The Penetration Site of n-Alkanols into Synaptosomal Plasma Membrane Vesicles Isolated from Bovine Cerebral Cortex

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=국문초록=

소의 대뇌피질로부터 분리된 Synaptosomal Plasma Membrane Vesicle에서의 n-Alkanols의 침투정도

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세포막에서 마취제의 작용점을 규명하기 위하여, 마취제의 많은 부분을 차지하는 n-Alkanol을 이용하여, 소의 synaptosomal plasma membrane vesicles(SPMV)에서 n-Alkanol의 침투 정도를 형광 probe를 이용한 형광소광법을 통하여 검색하였다.

n-Alkanols는 SPMV 외부 단층(outer monolayer)의 표면에 주로 분포하되 그 탄소수에 비례하여 소수성 부위에 분포되는 양이 증가되는 경향을 나타내었다(1-decanol은 제외). Methanol, Ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol 및 1-decanil은 SPMV 외부 단층의 표면(친수성 부위)에 분포되는 것에 비하여 각각 949, 416.8, 214.8, 90.3, 53.7, 15.20, 6.80, 2.00, 1.03 및 2.40 배가 된다는 것을 확인하였다. 1-decanol은 C_{10}인데도 불구하고 C_8인 1-octanol에 비하여 적은 양이 소수성 부위에 침투 분포한다는 것이 확인되었다.

Key Words: n-Alkanols, Penetration site, Native membranes, Fluorescence probe technique, Modified Stern-Volmer equation

INTRODUCTION

n-Alkanols are members of the large family of anesthetic agents whose biological potency correlates with lipid solubility. It is proposed that the site of action for n-alkanols is the cellular membrane[1]. It is suggested that acute n-alkanols administration act in a physical manner to cause the disordering of membranes and thereby increase membrane fluidity[1]. The molecules of n-alkanols dissolve into cell membranes and cause the lipid bilayer of the membrane to become less rigid in its structural arrangement[1]. 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[1]. The results of investigations on the effects of higher alkanols and the corresponding alkanes on membrane luciferases indicate that the anesthetic site could be hydrophobic pockets on membrane proteins rather than the lipid part of the membrane[2].

The membrane-fluidizing hypothesis of ethanol action 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[3,4]. Still, a large, diverse collection of physiological agonists produces the alterations in membrane fluidity as well as their specific ligand-receptor interaction[5].

At the present time, the exact mechanism(s) of action for ethanol and related alkanols in the central nervous system is not fully delineated.

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 isolated the synaptosomal plasma membrane vesicles (SPMV) from fresh bovine cerebral cortex, and examined the penetration site of n-alkanols into 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 the lipid bilayer structures.

EXPERIMENTAL 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[6].

3) Fluorescence measurements

The SPMV were suspended in phosphate buffered saline(PB, pH 7.4) containing NaCl 8 g/l, KCl 0.2 g/l, 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 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 μM, respectively. The mixture was stirred for 2 h at room temperature in order to reduce the concentration of tetrahydrofuran and dimethyl sulfoxide that might alter permeability of

SPMV 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 SPMV. The fluorescence measurements were carried out with a SPF-500C spectrofluorometer(SLM Instruments Inc., Champaogn-Urbana, IL, USA), equipped with a 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(SPMV 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:

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

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, τ_o is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of quencher, and $K = k_q \tau_o$ 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 SPMV. 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:

$$F_oF = 1 + fKP[Q]_T \cdots\cdots\cdots\cdots \text{equation 2}$$

In this equation, $[Q]_L$ and $[Q]_T$ are the concentrations of the quencher in the outer monolayers of the SPMV lipid bilayer structures and the total concentration of the quencher in the system, respectively. P is the partition coefficient, and f is a regional correction factor for uneven distribution of the quencher between the surface area and the interior area in the outer monlayers of the SPMV 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 SPMV gives the following:

$$F_o/F = 1 + f_s K_{ONS} P[Q]_T \cdots\cdots\cdots\cdots \text{equation 3}$$
$$F_o/F = 1 + f_i K_{AS} P[Q]_T \cdots\cdots\cdots\cdots \text{equation 4}$$

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 SPMV 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.

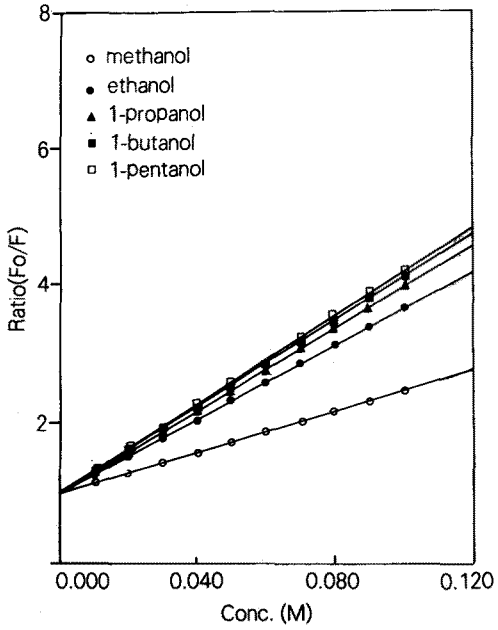

Fig. 1. Stern-Volmer plot of quenching of ONS fluorescence in SPMV by methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol. Lines were fitted by a least-squares analysis.

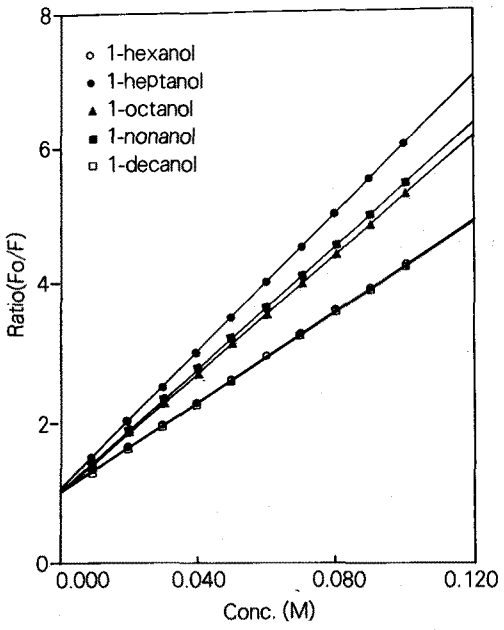

Fig. 2. Stern-Volmer plot of quenching of ONS fluorescence in SPMV by 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol and 1-decanol. Lines were fitted by a least-squares analysis.

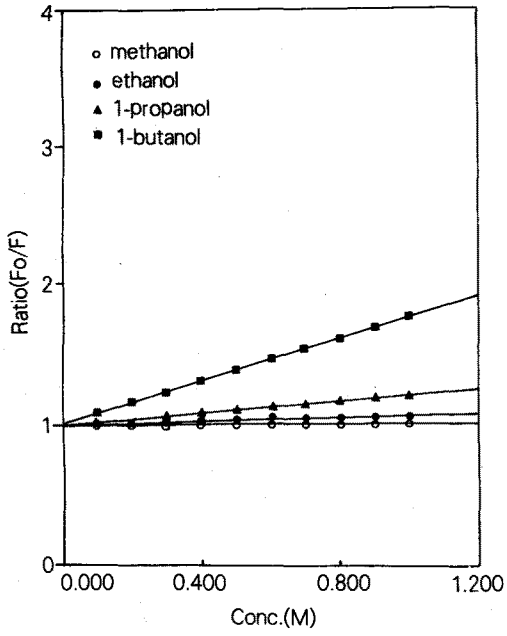

Fig. 3. Stern-Volmer plot of quenching of AS fluorescence in SPMV by methanol, ethanol, 1-propanol, and 1-butanol. Lines were fitted by a least-squares analysis.

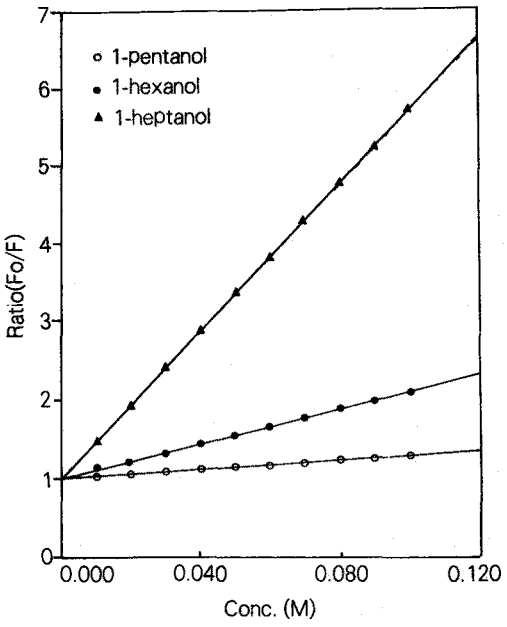

Fig. 4. Stern-Volmer plot of quenching of AS fluorescence in SPMV by 1-pentanol, 1-hexanol and 1-heptanol. Lines were fitted by a least-squares analysis.

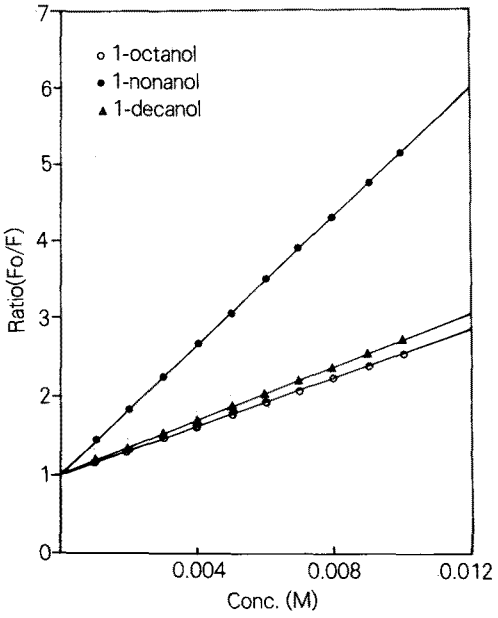

Fig. 5. Stern-Volmer plot of quenching of AS fluorescence in SPMV by 1-octanol, 1-nonanol and 1-decanol. Lines were fitted by least-squares analysis.

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

n-alkanols	Stern-Volmer Constant(1/M)[a]	
	ONS(S_o)	AS(S_i)
methanol	14.60	0.01
ethanol	26.40	0.06
1-propanol	29.40	0.20
1-butanol	31.10	0.76
1-pentanol	32.20	2.80
1-hexaonl	32.60	10.90
1-heptanol	50.10	46.80
1-octanol	42.70	152.60
1-nonanol	44.30	414.30
1-decanol	32.40	170.40

a); Values are taken from Fig. 1~5.

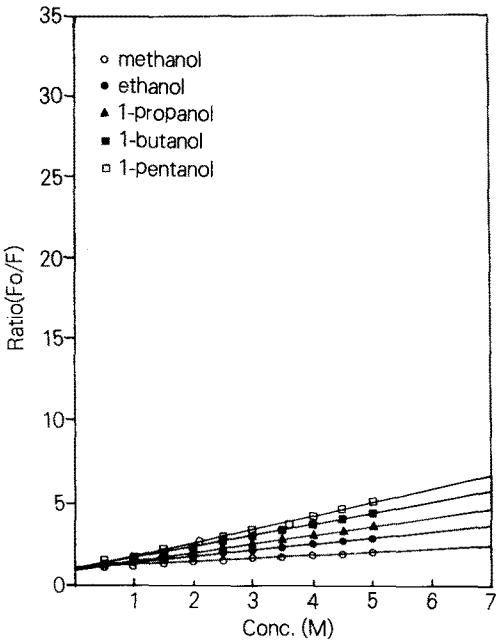

Fig. 6. Stern-Volmer plot of quenching of ONS fluorescence in DMSO and THF mixture(1 : 1) by methanol, ethanol, 2-propanol, 2-butanol and 1-pentanol. Lines were fitted by a least-squares analysis.

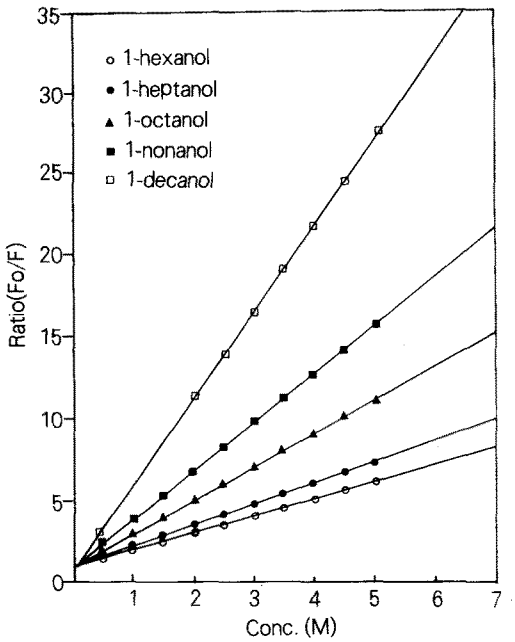

Fig. 7. Stern-Volmer plot of quenching of ONS fluorescence in DMSO and THF mixture(1 : 1) by 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol and 1-decanol. Lines were fitted by a least-squares analysis.

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

Stern-Volmer plots were also drawn for quenching of ONS and AS fluorescence by n-alkanols in 1 : 1 mixture of DMSO and THF(Fig. 6~9). 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 SPMV, these values can be substituted into equation 5. The values of f_s/f_i calculated by this method are listed in Table 3.

Table 2. Stern-Volmer Constant of Quenching of ONS and AS Fluorescence by n-alkanols in Dimethylsulfoxide and Tetrahydrofuran 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

a); Values are taken from Fig. 6~9.

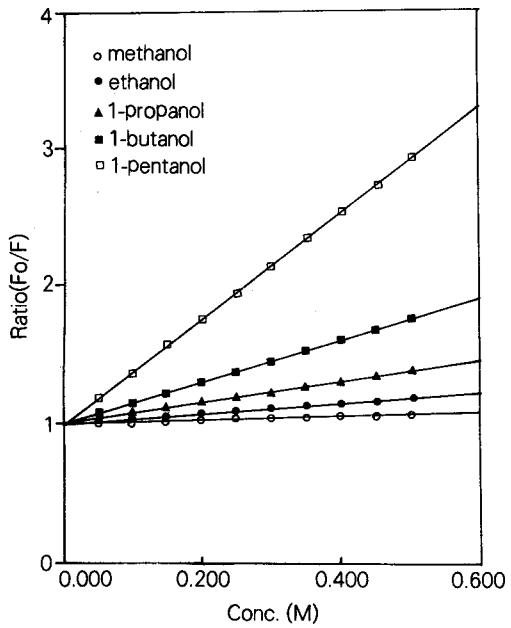

Fig. 8. Sten-Volmer plot of quenching of AS fluorescence in DMSO and THF mixture(1 : 1) by methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol. Lines were fitted by a least-squares analysis.

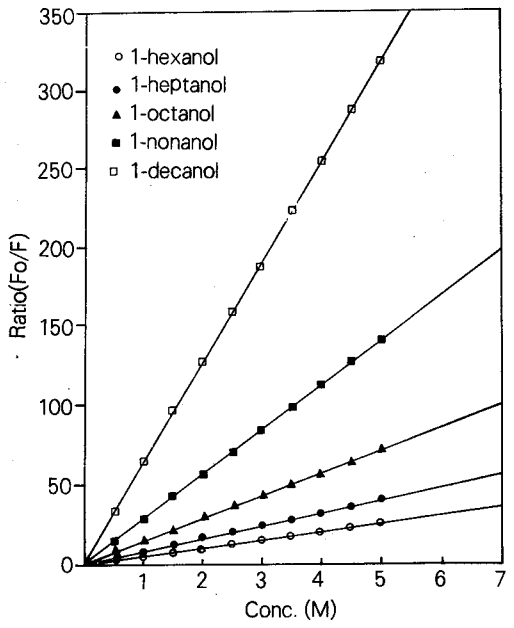

Fig. 9. Stern-Volmer plot of quenching of AS fluorescence in DMSO and THE mixture(1 : 1) by 1-hexanol, 1-heptanaol, 1-octanol, 1-nonanol and 1-decanol. Lines were fitted by a least-squares analysis.

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 SPMV($f/_sf_i$)

n-alkanols	Ratio[a)]
methanol	949.0
ethanol	416.8
1-propanol	214.8
1-butanol	90.3
1-pentanol	53.7
1-hexanol	15.20
1-heptanol	6.80
1-octanol	2.00
1-nonanol	1.03
1-decanol	2.40

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 table 1 and 2.

DISCUSSION

The processes that result in fluorescence quenching include excited state reactions, energy transfer, complex formation and collisional quenching. This type of quenching, known as collisional or dynamic quenching, results from collisional encounters between fluorophore and quencher during the lifetime of the excited state[8)]. This collisional 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 SPMV lipid bilayer structures(f_s/f_i) by the use of the modified Stern-Volmer equation(equation 5) were: Firstly, Stern-Volmer equation cannot be applied to heterogeneous conditions.

Secondly, there is a high possibility that THE and DMSO cannot be completely removed from SPMV suspension in spite of our best efforts. In addition, ONS, AS and n-alkanols were still present in the solutions of THE, 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 of preferential quenching of ONS and AS fluorescence by n-alkanols indicates that methanol is predominantly distributed on the surface area of the outer monolayers, while 1-nonanol has a greater accessibility to the hydrocarbon interior of the monolayer of the SPMV 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 indicated 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. n-Alkanols have been known to reduce the phase transition temperature of model membranes[9)]. Previous studies reported that n-alkanols increased the range and rate of the lateral motion of native and model membranes[10~14)]. Many investigators reported that n-alkanols increased the range of the rotational mobility of native and model membranes[15~21)]. Also, n-alkanols significantly increased the rate of the rotational mobility of native and model membranes[22~25)]. In summary, n-alkanols significantly increase the range and rate of the lateral and rotational motion of native and model membranes. The cut-off phenomenon was reached at 1-decanol, where fur-

ther increase in hydrocarbon length resulted in a decrease in fluidity of lipid bilayer structures of native and model membranes[12~14,20~25]. 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 fluidizing effect of n-alkanols have been related to changes in specific membrane functions, including neurotransmitter receptors for dopamine, norepinephrine, glutamate, and opioids; enzymes such as Na^+, K^+-ATPase, Ca^{2+}-ATPase, 5′-nucleotidase, acetylcholinesterase, and adenylate cyclase; the mitochondrial electron transport chain; and ion channels such as those for Ca ion[1]. 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 anesthestic 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. In fact, a large, diverse collection of physiological agonists produce the alterations in membrane fluidity as well as their specific ligand-receptor interaction [28]. n-Alkanols with lipid solubility will enter hydrophobic regions of both proteins and lipids in the core of the membrane[25]. Although it is too early to explain exactly how the hydrophobic interactions affects functions, it is reasonable to predict that disruption of protein function will result from disorder in any of the hydrophobic regions[25]. 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 effect 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 SPMV.

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