[Chem. Pharm. Bull. 24(6)1219—1225(1976)]

UDC 541.515.08.09 : 612.014.1

Motional State of Spin-labeled Stearates in Lecithin-Cholesterol Liposomes and Their Incorporation Capability[1]

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(Received September 10, 1975)

Electron spin resonance spectra of nitroxide-labeled stearates were investigated in the presence of dipalmitoyl or dimyristoyl lecithin-cholesterol liposomes. Beside the broad resonance lines, originated from the incorporated spin probes, the spectra were characterized with the sharp three resonance lines, which were attributed to the spin probes not incorporated in the liposomal membrane. The superposed spectra were analyzed in terms of the peak height ratio of the sharp and broad lines, and also of the "order parameter," derived from hyperfine splittings of the broad lines. The two quantities changed depending on the cholesterol content in the liposomes, but appeared to behave entirely different from each other: in the dipalmitoyl lecithin-cholesterol system, the spin probes were more incorporated in the membrane of higher motional state, while, in the dimyristoyl lecithin-cholesterol, they were less incorporated. The relation was discussed and compared with permeability property of the liposomal membranes.

Introduction

Since spin labeling techniques were introduced by McConnel and coworkers in 1965,[3] an increasing number of experiments employing various spin labels have been reported. Especially. the use of nitroxide-labeled stearate in the investigation of the phospholipid membranes has brought forth an interesting new methodology to explore the structure-function relationship of biological membranes. When the nitroxide-labeled stearates are incorporated into the membrane, broad resonance lines can usually be observed with electron spin resonance (ESR) spectroscopy. An analysis of this line-shape shows that the g-, and hyperfine tensors are axially symmetric, with principal values which can be quantitatively understood on the basis of a rapid rotation about the long axis of the extended stearic acid chain.[4] Thus, from the hyperfine tensors of the broad resonance lines, one may judge the motional character of its environment such as fluidity or permeability of the membrane.

It has often been reported, however, that the broad resonance line is accompanied by the "sharp three lines': according to Butler, *et al.*,[5] the sharp three lines can be seen among liposome preparations derived from various lecithins. It was suggested by them that the membrane fluidity might give some influence on the appearance of the sharp three lines. Gaffeney[6] also reported that the sharp lines were preferentially saturated at 100 mW of microwave power. While the sharp three lines are probably due to the spin probes undergoing rapid random motion *i.e.* to those not incorporated in the membrane, it seems to be of great interest to investigate why the spin probes are to be "excluded" from the membrane, or under what conditions they are favourably incorporated into the membrane. A comparative study

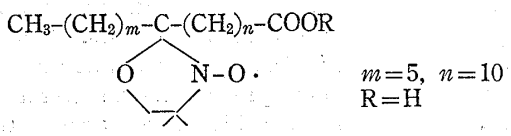
1) This work was reported at the 95th Annual Meeting of Pharmaceutical Society of Japan, Nishinomiya, April 1975.
2) Location: *Hongo 7-3-1, Bunkyo-ku, Tokyo.*
3) S. Ohnishi and H.M. McConnell, *J. Am. Chem. Soc.*, **87**, 2293 (1965); T. Stone, T. Bunkman, P. Nordio, and H.M. McConnell, *Proc. Nat. Acad. Sci. USA*, **54**, 1010 (1965).
4) W.L. Hubbell and H.M. McConnell, *J. Am. Chem. Soc.*, **93**, 314 (1971).
5) K.W. Butler, N.H. Tattrie, and I.C.P. Smith, *Biochim. Biophys. Acta*, **363**, 351 (1974).
6) B.J. Gaffeney, *Proc. Nat. Acad. Sci. USA*, **72**, 664 (1975).

of both the broad and the sharp resonance lines is expected to bring about some useful informations relating to this problem.

The purpose of the present work is to obtain further ESR evidences for the appearance of the sharp three lines as well as the broad ones with special interest in the motional character of various lecithin membranes, and to discuss its consequence.

Experimental

Materials and Experimental Procedure——Two model membranes were investigated: dipalmitoyl and dimyristoyl lecithins, purchased from Sigma Chemical Co., St. Louis, Mo. and Calbiochem., San Diego, Calif., respectively. Cholesterol, used as the additive to the lecithin, was obtained from Koso Chemical Co., Tokyo. The purity of the samples was checked by thin-layer chromatography on a silica gel plate with chloroform–methanol–water (65: 25: 5, by vol.) as the eluent. The 12-[N-oxyl-4′,4′-dimethyloxazolidine]-stearic acid (12SLS) and its methylester (12SLSM) were synthesized by the method of Waggoner, *et al.*[7] The 5-[N-oxyl-4′,4′-dimethyloxazolidine]-stearic acid (5SLS) was also synthesized by the method of Jost, *et al.*[8] The molecular formula of 12SLS is shown below:



The lecithin samples, cholesterol and spin-labels were stored, respectively, in chloroform at −20°. The method of preparation of spin-labeled liposomes is as follows: aliquotes of lecithin, cholesterol and spin-labeled stearate were mixed in a 15 ml glass tube, and subjected to rotary evaporation to coat the mixture of lecithin, cholesterol and spin-labeled stearate on the inner surface of the tube, and kept under vacuum for 1 hour to remove residual solvent. The content of spin probe was around 1 mole % of lecithin and that of cholesterol was ranged from 0 to 100 mole %.

A sufficient amount of distilled water or aqueous NaCl solution was then added to the tube so as to make the concentration of lecithin adjusted within $10^{-3}-3.5\times10^{-2}$ M. According to the phase diagram of the lecithin-water system due to Chapman,[9] our system may consist of fragments of lamellar phase dispersed in excess water, provided that each system is kept above the phase transition temperature of lecithin. The mixture was then agitated on a Vortex mixer for 60 seconds above 50° to give rise to liposomes.

The liposomal solution of different compositions of lecithin, cholesterol and spin-labeled stearate was transferred into a sealed capillary tube. ESR measurements were carried out at room temperature (18—20°) unless otherwise stated. A JEOL-PE-1 spectrometer with 100 kHz field modulation was used.

Results

Dipalmitoyl Lecithin-Cholesterol Liposome

The dipalmitoyl lecithin-liposomes containing various amounts of cholesterol and 0.86 mole % 12 SLS were prepared above 50° in 0.15M NaCl aqueous solution. After left standing for one hour at room temperature, a 40 μl liposomal solution was transferred in the capillary tube and subjected to ESR measurements. Preliminary experiments showed that the broad resonance signal is almost imperceptible with the liposome sample containing no cholesterol but the sharp three lines: it became perceptible with increase in the cholesterol-content in the liposome. In Fig. 1A is illustrated a typical ESR spectrum obtained from the sample containing 16 mole % cholesterol, while Fig. 1B shows that of the liposome containing an equimolar amount of cholesterol.

It is apparent from the figure that the spectra are superposed with the broad and sharp resonance lines, respectively. However, the intensity of the sharp lines was rather weak, in the equimolar cholesterol-containing liposome, while it appeared to be very strong with the lipo-

7) A.S. Waggoner, T.J. Kingzett, S. Rottschaefer, O.H. Griffith, and A.D. Keith, *Chem. Phys. Lipids*, **3**, 245 (1969).
8) P. Jost, L.J. Libertini, V.C. Hebert, and O.H. Griffith, *J. Mol. Biol.*, **59**, 77 (1971).
9) D. Chapman in G.B. Ansell, L.N. Hawthorne, and R.M.C. Dawson (Eds.), "Form and Function of Phospholipids," B. B. A. Library, Vol. 3, Elsevier Scientific Publ. Co., Amsterdam, 1973, p. 117.

some of less cholesterol or almost exclusively in the absence of cholesterol. When the liposome was subjected to dialysis against a 0.15M NaCl aqueous solution, the sharp lines were found to remain in the "dialyzate." Furthermore, these sharp lines entirely vanished when the liposome was treated with excess L-ascorbic acid: only the broad lines were alive, indicating that the spin probes are partially trapped or incorporated in the membrane. The observation may lead us to suggest that the sharp three lines are originated from the spin probes not incorporated in the liposomal membrane, and hence are tumbled freely in the environmental water region. Some spin-labels might, however, interact weakly with the surface of liposomes, because the sharp three lines were still perceptible with the pellet obtained by an ultra-centrifugation of the liposomal solution (100000 ***g***, 60 min).

Assuming that the sharp three lines are due to the excluded spin probes from the liposomal membrane, while the broad ones are from those incorporated, the corresponding ESR parameters were investigated over liposomal solutions consisted of various amounts of cholesterol. As a measure of the ability of excluding the spin-labeled stearate, the peak height ratio of the sharp and broad lines in the lower magnetic field, h_f/h_i, (see Fig. 1) was adopted.

On the other hand, the quantity, S, designated as the socalled "order parameter," was employed with respect to the broad signal as a relative index of the motional character or disorderliness of the membrane.[4,10] The quantity, S, is defined by

$$S = \frac{A_{//} - A_{\perp}}{A_{zz} - A_{xx}}$$

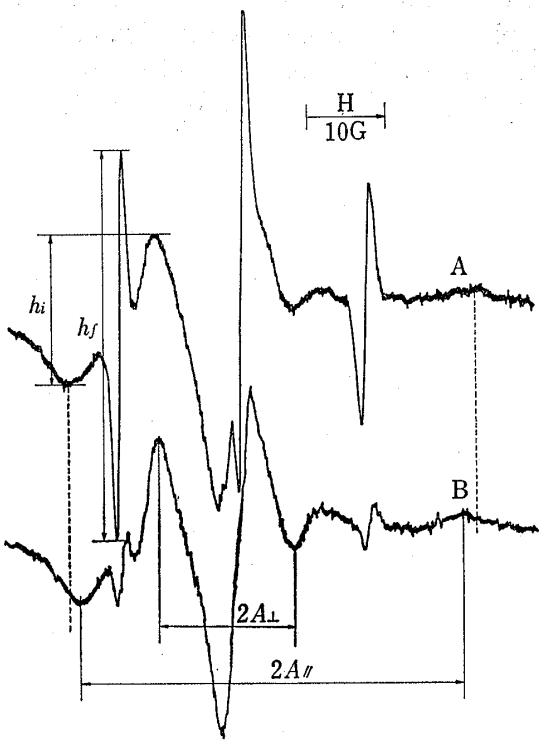


Fig. 1. ESR Spectra of Spin-labeled Stearate (12SLS) in the Presence of Dipalmitoyl Lecithin-Cholesterol Liposome in 0.15 M NaCl Aqueous Solution measured at Room Temperature

cholesterol : lecithin=0.16:1.0 (A), 1.0:1.0 (B)

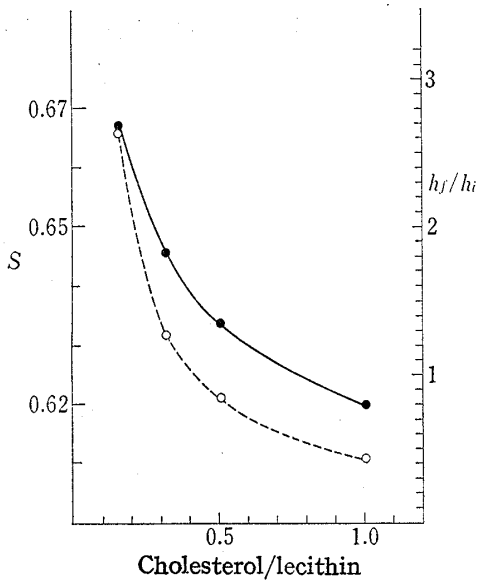


Fig. 2. The Relation Between the Order Parameter, S, or the Peak Height Ratio, h_f/h_i, and the Cholesterol-Dipalmitoyl Lecithin Mole Ratio

—●—: S, ---○---: h_f/h_i

10) A. Saupe, G. Englert, and A. Pova, *Advan. Chem. Ser.*, **No. 63**, 51 (1965); S. Schreier-Muccillo, D. Marsh, H. Dugas, H. Schneider, and I.C.P. Smith, *Chem. Phys. Lipids*, **10**, 11 (1973).

where $A_{//}$ and $A_{\perp}$ are the parallel and perpendicular components of hyperfine splittings (see Fig. 1), and A_{zz}, A_{xx} are the motional averages of the nitroxide hyperfine tensors in the directions parallel and perpendicular to its long molecular axis, and assumed to be 30.8 and 5.8 gauss, respectively.[4] Thus, when S is less than unity or $A_{//}-A_{\perp}<A_{zz}-A_{xx}$, the spin probe is regarded as more flexible.

Fig. 2 shows the relationship between h_f/h_i or S of the lecithin liposome and its cholesterol content. It is quite clear from Fig. 2 that both h_f/h_i and S monotonously decrease with increase of the cholesterol-content in the membrane. A behaviour of S similar to this has been reported on addition of cholesterol to dipalmitoyl lecithin liposome[10] without showing detailed data.

The lecithin-cholesterol interaction in water has been investigated by differential scanning calorimetry,[11] X-ray diffraction[12] and NMR.[13] While further informations are still required, it is currently believed that the addition of cholesterol to the dipalmitoyl lecithin in water causes a reduction in the cohesive forces between adjacent hydrocarbon chains of the lecithin, leading to a fluidization of the chains. The decay of S on addition of cholesterol, as observed in Fig. 2, may therefore be in harmony with such a concept. On the other hand, the observation that the quantity, h_f/h_i, also decreases on addition of cholesterol could be understood in such a way that the spin-labeled stearate favours a disordered membrane rather than to remain in an ordered one. The relationship of this kind was reproducible for any dipalmitoyl lecithin-cholesterol liposome left standing from 1 to 24 hours at room temperature.

Dimyristoyl Lecithin-Cholesterol Liposome

The dimyristoyl lecithin-cholesterol liposomes containing 2.4 mole % 12SLS were prepared in excess distilled water. After left standing for one hour at room temperature, ESR spectra of the liposomal solution were investigated. Typical spectra are shown in Fig. 3, where it is noticed that the sharp three lines are pronounced in the 30 mole % cholesterol-containing liposome (B) rather than in its absence (A) contrary to the case of dipalmitoyl lecithin liposome.

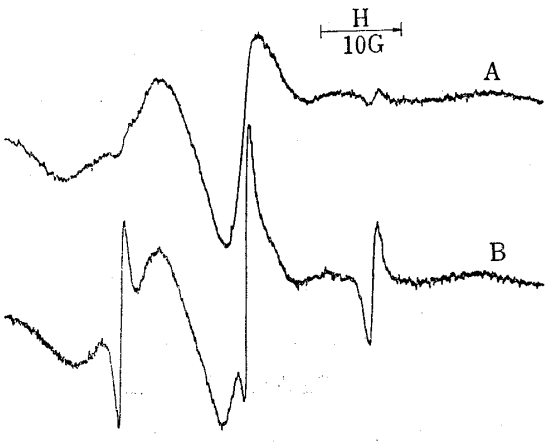


Fig. 3. ESR Spectra of the Spin-labeled Dimyristoyl Lecithin Liposome with (B) or without (A) Cholesterol in an Aqueous Solution measured at Room Temperature

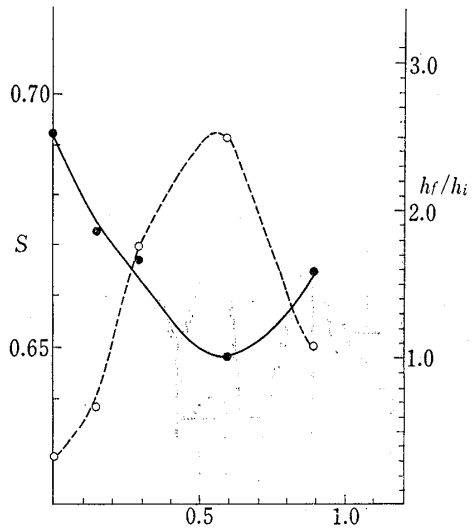


Fig. 4. The Relation between S or h_f/h_i and the Cholesterol-Dimyristoyl Lecithin Mole Ratio

—●—: S, ---○---: h_f/h_i

11) B.D. Ladbrooke, R.M. Williams, and D. Chapman, *Biochim. Biophys. Acta*, **150**, 333 (1968).
12) D. Chapman, "The Structure of Lipids by Spectroscopic and X-ray Techniques," Methuen, London, 1965.
13) A. Darke, E.G. Finer, A.G. Flook, and M.C. Fillips, *J. Mol. Biol.*, **63**, 265 (1972).

Since only the sharp three lines were present in the latter liposome (without cholesterol), it is quite clear that incorporation capability of 12SLS depends on lecithin.

The order parameter, S, as well as the peak height ratio, h_f/h_f, were plotted against the cholesterol-content in Fig. 4. It was found, then, that the two quantities behave in an entirely different manner from each other. When cholesterol was present in the liposome from 0 to 60 mole % of lecithin, the order parameter, S, decreased similarly as the dipalmitoyl case while the peak height ratio, h_f/h_i, appeared to increase, *i.e.* in disagreement with the dipalmitoyl lecithin-cholesterol system.

On further addition of cholesterol to the lecithin beyond 60%, the feature was reversed: S turned out to increase while h_f/h_i appeared to decay.

Thus, in the system of dimyristoyl lecithin-cholesterol, it must follow from the foregoing argument that the spin-labeled stearate does not favour the membrane of disordered state. The reversal relation between S and h_f/h_i was again reproducible with in 1—24 hours after preparations: it was time-independent.

Experimental Factors Affecting S and h_f/h_i Parameters

Before discussing the peculiar relation between S and h_f/h_i of lecithin liposome, some additional investigations may be mentioned concerning the effect of the sort of spin probe on the appearance of the respectively sharp and broad resonance lines in the spectra. The dipalmitoyl lecithin liposome was used for this purpose. Both 12SLSM and 5SLS are less soluble in the aqueous solution than 12SLS. The intensity of the sharp three lines was indeed weaker for 12SLSM and 5 SLS than for 12SLS, leading us to confirm that the sharp three lines may be due to the spin probes in the aqueous phase.

The appearance of the sharp three lines was temperature-dependent: the intensity was weaker to an extent when measured at 23° than at 15° with respect to the dimyristoyl lecithin liposome, indicating that the spin-labeled stearates are favourably incorporated in the liposomal membrane of thermally excited character (Fig. 5). The temperature-dependent incorporation of spin probe seems to be general. However, because of the measurements respectively above and below the phase transition temperature,[9] the interpretation should not be confused from that given at a constant temperature, *i.e.* that derived from the addition of cholesterol below the phase transition temperature. The effect of cholesterol addition on S and/or h_f/h_i above the phase transition temperature is now under investigations.

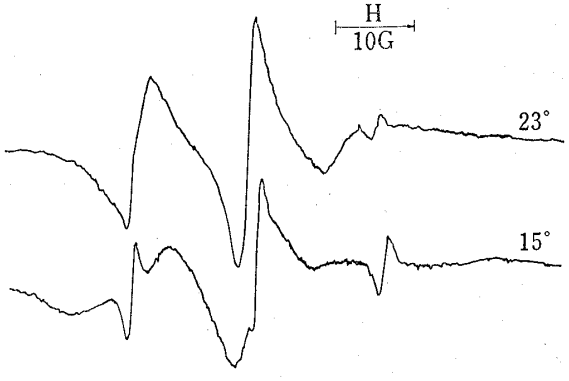


Fig. 5. The Temperature-Dependent ESR Spectra of the Spin-labeled Dimyristoyl Lecithin Liposome

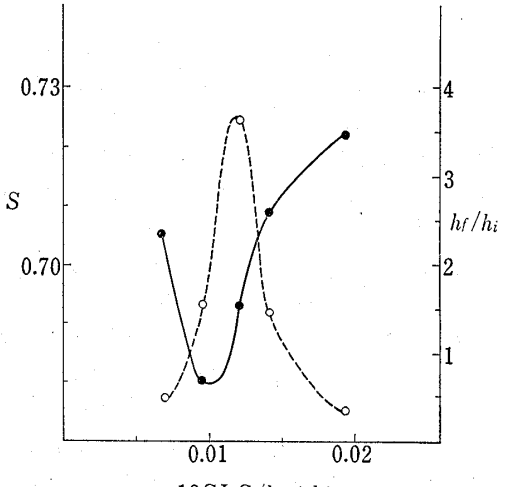


Fig. 6. The Relation between S or h_f/h_i and the 12SLS-Dimyristoyl Lecithin Mole Ratio

—●—: S, ---○---: h_f/h_i

The dimyristoyl lecithin liposomes containing various amounts of 12SLS were prepared in the absence of cholesterol, and changes in S and h_f/h_i were investigated against the concentration of 12SLS. The result is shown in Fig. 6, where it should be noted that the spin probes themselves have a drastic influence on S and h_f/h_i even if their concentration is very small, and moreover that S changes inversely with h_f/h_i again in an analogous manner as the cholesterol addition shown in Fig. 4. Thus, it goes from the result that the incorporation of spin-labeled stearate is favoured in the membrane of ordered state.

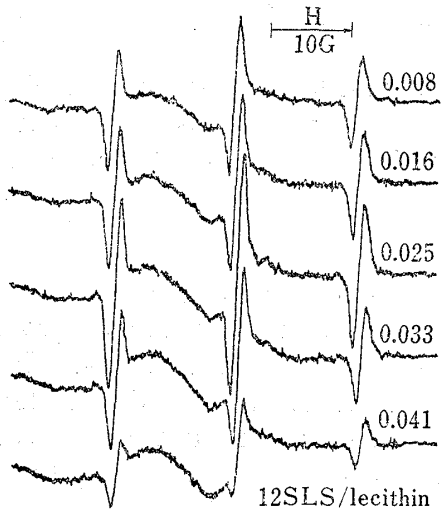


Fig. 7. ESR Spectra of Dipalmitoyl Lecithin Liposomes Containing Various Amounts of 12SLS

The effect of 12SLS addition was also investigated on the dipalmitoyl lecithin. Unfortunately, however, the resolution of broad lines was so poor to permit the calculation of S, although changes in h_f/h_j could qualitatively be judged from the spectra (Fig. 7). On addition of 12SLS, h_f/h_i seems to behave similarly as the case of dimyristoyl lecithin.

The effect of liposomal concentration on the appearance of spectra was investigated within 5.0×10^{-3}—3.5×10^{-2}M dipalmitoyl lecithin in the absence of cholesterol. The amount of 12SLS was kept constant at 1 mole % of lecithin. If the spin probes are to be partitioned between the liposomal membrane and the aqueous phase, it may well be expected that sharp three lines due to the spin probes in the aqueous phase should predominate in the lower concentration range of lecithin, because the spin probes may move to the aqueous phase having more room for them. In agreement with this, the sharp three resonance lines were almost imperceptible in the spectrum, leaving only the broad signal behind, at the higher concentration (3.5×10^{-2}M) of lecithin. The h_f/h_i can, therefore, be considered to be a measure of the partition of spin-labeled stearate between the liposomal membrane and the aqueous phase.

Discussion

The phase diagrams of the different chain length lecithin-water systems are essentially equivalent but characterized with different phase transition temperatures. For example, the dipalmitoyl lecithin in excess water "melts" at 41° while the dimyristoyl lecithin does around 23°,[9] showing that the lecithin of shorter chain length is "soft" or that water molecules can readily penetrate into the layers of the dimyristoyl lecithin even around room temperature. In accordance with this, one of the present authors[14] has already shown that the permeation of glucose through the dimyristoyl lecithin liposome is remarkably enhanced at the temperature range from 20 to 23°. The glucose permeation through the dipalmitoyl lecithin liposome was, accordingly, enhanced at 38—40° *i.e.* at its phase transition temperature in excess water. It seems very likely, therefore, that permeation property through the liposomal membrane is closely correlated with some disorderliness of the molecular assembly that constitutes a gel.

The addition of cholesterol to lecithin is regarded to promote the permeability of the membrane: studies on dipalmitoyl lecithin-cholesterol-water system by differential scanning calorimetry indicated that the transition temperature between the gel and the lamellar liquid crystalline phase is lowered by the addition of cholesterol.[9] The enhancing effect of cholesterol

14) K. Inoue, *Biochim. Biophys. Acta*, 339, 390 (1974).

incorporation on the glucose permeation was again confirmed by one of us[14] with dipalmitoyl and dimyristoyl lecithin liposomes, respectively. However, the effect was found to be highly complicated depending on the degree of cholesterol incorporation. In this connection, it is particularly interesting to note that the enhancing effect of cholesterol, in the case of dimyristoyl lecithin liposome, reaches maximum around 15 mole % in contrast to the dipalmitoyl lecithin liposome. As was shown already in the present work, the order parameter, S, reached minimum when cholesterol or even spin-labeled stearate was added to the dimyristoyl lecithin, although such a distinct tendency was not observed from the dipalmitoyl lecithin liposome at room temperature. Since the low order parameter is associated with a disordered state of the membrane, permeability property seems to be closely correlated with disorderliness of the membrane.

It might be desirable in the investigation of liposomal membrane that the spin probes do not perturb the membrane properties. However, te incorporation of a very small amount of spin-labeled stearate in the dimyristoyl lecithin appeared to cause S to vary to a remarkable extent. The dimyristoyl lecithin liposomal membrane seems, therefore, to be extremely structure-sensitive especially around room temperature.

The partition of spin probes between the the liposomal membrane and the surrounding aqueous phase can be determined, in principle, by the chemical potential of the spin probe in each phase. Here, it is assumed, for simplicity, that the motional and/or reorientional character of spin probe, as influenced by its surrounding plays a predominant role in the partition. Thus, a given spin probe may be incorporated more favourably in the membrane of higher disorderliness, because it has more freedoms there. The result shown in Fig. 2 indicated that h_f/h_i (an index of the partition of spin probe) decreases in parallel with S (a relative index of disorderliness of membrane). Thus, the relationship could be "naturally" interpreted.

On the other hand, it appeared to be "unnatural" in the case of dimyristoyl lecithin liposome: h_f/h_i changed reversely with S. The relation would imply that the spin probe favours an ordered membrane against the previous interpretation.

According to Chapman,[9] the lecithin-cholesterol-water system gives rise to no phase transition temperature: the system is regarded as an intermediate fluid condition between the gel and the lamellar liquid crystalline phase. If so, a number of fragmental phases would be present, in particular, in the dimyristoyl lecithin cholesterol-water system kept near the room temperature. The system is expected to be highly inhomogeneous and characterized with a lot of microphase boundaries. It is tentatively speculated that the unusual partition of spin-labeled stearate as observed from the dimyristoyl lecithin-cholesterol-water system is associated with such a highly inhomogeneous property of the liposomal membrane, and determined predominantly by the energy of incorporation although further investigations are obviously required for the elucidation of molecular mechanism.

Acknowledgement One of the authors (H. U,) wishes to thank Drs. H. Yamamoto and M. Setaka of this laboratory for their kind advices made during the course of the present work. He also should like to thank Takeda Chemical Co. for the financial support given to him.