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# Effect of Drug Substances on the Microviscosity of Lipid Bilayer of Liposomal Membrane

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**Abstract**□The microviscosities of the lipid bilayers of liposomal membranes of phospholipids were measured by the intermolecular excimer, formation method employing pyrene as a fluorescence probe, and the effects of *n*-alkanols and other local anesthetics on the microviscosity were investigated. The results showed that the *n*-alkanols and the other local anesthetics effectively lowered the microviscosity of the lipid bilayer of the dipalmitoyl phosphatidycholine liposomal membrane in proportion to the concentration of the additives. Moreover, there was a fairly good correlation between the local anesthetic activities and the microviscosity-lowering activities of these drugs. This results suggests that the nerve-blocking activity of local anesthetics might have some relation with their activity fluidizing the lipid bilayer of biomembrane.

**Keywords**□Microviscosity, liposome, phospholipid, *n*-alkanols, microviscosity-lowering activity, anesthetic.

The dynamic properties of the phospholipid bilayers of biomembranes are known to profoundly affect many of physiological functions associated with the biomembranes. The biomembranes are not simply mec hanical barriers to the cellular components, but dynamic structure intimately involved in the cellular functions such as component transports, enzyme activities, recognition, information transmission, *etc*[1,2]. Proper arrangement and orientation of the biomembrane components in the bilayer structure determine the physical characteristics of the membranes such as mechanical strength, flexibility, and permeation of solutes and water molecule across the membrane. Mobility of molecules in the biomembranes is as a whole expressed as the membrane fluidity. Fluidity is the property of a liquid that describes its ease of movement. Viscosity, the inverse of fluidity is the property describing the resistance of a liquid to movement. This fluidity concept for biomembranes was first introduced by Chapman *et el.*[3], and it has been suggested that the dynamic properties of the lipid bilayer of biomembranes affect the physiological functions of biomembranes, and there is a biosynthetic feedback mechanism by which a cell will attempt to retain a constant fluidity. Changes in the bilayer fluidity affected by either the cell itself or by some external influences can modulate normal cell functions[1,3,4].

Biomembranes are the sites of action of a variety of drugs. Some of the drugs are known to interact with specific protein sites on membranes. However, a large number of drugs are known which interact with non-specific sites on the membranes to excert their pharmacological activities. Such agents include tranquilizers, antidepressants, anticonvulsants, antihistamines, steroids, narcotics, sedatives, *etc.*[5-7]. Their mode of action involves not only passage through a hydrophobic barrier of biomembranes but also interaction with hydrophobic sites on target tissues, possibly the lipid bilayer of the biomembranes. There is a possibility that incorporation of a drug molecule into the lipid bilayer might have profound effect on the fluidity of the bilayer, and this might induce pharmacological activity through modification of the environment of the active-site proteins.

Reasearches on the biomembrane fluidity have involved a variety of techniques such as nmr spectroscopy[3], electron spin resonance spectroscopy[8,9], fluorscence spectroscopy[10-14], *etc.* Occasionally, the various techniques provide measurements for which the specific underlying molecular interactions are complex. Among these methods, the fluorescence method is one of the most widely used techniques. Environmentally sensitive fluorphores of varying structural complexity have been shown to report on most of those dynamic phenomena, both in natural as well as in model membrane and micellar systems. One major focus of these works has been aimed at the determination of the apparent membrane microviscosity. Unfortunately, many membrane microviscosity values reported for different fluorophores are often dependent upon the molecular characteristics of the probe. The most widely used luminescence techniques

for studying microviscosity are fluorescence depolarization[10, 11] and diffusion-dependent inter-and intra-molecular excimer formation methods[12-14].

The theoretical background for the measurement of viscosity from the intermolecular excimer formation of pyrene is as follows[15]. Photochemically excited pyrene molecules emit the monomer fluorescence, and also form an excimer with an other pyrene molecule which emitts the excimer fluorescence. The ratio of these two intensities of fluorescence is highly dependent upon the concentration of the probe and the viscosity of the medium. It has been shown that if the ratios versus the concentration of pyrene are plotted, the two lines intersect at a half-value point, and the concentration at this point is taken as the half-value concentration, Ch. Moreover, the following relation holds for Ch,

$$I_E/I_M = I_E^{max}/I_M^{max} \cdot C/C_h = C/C_h' \quad (1)$$

where $I_E$ and $I_M$ are the excimer and the monomer fluorescence intensities at the concentration, C, respectively, $I_E^{max}$ and $I_M^{max}$, the maximum intrinsic fluorescence intensities for the excimer and the monmer, respectively, and $Ch' = I_M^{max}/I_E^{max}$. Ch. It has been derived that

$$C_h' = (k_f + k_{rl})\,(3000\eta/8RT)\,(I_M^{max}/I_E^{max}) \quad (2)$$

where $k_f$ and $k_{rl}$ are the monomer fluorescence emission rate constant and the monomer raadiationless transition rate constant, respectively, and, the viscosity of the medium. Consequently, the following relation holds between Ch' value and the viscosity of the medium,

$$Ch' = 1/K\eta \quad (3)$$

where K is a constant for the system at a given temperature, which can be obtained empirically. Form the plot of $I_E/I_M$ versus the concentration of pyrene, C, the Ch' value can be obtained from the reciprocal of the slope, and the plot of Ch' versus viscositsy of the medium gives the reciprocal of the K value. The eq. (3) provides the basis for the measurement of the microviscosity of the lipid bilayer of the liposomal membrane and micellar interior from the meausurement of the excimr fluorescence of pyrene.

## EXPERIMENTAL

### *Chemicals*

Pyrene was purchased from Sigma Chemical Co. (USA.), and twice purified by recrystallization in ethanol. L-$\alpha$-Dimyristoyl-phosphatidylcholine (DMPC), L-$\alpha$-Dipalmitoylphosphatidylcholine (DPPC) and L-$\alpha$-Distearylphosphatidylcholine (DSPC) were also purchased from Sigma Chemical Co., and used without further purification. *n*-Alkanols were obtained from Shinyou Pure Chemical Co. (Jpn.), and local anesthetics were commercially supplied. All other reagents were of thee highest purity commercially available. Twice glass-distilled water was used.

### *Preparation of liposomes*

Appropriate phospholipid was dissolved in chloroform which was subsequently evaporated under vacuum leaving behind a thin film of dried lipid on the surface of flask. Trace of chloroform was removed by an overnight vacuum evaporation of the dried lipid film. Ten mM Tris buffer containing 100 mM KCl (pH 7.4) was added, the solution was vortexed to free the lipid film from the inside of the flask at 27°C for DMPC, 45°C for DPPC and 58°C for DSPC, which are several degrees above their phase transition temperatures, and multilamellar vesicle was obtained. The suspension was then sonicated with a ultrasonicator of DAWE instrument, Ltd. (United Kingdom) by immersing a metal probe directly into the suspension under nitrogen flow for 20 minutes, keeping the above-mentioned temperature with an iced water bath, and a unilamellar vesicle was prepared.

### *Fluorescence measurement*

The incorporation of pyrene was carried out by adding and aliquot of methanolic solution of pyrene ($2.5 \times 10^{-4}$ M) to the lipid dispersion, so that the final probe concentration was less than $2.5 \times 10^{-6}$ M. Various concentration of *n*-alkanols and other local anesthetics were added to the mixture. The mixtures were vigorously vortexed for the time enough to dissolved the additive. Blanks, always prepared under identical condition served as control for the fluormetric measurements. These measurements were carried out with a Perkin-Elmer luminescence spectrometer, model LS-5, provided was 338 nm with 15 nm excitation and 2.5 nm emission slits. The ratio of the excimer to the monomer fluorescence intensity was calculated from the 466 nm to 393 nm signal ratio.

### *Viscosity measurements*

The viscosities of the reference solvents were measured with Ostwalt viscometer in a thermostated bath at 25°C. The average values of at least three measurements were taken, and three values agreed within 0.1 cP or less.

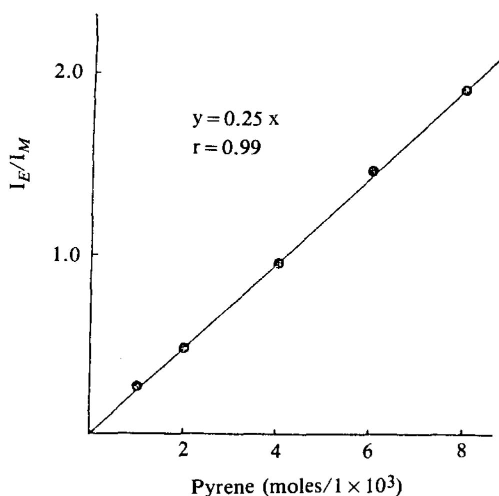


**Fig. 1. A plot of $I_E/I_M$ versus concentration of pyrene in methanol at 25°C.**
For $I_E$ and $I_M$, see eq. (1).

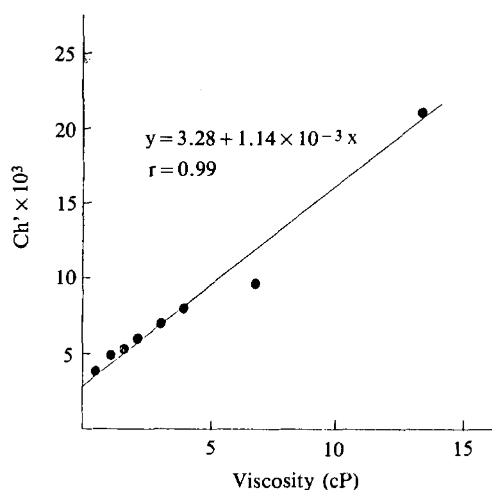


**Fig. 2. A plot of $C_h$' versus. kinematic viscosity.**
In order of increasing viscosity the solvents are: methanol, ethanol, n-propanol, n-butanol, n-pentanol, n-hexanol, n-octanol and n-decanol. For Ch', see eq. (2).

**Table I. Kinematic viscosity an Ch' value of various solvents at 25°C**

| Solvents | Ch' × 10³ (mole/*l*) | (cP) |
|---|---|---|
| Methanol | 4.17 | 0.57 |
| Ethanol | 5.02 | 1.33 |
| *n*-Propanol | 5.98 | 1.94 |
| *n*-Butanol | 6.24 | 2.46 |
| *n*-Pentanol | 7.09 | 3.30 |
| *n*-Hexanol | 7.80 | 4.51 |
| *n*-Octanol | 9.84 | 6.98 |
| *n*-Decanol | 19.68 | 13.81 |

## RESULTS AND DISCUSSION

### *Viscosity of pure solvent*

The ratios of relative intensity of the excimer to monomer fluorescence of pyrene, $I_E/I_M$ were measured and are plotted against the pyrene concentration in methanol solution in Fig. 1. The relation showed a good linearity. With all other solvents of homologous *n*-alkanols employed in this experiment, good linearities were obtained. The Ch' values were obtained from the reciprocal of the slopes of these plots. In Table I, the Ch' values for solvents at 25°C are listed with their corresponding kinematic viscosities. The plot of the Ch' values versus the viscosities of the solvents appeared to be linear as shown in Fig. 2. The slope of this line was $1.14 \times 10^{-3}$ at 25°C, and is equal to the quantity, 1/K in equation (3). The good linearity of this plot ascertains that this method would be a satisfactory method of determing the viscosity of the medium with an assumption that the linearity extends to the region of the viscosity of the object medium. From this calibration curve, the viscosity of a medium containing pyrene probe can be determined with the equation (3).

### *Microviscosity of the interiors of the lipid bilayers of liposomal membranes*

When pyrene is incorporated into the lipid bilayer of the liposomal membrane as a probe, it locates exclusively in the hydrocarbon region of the system because of its extreme hydrophobicity. As the formal concentration of pyrene is different from the actual concentration in the hydrocarbon region, the microscopic concentration, $C_\mu$ was employed for calculation in this study. $C_\mu$ was calculated by $C_\mu$ = $Cb/C_s$. $V_{hc}$' where $C_s$ is the lipid concentration, $C_b$, the bulk concentration of pyrene, and $V_{hc}$, the partial molar volume of the hydrocarbon region of the lipid, which was calculated as described in the ref.[16]. The calculated $V_{hc}$ values were 524.0 cm³/mole for DMPC, 556.4 for DPPC, and 588.8 for DSPC.

The ratios of the monomer to the excimer fluorescence intensity, $I_E/I_M$ versus the microscopic concentration in the liposomal systems of the phospholipids were measured. The relation showed a good linearity for each system. The Ch' value was calculated from the

reciprocal of the slope of the plot. The microviscosity value of the hydrocarbon region was calculated with the Ch' value, employing the equation (3). Four measrements were made for each value, and the variation of these measurements were within 10% range. The results of the measurements of the microviscosities of the hydrocarbon interiors of the lipid bilayers of phospholipid vesicles at 25°C were compiled in Table II. The result show that the microviscosities of the liposomal lipid bilayers of DMPC, DPPC, and DSPC were 73, 129, and 110 cP at 25°C, respectively. The lowerst value of DMPC vesicle was ascribed to the liquid crystalline state of the lipid layer at 25°C, while the other two are in crystalline state. The lower value of DSPC vesicle than that of DPPC vesicle was noticeble. This suggests that the length of the hydrocarbon chain of the fatty acids of the phospholipid might not have significan effectc on the fluidity in the lipid bilayers at crystalline state. The end portions of the hydrocarbon chains of the fatty acids might be in more randomnly distributed state than the portions near the hydrophilic head groups, and consequently, they might excert less resistance to flow.

**Table II. Microviscosity of the lipid bilayer of phospholipid liposomes at 25°C**

| Phospholipid | Microviscosity (cP) |
|---|---|
| DMPC | 73 |
| DPPC | 129 |
| DSPC | 120 |

### *Effects of n-alkanols and local anesthetics on the microviscosity of the lipid bilayer of liposomal membrane*

The microviscosity of the lipid bilayer of DPPC liposomal membrane in the presence of *n*-alkanols and other local anesthetics was measured, and the results were compiled in Table III, IV, and V. They significantly lowered the microviscosity in proportion to their concentration. The relations were obviously not linear; they were rather exponential. These results suggest that *n*-alkanols and the local anesthetics have significantly fluidizing effects on the lipid bilayer of liposomal membrane, and compounds with the more hydrophobic and longer hydrocarbon chain excert these effects more effectively. This might be due to the possibilities that the more hydrophobic compounds partition into the lipid layer more effectively, and incorporation of longer hydrocarbon chain compounds into the lipid bilayer induces less compact packing of the lamellar structure of the lipid bilayer.

### *Correlation between the microviscosity-lowering activities and local anesthetic concentrations*

Correleation between the microviscosity-lowering activities and local anesthetic activities of the *n*-

**Table III. Effects of methanol, ethanol,** *n*-propanol, *n*-butanol and *n*-pentanol on the microviscosity (cP) of the lipid bilayer of the DPPC liposome at 25°C

| Alkanols | Concentration (mM) 4 | 5 | 10 | 20 | 30 | 50 | 100 | 200 | 300 |
|---|---|---|---|---|---|---|---|---|---|
| Methanol | | | | | | 127 | 123 | 118 | 112 |
| Ethanol | | | | | 124 | 120 | 115 | 103 | |
| *n*-Propanol | | | 120 | 115 | 112 | 106 | | | |
| *n*-Butanol | | 121 | 113 | 104 | 96 | | | | |
| *n*-Pentanol | 107 | 116 | 105 | 97 | | | | | |

**Table IV. Effects of** *n*-hexanol, *n*-heptanol, *n*-octanol and *n*-decanol on the microviscosity (cP) of the lipid bilayer of the DPPC liposome at 25°C

| Alkanols | Concentration (mM) 0.1 | 0.2 | 0.3 | 0.5 | 0.7 | 1.0 | 2.0 | 3.0 | 4.0 |
|---|---|---|---|---|---|---|---|---|---|
| *n*-Hexanol | | | | | | 120 | 111 | 107 | 101 |
| *n*-Heptanol | | | | | 118 | 106 | 99 | 91 | |
| *n*-Octanol | | | | 113 | 107 | 98 | 92 | | |
| *n*-Decanol | 112 | 103 | 96 | 84 | | | | | |

**Table V. Effects of local anesthetics on the microviscosity (cP) of the lipid bilayer of DPPC liposome at 25°C**

| Drugs | Concentration (mM) 0.5 | 0.7 | 1.0 | 2.0 | 3.0 |
|---|---|---|---|---|---|
| Procaine HCL | | 123 | 117 | 111 | 106 |
| Lidocaine HCL | 119 | 110 | 101 | 93 | 88 |
| Benzyl alcohol | 125 | 121 | 120 | 118 | 115 |
| Menthol | 118 | 112 | 105 | 96 | 90 |
| Thymol | 109 | 105 | 96 | 90 | 81 |

**Table VI. Correlation between the nerve-blocking concentrations (NBC) and the concentrations of the drugs to reduce the microviscosity of the lipid bilayer of DPPC liposomes from 129 to 110 cP at 25°C**

| | Drugs | NBC (mole/$l$) | Conc. (mmole/$l$) |
|---|---|---|---|
| 1 | Methanol | 2.4 | 350 |
| 2 | Ethanol | $5.0\times10^{-1}$ | 150 |
| 3 | *n*-Propanol | $2.18\times10^{-1}$ | 40 |
| 4 | *n*-Butanol | $6.8\times10^{-2}$ | 15 |
| 5 | *n*-Pentanol | $2.1\times10^{-2}$ | 9 |
| 6 | *n*-Hexanol | $6.0\times10^{-3}$ | 1.9 |
| 7 | *n*-Heptanol | $1.75\times10^{-3}$ | 0.98 |
| 8 | *n*-Octanol | $1.0\times10^{-4}$ | 0.68 |
| 9 | *n*-Decanol | $1.0\times10^{-5}$ | 0.14 |
| 10 | Procaine HCL | $4.6\times10^{-4}$ | 1.8 |
| 11 | Benzyl alcohol | $2.0\times10^{-2}$ | 4 |
| 12 | Thymol | $2.2\times10^{-4}$ | 0.85 |
| 13 | Menthol | $5.8\times10^{-4}$ | 0.72 |

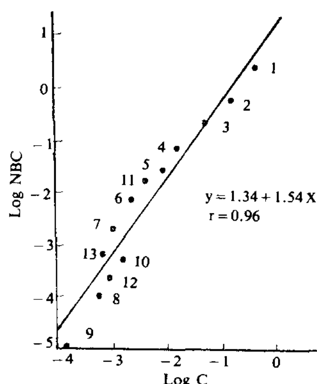


**Fig. 3. Double-logarithmic plot of the nerve-blocking concentration (NBC) and the microviscosity-lowering concentration (C) of n-alkanols and the other local anesthetics.**

1, methanol; 2, ethanol; 3, *n*-propanol; 4, *n*-butanol; 5, *n*-pentanol; 6, *n*-hexanol; 7, *n*-heptanol; 8, *n*-octanol; 9, *n*-decanol; 10, procaine HCl; 11, benzyl alcohol; 12, thymol; and 13, menthol.

alkanols and the other local anesthetics was examined. For this purpose, the concentraitons of these drugs necessary to lower the microviscosity of the lipid bilayer of the DPPC liposomal membrane from 129 cP to 114 cP, and the nerve-blocking concentration (NBC) of these compounds quoted from ref[7]. were compiled in Table VI. This table clearly shows that the more microviscosity-lowering activities, the less NBC of these drugs. The double-logarithmic plot of these two sets of data were plotted in Fig. 3, and a good linearity was obtained. This correlation suggests that the effect of local anesthetics on the lipid bilayer fluidity in biological membrane has some relation with the nerve-blocking activities of these drugs. Considering the diversity of the chemical structures of local anesthetic compounds, this mechanism is difficult to define with the results of experiments on the limited number and homolog series of local anesthetics. However, the possibility coud not be excluded that incorporation of local anesthetics into the lipid bilayer of biomembrane might fluidize the layer, and this in-

duce the perturbation of the conformation of the sodium channel protein and pharmacological activities. Further studies along this line would be interesting.

## ACKNOWLEDGEMENT

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