# Proton and Carbon-13 Nuclear Magnetic Resonance Studies of the Effects of Retinal on the Dynamic Structure and Stability of Lipid Bilayer

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The effects of retinal and vitamin A on the dynamic structure and stability of hen egg yolk lecithin bilayers have been studied by means of carbon-13 and proton NMR spectroscopies. $^{13}C$ spin-lattice relaxation and paramagnetic ion permeability studies on lecithin bilayers indicate a marked decrease in flexibility of the lipid acyl chain and a breakdown of membrane impermeableness to ion by the intercalated *all-trans*- and 11-*cis*-retinal, whereas the effect of incorporated vitamin A on the fluidity of bilayers is small and its impermeableness to ion remains effective even in the presence of higher concentration of vitamin A. The experimental results are discussed in connection with the mechanism of the permeability change in photoreceptive disk membrane.

Rod outer segments are organelles in which the photon is captured by rhodopsin, the visual pigment of the retinal photoreceptor cells for black and white vision in the eye. Rhodopsin is composed of the vitamin $A_1$ aldehyde isomer 11-*cis*-retinal and the lipoprotein opsin, in which the former is bonded through a Schiff base linkage to the ε-amino group of a lysine residue. Upon exposure to light, rhodopsin bleaches through a series of transformations and finally releases *all-trans*-retinal from opsin into lipid medium in retina.[1] During these events the ionic permeability of the disk membrane is altered and thus the photoreception is translated into an electrical response.[2]

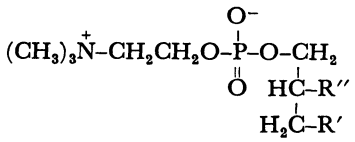
Much is known about bleaching process of rhodopsin,[1] while the mechanism of the translation of photoinformation to the neural system has so far remained almost an event of conjecture. The fact, which seems established, is that the absorption of a single photon by rhodopsin leads to a reduction of the sodium influx[2-4] and consequent hyperpolarization of the disk membrane.[5]

There are considerable interests in the role of membrane lipid in these photochemical events; almost half of the retina rod outer segment fragments is lipid on a weight basis and most of lipid is phospholipid.[6] Phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) are the major phospholipids of bovine rod outer segments, making up about 39, 40, and 13%, respectively, of the lipid phosphorus. The major fatty acids in PE and PS are 18 : 0 and 22 : 6, while PC is made up predominantly of 16 : 0 and 22 : 6, and all of the phospholipids contain high levels of polyunsaturated fatty acids.[6,7] Rhodopsin is considered to be an integral membrane protein and at least a part of the protein is embedded within the membrane interior. The interactions between rhodopsin and phospholipid including artificially reconstructed model membranes have been studied by many authors,[8-15] while those between retinal isomers and phospholipid have not been studied well. Retinal has been thought to decompose some biomembranes,[16] especially, *all-trans*- and 11-*cis*-retinal hemolyse rabbit erythrocytes.[17] Retinal isomers may, therefore, affect the arrangement of lipid membrane and *all-trans*-retinal released from rhodopsin plays significant roles in alteration of ionic permeability of plasma membrane.

By the way, the chromophore *all-trans*-retinal released from bleached pigment can be recycled with newly incorporated one *in vivo* to rhodopsin regeneration.[18] Rhodopsin regeneration is believed to occur *via* two discrete reactions: isomerization of *all-trans*-retinal to 11-*cis*-retinal and binding of 11-*cis*-retinal with opsin. *In vivo*, little free retina is found in the photoreceptor cell in contrast to the presence of retinylidene phosphatidylethanolamine.[19-21] Shichi and Somers[18,22,23] have found that phospholipid stimulates the photo-regeneration of rhodopsin from opsin and *all-trans*-retinal. The enhancement of stereoselective isomerization of retinal by an addition of phosphatidylethanolamine has been demonstrated *in vivo* experiment[24] and a series of molecular orbital calculations.[25] It was pointed out[22] that the role of phosphatidylethanolamine in retina are (i) to maintain the integrity of the visual pigment and (ii) to participate in the regenaration of rhodopsin after bleaching, although the latter role is still a matter of speculation.[7,26,27]

In this paper, the interactions between model membrane lipid and retinal and vitamin A were studied by observing the proton and carbon-13 nuclear magnetic resonance spectra. The locations of retinal compounds in the membrane and their effects on the dynamic structure and stability of membrane were investigated by measuring the carbon-13 spin-lattice relaxation time and by monitoring the ionic permeability of the membrane by proton spectra. The determinations of the position of retinals in the lipid bilayers and their effects on the membrane properties are expected to give valuable informations required for the elucidation of the visual process, including the ion permeation and rhodopsin regeneration. As pigments, we will choose *all-trans*- and 11-*cis*-retinal, and vitamin A: the former two are compounds participating in a visual cycle, and the latter one was chosen for the sake of comparison with the formers.

Hen egg yolk phosphatidylcholine (egg yolk lecithin, EYL) bilayer will be used as a model membrane of disk. Use of phosphatidylethanolamine, in spite of one of major phospholipid constituents of rod outer segment's membrane, will be excluded, since it forms Schiff base with retinal. The formation of Schiff base may shade off our purposes. Egg yolk lecithin has several advantages as a lipid membrane system for a model study of disk membrane. Needless to say phosphatidylcholine is one of major constituents of lipid of rod outer segments. The structure of EYL is,



where the predominant esterified residues of fatty acids R′ are palmitic (16 : 0) and stearic (18 : 0), and those of R″ are oleic (18 : 1) and linoleic (18 : 2).[28,29] Long-chain esterified fatty acids with varying degree of unsaturation amounts to 12% are also contained in EYL. EYL thus contains high levels of polyunsaturated fatty acids like a disk phospholipid, and EYL bilayer is in the liquid crystalline states at physiological temperature corresponding to the state of disk membrane. The kinetic study of interconversion from metarhodopsin I to II in a rhodopsin recombinant indicates comparable rate of interconversion in rod outer segments membrane and rhodopsin-EYL bilayers.[30] Thus, EYL could be convinced for a pertinent model for phospholipids of rod outer segments.

## Experimental

Vitamin A was purchased from Riken Vitamin Co. Ltd., Tokyo. The crystal of *all-trans*-retinal was prepared by oxidation of vitamin A with manganese dioxide in petroleum ether. The crystal of 11-*cis*-retinal was prepared by successive procedures,[31] *i.e.*, photoisomerization of *all-trans*-retinal in ethanol at 0 °C, fractionation of retinal isomers on an alumina column by eluting with petroleum ether, and seeded recrystallization at −20 °C. Details of preparation of these retinal compounds were described elsewhere.[32]

Egg yolk lecithin was isolated from fresh hen egg yolk according to the procedure of Singleton *et al.*[33] The purity of EYL was checked by thin-layer chromatography. Paramagnetic reagents, $Eu(NO_3)_3 \cdot 6H_2O$, $K_3[Fe(CN)_6]$, and $MnSO_4$ were purchased from Nakarai Chemicals, Ltd., Kyoto. Before use, $Eu(NO_3) \cdot 6H_2O$ were treated by repeated process of dissolving in deuterium oxide and freeze-drying to exchange $H_2O$ with $D_2O$.

Lecithin dispersions (10% w/v) were prepared by shaking the dry EYL with 0.05 M KCl/$D_2O$ solution (pH 5.6±0.2). The dispersions were then sonicated under cooling by ice/water until translucence (about 20 min) with an Ultrasonic Disruptor Model UR-200P (Tomy Seiko Co. Ltd., Tokyo, frequency 20 kHz, output power 200 W). The resultant vesicle solutions were thoroughly deoxygenated by bubbling argon gas, and were centrifuged (10000 $min^{-1}$ for 1 h) to remove titanium fragment from sonicator's microtip and to remove multilayer liposomes. The vesicle solution was transfered under argon to NMR glass-tubes (5 mm for $^1H$ and 8 mm for $^{13}C$ NMR samples) and the NMR spectra were measured immediately after the preparation. Retinal compound-lipid mixed vesicle solution was prepared by first co-dissolving both components in chloroform followed by evaporating the solvent and then treating the resulting dry mixture in a similar manner as described above. KCl was not added to the sample for the proton NMR study of cationic permeability. In this case pH of sample was 7.0. The total concentrations of lipid and retinal-compounds were about 5% (w/v) for proton and 10—20% (w/v) for carbon-13 NMR samples. It has been reported that lecithin concentration over the range of 10—20% (w/v) and changes of pH from 2.5 to 8.0 did not have a significant effect on the carbon-13 relaxation time.[34] Proton and carbon-13 NMR spectra were measured using a JEOL JNM PS-100 spectrometer operating at 100 and 25 MHz, respectively. Proton spectra were measured in a continuous-wave mode. In the $^1H$ NMR experiment of ionic permeability, $1—2 \times 10^{-2}$ mol $dm^{-3}$ paramagnetic compounds were added to the vesicle dispersion.

In the measurement of carbon-13 spectrum, the spectrometer was equipped with a JEOL JNM PFT-100 Fourier transform system, internal deuterium lock, noise-modulating proton-decoupler, JEOL JEC-6 computer (12K), and JEOL CM-219 IC core (8K) as an auxiliary memory device. A width of frequency domain spectra was 5000 Hz and a data file was 4096 points. The normal spectra were recorded after 1024 or 2048 pulses. The pulse sequence was repeated after a delay time at least five times greater than the largest spin-lattice relaxation time to be measured in order to avoid the effect of saturation. The duration time for 90° pulse was about $2 \times 10^{-5}$ s. Chemical shifts were measured as down field shifts from external tetramethylsilane (TMS). Carbon-13 spin-lattice relaxation time ($T_1$) was determined by fast inversion recovery method[35] with a waiting time 5s in order to circumvent the required waiting period. $T_1$ values for several carbon atoms were checked by the so-called inversion recovery method[36] and were found to be in agreement with the fast inversion recovery determination within experimental error (±10% for well-resolved large peaks and ±15% for less-resolved and small peaks). The sample temperature was kept at 36±1 °C. The preparations of samples and NMR measurements were carried out in the dark. Electron micrographs of sonicated aqueous dispersions of EYL vesicle were observed on a copper sheet mesh soiled by a drop of 2% tungstophosphoric acid with a JEOL JEM-7A type electron microscopy. Ultraviolet spectra were observed on a Beckman-25 spectrometer.

## Results

*Electron Micrograph.* Electron micrographs of sonicated dispersions of EYL indicated the presence of a distribution of vesicle sizes but the most of vesicle have a narrow range of sizes (200—300 Å in diameter). The addition of 20 mol% 11-*cis*-retinal shifted and spread the distribution of vesicle sizes (300—500 Å in diameter). The formations of multilayer or large aggregate with diameter greater than 500 Å were found to be scarce but not negligible. Thus the incorporation of retinal causes the expansion of vesicle size although most of the vesicles remained small. The structural invariableness of retinals and vitamin A before and after the sonication in an EYL dispersion was confirmed by observing ultravilet spectra. Furthermore no major differences were noted between the electron micrographs of sonicated dispersions, even the dispersions with retinals and vitamin A, observed at immediately after and 3—4 d after the preparation.

*Carbon-13 Spin-lattice Relaxation Time.* The $^{13}C$ spin-lattice relaxation times for EYL, EYL with 20 mol% incorporated *all-trans*- and 11-*cis*-retinal, and vitamin A are shown in Table 1, where the carbons are listed along the lipid backbone. Since the EYL's esterified fatty acids are heterogeneous, there is some ambiguity in the exact position of the carbon on the fatty acyl chain. Spectral assignments were followed to those made previously.[34] No detectable chemical shifts differences over the experimental error were found between EYL alone and EYL with incorporated

TABLE 1. VALUES OF $^{13}C$ $NT_1$[a,b] FOR EGG YOLK LECITHIN(EYL)-RETINALS AND -VITAMIN A BILAYERS IN $5 \times 10^{-2}$ mol dm$^{-3}$ KCl/$D_2O$ SOLUTIONS AT 36 °C[c]

| Carbon | $^{13}C$ $NT_1$/s | | | |
|---|---|---|---|---|
| | EYL | EYL+20 mol% *all-trans*-Retinal | EYL+20 mol% 11-*cis*-Retinal | EYL+20 mol% Vitamin A |
| $-N(\underline{C}H_3)_3$ | 1.89 | 1.50 (0.80) | 1.41 (0.75) | 1.74 (0.92) |
| $\underline{C}=O$ | 1.8[d] | 1.4[d] (0.78) | 1.4[d] (0.78) | 1.9[d] (1.05) |
| $-\underline{C}H_2CO_2-$ | 0.48 | 0.42 (0.88) | —[e] | —[e] |
| Main fatty acyl $(\underline{C}H_2)_n$ | 0.96 | 0.66 (0.69) | 0.72 (0.75) | 0.84 (0.88) |
| $-\underline{C}H_2-CH=CH-$ | 0.96 | 0.60 (0.62) | 0.70 (0.73) | —[e] |
| $-CH=\underline{C}H-CH_2-CH_2-$ | 0.58 | 0.40 (0.69) | 0.49 (0.84) | 0.52 (0.90) |
| $-CH=\underline{C}H-CH_2-\underline{C}H=CH-$ | 0.67 | 0.54 (0.81) | 0.66 (0.99) | 0.65 (0.97) |
| $-CH=CH-\underline{C}H_2-CH=CH-$ | 1.20 | 0.92 (0.77) | 0.70 (0.58) | 0.96 (0.80) |
| $-\underline{C}H_2-CH_2-CH_3$ | 1.46 | 1.14 (0.78) | 0.78 (0.53) | —[e] |
| $-\underline{C}H_2-CH_3$ | 3.00 | 2.20 (0.73) | 1.80 (0.60) | 2.80 (0.93) |
| $-\underline{C}H_3$ | 9.60 | 5.64 (0.59) | 5.40 (0.56) | 7.80 (0.81) |

a) Here, $T_1$ is the spin-lattice relaxation time and $N$ is the number of protons attached to the carbon. The measured $T_1$ values correspond to the underlined carbons. b) Estimated maximum error in $T_1$ is $\pm 15\%$. c) The values shown in parentheses are the $T_1$ ratios of EYL-additive mixture to EYL alone. d) The values shown are $T_1$'s. e) Not measured accurately.

retinals and vitamin A. At the concentrations employed here, resonance intensities of the retinal additives were too weak for an accurate determination of their relaxation times. The $^{13}C$ relaxation times for some carbon resonances of EYL also could not be measured accurately due to their low signal to noise ratios and are not included in Table 1. Since no major differences were noted between normal spectra observed at the beginning and the end of a set of inversion-recovery experiment, and between micrographs observed at immediately after and 3—4 d after the preparation of dispersion, an extent of vesicle fusion during the course of NMR experiment may be small.

The decrease in the rate of motion and in the acyl chain disorder of EYL will result in smaller $NT_1$ values, although an interpretation of $^{13}C$ $T_1$ value is hampered by the fact that the relaxation rate is determined by the anisotropy of molecular motion as well as the rate of motion.[37] Here $N$ is the number of protons bonded to a given carbon atom. For dispersions of EYL alone and EYL with retinals and vitamin A in bilayers, $NT_1$ values increase from the glycerol carbons toward both the $N^+(CH_3)_3$ group and the terminal methyl carbon of acyl chains, indicating that there are increases in the mobility along the lipid chain in going from the glycerol carbons to the terminal methyl groups. These observations are in agreement with previous $^{13}C$ relaxation studies of lecithin dispersions.[34,38]

Inspection of $^{13}C$ $T_1$ values of EYL listed in Table 1 reveals that the effects of added retinals and vitamin A on $T_1$ values of EYL are relatively small but almost all $T_1$ values decrease, namely the motion of EYL molecule is slowed down by additions of retinals and vitamin A. It is noticeable that the reductions of $T_1$ values of acyl chain carbons are larger in EYL–retinal systems than in EYL–vitamin A system. In the former systems, the carbons of methyl terminal region of acyl chain ($-CH_2\underline{C}H_2CH_3$) show especially larger $T_1$ reductions than the other part of EYL molecules, indicating that the retinal molecules locate rather preferentially at the acyl chain region than at the choline polar region of the lipid bilayer. Since retinal is not soluble in water alone, retinal molecule may be solubilized and incorporated into the acyl chain region of lipid bilayer by the hydrophobic interaction between retinal and lipid acyl chain.

On the other hand, the reductions of $NT_1$ values for EYL–vitamin A system are relatively small and not suggesting any localization of vitamin A molecule at special region in the lipid bilayer. Vitamin A may be allowed to stay near the polar hydrophilic site of lipid bilayer, exposing its hydroxyl group to aqueous medium and extending its hydrophobic part into nonpolar region of lipid bilayer.

For a comparison, $^{13}C$ $T_1$ values for EYL alone and EYL with 22 mol% *all-trans*-retinal were also measured in $CDCl_3$ solutions, where lecithin molecules exist as spherical inverted micelles. The results are shown in Table 2. As reported previously,[38] $NT_1$ values of the chain resonances in $CDCl_3$ are longer than those for the corresponding nuclei in bilayers in $D_2O$ and there is a marked increase in $NT_1$ values from carbonyl carbon toward the terminal methyls. The $NT_1$ value of choline methyl resonance is similar to that of $OOC-CH_2-$ methylene carbon resonance in $CDCl_3$ solution, contrasting with the results in $D_2O$ solution where choline methyl $NT_1$ value is much larger than $OOC-CH_2-$ methylene carbon value. The small $NT_1$ value for choline methyl groups may result from tight packing of these groups in the inverted micellar structure.[38] In $CDCl_3$ solutions, the inverted micellar structure of EYL is not perturbed by *all-trans*-retinal, since $NT_1$ values are not changed greatly by an addition of it. Furthermore the resonances corresponding to the retinal carbons are sharper in $CDCl_3$ solution than in $D_2O$ solution. Thus, there isn't any specific interaction between EYL and retinal in $CDCl_3$ solution. These results also confirm that the nature of interaction between EYL and retinals and vitamin A in $D_2O$ solution is essentially hydrophobic.

TABLE 2. VALUES OF $^{13}C$ $T_1$[a] FOR EGG YOLK LECITHIN(EYL) IN $CDCl_3$ AT 37 °C

| Carbon | EYL | | EYL+22 mol% *all-trans*-Retinal | | $T_1$ Ratio[c] |
|---|---|---|---|---|---|
| | $T_1$/s | $NT_1$/s[b] | $T_1$/s | $NT_1$/s[b] | |
| $-N(\underline{C}H_3)_3$ | 0.15 | 0.44 | 0.14 | 0.41 | 0.93 |
| $\underline{C}=O$ | 2.68 | | 2.31 | | 0.86 |
| $-\underline{C}H_2CO_2-$ | 0.23 | 0.46 | 0.30 | 0.59 | 1.29 |
| Main fatty acyl $(\underline{C}H_2)_n$ | 0.94 | 1.87 | 1.11 | 2.21 | 1.18 |
| $-\underline{C}H_2-CH=CH-$ | 1.08 | 2.15 | 1.24 | 2.49 | 1.16 |
| $-CH=\underline{C}H-CH_2-\underline{C}H=CH-$ | 1.50 | 1.50 | 1.10 | 1.10 | 0.73 |
| $-CH=CH-\underline{C}H_2-\underline{C}H=CH-$ | 1.39 | 2.78 | 1.42 | 2.84 | 1.02 |
| $-\underline{C}H_2-CH_2-CH_3$ | 2.53 | 5.06 | 2.77 | 5.54 | 1.09 |
| $-\underline{C}H_2-CH_3$ | 3.49 | 6.99 | 3.51 | 7.02 | 1.00 |
| $-\underline{C}H_3$ | 4.70 | 14.10 | 5.05 | 15.15 | 1.07 |

a) Here, $T_1$ is the spin-lattice relaxation time of the underlined carbon. Estimated maximum error in $T_1$ is $\pm 10\%$. b) $N$ is the number of protons attached to the carbon. c) The observed $T_1$ values of EYL under the coexistence of 22 mol% *all-trans*-retinal relative to those obtained for EYL alone.

*$^1H$ NMR Studies on Ionic Permeability of Bilayers.* The stability and vesicular nature of the lipid has been further investigated using $^1H$ NMR. The ionic permeability of lipid bilayers can be correlated with the membrane fluidity as described by $^{13}C$ spin-lattice relaxation times. In this paper, we studied the effect of the incorporation of retinals and vitamin A on the permeability of EYL vesicles using a paramagnetically induced variation of choline methyl proton resonance. It had previously been shown for $^1H$, $^{13}C$, and $^{31}P$ NMR that an addition of paramagnetic ion to lipid bilayer systems results in broadening or shifting of resonances from nuclei near the bilayer surface.[39,40] Paramagnetic ions can hence be used to distinguish the internal and external surface of a phospholipid vesicle by selectively adding the paramagnetic ion to the interior or exterior of the vesicle. Furthermore a variation of resonances induced by paramagnetic ion can be used to study the ionic permeability of lipid bilayers. Here, we used $Eu^{3+}$ and $Fe(CN)_6^{3-}$ ions as the shift reagents and $Mn^{2+}$ ion as the broadening reagent.

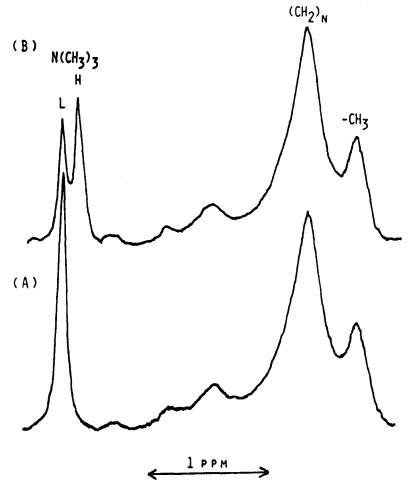



Fig. 1. 100 MHz proton NMR spectra of: (A) sonicated egg yolk lecithin vesicles (5 w/v%) in deuterium oxide solution and (B) sample A plus $10^{-2}$ mol $dm^{-3}$ $Eu^{3+}$ outside the vesicles. The spectra weretaken at 36 °C.

Figure 1 illustrates the effects of shift reagent $Eu(NO_3)_3$ as typical example. Spectral assignments are taken from those made previously.[40] Spectra A and B were obtained from the same sample without and with $Eu(NO_3)_3$. Upon addition of the $Eu(NO_3)_3$ to sample A, the choline methyl resonance splits into a doublet, while the other resonances are hardly affected. The high-field, shifted(H) and low-field, non-shifted(L) parts of the choline methyl resonance correspond to choline $N(CH_3)_3$ protons on the outside and the inside of the lecithin bilayers, respectively. The intensity ratio L : H of the non-shifted and shifted choline methyl resonance were 1 : 1.4. Assuming that the packing densities of both outside and inside layers of vesicle bilayers are equal and the thickness of the membrane is 37 Å,[40] the diameter of vesicle is estimated from the intensity ratio to be about 480 Å, which is slightly larger than that of prominent particles found by electron micrograph.

If the bilayers are permeable to $Eu^{3+}$ and $Fe(CN)_6^{3-}$ ions, the inward-facing choline methyl groups are exposed to these ions, and the intensity of the shifted signal increases while that of the non-shifted signal correspondingly decreases with progress of ion permeation. When $Mn^{2+}$ is added to the permeable bilayers, the resonance of only inward-facing choline methyl group may be firstly observed, but all finally disappears from $^1H$ NMR spectrum.

The intensity ratio of the choline methyl doublet shown in Fig. 1B remained practically constant for two days, indicating that $Eu^{3+}$ cation is impermeable through EYL bilayers. Same impermeableness was also observed for $Mn^{2+}$ and $Fe(CN)_6^{3-}$ ions. We have investigated the influences of incorporated retinals and vitamin A in the concentration range 5—33 mol% on the ionic permeability of EYL bilayers. It was found that an addition of vitamin A even at the concentration of 33 mol% does not disturb the impermeableness of bilayers which are effective ion barriers. Furthermore EYL vesicle containing less than 20 mol% *all-trans*- or 11-*cis*-retinal was found to form bilayers stable on the time scale of our experiments and also practically

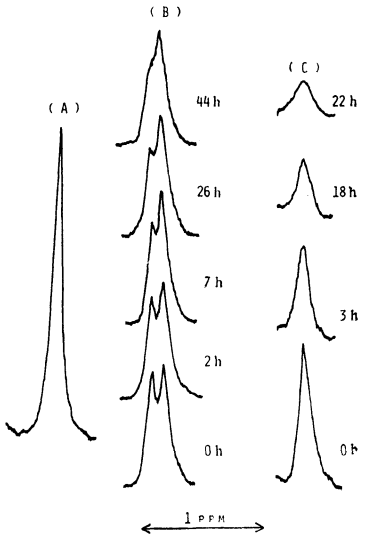



Fig. 2. Time dependence of choline methyl resonance of sonicated egg yolk lecithin vesicles (5 w/v%) with 33 mol% incorporated *all-trans*-retinal in deuterium oxide solution at 36 °C. (A) spectrum before adding the paramagnetic ions; (B) spectra of sample A just after (0 h) and 2, 7, 26, and 44 h after adding the $Eu(NO_3)_3$ ($10^{-2}$ mol $dm^{-3}$); and (C) spectra of sample A just after (0 h) and 3, 18, and 22 h after adding the $MnSO_4$ ($10^{-2}$ mol $dm^{-3}$).

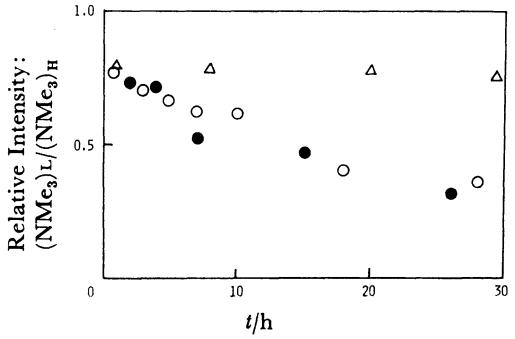



Fig. 3. Time dependence of intensity ratio of low-field(L) to high-field(H) choline methyl resonances for egg yolk lecithin(EYL) with 20 mol% incorporated *all-trans*-retinal plus $10^{-2}$ mol $dm^{-3}$ $Eu^{3+}$ (△), EYL with 33 mol% incorporated *all-trans*-retinal plus $10^{-2}$ mol $dm^{-3}$ $Eu^{3+}$ (●), and EYL with 33 mol% incorporated *all-trans*-retinal plus $2\times10^{-2}$ mol $dm^{-3}$ $Fe(CN)_6^{3-}$ (○).

impermeable to $Eu^{3+}$, $Fe(CN)_6^{3-}$, and $Mn^{2+}$ ions.

On the contrary, less but significant ion permeation was found with the incorporation of over 20 mol% *all-trans*- and 11-*cis*-retinal to the system. Figure 2 shows the change of the choline methyl resonance with time after adding $Eu^{3+}$ and $Mn^{2+}$ ions to EYL dispersion with 33 mol% incorporated *all-trans*-retinal. The rate of penetration of ions across the bilayers is reflected in the rate of the spectral change. Time dependence of the intensity ratio of low-field to high-field choline methyl resonances after the addition of $Eu^{3+}$ and $Fe(CN)_6^{3-}$ for EYL–*all-trans*-retinal systems are shown in Fig. 3. The increased amount of incorporated *all-trans*-retinal results in the increased rate of permeation of ion. Similar plots were also obtained for EYL–11-*cis*-retinal systems.

The $^1H$ results show that the incorporations of *all-trans*- and 11-*cis*-retinal but not vitamin A disturb the integrity of the lipid bilayers and cause the formation of a channel through which the ion is allowed to permeate.

## Discussion

The results of $^1H$ permeability studies parallel those of $^{13}C$ relaxation studies. The $^{13}C$ and $^1H$ NMR studies have shown a decrease in flexibility of the lipid acyl chain and a breakdown of membrane impermeableness to ion caused by the intercalation of *all-trans*- and 11-*cis*-retinal, whereas the effect of vitamin A on the fluidity of bilayers is small and its impermeableness to ion remains effective even in the presence of higher concentration of vitamin A. From the results reported here, it is clear that retinal and vitamin A are incorporated into EYL bilayers and they expand the bilayers slightly without significant disruption of overall vesicular structure. These incorporated molecules may be situated in the bilayer orienting its polyene chain nearly parallel to the lipid acyl chain. *All-trans*- and 11-*cis*-retinal molecules stay preferentially at the acyl chain region due to the hydrophobic interaction. On a molecular level, incorporations of *all-trans*- and 11-*cis*-retinal, which have inflexible polyene chain and bulky ionone ring, stiffen the flexible chain of lipid molecules surrounding the intercalated retinal molecules and disrupt the packing of the hydrocarbon region of phospholipid bilayers. In other words, the incorporated retinal molecules obstruct the formation of stable association of lipid acyl chains, which is considered to be responsible for the impermeableness of lipid membrane to ion.[41] The significant difference was not observed between the effects of incorporated *all-trans*- and 11-*cis*-retinal on the structure and stability of bilayers.

The position of vitamin A molecule, on the other hand, may be not restricted only at the hydrocarbon region, but it can interject further into the polar hydrophilic region of the EYL bilayers than does retinal, since the former has the hydroxyl group. Vitamin A seems to be alike cholesterol, in respect that the intercalation of the latter does not cause any appreciable change in $^{13}C$ spin-lattice relaxation time of EYL[42] and further the incorporation of the latter is known to decrease the permeability of phospholipid bilayers to $Na^+$, $K^+$, and $Cl^-$.[43] The cholesterol hydroxyl, which is on the bulky steroid ring, is found at the position of the ester carbonyl of phospholipid.[44] Vitamin A hydroxyl, which attaches to the nonbulky polyene chain, is expected to penetrate as far into the hydrophilic region or parallel, at least, that occupied by the hydroxyl of incorporated cholesterol. In this situation, the effect of incorporated vitamin A molecule on the structural integrity and dynamic nature of lipid bilayers is not expected to be large, because the lipid head group and acyl chain end region near the head have essentially high ordering and low mobility and vitamin A hydroxyl is not effective to disrupt the strong electrostatic interac-

tion between lecithin head groups.

Several studies[16,17] have indicated that vitamin A as well as retinal are effective in lysis of erythrocytes. These results are not necessarily to match well with whole of our results observed here, since the erythrocytes membrane contains not only phospholipid but also lipoproteins and glycolipids. In that case, vitamin A and retinal can affect the arrangement of lipid and/or protein molecules in the membrane. It is not clear whether retinal acts on the same site on the erythrocyte membrane as vitamin A.

The experimental results described above give some suggestions meaningful in physiology; the incorporated retinal resides inside of the lipid bilayer and causes the ionic permeability change of the membrane. Hence the released *all-trans*-retinal from the photobleached pigment is suggested to be able to effect an increase in the ionic permeability of the disk membrane. Namely, it is not unreasonable to assume that *all-trans*-retinal released from photobleached pigment affects the arrangement of lipid and plays significant or at least some role in the alteration of ionic permeability of disk membrane, and in this way it participates the transmission of photosignal to the neural system. Since the chromophore 11-*cis*-retinal in the unbleached visual pigment is fixed to and surrounded by an opsin through Schiff base linkage, its ability to increase in permeability of membrane may be less important in visual process.

It is known that the thermal stability of rhodopsin, the completion of the photolytic cycle, and the regeneration of rhodopsin from opsin and retinal require the presence of a lipid membrane.[44] The experimental fact that hydrophobic retinal molecule can stay stable in the lipid bilayer in aqueous dispersion supports the idea that the isomerization of *all-trans*-retinal to 11-*cis*-form and the regeneration of rhodopsin from opsin and retinal take place in the disk membrane.

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