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The Region of Distribution of Barbiturates in Synaptosomal Plasma Membrane Vesicles Isolated from Rat Brain as Studied by Fluorescence Quenching[1]

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ABSTRACT

The relative distribution ratio of barbiturates between hydrocarbon interior and surface region of outer monolayer of synaptosomal plasma membrane vesicles (RSPMV) isolated from rat whole brain was determined by employing the fluorescent probe technique. The two fluorescent probes N- octadecylnaphthyl-2-amine-6-sulfonic acid (ONS) and 12-(9-anthroyloxy) stearic acid (AS) were utilized as probes for hydrocarbon interior and surface of outer monolayer of RSPMV, respectively. The Stern-Volmer equation for fluorescent quenching was modified to calculate the relative distribution ratio. The analysis of preferential quenching of these probes by barbiturates indicates that pentobarbital, hexobarbital, amobarbital and phenobarbital are predominantly distributed on the surface region, whereas thiopental sodium has an accessibility to the hydrocarbon interior of the outer monolayer of the RSPMV. From these results, it is strongly suggested that the more effective penetration into the hydrocarbon interior of the outer monolayer of the membrane lipid bilayer could result in higher general anesthetic activity.

Key Words: Barbiturates, Fluorescence quenching, Modified Stern-Volmer equation, Biomembranes

INTRODUCTION

Barbiturates are among the oldest drugs to treat anxiety and related states. While they have been largely replaced by benzodiazepines as anxiolytics, hypnotics and sedatives, barbiturates are still essential drugs in antiepileptic therapy and in general anesthesia. Despite the long and widespread use of barbiturates and their clear potential for abuse, their precise molecular mechanism of action has not yet been understood.

The purpose of this research is three-fold: (i) to provide a basis for studying the molecular mechanism of action of barbiturates through the investigation of the primary site of action of the drugs; (ii) to develop a fluorescence analysis in detecting the region of distribution of the drugs and various substances at cellular level; and (iii) to contribute to an understanding of the behavior of synaptosomal plasma membrane vesicles (RSPMV) isolated from rat whole brain. In the present study, we examined the relative distribution ratio of barbiturates between hydrocarbon interior and surface re-

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gion of outer monolayer of RSPMV, employing two fluorescent probes N-octadecylnaphthyl-2-amine-6-sulfonic acid (ONS) and 12-(9-anthroyl-oxy) stearic acid (AS). The analysis of preferential quenching of these probes by barbiturates revealed the relative accessibility of the drugs into hydrocarbon interior of the outer monolayer of RSPMV.

MATERIALS AND METHODS

Chemicals

The fluorescent probes, ONS and AS were purchased from Molecular Probes, Inc. (Junction City, OR., USA). Barbiturates were obtained from Shinjun Pharmaceutical Co. (Seoul Korea). 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 purchased from commercially available sources and were of the highest quality available. Water was deionized.

Membrane preparation

RSPMV were isolated from the whole brain of Mature male Sprague-Dawley rat (weighing 180~250 g) using Ficoll and sucrose gradient centrifugation as described previously (Yun *et al.*, 1990a; Yun and Kang, 1990). Briefly, rat brains (20 g) were rapidly removed following decapitation, suspended in ice-cold 0.32 M sucrose and 3 mM HEPES at pH 7.5 (SH). The whole brains were homogenized with 10 strokes of a tissue grinder and then centrifugated at 1,200×g for 5 min. The pellet (P-1) was saved and the supernatant (S) was centrifugated at 17,300×g for 12 min. This second pellet (P-2) was washed once with SH and recentrifuged at 17,300×g for 12 min. The pellet (washed P-2) was resuspended in SH, layered over 7.5 % (w/v) and 12% (w/v) Ficoll in SH, and centrifuged at 75,000×g for 60 min. Particles were resolved into the following fractions: ① particles dispersed in 7.5% (w/v) Ficoll in SH, ② particles at 7.5% (w/v)~12% (w/v) interface, ③ particles dispersed in 12% (w/v) Ficoll in SH, and ④ the pellet (P-3). The fraction 2 was saved, diluted with SH, and centrifuged at 18,120×g for 20 min. The synaptosomal pellet was lysed by resuspension in 6 mM-Tricine (pH 8.1) and lysis was completed by stirring for 90 min. The suspension was layered over discontinuous density gradients consisting of 10, 20, 25 and 32.5% (w/v) sucrose containing 3 mM-HEPES, pH 7.5 and centrifuged at 16,400×g for 160 min. The RSPMV-A (10~20%), RSPMV-B (20~25%) and RSPMV-C (25~32.5%) membrane bands were collected, diluted with water, and centrifuged at 30,620×g for 20 min. The pooled RSPMV (RSPMV-B+RSPMV-C) were also resuspended in 0.32 M sucrose, 1 mM $MgSO_4$, 0.5 mM EDTA, 5 mM Tris-SO_4, pH 7.4 (buffer A) at a protein concentration of 5~10 mg/ml. For the fluorescence measurements, the pooled RSPMV were resuspended in phosphate-buffered saline (PBS, pH 7.4) containing 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g $Na_2HPO_4 \cdot 7H_2O$ and 0.48 g/l HEPES.

The purity of RSPMV was determined by enzymatic and morphological standard. The specific activities of Na,K-ATPase (Yun and Kang, 1990; Yun *et al.*, 1990a), acetylcholinesterase (Ellman *et al.*, 1961) and 5′-nucleotidase (Yun and Kang, 1990; Yun *et al.*, 1990a) were about 6-, 2.5- and 3-fold, respectively, enriched in the plasma membrane fraction as compared to crude homogenates. Electron microscopic examination also showed that the membranes were in vesicular form (Yun *et al.*, 1990a). Protein was determined by the procedure of Lowry *et al.* (1959) using BSA as a standard.

Fluorescence measurements

Fluorescence measurements were performed as described earlier (Kim *et al.*, 1993). RSPMV were suspended in PBS to a concentration of 1 mg of protein/ml. Barbiturates were dissolved in a minimum volume of 0.1 N-NaOH, diluted with deionized water and the pH adjusted to 9 to 10. Solutions were prepared immediately before use. Barbiturates, at the concentrations indicated, were added directly to membranes resuspended in PBS. The pH of the buffered RSPMV solution was not changed significantly by addition of barbiturates.

Stock solutions of AS (1 mM) in tetrahy-

drofuran (THF) and ONS (1 mM) in dimethylsulfoxide (DMSO) were made and kept in a cold dark place. The incorporation of these 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 THF and DMSO that might alter permeability of biomem. 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 and might oxidize biomembranes. The fluorescence measurements were carried out with an SPF-500C spectrofluorometer (SLM Instruments Inc., Champaign-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 2 min. ONS was excited 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 (RSPMV suspensions without fluorescent probes), prepared under identical conditions, served as controls for the fluorometric measurements. All experiments were carried out at least five times and the averages of these values were obtained. These relative values did not differ by more than ± 5 % from each value measured. All measurements were performed at 37 ± 0.1℃.

Calculations

The relative distribution ratio of barbiturates between hydrocarbon interior and surface region of outer monolayer of RSPMV was calculated by modified Stern-Volmer equation.

RESULTS

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

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K[Q] \qquad \text{equation 1}$$

In this equation, F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of quencher, and $K = k_q \tau_0$ is the Stern-Volmer quenching constant. A plot of F_0/F versus [Q] yields an intercept of one on the y axis and a slope equal to K. The primary goal of this work has been to evaluate the relative ratio of distribution of barbiturates between the surface and hydrocarbon interior region of the outer monolayer of the RSPMV. In the present study, the Stern-Volmer equation was modified for uneven distribution of the quencher in the RSPMV. For water-soluble barbiturates, the concentration in the aqueous phase is excessively larger than that in the lipid bilayer at pH 7.4. Consequently, $[Q]_L \cong P[Q]_T$ and equation 1 can be modified:

$$F_0/F = 1 + fKP[Q]_T \qquad \text{equation 2}$$

where $[Q]_L$ and $[Q]_T$ are the concentrations of the quencher in the outer monolayer of the lipid bilayer structures of RSPMV 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 and the interior region in the outer monolayer of the RSPMV. 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 hydrocarbon region of the outer monolayer of the membrane lipid bilayer structures (Yun *et al.*, 1990b). Hence, ONS or AS quenching in the RSPMV gives the following:

$$F_0/F = 1 + f_s K_{ONS} P[Q]_T \qquad \text{equation 3}$$
$$F_0/F = 1 + f_i K_{AS} P[Q]_T \qquad \text{equation 4}$$

where 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 and the interior region of the outer monolayer of the biomembranes and becomes the concentration gradient of the quencher between these two regions.

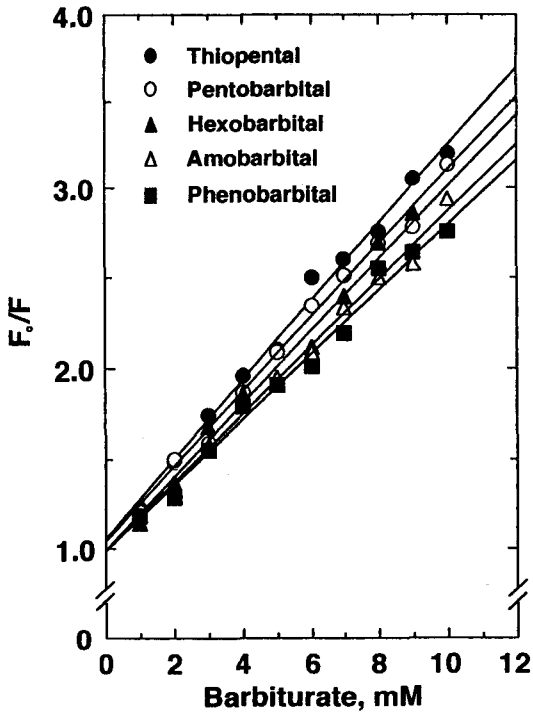

Fig. 1. Stern-Volmer plot of quenching of ONS fluorescence in RSPMV by thiopental sodium, pentobarbital, hexobarbital, amobarbital and phenobarbital. Lines were fitted by a least-squares analysis.

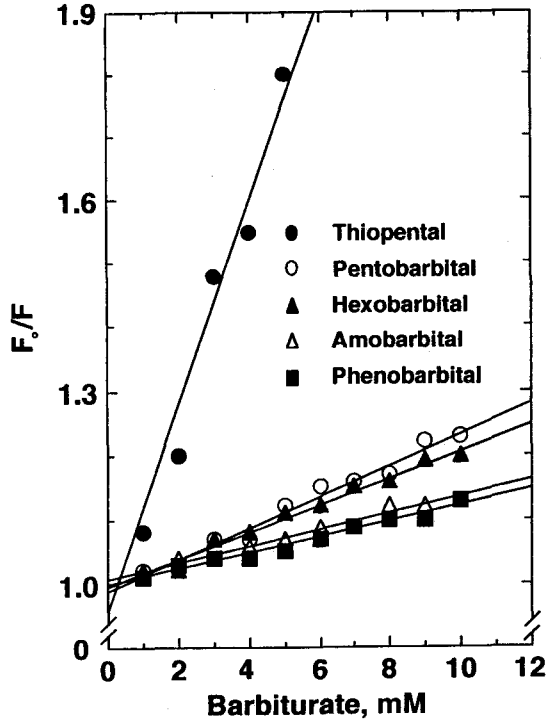

Fig. 2. Stern-Volmer plot of quenching of AS fluorescence in RSPMV by thiopental sodium, pentobarbital, hexobarbital, amobarbital and phenobarbital. Lines were fitted by a least-squares analysis.

Table 1. S_s and S_i values in synaptosamal plasma membrane vesicles (RSPMV) isolated from rat whole brain

Barbiturates	SPMV[a]	
	$S_s(M^{-1})$	$S_i(M^{-1})$
Thiopental sodium	220.0	159.5
Pentobarbital	206.4	24.4
Hexobarbital	202.7	21.1
Amobarbital	188.4	13.0
Phenobarbital	181.6	12.4

[a]Values are taken from Figures 1 and 2.

Table 2. Stern-Volmer constant of quenching of ONS and AS fluorescence by barbiturates in dimethylsulfoxide and tetrahydrofuran mixture (1 : 1)

Barbiturates	Stern-Volmer Constant(M^{-1})[a]	
	K_{ONS}	K_{AS}
Thiopental sodium	9.4	29.6
Pentobarbital	7.5	17.4
Hexobarbital	7.3	16.1
Amobarbital	4.9	13.8
Phenobarbital	3.5	12.0

[a]Values are taken from Figures 3 and 4.

The plots of F_o/F vs. $[Q]_T$ of equation 3 are shown in Fig. 1. And the plots of F_o/F vs. $[Q]_T$ of equation 4 are shown in Fig. 2. From these lines, f_s/f_i can be obtained:

$$f_s/f_i = \frac{S_s K_{AS}}{S_i K_{ONS}} \qquad \text{equation 5}$$

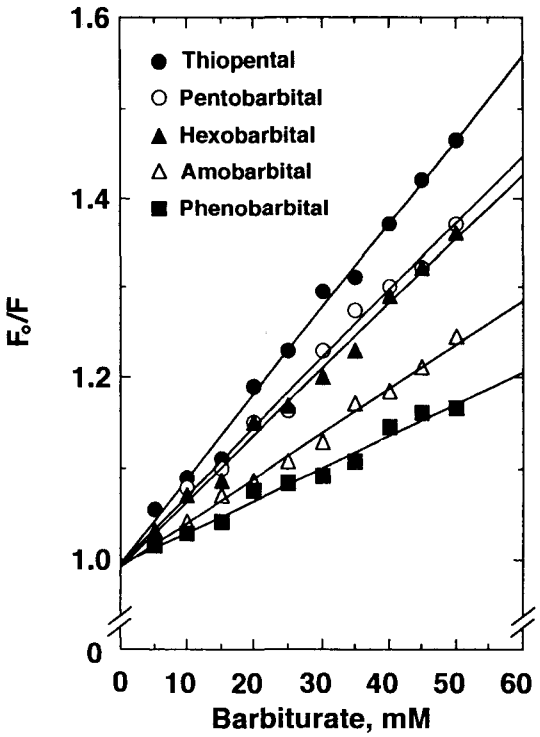

Fig. 3. Stern-Volmer plot of quenching of ONS fluorescence in DMSO and THF mixture (1:1) by thiopental sodium, pentobarbital, hexobarbital, amobarbital and phenobarbital. Lines were fitted by a least-squares analysis.

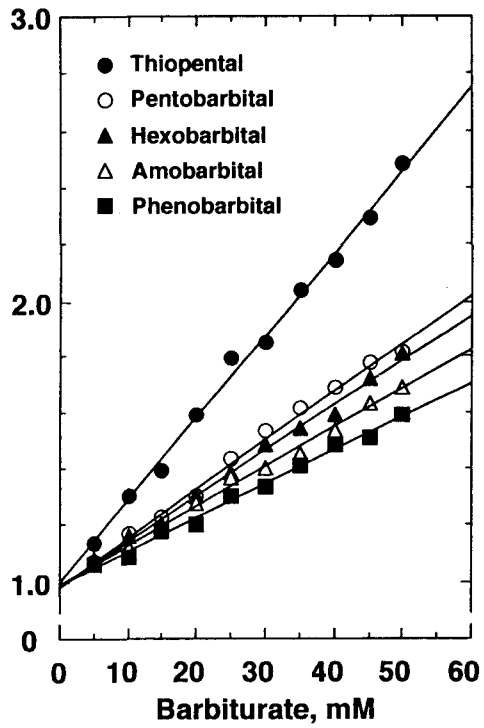

Fig. 4. Stern-Volmer plot of quenching of AS fluorescence in DMSO and THF mixture (1:1) by thiopental sodium, pentobarbital, hexobarbital, amobarbital and phenobarbital. Lines were fitted by a least-squares analysis.

In this equation, S_S and S_I are the slopes of the plots of equations 3 and 4, respectively, and are listed in Table 1. For water-insoluble barbiturates, $[Q]_L \cong P[Q]_T$, and equation 5 could be easily reached.

Stern-Volmer plots were also drawn for quenching of ONS and AS fluorescence by barbiturates in 1:1 mixture of DMSO and THF (Figs. 3 and 4). 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 biomembranes, 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 3. Ratios (f_s/f_i) of the concentration of barbiturates in the surface region to the concentration in the hydrocarbon interior of the outer monolayer of synaptosomal plasma membrane vesicles (RSPMV) isolated from rat whole brain (f_s/f_i)

Barbiturates	Ratios[a]
	RSPMV
Thiopental sodium	4.3
Pentobarbital	19.6
Hexobarbital	21.2
Amobarbital	40.8
Phenobarbital	50.2

[a]The ratio values were obtained from equation 5 where the values of S_S, S_I, K_{ONS} and K_{AS} are those as shown in Tables 1 and 2.

DISCUSSION

The 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 (Yun *et al.*, 1990b; Kim *et al.*, 1993). Therefore, the extent of fluorescence quenching depends upon the effective concentration of the barbiturates surrounding the fluorophore.

The reasons for calculating the relative ratio (f_s/f_i) of distribution of the concentrations of barbiturates in the surface region to those in hydrocarbon interior of the outer monolayer of RSPMV by the modified Stern-Volmer equation (equation 5) were: (i) Because the Stern-Volmer equation for fluorescence quenching is a rule which can be applicable to homogeneous solutions, not to heterogeneous conditions used in the present study, we modified the Stern-Volmer equation, (ii) The method utilized in this and previous experiments (Yun *et al.*, 1990b; Kim *et al.*, 1993) is based on the assumption that the modified Stern-Volmer equation is a rule which can be applicable to heterogeneous solutions and (iii) There is a good possibility that THF and DMSO cannot be completely removed from the suspension of RSPMV in spite of our best efforts. In addition, ONS, AS and barbiturates were still present in the solutions of THF, DMSO and water.

The analysis of preferential quenching of ONS and AS fluorescence by barbiturates revealed that the penetration of barbiturates into hydrocarbon interior increased with their lipid solubility and general anesthetic efficacy. This indicates that pentobarbital, hexobarbital, amobarbital and phenobarbital are predominantly distributed on the surface region of the outer monolayer, while thiopental sodium has an accessibility to the hydrocarbon interior of the monolayer of the RSPMV. We also reported that the major distribution region of barbiturates in the model membranes of phosphatidylcholine (BSPMVPC) (Kim *et al.*, 1993) and phosphatidylethanolamine (BSPMVPE) (Kim *et al.*, 1993) extracted from synaptosomal plasma membrane vesicles (BSPMV) of bovine brain cortex was the surface of the outer monolayer of the model membranes. The results of the present study, together with our previous report, (Kim *et al.*, 1993) demonstrated an interesting phenomenon that barbiturates have a strong tendency to distribute to the surface region of the outer monolayer of the RSPMV, BSPMVPC and BSPMVPE than to the hydrocarbon region of the membranes' outer monolayer. This may be due to electrostatic attraction between the surface of the outer monolayer of membrane which is positively charged and the barbiturates which are negatively charged in aqueous media.

The relative penetrabilities of individual barbiturates into the hydrocarbon region were identical to our previous study (Kim *et al.*, 1993). However, the important point is the different degree of penetrability of barbiturates into RSPMV (in this study), BSPMVPC (Kim *et al.*, 1993) and BSPMVPE (Kim *et al.*, 1993) in terms of the amount of penetration of the drugs into the hydrocarbon region of outer monolayer of membranes. The penetrability of barbiturates in RSPMV, BSPMVPC and BSPMVPE was in the following order: RSPMV >BSPMVPC>BSPMVPE. According to previous studies (Kang, 1990; Kang *et al.*, 1992), the relative fluidity of BSPMV, BSPMVPC and BSPMVPE in the relation to the range and the rate of the lateral and the rotational mobility was in the order of BSPMVPE, BSPMVPC, BSPMV. Thus, it seems likely that there might be a correlation between intrinsic membrane fluidity and penetrability of the barbiturates into the hydrocarbon region of the outer monolayer of native and model membranes. The results of this study and previous study (Kim *et al.*, 1993) strongly suggest that the penetrabilities of the barbiturates into hydrocarbon region of outer monolayer are inversely proportional to intrinsic fluidity of native and model membranes. It is highly probable that the membrane fluidity and the penetrability of drugs to the membrane are directly proportional. In this respect, barbiturates seem to be an exception and the reasons for this can be delineated as follows. The penetrability of barbiturates into the RSPMV may be amplified by the presence

of proteins (the lipid-protein interactions) which are found to be tightly associated with lipids through covalent or noncovalent bonds. Phosphatidylcholine is generally positively charged and this results in attracting more barbiturates. BSPMVPE is less attractive to barbiturates in comparison with BSPMVPC, due to its neutrality and bearing no charge.

The penetrabilities of individual barbiturates into the hydrocarbon region of the outer monolayer of the RSPMV were in the order of thiopental sodium, pentobarbital, hexobarbital, amobarbital, phenobarbital. Barbiturates have been known to reduce the phase transition temperature of pure phospholipid model membranes (Lee, 1976). Previous studies reported that barbiturates increased the range and the rate of the lateral and the rotational motion of BSPMV (Kang, 1990). In particular, barbiturates have a greater increasing effect on rotational diffusion range of the outer monolayer of native membranes as compared to the inner monolayer of the membranes (Kang, 1990). It is strongly suggested that the more effective penetration of barbiturates into the hydrocarbon region of the outer monolayer of the native membranes could result in higher disordering effects on the hydrophobic core of RSPMV and possibly a greater general anesthetic activity.

In detecting the site of distribution or the binding site of drugs, a radiolabelled isotope has been widely used. However, this method bears several disadvantages. It not only requires expensive laboratory equipments but also has a difficulty in disposition of waste materials. Hence it has become a serious contributing factor to environmental pollution. On the contrary, the method adopted in this research, namely the fluorescence analysis, requires only a minimal experimental expense and is very simple and easy in itself. To our knowledge, the results presented herein are unprecedented in demonstrating the region of distribution of barbiturates in RSPMV by specifically employing fluorescence probe technique.

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=국문초록=

Barbiturates가 생체세포막 외측 단층의 소수성 부위와 친수성 부위에 분포되는 상대적 비율

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윤 일·이 병 우

Barbiturates의 분자적 약리학적 작용기전 연구에 기초자료를 제공키 위하여 본연구를 수행하였다. 분자적 약리작용 기전 연구에서는 무엇보다도 선행되어야 하는 것이 barbiturates가 신경세포막에서 어느 부위에 주로 분포되는가를 알아내는 데에 있다. 쥐(Rat)의 뇌로부터 분리한 synaptosomal plasma membrane vesicles (RSPMV)를 분리한 후 이 RSPMV 외측 단층(outer monolayer)의 소수성 부위와 친수성 부위에 barbiturates가 분포되는 경향을 형광 probe 법으로 검색하였다. 세포막 외측 단층의 친수성 부위에 분포되는 형광 probe N-octadecylnaphthyl-2-amine-6-sulfonic acid (ONS)와 소수성 부위에 분포되는 형광 probe 12-(9-anthroyloxy)stearic acid (AS)를 각각 봉입한 후 형광소광법으로 barbiturates의 분포를 측정한 결과는 다음과 같다.

1) 대부분의 barbiturates가 RSPMV 외측 단층의 친수성 부위(표면)에 분포되고 소수성 부위 (hydrophobic region)에 극히 소량만이 분포된다는 것을 확인하였다.

2) 마취효과를 크게 일으키는 barbiturates일수록 소수성 부위에 분포되는 양이 증가하였다. barbiturates 종류에 따른 RSPMV 외측 단층 소수성 부위에의 분포 크기는 thiopental sodium>pentobarbital>hexobarbital>amobarbital>phenobarbital의 순위였다.