, at lower digitonin/cholesterol ratios, toward formation of the stable complex, on the other hand, we noticed that there appeared "aggregated" species of digitonin and cholesterol in which molecular ordering of the latter is highly distorted by interaction with the former. By increasing the amount of digitonin, those species were converted into the stable complex. For saponins with a different sugar moiety, the extent of the complex formation was rather low. Therefore, it is concluded that the configuration of the sugar moeity in saponins plays a dominant role in the specific interaction with cholesterol.

Experimental Section

Materials. The SA-d_3 probe was supplied by MSD Japan Ltd. and used without further purification. Nondeuterated cholesterol was obtained from Wako Pure Chemical Industries, Ltd., and recrystallized twice from benzene before use. Egg yolk lecithin was extracted from fresh egg yolk by the procedure of Bligh & Dyer (1959) with minor modification and purified by alumina and silicic acid chromatography. Lipid phosphorus was analyzed by the conventional way in chloroform solution, and the solution was tightly closed and stored at −20 °C before use. Digitonin and desglucodigitonin were separated and purified from commercial digitonin (Merck) by droplet countercurrent chromatography (Ogihara et al., 1976) using the solvent system of chloroform–methanol–water–propanol (9:12:8:1 by volume). Glucosylgalactosyldigitogenin was prepared from the hydrolysate of digitonin and purified by the same procedure. Chol-d_6 was synthesized from commercially available 3-*O*-acetoxy-22,23-bisnor-5-cholenic acid. Details of the procedure will be published elsewhere. The purity of glycosides and deuterated cholesterol was established by ^1H and ^{13}C NMR spectra and by high-performance thin-layer chromatography (Merck). In some instances, we also used ^2H-depleted water from Aldrich Co.

Preparation of Multibilayers of Egg Yolk Lecithin. Deuterated cholesterol or stearic acid probes were weighed directly into 10-mm o.d. NMR tubes. The required amount of egg yolk lecithin in chloroform was transferred into the NMR tube volumetrically and vortexed. Under a stream of nitrogen, approximately three-quarters of the chloroform was evaporated. A dimethyl sulfoxide solution of saponin was added to the concentrated chloroform solution. These solvents were evaporated under a stream of nitrogen and then removed by placing the sample under vacuum. Lamellar multibilayers of lecithin were prepared by adding excess distilled water or ^2H-depleted water and shaking vigorously in a vortex mixer for several minutes, followed by freezing and thawing. Alternatively, digitonin in dimethyl sulfoxide was directly incorporated into the sonicated lecithin vesicles. The vesicles were vortexed several times at room temperature and stored at −20 °C. Before use of the multibilayers, freezing and thawing were repeated several times.

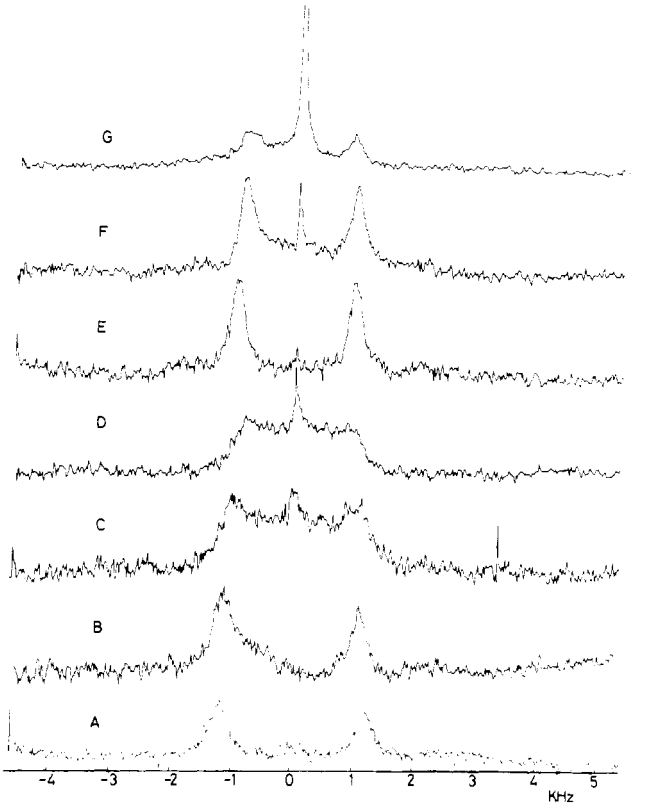

FIGURE 1: 15.28-MHz ^2H NMR spectra of SA-d_3 (14 mol %) intercalated in the multibilayers of egg yolk lecithin containing cholesterol (16 mol %) and/or digitonin (48 000–159 000 transients). (A) Lecithin only; (B–D) digitonin/cholesterol ratio 0.3, 0.35, and 1.0, respectively; (E–G) lacking cholesterol, the amount of digitonin is the same as that of the digitonin/cholesterol ratio 0.5, 0.75, and 1.0, respectively.

^2H NMR Spectroscopy. ^2H NMR spectra at 15.28 MHz were recorded by a JEOL PFT-100/EC-100 spectrometer. A 90° pulse requiring 28 μs was used to accumulate free induction decays with repetition times of 0.3 s. A delay time, 60 μs (in some instances 200 μs), was used between the end of the pulse and the acquisition of the first data point. All 15.28-MHz spectra were obtained by using 4K data points and an 8–10-kHz spectral width. The ^{19}F signal of perfluorobenzene, contained in a 2-mm o.d. tube, was used for field-frequency stabilization. ^2H NMR spectra at 46.06 MHz were recorded by a Bruker CXP-300 spectrometer, installed at the National Research Council of Canada, with the quadrupole-echo method (Davis et al., 1976). A 90° pulse requiring 7 μs was used with a spectral width of 125 kHz and 2K data points. Samples in 10-mm o.d. tubes were placed in the transverse solenoid coil configuration. No lock system for this spectrometer was used.

Results

Stearic Acid (SA-d_3) Probe. Figure 1 demonstrates that digitonin caused a distinct spectral change in the ^2H NMR spectra of SA-d_3 intercalated in multibilayers in the presence of cholesterol (16 mol %). To clarify the phase behavior, we plotted, in Figure 2, the quadrupole splittings against the digitonin/cholesterol ratio (open circles) together with those obtained in the absence of cholesterol (closed circles) vs. the equivalent amount of digitonin corresponding to the cholesterol-containing sample. Interestingly, there are sudden decreases in the quadrupole splitting at levels of the digitonin/cholesterol ratio around 0.35 and 1.0. After the first transition at 0.35, the quadrupole splitting was decreased to

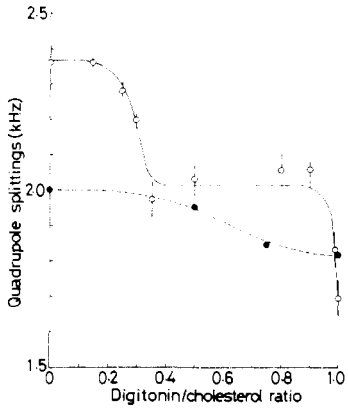

FIGURE 2: A plot of the quadrupole splittings of the SA-d_3 probe vs. the digitonin/cholesterol ratio: (○) in the presence of cholesterol; (●) in the absence of cholesterol (for the latter, the abcissa is expressed by the equivalent amount of digitonin as that containing cholesterol).

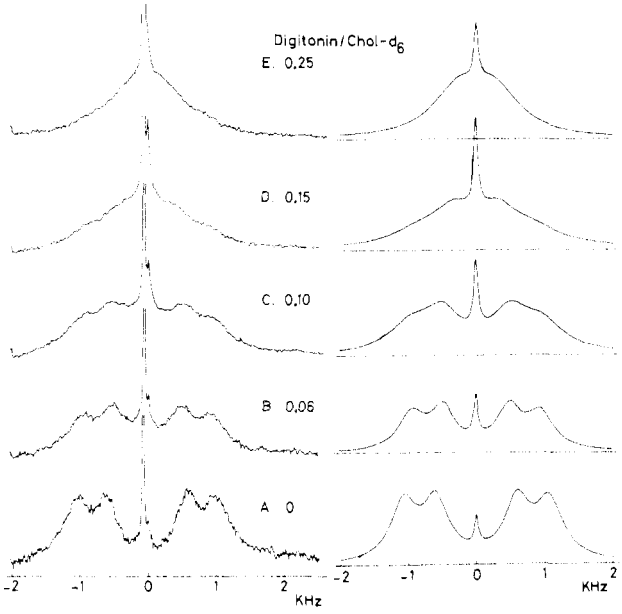

FIGURE 3: 15.28-MHz ^{2}H NMR spectra (left) of Chol-d_6 (16 mol %) in the multibilayers of egg yolk lecithin containing various amounts of digitonin (56 000–155 000 transients) and simulated spectra (right). (A) No digitonin; digitonin/cholesterol ratio (B) 0.06, (C) 0.10, (D) 0.15, and (E) 0.25.

the value which is almost the same as that obtained in the absence of cholesterol. It appears that the second transition corresponds with formation of the 1:1 digitonin–cholesterol complex. Obviously, the line widths were broadened by a factor of 2 at the equimolecular digitonin/cholesterol ratio, compared with those of the digitonin-free sample. Furthermore, the central peak appeared exclusively for the samples containing digitonin over the digitonin/cholesterol ratio 0.35 (Figure 1). In the absence of cholesterol, however, this peak appeared for samples containing higher amounts of digitonin; the intensity was increased suprisingly high for the sample containing digitonin equivalent to the digitonin/cholesterol ratio 1.0 (Figure 1G).

Cholesterol (Chol-d_6) Probe. Figure 3A shows that two (two splittings, four peaks) of the quadrupole splittings were observed for Chol-d_6 in the multibilayers. These two quadrupole splittings, with equal intensities, arose from different orientations of the C–^{2}H vectors, in 26- and 27-deuterated methyl groups, to the normal of bilayers. The concentration dependence of the two types of quadrupole splittings was sigmoidal, in contrast to the case of [3α-^{2}H]cholesterol by

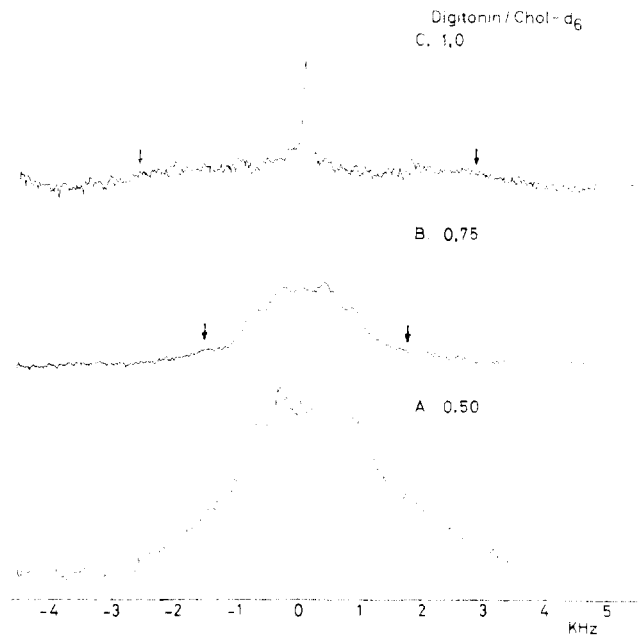

FIGURE 4: 15.28-MHz ^{2}H NMR spectra of Chol-d_6 (16 mol %) in the multibilayers of egg yolk lecithin (15 000–16 000 transients). Digitonin/cholesterol ratio: (A) 0.5, (B) 0.75, and (C) 1.0.

Oldfield et al. (1978a). The concentration of Chol-d_6 in this study was usually 16 mol % except for several cases (20 and 33%). The quadrupole splittings in this case were 1.20 and 2.2 kHz for the inner and outer pairs, respectively (Figure 3A).

Figure 3 shows the ^{2}H NMR spectra of Chol-d_6 at lower digitonin/cholesterol ratios ($\leq$0.25). The line shape of the ^{2}H NMR spectra was altered drastically with increasing digitonin/cholesterol ratios, although a slight change was noted for the ^{2}H NMR spectra of the SA-d_3 probe in this region (Figures 2 and 3). Clearly, the use of the Chol-d_6 probe is more sensitive at detecting digitonin–cholesterol interaction compared with that of SA-d_3. Surprisingly, digitonin caused a decrease of the quadrupole splittings of Chol-d_6. It appears that the extent of the decrease in the order parameter (Seelig, 1977; Mantsch et al., 1977) together with concomitant line broadening is proportional to the amount of digitonin present in the bilayers. A more quantitative estimate of the order parameters will be given later on the basis of spectral simulation.

At higher digitonin/cholesterol ratios (>0.35), on the other hand, the ^{2}H NMR spectra of Chol-d_6 exhibited quite different features (Figure 4). As shown in Figure 4A, the ^{2}H NMR spectrum of Chol-d_6 at the digitonin/cholesterol ratio 0.5 gave rise to a broad envelope in addition to the narrow components (the quadrupole splittings of which are 0.75 and 1.6 kHz for the inner and outer pairs, respectively) already seen at lower digitonin/cholesterol ratios. It becomes apparent, by examining the ^{2}H NMR spectra at higher digitonin/cholesterol ratios (parts B and C of Figure 4), that this evelope arises from components of the increased quadrupole splittings. The increased quadrupole splittings, appearing outside the narrow components (see the arrows in Figure 4), were 3.3 and 5.3 kHz for the sample at the digitonin/cholesterol ratios 0.75 and 1.0, respectively. It is interesting to note that the peak intensities of the narrow components were suppressed considerably in accordance with increasing proportions of digitonin (parts B and C of Figure 4). Obviously, these features were caused by formation of the more rigid 1:1 complex, especially at a digitonin/cholesterol ratio around 1.0. We were unable to observe ^{2}H signals arising from wider components than 10 kHz, even if they existed, because of our instrumental limi-

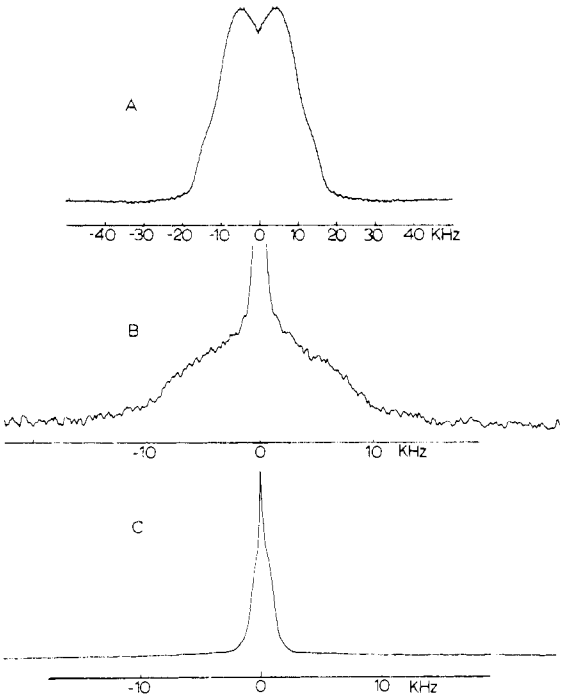

FIGURE 5: 46.06-MHz ^2H quadrupole-echo spectra of (A) polycrystalline Chol-d_6, (B) 10 mol % Chol-d_6 in the presence of equimolecular digitonin in the multibilayers, and (C) 20 mol % Chol-d_6 in the presence of digitonin (digitonin/cholesterol ratio 0.15); 15 000–36 000 transients; $\tau_1 = \tau_2 = 46$ μs for (A) and (B), and $\tau_1 = \tau_2 = 20$ μs for (C).

tations (delay time 60 μs and spectral width 10 kHz) with the 15.28-MHz spectrometer. To overcome this difficulty, we recorded a 46.06-MHz ^2H NMR spectrum of Chol-d_6 by means of the quadrupole–echo technique at the equimolecular digitonin/cholesterol ratio (Figure 5B). The quadrupole splitting of the broad component was nearly 14 kHz, which was increased ~1 order of magnitude over that in the absence of digitonin. Further, this value is rather close to that of polycrystalline Chol-d_6, 9 and 28 kHz, as shown in Figure 5A.

Figure 5C illustrates the quadrupole–echo ^2H NMR spectrum of Chol-d_6 at lower digitonin/cholesterol ratios (0.15; 20 mol % Chol-d_6) to clarify the possibility of whether the broad components were lost by the 15.28-MHz spectrometer. Obviously, only the narrow components were seen in this case (and also at 0.30; data not shown), which proved that the previous data were correctly recorded (see Figure 3D).

Saponins with a Reduced Number of Sugar Moieties. Figure 6 demonstrates that saponins with a reduced number of sugars induced less remarkable changes in the ^2H NMR spectra of Chol-d_6 compared with those of digitonin (at lower saponin/cholesterol ratios such as 0.15). Such a difference, as to the extent of the interaction with cholesterol, can be more clearly seen by ^2H NMR spectra taken at an elevated temperature (47 °C). Figure 7A shows that the ^2H NMR spectrum of Chol-d_6 was extremely broadened by digitonin at 47 °C, even at very low digitonin/cholesterol ratios. This change was irreversible. It is obvious that this change was caused by formation of the rigid complex as found at higher digitonin/cholesterol ratios (the use of a rather long delay time here, 200 μs, prevented us from observing the broad envelope as in Figure 4). As shown in Figure 7B, the ^2H NMR spectrum of Chol-d_6 in the presence of desglucodigitonin (II) was extremely broadened with an obvious reduction in the quadrupole splittings. The quadrupole splittings for glucosylgalactosyldigitogenin (III) (Figure 7) were slightly reduced at 47 °C, but the spectral pattern was found to be reproducible

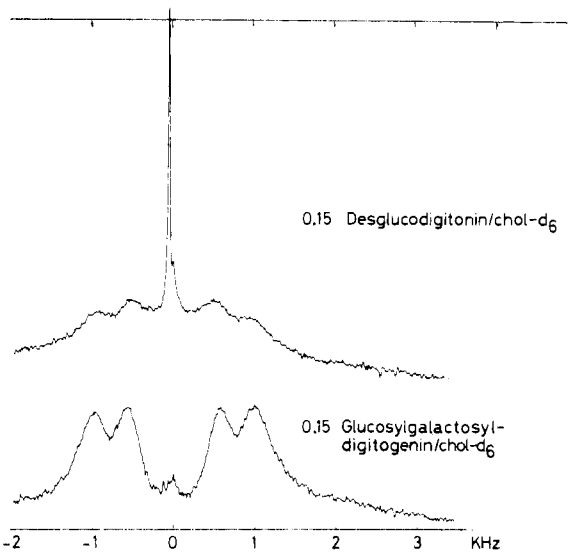

FIGURE 6: 15.28-MHz ^2H NMR spectra of Chol-d_6 (16 mol %) in egg yolk lecithin multibilayers containing digitonin analogues. Saponin/cholesterol ratio 0.15, and 160 000 transients. Top: desglucodigitonin. Bottom: glucosylgalactosyldigitogenin.

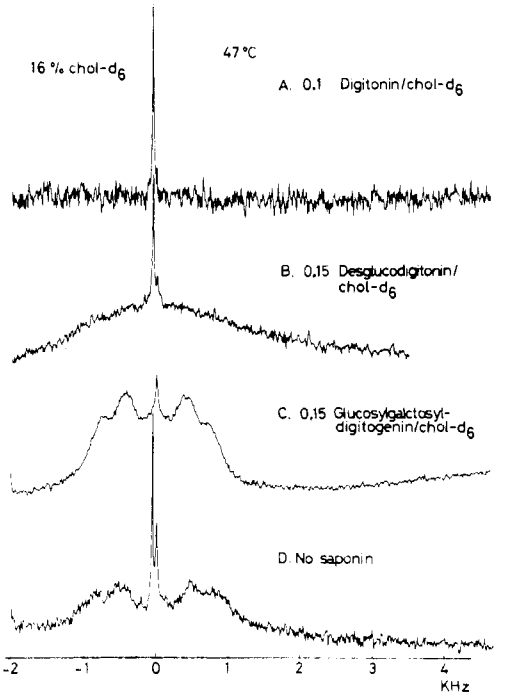

FIGURE 7: 15.28-MHz ^2H NMR spectra of Chol-d_6 (16 mol %) in the multibilayers of egg yolk lecithin in the presence of saponins at 47 °C, 9000–55 000 transients. (A) Digitonin/cholesterol ratio 0.10; (B) desglucodigitonin/cholesterol ratio 0.15; (C) glucosylgalactosyldigitogenin/cholesterol ratio 0.15; (D) no saponin.

after III was cooled to room temperature.

Incorporation of Digitonin through Single Vesicles. We tried to incorporate digitonin into the multibilayers by adding dimethyl sulfoxide solution to sonicated single vesicles containing 16 mol % Chol-d_6. After repeating the process of freezing and thawing several times, we obtained the ^2H NMR spectra exhibiting powder pattern signals, as shown in Figure 8. The spectra are very similar to the cases of direct mixing, except for the presence of a higher proportion of the central lines ascribable to the "small vesicles" (Stockton et al., 1974) or micelles.

Order Parameters and Spectral Simulation. The ^2H NMR quadrupole splittings, D_q's, are related to the order parameter,

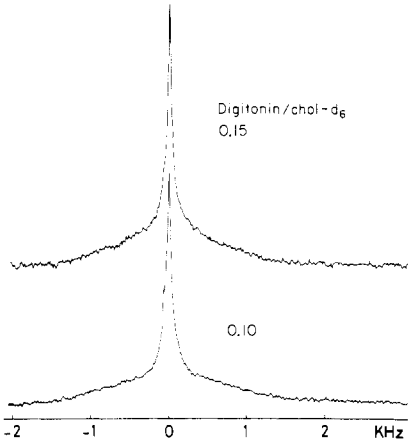

FIGURE 8: 15.28-MHz ^{2}H NMR spectra of Chol-d_6 (16 mol %) in the multibilayers of egg yolk lecithin. Digitonin in dimethyl sulfoxide was incorporated into sonicated vesicles, which were converted to the multibilayers (26 000 transients). Digitonin/cholesterol ratio: 0.15 (top); 0.10 (bottom).

Table I: Changes in Order Parameters of SA-d_3 Due to the Presence of Digitonin

digitonin/cholesterol ratio	S_{CD}	S_{mol}	rel decrease of order parameters ($\Delta S/S$)
0	0.0185 ± 0.02	0.111 ± 0.01	0
0.25	0.0178 ± 0.02	0.107 ± 0.01	0.04
0.5	0.0158 ± 0.04	0.095 ± 0.02	0.14
1.0	0.0133 ± 0.04	0.080 ± 0.02	0.28
no cholesterol and digitonin	0.0157 ± 0.02	0.094 ± 0.01	0
digitonin only[a]	0.0142 ± 0.04	0.087 ± 0.02	0.07

[a] The amount of digitonin is the same as that of the digitonin/cholesterol ratio 1.0.

S_{CD}, for the deuterium–carbon bond according to (Seelig, 1977; Mantsch et al., 1977)

$$D_q = (3/4)(e^2qQ/h)S_{CD} \quad (1)$$

where the quadrupole coupling constant e^2qQ/h is usually taken as 170 kHz for deuterium in sp^3 hybridization. The molecular order parameter, representing segmental order, for the methyl group is given by (Stockton & Smith, 1976)

$$S_{mol} = 6S_{CD} \quad (2)$$

The order parameters for SA-d_3 thus obtained are summarized in Table I. For the ^{2}H NMR spectra of Chol-d_6 in the presence of digitonin at lower digitonin/cholesterol ratios, it may be more useful to deduce the order parameters, S_{CD}'s, by spectral simulation of the powder pattern. The curve-fitting procedure by a digital computer was performed on the basis of the formula for powder pattern signals (Stockton et al., 1976)

$$g(w) = \int_0^{\pi/2} \frac{\sin\Theta\, d\Theta}{(1/T_2)^2 + [w \pm (3/8)(e^2qQ/h)S_{CD}(3\cos^2\Theta - 1)]} \quad (3)$$

where $1/T_2$ is the line width and Θ stands for the angle between the applied field and the normal to the bilayers. We assumed that the ^{2}H NMR signals of the 26- and 27-deuteriums in Chol-d_6 have different order parameters and line widths. In addition, we also included the signal from the small vesicles by

$$g(w') = \frac{1}{(1/T_2)^2 + (w')^2} \quad (4)$$

The simulated spectra, by superpositioning of two kinds of powder patterns in addition to the central peak, thus obtained are given to the right of the experimental ones (Figure 3). Comparison between the experimental and simulated spectra is very satisfactory.[2]

The relative decrease of the order parameters, $\Delta S/S$, in which S is taken as the value in the absence of digitonin, is shown in Table II. With a few exceptions, these values ($\Delta S/S$ values) are found to be generally larger for the inner pair than for the outer pair. Table II also includes the $\Delta S/S$ values for the sample containing 33 mol % cholesterol. Clearly, these values are very close to those of the 16 mol % sample if compared at the same digitonin/cholesterol ratios. Thus, the value $\Delta S/S$ does not depend on the absolute amount of Chol-d_6 but mainly on the digitonin/cholesterol ratio.

Discussion

Characterization of Digitonin–Cholesterol Interaction. The use of Chol-d_6 proved that specific interaction between digitonin and cholesterol occurs in the multibilayers (Figures 5–7). Therefore, it is clear that cholesterol is the target for the action of digitonin, in agreement with previous data (Glauert et al., 1962; Shany et al., 1974; Nakamura et al., 1979). It follows from the phase diagram obtained by the SA-d_3 probe that digitonin–cholesterol interaction can be described by the following three different stages, depending on the digitonin/cholesterol ratio.

(1) Aggregated Species (Digitonin/Cholesterol Ratio 0–0.35). We found that the quadrupole splitting of the SA-d_3 probe decreased with increasing proportions of digitonin, reaching a plateau, the quadrupole splitting of which is very close to that observed in the absence of digitonin and cholesterol. This change is well explained by segregation of cholesterol from lipids within the multibilayers, caused by interaction with digitonin, as viewed by the change in the molecular order parameter (Table I). Before reaching this plateau, however, the amount of Chol-d_6 is much in excess over that of the digitonin present in the bilayers. Nevertheless, a distinct change in the ^{2}H NMR spectra of Chol-d_6 is evident due to the specific interaction with digitonin. Clearly, this change arises from a rapid exchange process between free and associated states with digitonin, because the individual signals of the two states cannot be distinguished in both Chol-d_6 and SA-d_3 probes. Table II shows that the order parameters of Chol-d_6 in this case are decreased roughly in proportion to increasing amounts of digitonin. At the digitonin/cholesterol ratio 0.25, the relative decrease in the order parameters ($\Delta S/S$) reached 0.58, as shown in Table II (16 mol % Chol-d_6). This result is rather surprising, since digitonin–cholesterol interaction caused cholesterol to result in molecular disordering instead of the expected molecular ordering due to the complex formation. Thus, cholesterol–digitonin interaction at this stage does not mean the formation of the stable complex

[2] Alternatively, there appears another possibility that the spectral feature mainly arises from the nonzero asymmetry parameter (Chiba, 1962) owing to that the molecular motion is no longer symmetric. This possibility, however, is easily ruled out by our finding that cholesterol at this stage is not strongly complexed with digitonin but is rapidly exchanging with free cholesterol within the bilayers, as described in detail under Discussion. Furthermore, our treatment is also justified by the fact that the order parameters thus obtained are very close to those obtained directly from the ^{2}H NMR spectra of Chol-d_6 at higher digitonin/cholesterol ratios (see Figure 4 and footnote 3).

Table II: Order Parameters and Line Widths of ^2H NMR Spectra of Chol-d_6 Obtained by Spectral Simulation

concn of Chol-d_6 (mol %)	saponin	saponin/Chol-d_6 ratio	temp (°C)	inner pair			outer pair		
				order parameter	$\Delta S/S$	change of line width	order parameter	$\Delta S/S$	change of line width
10		0	28	0.0045			0.015		
16		0	28	0.0106	0	1[a]	0.019	0	1[a]
16	I	0.06	28	0.0094	0.11	1	0.017	0.12	1
16	I	0.10	28	0.0087	0.18	1.4	0.017	0.12	1.6
16	I	0.10[b]	28	0.0037	0.65	1.6	0.012	0.37	2.0
16	I	0.15	28	0.0056	0.47	1.5	0.015	0.19	2.2
16	I	0.15[b]	28	0.0037	0.65	1.8	0.012	0.37	3.4
16	I	0.25	28	0.0044	0.58	1.6	0.008	0.58	2.0
16	II	0.15	28	0.0087	0.18	1.4	0.018	0.06	1.7
16	III	0.15	28	0.0100	0.06	0.9	0.018	0.06	0.8
16		0	47	0.0085	0	1[c]	0.017	0	1[d]
16	II	0.15	47	0.0060	0.31	1.3	0.014	0.20	1.3
16	III	0.15	47	0.0073	0.11	0.7	0.013	0.21	1.4
33		0	28	0.015	0	1[e]	0.020	0	1[f]
33	I	0.10	28	0.013	0.15	0.72	0.019	0.06	0.75
33	I	0.15	28	0.011	0.33	0.90	0.017	0.18	0.75

[a] Line width 540 Hz. [b] Digitonin incorporated into sonicated vesicles and then converted to the multibilayer system by freezing and thawing. [c] Line width 700 Hz. [d] Line width 800 Hz. [e] Line width 860 Hz. [f] Line width 800 Hz.

whose stoichiometry is well-defined. This should be described as aggregated species of digitonin and cholesterol where free cholesterol is rapidly exchanging with cholesterol in the vicinity of digitonin.

(*2*) *The Intermediate Complex* (*Digitonin/Cholesterol Ratio 0.35–0.9*). Most of the cholesterol, segregated from lipid sites, should be in direct contact with digitonin, since the molecular ordering of the lipid phase is very close to that of the cholesterol- and digitonin-free lipids (Figure 2). It appears that cholesterol complexed with digitonin results in preventing the lipid phase from further disordering by digitonin, as compared with the case of cholesterol-free lipids. Here the more rigid complex is formed between digitonin and cholesterol, as manifested by the presence of the portion of the increased quadrupole splitting, in addition to the presence of the aggregated species (the narrow components).[3] It is likely that, in view of the spectral features, the complex is not composed of single species but is a mixture of a rather broad distribution of various degrees of the complexed states. The rate of the exchange among those species, however, should be much slower compared with the NMR time scale. The noticeable suppression of the peak areas of the narrow components (Figure 4) can be well explained by conversion of the aggregated species (the narrow components) into ones with remarkably increased quadrupole splitting which is unable to be seen by the 15.28-MHz spectrometer. Naturally, this observation is consistent with a change toward formation of the 1:1 complex.

(*3*) *The Equimolecular Complex*. It follows from Figure 5B that the equimolecular complex has a characteristic of the solid state as in digitonides because the order parameter is increased by an order of magnitude over that of the digitonin-free sample. This observation implies that even the side-chain methyl groups of cholesterol are immobilized by formation of the rigid complex with digitonin, in spite of the most mobile positions being the ^2H labeled ones. In other words, the terminal methyl groups of the side chain are also involved in stabilizing the digitonin–cholesterol complex. It should be emphasized that this immobilized complex is still *in* the bilayers, because the ^2H NMR spectrum of the SA-d_3 probe is also highly perturbed by the presence of the 1:1 complex.

Molecular Orders of Bilayers. It was shown that digitonin disturbed the organization of the lipids in the multibilayers of egg yolk lecithin (parts E–G of Figure 1). Obviously, digitonin fluidizes the lipid structures, as manifested by the decrease in the molecular order parameter of the SA-d_3 probe (Stockton et al., 1976; Stockton & Smith, 1976; Smith et al., 1978) (Table I) together with the formation of a considerable proportion of small vesicles or micelles (Figure 1G). The latter effect may be caused by a detergent property of digitonin. It was shown by Stockton et al. (1976) that a single peak appears when the sizes of such particles are small enough (~300 Å in diameter) to allow isotropic rotation with a correlation time shorter than 10^{-6} s. A more pronounced effect of digitonin, however, can be seen in the presence of cholesterol in the multibilayers. As pointed out already, the first reduction in the molecular order parameters of SA-d_3 was due to the segregation of cholesterol from bilayers. The second reduction in the molecular ordering is obviously caused by the formation of the 1:1 complex, because the noticeable change of the ^2H NMR spectrum of Chol-d_6 was associated. Thus, it is clear that the presence of the rigid complex results in the disordering of the bilayer structure.

The present finding is in parallel with the cases of gramicidine A′–lipid interaction (Rice & Oldfield, 1979) and protein–lipid interaction in reconstituted membranes (Seelig & Seelig, 1978; Oldfield et al., 1978b; Kang et al., 1979b) and in intact cell membranes of *Escherichia coli* (Davis et al., 1979; Kang et al., 1979a). On the contrary, a number of EPR spin-label studies showed that protein incorporated in lipids yielded a portion of the immobilized (boundary) lipids which are in intimate contact with the protein surface (Jost et al., 1973a,b, 1977; Marsh et al., 1978; Knowles et al., 1979), which is consistent with some early ^2H works (Dahlquist et al., 1977; Longmuir et al., 1977). This apparent conflict might be compromised by taking into account the difference in time scale between the two kinds of methods. Namely, the boundary lipid is in motion and disordered on the ^2H NMR time scale ($\geq 10^5$ s^{-1}) but relatively rigid as viewed by EPR (~10^8 s^{-1}) (Kang et al., 1979b). Thus, the boundary lipid

[3] In parts A and B of Figure 4 the order parameters of the narrow components are estimated directly as 0.005 and 0.12 for the inner and outer pairs, respectively. These values are comparable with those obtained by the spectral simulation for the case of the digitonin/cholesterol ratio 0.25 (0.0044 and 0.0080). Thus, it is concluded that the property of the narrow component is essentially the same as that of the aggregated species.

might be completely disordered, and ^2H NMR experiments have been interpreted in terms of a lipid population around the protein which has a lower order but is in fast exchange with the lipid of the bilayers (Seelig & Seelig, 1978). In this connection, it is interesting to compare our result with the finding by the EPR spin-label method in which saikosaponins caused an increase in the order parameters of the erythrocyte and erythrocyte ghost membranes (Abe et al., 1978). Further, the conformational disorder as judged by the ^2H order parameter does not imply that the bilayer is more fluid (Seelig & Seelig, 1978). In fact, the line broadening of the SA-d_3 probe by a factor of 2 occurred at the same time and should be ascribed to the immobilization, as discussed.

Role of the Sugar Moiety. In view of the molecular structure of digitonin, the hydrophobic aglycon moiety might be the major interaction site with cholesterol within the bilayers. It is likely that the sugar moiety should be located outside of the bilayers, far away from the major interaction site. Nevertheless, the configuration of the sugar moiety is very important in the aspect of stabilizing the saponin–cholesterol complex, as manifested by the striking difference in the relative decrease of the order parameter caused by saponins with a variety of sugar moieties (saponin/cholesterol ratio 0.15). Namely, the extent of the perturbation by the saponins with deletion of the terminal glucosyl group (desglucodigitonin, II) and of the xylosyl and glucosylgalactosyl moieties (glucosylgalactosyldigitogenin, III) is much more diminished in comparison with that of digitonin. The relative decreases in the order parameters of Chol-d_6 are 0.47 and 0.19 (digitonin), 0.18 and 0.06 (II), and 0.06 and 0.06 (III), respectively, for the outer and inner pairs. Thus, the configuration of the terminal sugar moiety plays a crucial role even at the initial stage as in the aggregated species between saponins and cholesterol.

At an elevated temperature, an accelerated rate of diffusion of cholesterol within the bilayers may be favorable for the formation of such a rigid complex as that observed at higher digitonin/cholesterol ratios. This view is supported by the observation of the irreversible change in the ^2H NMR spectra after the sample of digitonin/cholesterol ratio 0.15 (Figure 8) was heated. Still, it is surprising that only 0.1 of digitonin with respect to cholesterol causes such a drastic change of Chol-d_6 in the bilayers. For the cases of the digitonin analogues, however, the spectral change is moderate; the relative decrease in the order parameter ($\Delta S/S$) of Chol-d_6 is roughly by a factor of 2 or 3 when heated up to 47 °C. Undoubtedly, these findings indicate that the configuration of the terminal sugar groups involving xylose and glucose is crucially important to facilitate the formation of the stable complex.

Biological Significance. It is well-known that polyene antibiotics such as amphotericin B, filipin, and nystatin selectively attack fungi and other cells having a sterol-containing membrane, forming a pore permeable to ions, water, and nonelectrolytes (Andreoli & Monahan, 1968; Holz & Finkelstein, 1970; Dennis et al., 1970; Kinsky, 1970). The pore formation by amphotericin B is believed to be caused by the formation of a circular arrangement of eight amphotericin B molecules interdigitated by eight cholesterol molecues (De Kruijff & Demel, 1974). Digitonin, on the other hand, yielded an evenly folded surface as observed by an electron microscope (Ohtsuki, private communication). Regardless of the difference in morphology, we found that there appears to be a common feature in the ^2H NMR spectra of Chol-d_6 induced by saponins and polyene antibiotics; these substances generally induced a reduction in the order parameters of Chol-d_6 (unpublished experiments). The effect by polyene antibiotics, however, is much less than that of digitonin but is to the same extent as that of desglucodigitonin. It is also interesting to note that the irreversible change in the ^2H NMR spectrum of Chol-d_6 was induced by the presence of amphotericin B at the elevated temperature (47 °C). Therefore, the obvious decrease in the order parameter of Chol-d_6 should be a general feature for such substances which interact with cholesterol.

Among the three kinds of saponins, digitonin surpasses all others in hemolytic activity (Nojima et al., unpublished experiments). This activity is in parallel with the extent of interaction ($\Delta S/S$) with cholesterol. Very recently, Nakamura et al. (1979) showed that digitonin induced a noticeable change in the permeability of glucose in vesicles of egg yolk lecithin containing cholesterol but not in the case of cholesterol-free vesicles. Thus, it is likely that the formation of the immobilized complex in the membranes might be related to the hemolytic activity. One possibility is that lipids surrounded by such stable complexes might be easily removed, resulting in lysis. The formation of the stable complex, however, is not always restricted to the case of the equimolecular mixture. Even at lower digitonin/cholesterol ratios, we observed the appearance of the stable complex at 47 °C. This result suggests that the stable complex might be formed to some extent at physiological temperature when a very low amount of digitonin attacks the erythrocyte membrane containing ~27% cholesterol (Rouser et al., 1968). Naturally, a further study using erythrocyte membranes is necessary to confirm our view.

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Mechanism of Exchange of Cytochrome b_5 between Phosphatidylcholine Vesicles†

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ABSTRACT: The intervesicle exchange of cytochrome b_5 has been studied by fluorescence quenching. The binding of cytochrome b_5 to 1,2-bis(9,10-dibromostearoyl)-*sn*-glycerol-3-phosphorylcholine vesicles results in a quenching of cytochrome b_5 fluorescence whereas the fluorescence is enhanced upon binding to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine vesicles. This difference in cytochrome b_5 fluorescence upon binding was used to study the kinetics of cytochrome b_5 intervesicle exchange between the "quenching" and "enhancing" vesicles. Separation of the two cytochrome b_5–vesicle complexes by density gradient centrifugation provided direct evidence for cytochrome b_5 intervesicle exchange. Both the fluorescence assay and the density gradient assay yield the same value for the extent of cytochrome b_5 exchange, obtained after equilibration, between the two types of vesicles. Both experiments also indicate that cytochrome b_5 binds in a reversible fashion and has an equal affinity for the two types of vesicles. The kinetics of the exchange process are consistent with a mechanism involving the transfer of cytochrome b_5 through the aqueous phase and rule out a mechanism involving vesicle collision.

Considerable recent interest has been focused on the phenomenon of intermembrane transfer or exchange of membrane components. Such processes may prove to be of importance in membrane biosynthesis and may, in part, account for the observed heterogeneity in turnover rates of membrane lipids and proteins (Omura et al., 1967). Intermembrane exchange of lipids has been described in a number of model systems, occurring both spontaneously (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Doody et al., 1978; Roseman & Thompson, 1979) or in the presence of exchange proteins (Wirtz, 1974). Spontaneous intermembrane transfer of some membrane proteins has also been demonstrated recently in vitro. The exchange of cytochrome b_5 (Roseman et al., 1977) and cytochrome b_5 reductase (Enoch et al., 1977) between

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