N.m.r. study of egg yolk lecithin in aromatic solvents. Magnetic nonequivalence in the methylene protons of the fatty acyl chains.

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^1H and ^{13}C n.m.r. chemical shifts and also spin-lattice relaxation times of phospholipids, mainly egg yolk lecithin, were measured in organic solvents, especially aromatic solvents. The use of aromatic solvents promotes the difference in the magnetic shielding environment of the internal methylene protons of the fatty acyl chains and as a result, a doublet peak for the methylene groups was observed in the solvents, especially mesitylene. The ^1H and ^{13}C n.m.r. T_1 measurements indicate the remarkable gradient in the motional freedom along the fatty acyl chains as well as the marked decrease in the motional freedom of the polar head group and also, the high-field component of the doublet peak for internal methylene protons has a shorter T_1 value than the low-field component. Thus, it was concluded that the low-field component in the doublet comes from the methylene protons located relatively in the neighbourhood of the carbonyl groups and the high-field component the methylene protons located relatively in the neighbourhood of the terminal methyl groups of the fatty acyl chains.

INTRODUCTION

A philospholipid bilayer is known to be a common structural element of biomembrane systems and to clarify the physical state of the hydrocarbon region of the fatty acyl chains seems to be important in connection with the membrane function and cell viability[1]. For the purpose, it is very useful to detect the differences in the environment among the methylene groups in the fatty acyl chains.

Recently Roberts *et al.*[2,3] have found a large chemical shift difference between the α-methylene groups of the two fatty acyl chains of phospholipids in Triton X-100/phospholipid mixed micelles using ^1H n.m.r. spectroscopy and have suggested that phospholipid molecules adopt a unique conformation in the miceller environments. In the conformation, the *sn*-1 α-methylene protons had indistinguishable chemical shifts and were in a more hydrophobic (shielded) environment than the strongly differentiated protons of the *sn*-2 α-methylene group. However, only the broad and asymmetric lineshape has been observed for the main methylene peak of the fatty acyl chains using 360 MHz n.m.r. apparatus[4].

The n.m.r. spectral behaviour of *n*-alkane chains would be the basis for the understanding of the behaviour of the fatty acyl chains in lecithin. Liu[5] has observed that the singlet proton peak associated with the methylene groups of *n*-alkane chains is split into a doublet in 1-chloronaphthalene solutions for chains larger than 15 carbons. Ando and Nishioka[6] have interpreted this phenomenon in terms of non-averaged ^1H chemical shift of the methylene protons caused by the strong steric interaction between the *n*-alkane molecule and the solvent on the basis of theoretical calculations of the ^1H chemical shifts of methylene and methyl protons of *n*-alkanes. Campa *et al.*[7] have observed doublet peaks for the methylene protons of *N*-(*n*-alkyl) maleimide in benzene when $n \geqslant 10$ and interpreted this result in terms of the same mechanism as in the case of *n*-alkane. Most recently, Winnik *et al.*[8] have reported three broad peaks for internal methylene protons of the octadecyl ester of benzophenone-4-carboxylic acid, in 1-chloronaphthalene and have suggested that these splittings are due to solvation effects by the aromatic solvent associated with the chain ends. Thus, the use of aromatic solvent promotes the difference in the magnetic shielding environment of the internal methylene groups.

In this paper, ^1H and ^{13}C n.m.r. chemical shifts and also spin-lattice relaxation times of phospholipids, mainly egg yolk lecithin, were measured in organic solvents, especially aromatic solvents, to clarify the physical state, including the motional freedom of the fatty acyl chains of the phospholipid molecule through a detailed analysis of the doublet peak observed for the internal methylene groups.

EXPERIMENTAL

Egg yolk lecithin (EYL) was isolated from fresh egg yolk according to the method of Singleton *et al.*[9] and the purity was checked by thin-layer chromatography. When EYL was dissolved in the solvent, an insoluble-fraction remained in all the solutions. The insoluble white powder was eliminated by centrifugation and the clear supernatant solutions were used for n.m.r. measurements. The ^1H n.m.r. measurements were carried out at least two times for each solution which was prepared twice by the same procedure. The spectra were reproducible within experimental error. Dipalmitoyl lecithin (DPL) was obtained from Fluka AG, Buchs, and *n*-docosane, mono- and triolein from Tokyo Kasei Company, Ltd. The solvents, CCl_4, toluene, *p*-xylene, mesitylene and 1-chloronaphthalene were also purchased from Tokyo Kasei

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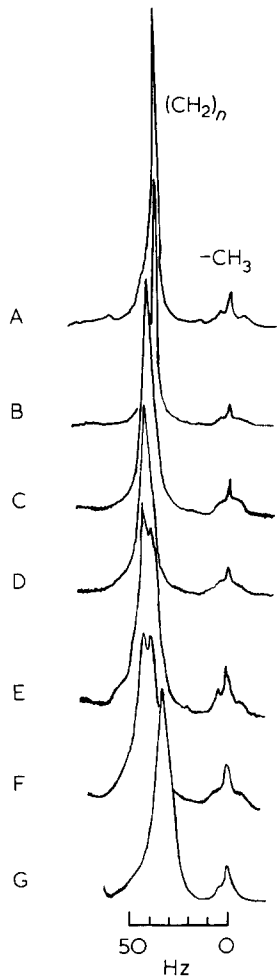


Figure 1 ^{1}H n.m.r. spectra of hydrocarbon region of egg yolk lecithin in various organic solvents at 25°C and at 10 w/v %. Solvents; (A) CCl_4, (B) $CDCl_3$, (C) C_6D_6, (D) toluene, (E) p-xylene, (F) mesitylene and (G) 1-chloronaphthalene. The chemical shift of the terminal methyl protons is taken as a standard reference

Company, Ltd., and $CDCl_3$ and C_6D_6 from CEA, France. The n.m.r. spectra were recorded with either a JOEL PS-100 spectrometer equipped with PFT-100 Fourier transform unit operating at 100 MHz (^{1}H) or at 25.14 MHz (^{13}C). ^{1}H and ^{13}C n.m.r. spin-lattice relaxation times, T_1s, were measured by using the standard inversion recovery [180° ~ τ ~ 90°] technique, where τ is the delay time between the 180° and 90° pulses. Prior to the measurement of the ^{1}H n.m.r. T_1, the atmospheric oxygen dissolved in the samples was removed by several freeze–pump–thaw cycles in an n.m.r. tube and then, the sample tube was sealed off under a vacuum, whereas the samples were not degassed in the ^{13}C n.m.r. T_1 measurements. The estimated errors in T_1 values were approximately ±10%. The n.m.r. measurements were carried out at 25°C except for the temperature variable experiments.

RESULTS AND DISCUSSION

Appearance of the doublet peak for methylene protons of egg yolk lecithin

The formation of inverted micellar structures in which the polar head groups are tightly packing was presumed in all the organic solvents used here since the observed choline–methyl proton signals of EYL molecules were much broader than those observed in D_2O solution[10,11]. The presumption will be supported from the T_1 observation of the lecithin as described below.

Figure 1 shows the ^{1}H n.m.r. spectra of the hydrocarbon chain region of EYL in some organic solvents. The methylene protons gave a single sharp signal in CCl_4 and $CDCl_3$ solutions, while an asymmetric lineshape was observed in C_6D_6 solution. The phenomenon of the latter is clearly evident in the toluene solution. In *p*-xylene and mesitylene solutions the methylene signal splits clearly into a doublet peak. The resonance position of the high-field component in the doublet measured from the terminal methyl peak of the fatty acyl chain is coincident with that of the single methylene peak in CCl_4 and $CDCl_3$, and the intentsity ratio of high- to low-field components in the doublet increases as number of substituent methyl groups on the benzene ring increases. Thus, a doublet peak was also observed in the ^{1}H n.m.r. spectra of internal methylene groups of the fatty acyl chain of EYL in some aromatic solvents similar to the appearance of the doublet peak of *n*-alkanes in l-chloronaphthalene[5] although only line broadening of the methylene signal was observed in the 1-chloronaphthalene solution of EYL.

Moreover, the temperature and concentration dependences of the doublet methylene peak were examined in mesitylene as shown in *Figure* 2. The relative intensity of the high-field component decreases with rise in temperature or with dilution. And at 1.3 w/v% solution further components clearly appear at upfield of the doublet. Thus, the methylene peak was not always observed as a doublet even in mesitylene, which means the presence of more than two different magnetic environments in the fatty acyl chain of EYL in some experimental conditions.

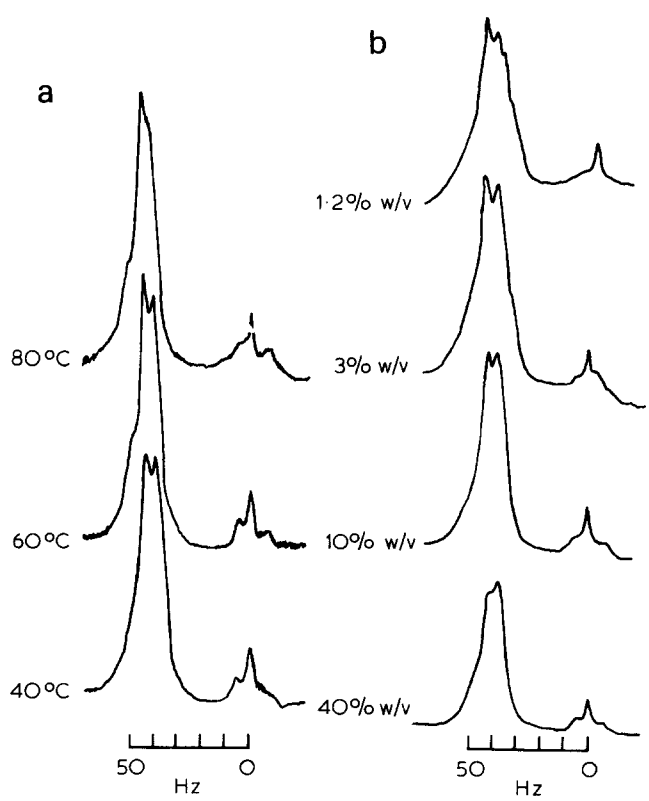


Figure 2 Temperature (a) and concentration (b) dependence of ^{1}H n.m.r. spectra of egg yolk lecithin in mesitylene. (a) Concentration, 10 w/v %; (b) temperature, 25°C. The chemical shift of the terminal methyl protons is taken as a standard reference

Table 1 T_1 relaxation times (s) of protons in egg yolk lecithin in $CDCl_3$, C_6D_6 and mesitylene at 25°C

Proton	$CDCl_3$ (10 w/v %)	C_6D_6 (10 w/v %)	$(CH_3)_3C_6H_3$ (30 w/v %)
$-\underline{CH}=\underline{CH}-$	1.3	1.7	–
$PO\underline{CH_2}\underline{CH_2}$	0.05	0.11	–
$-\underline{CH_2}OCO$ or $\underline{CH_2}N^+$	0.12	0.11	–
$(\underline{CH_3})_3\underline{N}^+$	0.11	0.13	0.12
$=\underline{CH}-\underline{CH_2}-CH=$	0.88	1.5	–
$-\underline{CH_2}CO$	0.51	0.39	–
$-\underline{CH_2}CH=$	0.76	0.70	–
$-(\underline{CH_2})_n-$			
Low Field	–	0.49	0.49
High Field	0.92	0.78	0.64
$-\underline{CH_3}$	2.3	2.1	1.8

* An estimate of the error is approximately ±10% for T_1

^1H n.m.r. spin-lattice relaxation times of egg yolk lecithin

Table 1 summarizes the ^1H n.m.r. T_1 values of EYL in $CDCl_3$, C_6D_6 and mesitylene solutions. The assignment of ^1H n.m.r. spectra of EYL was carried out according to refs. 12, 13 and 14. T_1 values for small peaks of EYL were not calculated in proton containing solvents, mesitylene because of the relatively large experimental error. $N^+\underline{CH_3})_3$ T_1 values in these solvents is 0.11–0.13 s, which are considerably shorter than that of sonicated DPL in D_2O (0.26 s) in 25°C [10,15]. The T_1 values for $\underline{CH_2}OCO$, $\underline{CH_2}N^+$, $PO\underline{CH_2}\underline{CH_2}$ and $\underline{CH_2}CO$ protons are considerably shorter than those for internal methylene protons in the fatty acyl chains.

It should be noted that there is a marked difference in the ^1H T_1 values between high- and low-field components in the doublet peak for internal methylene protons of EYL in C_6D_6 and mesitylene solutions. Although the doublet peak was not clearly observed in the ^1H n.m.r. spectrum of the C_6D_6 solution, the presence of two or more peak components in the methylene signal was confirmed in the partly relaxed ^1H spectra as shown in *Figure* 3. As one of the examples, two ^1H T_1 values for the methylene peak in the C_6D_6 solution were obtained by plotting the intensities of the corresponding peaks to the resonance positions of the doublet peak in mesitylene against the delay time, τ. T_1 value for the high-field component is longer than that for the low-field one by 0.15 and 0.29 s in mesitylene and C_6D_6 solutions, respectively. The T_1 value of the single methylene peak in $CDCl_3$ is still longer than that of the high-field component in mesitylene and C_6D_6 solutions.

^{13}C n.m.r. spectra and ^{13}C spin-lattice relaxation times of egg yolk lecithin

Figure 4 shows ^{13}C n.m.r. spectra of EYL in $CDCl_3$ and mesitylene solutions. The assignments were carried out according to refs. 13, 16 and 17. Two marked differences between the spectra in these solutions could be identified. One is the absence of the chemical shift difference between the carbonyl resonances of α and β-fatty acyl chains in mesitylene, although a large chemical shift difference between these resonances, 0.4 ppm, was observed in $CDCl_3$. Another is a considerable line broadening of the $\underline{CH_2}CO_2$ peak in mesitylene compared with that in $CDCl_3$. The ^{13}C n.m.r. chemical shifts and ^{13}C T_1 values observed in these solvents were listed in *Table* 2. Also the plots of NT_1 vs. approximate fatty acyl chain position are given in *Figure* 5 where N is the number of protons bonded to a given carbon. In both the $CDCl_3$ and mesitylene solutions, there is a marked decrease in the motional freedom of polar head groups from $\underline{CH}OCOR$ to $N^+\underline{CH_3}$ groups, while there is an increase in the mobility along the fatty acyl chain toward the terminal methyl group[16].

In connection with the appearance of the doublet methylene peak in the ^1H n.m.r. spectra of EYL in mesitylene, a comparison of the ^{13}C T_1 behaviour of EYL in $CDCl_3$ with that in mesitylene was done. The difference in the motional freedom was not observed for the polar head group, that is, $\underline{CH}OCOR$, $\underline{CH_2}OP$ choline, $\underline{CH_2}OP$ glycerol, $N^+\underline{CH_2}$ and $N^+\underline{CH_3}$ carbons between in both solvents. NT_1 values of the carbonyl and $\underline{CH_2}CO_2$ groups in mesitylene, however, are considerably shorter than those in $CDCl_3$. This trend may be correlated with the marked line broadening of the $\underline{CH_2}CO_2$ group in mesitylene as suggested above. This means that the mobilities of the carbonyl group and methylene groups which are in the neighbourhood of the carbonyl group along the fatty acyl chain are considerably restricted compared with those in $CDCl_3$. Also NT_1 of main methylene carbons in mesitylene is shorter than in $CDCl_3$.

Interpretation of the doublet methylene peak of egg yolk lecithin in aromatic solvents

The doublet peak for internal methylene protons in the fatty acyl chains of EYL was observed in aromatic

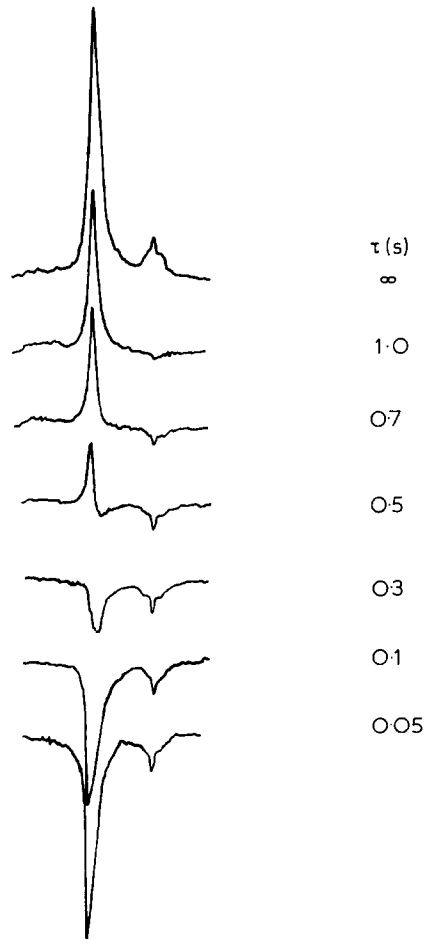


Figure 3 Partially relaxed ^1H n.m.r. spectra of egg yolk lecithin in C_6D_6 at 25° C, where τ is the delay time between the 180° and 90° pulses

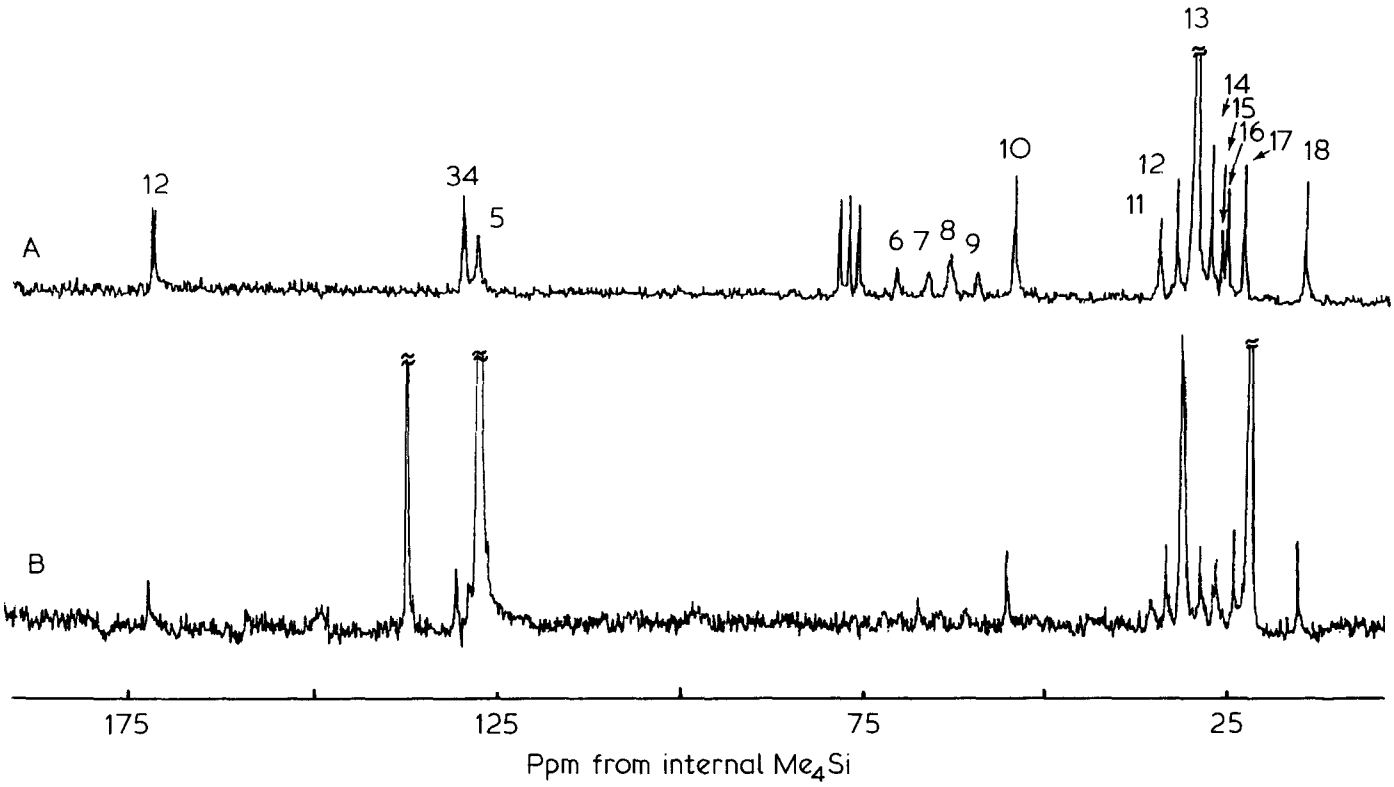


Figure 4 ^{13}C n.m.r. spectra of egg yolk lecithin in (A) $CDCl_3$ and (B) mesitylene at 25°C. The assignment of the peaks is shown in *Table 2* and the chemical shift is represented downfield from internal Me_4Si

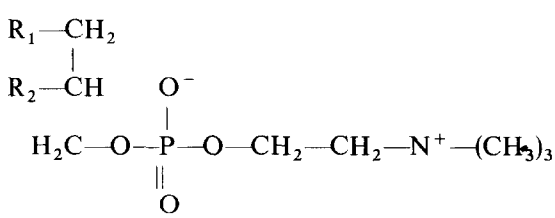
Table 2 T_1 relaxation times[a] of ^{13}C nuclei in egg yolk lecithin in 30 w/v % $CDCl_3$ and mesitylene solutions at 25°C

Peak[b] No.	Assignment	Chemical shift (ppm)[c]		T_1 (s)	
		$CDCl_3$	$(CH_3)_3C_6H_3$	$CDCl_3$	$(CH_3)_3C_6H_3$
1	Carbonyl α	173.5	173.2	2.4	1.2
2	β	173.1			
3	$-CH=\underline{C}HCH_2CH_2-$	130.1	130.1	0.97	0.70
4		129.9			
5	$-CH=\underline{C}HCH_2\underline{C}H=$	128.4	128.4	1.2	–
6	$\underline{C}HOCOR$	71.1	71.4	0.11	0.12
7	$\overline{N^+}\underline{C}H_2-$	66.6	66.7	0.05	0.08
8	$\underline{C}H_2OP$ glycerol $\underline{C}H_2OCOR$	63.4	63.9	0.09	0.04
9	$\underline{C}H_2OP$ choline	59.6	59.9	0.09	0.07
10	$\overline{N^+}(\underline{C}H_3)_3$	54.7	54.5	0.14	0.15
11	$-\underline{C}H_2CO_2-$	34.5	34.6	0.35	0.10
12	$-\underline{C}H_2CH_2CH_3$	32.1	32.5	1.8	2.0
13	$-(\underline{C}H_2)_n-$	29.9	30.5	0.74	0.57
14	$-CH_2\underline{C}H_2CH=CH-$	27.5	27.8	0.76	0.78
15	$-CH=\underline{C}HCH_2CH=CH-$	26.0	26.0	0.82	0.94
16	$-\underline{C}H_2CH_2CO_2$	25.2	25.6	0.42	0.33
17	$-\underline{C}H_2CH_3$	22.8	23.2	2.2	2.2
18	$-\underline{C}H_3$	14.1	14.4	3.2	2.7

[a] An estimate of the error is approximately ±10%
[b] Corresponds to labelling in *Figure 4*
[c] In ppm downfield from internal tetramethyl silane

solvents, especially mesitylene.

The structure of EYL is:



where R_1 are predominantly palmitic (16:0) or stearic (18:0) fatty acyl chains and R_2 are predominantly oleic (18:1) or linoleic (18:2) fatty acyl chains[18,19] and the fatty acid composition is reported as 37.7, 9.2, 32.9 and 17.0 in terms of mol% of acid, respectively[9]. Therefore, since there is a possibility that the existence of a heterogeneous distribution of esterified fatty acids in EYL might cause the doublet peak for the internal methylene protons in the ^1H n.m.r. spectrum of EYL in aromatic solvents, the ^1H n.m.r. spectrum measurement of only DPL was tried in

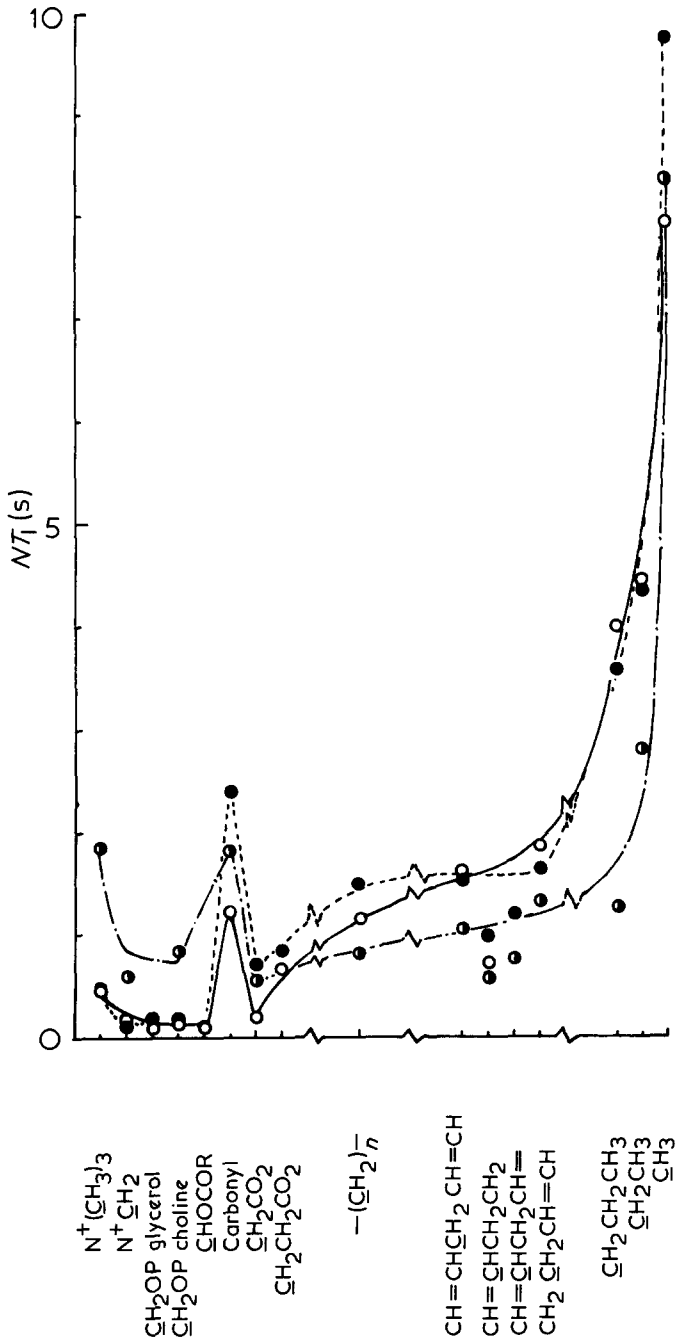


Figure 5 Plots of NT_1 *vs.* approximate fatty acyl chain position of egg yolk lecithin where N is the number of hydrogens bonded to the carbon nucleus in mesitylene; —○—, $CDCl_3$; — —●— —, D_2O, — - ◑ - —[16]

mesitylene and the spectrum was shown in *Figure* 6. The gel–liquid crystalline phase transition temperature for EYL is −5°C in contrast with DPL for which the transition occurs at 41°C in D_2O solution[20]. Therefore, EYL is in the liquid crystalline state, while DPL is in the gel state in D_2O at 25°C. It seems likely that this difference in the gel–liquid crystalline phase transition temperature also occurs in the mesitylene solution because the ^1H n.m.r. resonance from DPL disappears at 25°C and appears at 60°C, although the spectrum of EYL can be observed at 25°C. In the spectrum of the benzene solution of DPL, a similar trend to the mesitylene solution was observed. A more detailed measurement requires to determine the temperature for EYL and DPL gel–liquid crystalline phase transition or other phase transition in the mesitylene solution. Although the doublet for the main methylene peak of the fatty acyl chains of DPL was not clearly observed in mesitylene solution at 60°C, the peak is broad and asymmetric and the line shape is closely similar to that of EYL in mesitylene observed at 80°C. Thus it is concluded that the effect of the heterogeneous distribution of esterified fatty acids in EYL is small and other causes must be taken into account for the doublet peak appearance.

Since relative peak intensities of two components depend on the number of substituent methyl groups in the aromatic solvents, the appearance of the doublet methylene peak is not due to the difference in the magnetic shielding environment between two fatty acyl chains of EYL. ^1H T_1 data show that the high-field component has a longer T_1 than the low-field component in C_6D_6 and mesitylene solutions. However, the T_1 value for the former component in these solvents is still shorter than that for the single methylene peak in $CDCl_3$. ^{13}C T_1 measurement indicates a remarkably reduced motional freedom in the neighbourhood of the carbonyl groups along the fatty acyl chain in the mesitylene solution because NT_1 values of carbonyl, $\underline{C}H_2CO_2$ and $\underline{C}H_2CH_2CO_2$ carbons are markedly shorter in the mesitylene solution of EYL than those in the $CDCl_3$ solution. We can conclude that the

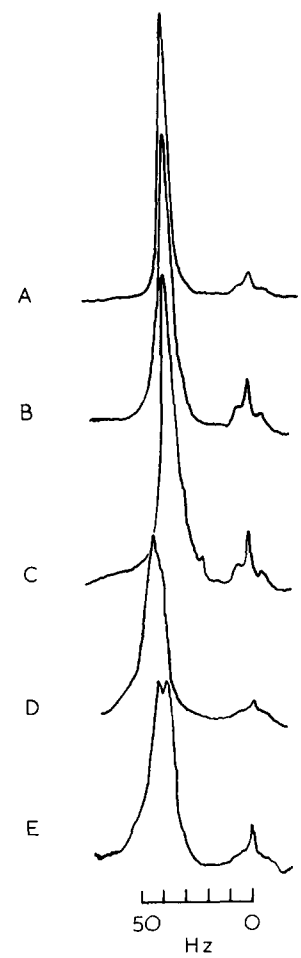


Figure 6 ^1H n.m.r. spectra of hydrocarbon region of (A) $N-C_{22}H_{46}$, (B) mono-olein, (C) triolein, (D) dipalmitoyl lecithin and (E) egg yolk lecithin in mesitylene at 25°C. Concentration, 10 w/v %; (D) was observed at 60°C. Chemical shift of the terminal methyl protons is taken as a standard reference

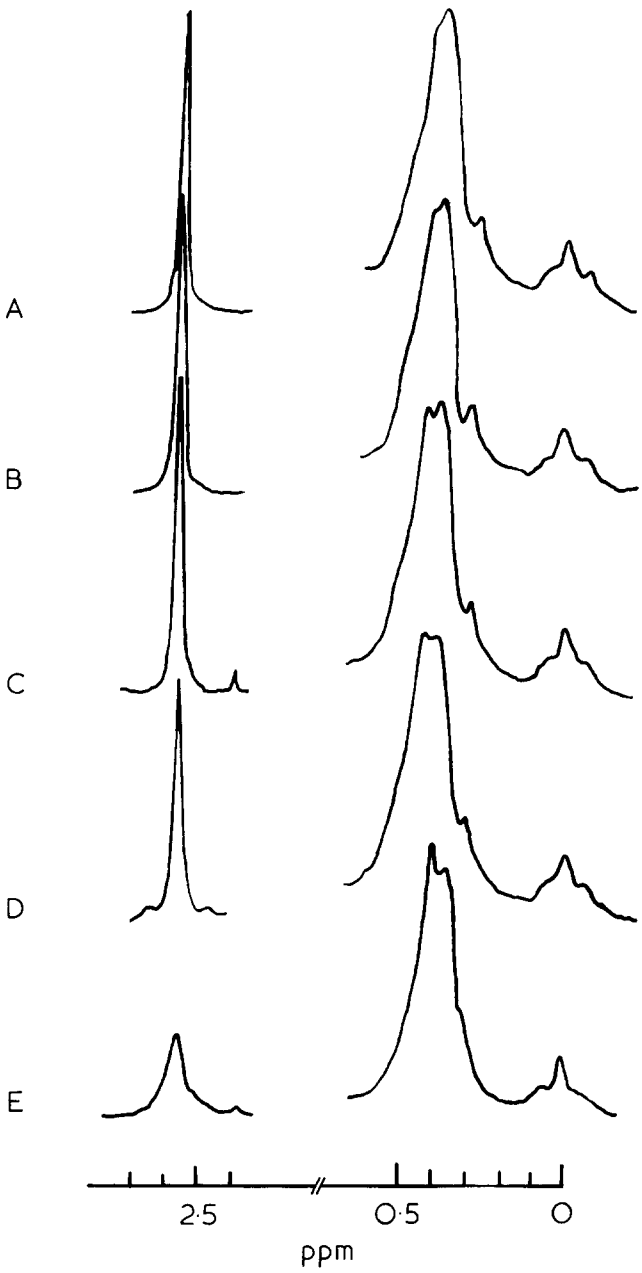


Figure 7 Variation of ^{1}H n.m.r. spectra of egg yolk lecithin in mesitylene at 25°C with increasing the amounts of water. Concentration; c.a., 5 w/v %. Water of (A) 0.05, (B) 0.03, (C) 0.02, (D) 0.01 and (E) 0 ml was added to the lecithin solution of 0.5 ml

low-field component in the doublet peak comes from the methylene protons located relatively in the neighbourhood of the carbonyl groups and the high-field component the methylene protons located relatively in the neighbourhood of the terminal methyl groups of the fatty acyl chains in aromatic solvents. The exchange between these two components is slow enough to permit the observation of doublet peak.

In order to examine whether the appearance of the doublet peak in mesitylene is inherent to the lecithin solution or not, ^{1}H n.m.r. spectra of n-docosane, mono- and triolein were observed in the solvent. The spectra of the hydrocarbon region were shown in *Figure* 6, together with the EYL spectrum. Although the line shape of the methylene peak of n-docosane, mono- and triolein is slightly asymmetric and broad compared with the single methylene signal of EYL in $CDCl_3$, the doublet peak was not observed. The methylene peak position is coincident with that of the high-field component of the doublet of EYL in mesitylene. Thus, the remarkable magnetic shielding gradient along the hydrocarbon chain seems to be characteristic of the mesitylene solution of lecithin. The formation of inverted micellar structures of lecithin in which the polar head group is tightly packed might be one of the causes. However, it should be emphasized that the inverted micellar structures would be slightly different from each other between aromatic solvents and in $CDCl_3$. The presence of chlorine atoms in the solvent molecule might slightly change the inverted micellar structure of the lecithin because of the electrostatic interaction between the polar groups of the lecithin and the choline atoms. The line broadening of internal methylene protons of the fatty acyl chain in 1-chloronaphthalene might be also due to this interaction.

At this stage, the origin of the magnetic shielding of the doublet peak is not conclusive, but one possibility may be due to a difference in the ring current shielding effect from the aromatic solvent between the methylene protons located relatively in the neighbourhood of the carbonyl groups and those located relatively in the neighbourhood of the terminal methyl groups of the fatty acyl chain and another may be due to the non-averaged methylene ^{1}H-chemical shift in the restricted state which is intrinsic of the chain[6]. However, the difference in the physical state of a fatty acyl chain of lecithin was in any event clearly observed in aromatic solvents.

The doublet peak behaviour of internal methylene protons of egg yolk lecithin upon addition of water to the mesitylene solution

Small amounts of water were added to the mesitylene solution of EYL in order to examine the change in the physical state of the fatty acyl chains through a change in the structure of the micelle with tightly packing head groups[12,14,21,22]. As shown in *Figure* 7, on progressive additions of water to the mesitylene solution, the relative intensity of the low-field component in the doublet peak decreases, together with the decrease in the line-width of the $N^+(CH_3)$ peak. This indicates that a loose packing of the micelle causes an increase in the motional freedom of the methylene protons located relatively in the neighbourhood of the carbonyl groups of the fatty acyl chain. However, it seems that the averaging of the magnetic shielding environments of the chain is limited as may be seen from the broadening of the methylene peak. methylene peak.

CONCLUSION

It was concluded that the low-field component in the doublet peak observed for the internal methylene protons in the fatty acyl chains of egg yolk lecithin in aromatic solvents, especially mesitylene, comes from the methylene protons located relatively in the neighbourhood of the carbonyl groups and the high-field component for the methylene protons relatively located in the neighbourhood of the terminal methyl groups of the fatty acyl chain in aromatic solvents. This is based on the following:
(i) since relative peak intensities of two components depend on the number of substituent methyl groups in the aromatic solvents, the appearance of the doublet methylene peak is not due to the difference in the magnetic

shielding environment between two fatty acyl chains of egg yolk lecithin;
(ii) a similarity in the line shape of the internal methylene proton peak was observed between egg yolk lecithin and diparmitoyl lecithin in mesitylene solution, which indicates a minor effect of the heterogeneous distribution of esterified fatty acids in egg yolk lecithin on the appearance of the doublet;
(iii) the ^{1}H and ^{13}C n.m.r. spin-lattice relaxation time measurements indicate the remarkable gradient in the motional freedom along the fatty acyl chains as well as the marked decrease in the motional freedom of the polar head group and also the high-field component of the doublet peak for internal methylene protons has a shorter T_1 value than the low-field component.

A spectral change caused from progressive additions of water in the mesitylene solution of egg yolk lecithin was concluded to mean an increase in the motional freedom of the methylene protons located relatively in the neighbourhood of the carbonyl groups of the fatty acyl chains.

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