The Penetration of n-Alkanols into Model Membranes of Cholesterol plus Phospholipids Extracted from Brain Membranes

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=국문초록=

n-Alkanols가 Cholesterol과 인지질들로서 제제한 인공세포막에서의 침투정도

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신선한 소의 대뇌피질로부터 synaptosomal plasma membrane vesicles(SPMV)를 분리하고 이 SPMV로부터 추출한 총지질(cholesterol과 각종 인지질 함유)로서 제제한 인공세포막(SPMVTL)에서의 n-alkanols 침투 정도를 형광 probe를 이용한 형광 소광법을 통하여 검색하였다. n-alkanols는 SPMVTL 외부 단층(outer monolayer)의 표면에 주로 분포하되 그 탄소수에 비례하여 소수성 부위에 분포되는 양이 증가되는 경향을 나타내었다(1-decanol은 예외). Methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-hepatnol, 1-cotanol, 1-nonanol 및 1-decanol은 SPMVTL 외부 단층 표면(친수성 부위)에 분포되는 것이 소수성 부위에 분포되는 것에 비하여 각각 650, 288, 151.6, 69.5, 36.8, 11.9, 4.8, 1.6, 0.74, 2.1배가 된다는 것을 확인하였다. 1-decanol은 C_{10}인데도 불구하고 C_8인 1-octanol에 비하여 적은 양이 소수성 부위에 침투 분포된다는 것이 확인되었다. 또한 n-alkanols는 저자등이 이미 보고한 SPMV에서의 경우보다도 본 연구에서의 SPMVTL의 경우가 현저하게 많은 양이 소수성 부위로 침투 분포된다는 것도 확인되었다.

Key Words: n-Alkanols, Penetration site, Cholesterol plus phosphoipid model membranes, Fluorescence probe technique, Modified Stern-Volmer equation

INTRODUCTION

n-Alkanols are neutral compounds and are members of the large family of anesthetics whose biological potency correlates with lipid solubility[1]. The exact mechanisms by which ethanol and similar drugs cause depression of the central nervous system(CNS) and the subsequent behavioral manifestations of intoxication remain undefined. In recent years, a great deal of research has focused on the effects of n-alkanols on the physical properties of native and model membranes[1]. Many different physicochemical techniques have been used to provide evidence that n-alkanols including have a

biophysical action on cell membranes that can often be described as a disordering or fluidizing effect[1].

The membrane-fluidinzing hypothesis of ethanol in the central nervous system is now being strongly challenged by recent data showing that ethanol specifically and selectively affects the function of the γ-aminobutyric acid-coupled chloride channel[2]. Still, a large, diverse collection of physiological agonists produces the alterations in membrane fluidity as well as their specific ligand-receptor interaction[3].

At the present time, the exact mechanism(s) of action for ethanol and related alkanols in the central nervous system is unclear. The investigation of the primary site of action of n-alkanols can provide a basis for studying the mechanism of action of these drugs. In the present study, we examined the penetration site of n-alkanols into model membranes of total lipids (SPMVTL) extracted from synaptosomal plasma membrane vesicles(SPMV), employing two fluorescent probes N-octadecyl naphthyl-2-amine-6-sulfonic acid(ONS) and 12-(9-anthroyl) stearic acid(AS). The analysis of preferential quenching of these probes by n-alkanols revealed the relative accessibility of these drugs to the hydrocarbon region of the outer monolayers of SPMVTL lipid bilayer structures.

MATERIALS AND METHODS

1) Materials

The fluorescent probe, 12-(9-anthroyl) stearic acid(AS), was purchased from Sigma Chemical Co.(St. Louis, MO, USA). The other fluorescent probe, N-octadecyl naphthyl-2-amine-6-sulfonic acid(ONS) was synthesized by Dr. Yun(Department of Dental Pharmacology and Biophysics, College of Dentistry, Pusan National University) and was kindly donated to us. n-Alkanols were purchased from Fluka(Buchs, Switzerland). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid(Hepes), Ficoll(70,000 M.W.), and bovine serum albumin(BSA) were purchased from Sigma Chemical Co.(St. Louis, Mo, USA). All other reagents were of the highest quality available. Water was double-distilled.

2) Membrane preparation

The SPMV were isolated from bovine cerebral cortex and characterized as described previously[4]. Lipids were extracted from the SPMV as described earlier[5]. The cholesterol to phospholipid molar ratio was 0.60±0.01. Large unilamellar liposomes(SPMVTL; 0.7 mg of total lipids/ml, pH 7.4) were prepared by the method described earlier[6].

3) Fluorescence measurements

The SPMVTL were suspended in phosphate buffered saline(PBS, pH 7.4) containing NaCl 18 g/l, KCl 0.2 g, KH_2PO_4 0.2 g/l, Na_2HPO_4 $7H_2O$ 1.15 g/l and N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid(Hepes) 0.48 g/l to a concentration of 1 mg protein ml^{-1}. n-Alkanols, at the concentrations indicated, were added directly to the membranes resuspended in PBS. The pH of the buffered sample was not changed significantly by addition of n-alkanols.

Stock solutions of AS in tetrahydrofuran (THF, 1 mM) and ONS in dimethyl sulfoxide (DMSO, 1 mM) were made and kept in a cold dark place. The incorporation of the probes was carried out by adding aliquots of the stock solutions to the membrane so that the final concentrations of AS and ONS were 10 and 20 mM, respectively. the mixture was stirred for 2 h at room temperature in order to reduce the concentration of THF and DMSO that might alter permeability of SPMVTL lipid bilayer. Also, the mixture was bubbled by dry nitrogen for 5 min

with every 20-min interval in order to eliminate oxygen that might be a quencher such as n-alkanols and might denaturalize SPMVTL. The fluorescence measurements were carried out with a SPF-500C spectrofluorometer(SLM Instruments Inc., Champaoga-Urbana, IL, USA), equipped with thermostated cell holder, and performed at pH 7.4 Before the fluorescence spectra were obtained, all samples were degassed by bubbling dry nitrogen through the solution for at least 30 min. Excitation of ONS was at 360 nm and emission was read at 420 nm. The fluorescent probe AS was excited at 386 nm and its emission recorded at 440 nm. Blanks (SPMVTL solutions not incorporated with fluorescent probes), prepared under identical conditions, served as controls for the fluorometric measurements. All experiments were carried out at least five times and the values were the average of these experiments. The relative values did not differ by more than ±5%. All measurements were performed at 37±0.1℃.

RESULTS

Fluorescence quenching in homogeneous solution has been described in terms of the Stern-Volmer equation:

$$F_o/F = 1 + k_q t_o [Q] = 1 + K[Q] \quad \cdots\cdots \text{equation 1}$$

In this equation F_o and F are the fluorescence intensities in the absence and presence of the quencher, respectively, k_q is the bimolecular quenching constant, t_o is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of quencher, and $K = k_q t_o$ is the Stern-Volmer quenching constant. A plot of F_o/F versus [Q] yields an intercept of one on they axis and a slope equal to K. In the present study, the Stern-Volmer equation was modified for uneven distribution of the quencher in the SPMVTL. For water-soluble n-alkanols, the concentration in the aqueous phase is excessively larger than that in the lipid bilayer at pH 7.4. Consequently, $[Q]_L = P[Q]_T$ and equation 1 can be modified:

$$F_o/F = 1 + fKP[Q]_T \quad \cdots\cdots \text{equation 2}$$

In this equation $[Q]_L$ and $[Q]_T$ are the concentrations of the quencher in the outer monolayers of the SPMVTL lipid bilayer structures and the total concentration ot the quencher in the system, respectively, P is the partition coefficient, and is a regional correction factor for uneven distribution of the quencher between the surface area and the interior area in the outer monolayers of the SPMVTL lipid bilayer structures. Studies have shown that the most probable position of the naphthalene sulfonate moiety of the ONS molecule is at the surface of membrane's outer monolayer, and the anthroyl moiety of the AS molecule is most likely located in the hydrocarbon region of the outer monolayers of the membrane lipid bilayer structures[7]. Hence, ONS or AS quenching in the SPMVTL gives the following:

$$F_o/F = 1 + f_s K_{ONS} P[Q]_T \quad \cdots\cdots \text{equation 3}$$

$$F_o/F = 1 + f_i K_{AS} P[Q]_T \quad \cdots\cdots \text{equation 4}$$

In these equations K_{ONS} and K_{AS} are the Stern-Volmer constants of the fluorescence quenching of ONS and AS by the quencher, respectively. F_S/F_i is the ratio of the regional correction factors in the surface area and the interior area of the outer monolayers of the SPMVTL lipid bilayer structures, and becomes the concentration gradient of the quencher between these two areas.

The plots of F_o/F vs. $[Q]_T$ of equations 3 is shown in Fig. 1 and Fig. 2. And the plots of F_o/F vs.$[Q]_T$ of equations 4 is shown Fig. 3, 4, and Fig. 5. From this line, f_s/f_i can be obtained:

$$F_S/F_i = \frac{S_S K_{AS}}{S_i K_{ONS}} \quad \cdots\cdots \text{equation 5}$$

In this equation S_S and S_i are the slopes of the

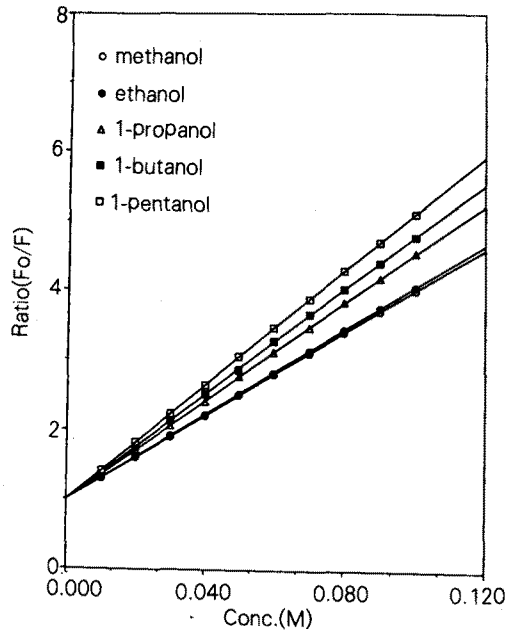

Fig. 1. Stern-Volmer plot of quenching of ONs fluorescence in SPMVTL by methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol. Lines were fitted by a least-squares analysis.

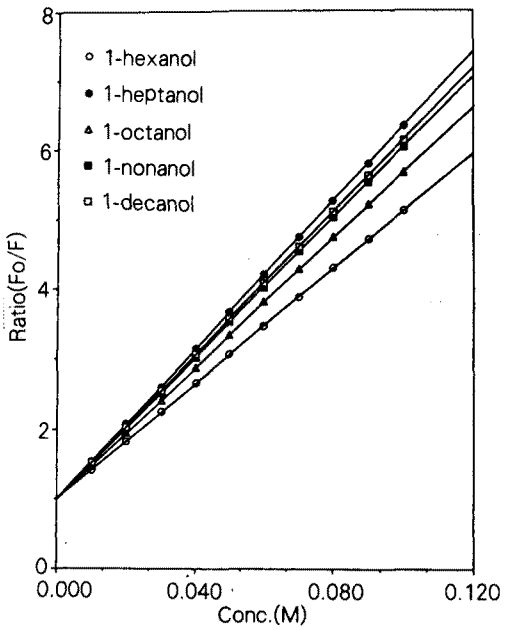

Fig. 2. Sterm-Volmer plot of quenching of ONS fluorescence in SPMVTL by 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol and 1-decanol. Lines were fitted by a least-squares analysis.

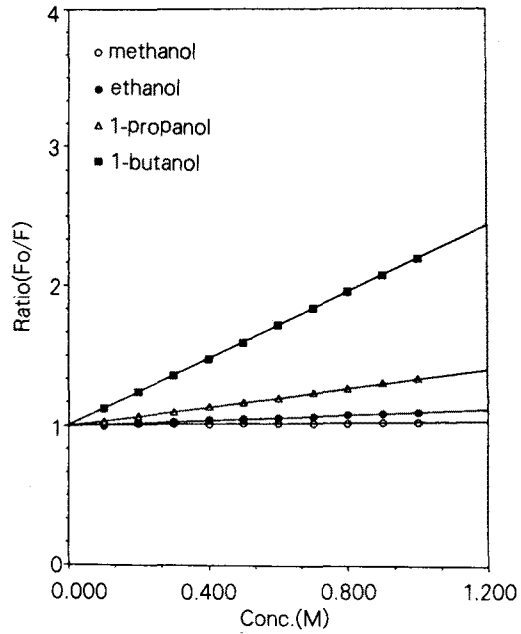

Fig. 3. Stern-Volmer plot of quenching of AS fluorescence in SPMVTL by methanol, ethanol, 1-propanol, and 1-butanol. Lines were fitted by a least-squares analysis.

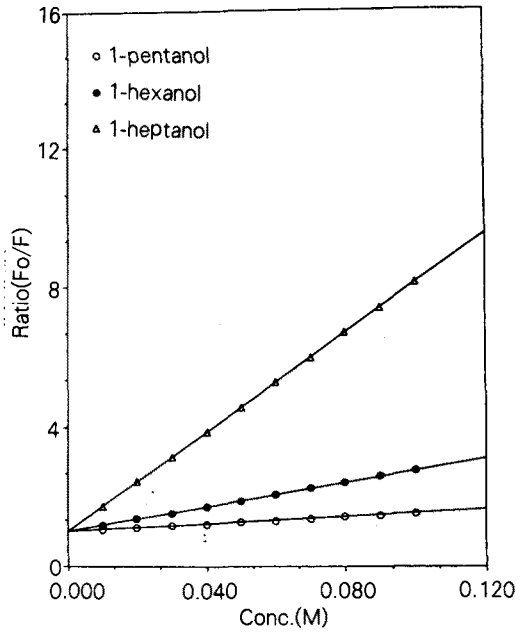

Fig. 4. Stern-Volmer plot of quenching of AS fluorescence in SPMVTL by 1-pentanol, 1-hexanol and 1-heptanol. Lines were fitted by a least-squares analysis.

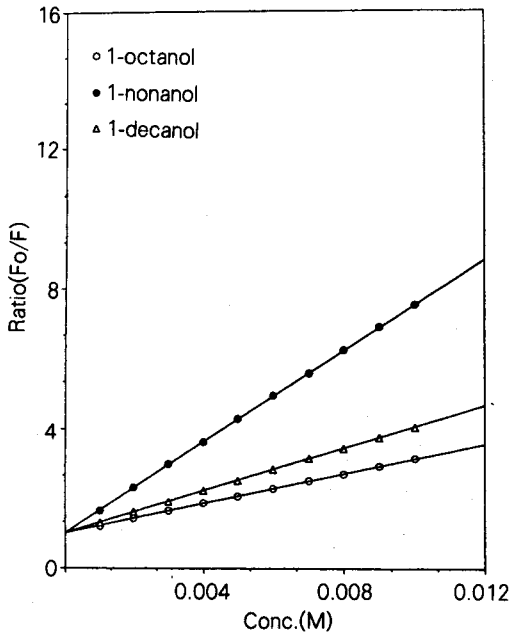

Fig. 5. Stern-Volmer plot of quenching of AS fluorescence in SPMVTL by 1-octanol, 1-nonanol and 1-decanol. Lines were fitted by a least-squares analysis.

Table 1. Stern-Volmer Constant of Quenching of ONS and AS Fluorescence by n-alkanols in SPMVTL Liposomes

n-alkanols	Stern-Volmer constant(1/M)[a]	
	ONS(S_s)	AS(S_i)
methanol	30.00	0.03
ethanol	30.40	0.10
1-propanol	35.30	0.34
1-butanol	37.80	1.20
1-pentanol	41.00	5.20
1-hexanol	41.20	17.60
1-heptanol	53.40	17.20
1-octanol	46.80	212.70
1-nonanol	50.40	654.30
1-decanol	51.40	302.60

a); Values are taken from Fig. 1, 2, 3, 4, and 5.

plots of equations 3 and 4, respectively, and are listed in Table 1. For water-insoluble n-alkanols, $[Q]_L=[Q]_T$, and equation 5 could be easily reached.

Table 2. Stern-Volmer Constant of Quenching of ONS and AS Fluorescence by n-alkanols in Dimethylsulfoxide and Textrahydrofuran Mixture(1 : 1).

n-alkanols	Stern-Volmer constant(1/M)[a]	
	K_{ONS}	K_{AS}
methanol	0.20	0.13
ethanol	0.38	0.36
1-propanol	0.52	0.76
1-butanol	0.68	1.50
1-pentanol	0.82	3.83
1-hexanol	1.02	5.18
1-heptanol	1.26	8.03
1-octanol	2.00	14.28
1-nonanol	2.92	28.07
1-decanol	5.14	63.33

Deta were adapted from out previous study(reference unmber, 9).

Table 3. Ratio of the Concentration of n-alkanols in the Surface Area to the Concentration in the Hydrocarbon Interior of the Lipid Bilayer of SPMVTL Liposomes(f_s/f_i).

n-alkanols	Ratio[a]
methanol	650.0
ethanol	228.0
1-propanol	151.6
1-butanol	69.5
1-pentanol	36.8
1-hexanol	11.90
1-heptanol	4.80
1-octanol	1.60
1-nonanol	0.74
1-decanol	2.10

a); The ratio values were obtained from equation (5) where the values of K_{ONS}, K_{AS}, S_S and S_i were those as shown in tables 1 and 2.

Our previous study, Stern-Volmer plots were drawn for quenching of ONS and AS fluorescence by n-alknols in in 1 : 1 mixture of DMSO

and THF[9]. The slopes of these plots yield the K_{ONS} and K_{AS} values, and the values are listed in Table 2. Assuming K_{ONS} and K_{AS} values in bulk solution are not much different from the values in the SPMVTL, these values can be substituted into equation 5. The values of F_s/F_i calculated by this method are listed in Table 3.

DISCUSSION

Type of quenching in this study, known as collisional or dynamic quenching, results from collisional encounters between fluorophore and quencher during the lifetime of the excited state[7]. This quenching is due to the interaction with fluorescent probe and hydroxyl group of n-alkanols. Therefore, the extent of fluorescence quenching depends upon the effective concentration of the n-alkanols surrounding the fluorophore.

The reasons for calculating the ratio of the concentrations of n-alkanols in the surface area to those in hydrocarbon interior of the outer monolayers of the SPMVTL lipid bilayer structures(f_s/f_i) by the use of the modified stern-Volmer equation(equation 5) were:

Firstly the Stern-Volmer equation cannot be applied to heterogeneous conditions. Secondly, there is a high possibility that THF and DMSO cannot be completely removed from SPMVTL suspension in spite of our best efforts. In addition, ONS, AS and n-alkanols were still present in the solutions of THF, DMSO and water.

In accordance to the analysis of preferential quenching of ONS and AS fluorescence by n-alkanols the penetration of n-alkanols to hydrocarbon interior increased directly proportional to their number of carbons. The analysis for uneven distribution of n-alkanols in the surface and hydrocarbon region of the outer monolayers that methanol is predominantly distributed on the surface area, while 1-nonanol has a greater accessibility to the hydrocarbon interior of the monolayer of the SPMVTL lipid bilayer structures. The cut-off phenomenon was reached at 1-decanol, where further increase in hydrocarbon length resulted in a decrease in the amount of penetration(Table 3). this indicates that the penetration in the hydrocarbon interior of the outer monolayers of the SPMV lipid bilayer structures of the n-alkanols increases with an increase in their carbon chain length and lipid solubility(except 1-decanol). We reported that the major distribution area of n-alkanols in the SPMV was the surface of the outer monolayers of the native membrane lipid bilayer structures[9]. Our study extended from the above research demonstrated a very interesting phenomenon in the SPMVTL. This result is in agreement with that of our study, indicating n-alkanols have a large distribution on the surface area of the outer monolayers of the SPMV and SPMVTL. However, the important point is the different abilities of n-alkanols between SPMV and SPMVTL in terms of the amount of penetration of the general anesthetics into the hydrocarbon region of the outer monolayers of the membrane bilayers. The ability of n-alkanols to penetrate into the hydrocarbon interior of the outer monolayers of the SPMVTL was larger than do into the SPMV. The reasons of the different penetrability of the general anesthetics can be analyzed as follows: (i) the penetrating effects of n-alkanols into the SPMV may be inhibited by the presence of proteins(the lipid-protein interactions) which are found to be tightly associated with lipids through covalent or noncovalent bonds, and (ii) gangliosides may have a significant role in the penetrability of the n-alkanols. n-Alkanols have been known to reduce the phase

transition temperature of model membranes[10]. Previous studies reported that n-alkanols increased the range and rate of the lateral motion of native and model membranes[11~14].

Many investigators reported that n-alkanols increased the range of the rotational mobility of native and model membranes[15~20]. Also, n-alkanols significantly increased the rate of the rotational mobility of native and model membranes[21~24]. Hence, it is strongly suggested that the more effective penetration into the lipid bilayer could result in higher perturbation of the fluidity of the hydrophobic core of SPMV and possibly higher general anesthetic activity. The enhanced fluidity of neuronal membranes produces by n-alkanols may alter such events as ion fluxes across the membrane, conformational changes in enzymes, or neurotransmitter mechanisms[25]. In contrast, ethanol may also have direct effects on receptor and transport molecules associated with the cell membrane[26]. For example, acute ethanol exposure has been reported to increase the number of GABA-receptors, which is consistent with the ability of GABA-mimetics to intensify many of the acute effects of alcohol[26]. There is also evidence that ethanol has effects on sodium dependent calcium uptake not accounted for by changes in bulk membrane fluidity[26].

The mechanism (s) of action of ethanol and the processes underlying the development of ethanol tolerance and dependence have been areas of active interest in our laboratories. Our approach has been largely focused on the biophysical properties of ethanol. The results indicate that a relationship exists between the ability of n-alkanols to penetrate the membrane bilayer and their general anesthetic activity. With increasing frequency, it is being recognized that the physical state of fluidity of the membrane has major biological significance. The fluidity of membrane lipids surrounding integral proteins can not only affect their conformation, but it can also regulate their activity[27]. The old concept that proteins carry the specific functions and lipids merely provide the proper fluidity does not hold any longer. At present, most investigators agree to this view that both the lipids and the proteins influence each other in their dynamics and each of them can bear specific functions. n-Alkanols with lipid solubility will enter hydrophobic regions of both proteins and lipids in the core of the membrane[24]. Although it is too early to explain exactly how the hydrophobic interactions affects functions, it is reasonable to predict that disruption of prtein function will result from disorder in any of the hydrophobic regions[24]. The function of membrane proteins may be modulated secondarily to changes in membrane fluidity. Conversely, there is also a possibility that n-alkanols may have a direct effects on certain receptors, receptor-gated ion channels, or membrane-bound enzymes, and then on membrane lipids. It may well be that n-alkanols concurrently interact with membrane lipids and membrane-bound proteins. At present, it may be premature to take sides in the controversy about whether membrane lipids or membrane proteins are site of n-alkanols action.

To the best of our knowledge, the results presented herein are the first to demonstrate the penetration site of n-alkanols into SPMVTL.

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참 고 문 헌

1) Goldstein DB. *The effects of drugs on membrane*

fluidity. Ann Rev Pharmacol Toxicol 1984; 24: 43-64.

2) Gonzales RA, Hoffman PL. *Receptor-gated ion channels may be selective CNS targets for ethanol. Trends Pharmacol Sciences 1991; 12: 1-3.*

3) Manevich EM, Koiv A, Jarv J, et al. *Binding of specific ligands to muscarinic receptors alters the fluidity of membrane fragments from rat brain. A fluorescence polarization study with lipid-specific probes. FEBS Letters 1988; 236: 43-46.*

4) Yunn I, Kim YS, Yu SH, et al. *Comparison of several procedures for the preparation of synaptosomal plasma membrane vesicles. Arch Pharm Res 1990; 13: 325-329.*

5) Yun I, Kang JS. *The general lipid composition and aminophospholipid asymmetry of synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. Mol Cells 1990; 1: 15-20.*

6) Yun I, Kang JS. *Effects of barbiturates on transbilayer fluidity domains of phospholipid model membrane monolayers. Korean J Pharmacol 1992; 28: 103-114.*

7) Yun I, Jung IK, Kim IS, et al. *The penetration of local anesthetics into the synaptosomal plasma membrane vesicles isolated from bovine brain. Asia Pacific J Pharmacol 1990; 5: 19-26.*

8) Jain MK, Mw NM. *Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer. III Phase transition in lipid bilayer. J Membrane Biol 1977; 34: 157-201.*

9) Kim IS, Baik SW, Chung KS. *The penetration site of n-alkanols into synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. J Korean Socity of Anesthesiologists 1993; 6: in press.*

10) Yun I, Yang MS, Kim IS, et al. *Bulk vs. transbilayer effects of ethanol on the fluidity of the plasma membrane vesicles of cultured Chinese hamster ovary cells. Asia Pacific J Pharmacol 1993; 8: 25-29.*

11) Yun I, Chung IK, Park YM, et al. *Effects of ethanol on the fluidity of plasma membrane vesicles isolated from cultured mous myeloma cell line Sp 2 /0-Ag 14. Korean J Pharmacol 1993; 29: in press.*

12) Chung IK, Kang JS, Kim BS, et al. *The effect of n-alkanols on the lateral diffusion of synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. Korean J Pharmacol 1993; 29: in press.*

13) Chung IK, Kim HI, Kim JR, et al. *Effects of n-alkanols on the lateral diffusion of model membranes of total lipid fracion extracted from brain membranes. Korean Biochem J 1993; 25: in press.*

14) Chung IK, Kim JS, Kim MH, et al. *Effects of n-alkanols on the lateral diffusion of total phosphospholipid fraction extracted from brain membranes. Arch Pharm Res 1993; 16: in press.*

15) Perlman BJ, Goldstein DB. *Genetic influence of the central nervous system depressant and membrane-disordering actions of ethanol and sodium valporate. Mol Pharmacol 1984; 26: 547-552.*

16) Harris RA, Baxter DM, Mitchel MA, et al. *Physical properties and lipid compositin of brain membranes from ethanol tolerant-dependent mice. Mol Pharmacol 1984; 25: 401-409.*

17) Harrsi RA, Bruno P. *Membrane disordering by anesthetic drugs: Relationship to synaptosomal sodium and calcium fluxes. J Neurochem 1985; 44: 1274-1281.*

18) Harris RA, Schroeder F. *Ethanol and the physcial properties of brain membranes. Mol Pharmacol 1981; 20: 128-137.*

19) Yun I, Kang JS. *Transbilayer effects of n-alkanols on the fluidity of phospholipid model membranes. Arch Pharm Res 1992; 15: 152-161.*

20) Yun I, Kang JS. *Transbilayer effects of n-alkanols on the fluidity of total lipids extracted from synaptosomal plasma membrane vesicles. Korean J Pharmacol 1992; 28: 103-114.*

21) Yun I, Kang JS. *Effects of n-alkanols on the fluidity of native and model membranes. Biochim Biophys Acta 1993; 1208: in press.*

22) Chung YZ, Kang JS, Kim SH, et al. *Effects of n-alkanols on the rotational relaxation time of 1, 6-diphenyl-1, 3, 5-hexatriene in the synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. Arch Pharm Res 1993; 16: in press.*

23) Chung YZ, Choi CH, Park HS, et al. *Effects on n-alkanols on the rotational relaxation time of 1, 6-diphenyl-1, 3, 5-hexatriene in the model membranes of total lipid fraction extracted from brain membranes. Korean Biochem J 1993; 25: in press.*

24) Chung YZ, Son HC, Kim BS, et al. *Effects of n-alkanols on the rotational relaxation time of 1, 6-diphenyl-1, 3, 5-hexatriene in the model membranes of phospholipids extracted from brain membranes. Mol Cells 1993; 3: in press.*

25) Lee NM, Smith AS. *Toxicology of CNS Depressants. lst ed. Boca Raton: CRC Press. 1986; 35-67.*
26) Katzung BG. *Basic and clinical Pharmacology. 4th ed, Singapore: Simon & Schuster Asia Pte Ltd. 1989; 278-279.*
27) Houslay MD, Stanley KK. *Dynamics of biological membranes. lst ed, New YorkL John Wiley & Son. 1982; 92-151.*