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Interaction Between Barbiturate and Membrane Components

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Abstract□Intermolecular interaction between barbiturates and membrane components such as phospholipid and cholesterol were investigated on ^{1}H-NMR spectra and infrared spectra. According to previous reports, barbiturates interacted with phospholipid through intermolecular hydrogen bonds. We also investigated this observation using dipalmitoylphosphatidylcholine (DPPC) as phospholipid in deuterochloroform, and characterized quantitatively. Also, the observed drug could interact with cholesterol which is one of the major components of biomembranes through hydrogen bonds. It was the carbonyl groups of barbiturate and the hydroxyl group of cholesterol that formed hydrogen bond complex. In addition to spectroscopic studies, we investigated the direct effect of phenobarbital on lipid multibilayer vesicles, whose compositions were varied, by calorimetric method. Phenobarbital caused a reduction in the temperature of phase transition of vesicles.

These studies may provided a basis for interpreting the mode of action of barbiturates.

Keywords□Barbiturates, dipalmitoylphosphatidylcholine (DPPC), cholesterol, hydrogen bonds, ^{1}H-NMR spectra, infrared spectra, differential scanning calorimetry (DSC).

Barbiturates are general depressants and particularly specific to central nervous system (CNS)[1]. By previous reports[2-4], these CNS-acting drugs could interact with other molecules through hydrogen bond involving the N-H protons and the carbonyl oxygens. Novak *et al.*, found that barbiturates formed forceful hydrogen bond complex with phosphatidylcholine, a major lipid component of mammalian brain membranes, through the nitrogen bond proton of the barbiturate and the orthophosphate moiety of the phosphatidylcholine molecule[5,6]. This observation supported that the perturbation of membrane by barbituric acid derivatives might result in pharmacological activity. But, aspects of mechanism of action, this intermolecular interaction with phospholipid have not yet been investigated quantitatively, and mammalian membrane contained large amount of cholesterol; from 0 mol% to 50 mol% of lipid[7]. The role of cholesterol seems very important, but its exact role in the membrane remains unknown.

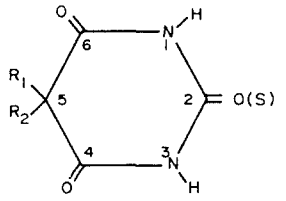
Former investigators reported[8] that phenobarbital as one of the general anesthetics could selectively perturbed lipid multibilayer vesicles, which contained large amount of cholesterol; 33 mol% of lipid. Therefore, the importance of cholesterol in the study on the mode of action of this CNS-acting drug may well be empasized.

We investigated again the effect of phenobarbital on the lipid multibilayer vesicles, using differential scanning calorimetry (DSC) and analyzed quantitatively the interaction of four different barbiturates with dipalmitoylphosphatidylcholine (DPPC) as membrane phospholipid, by nuclear magnetic resonance spectroscopy. Especially, we represented here direct evidence for an association *in vitro* between cholesterol and barbiturates, using infrared spectroscopy as the analytical tool.

MATERIALS AND METHODS

Materials

DPPC was purchased from Sigma Chemical Co. and was used without further purification. Cholesterol was purchased from Nakarai Chemical Ltd., Japan and was recrystallized from absolute ethanol. Phenobarbital, hexobarbital and amobarbital were generously provided by Chong Ken Dang Co., Thiopental and allobarbital were generously provided by Choong Wae Pharm. Co.. Phenobarbital and thiopental were obtained as sodium salts. To perpare the free barbituric acid derivatives, aqueous solutions of the salts were acidified with diluted hydrochloric acid and were extracted with chloroform. After evaporation of the extracts, various powders were obtained. All barbituric acid derivatives were recrystallized twice from hot water and dried overnight in *vacuo* over



	1-N	2-C
phenobarbital	H	O
allobarbital	H	O
amobarbital	H	O
thiopental	H	S
hexobarbital	CH_3	O

Fig. 1. Strutures of barbituric acid derivatives. In convenience, only strutural features are shown.

P_2O_5 at 120°C The purity of barbituric acid derivatived were checked by thin layer chromatography on silica gel plates, using solvents system composed of chloroform-acetone (9:1). The structural formulae of these molecules are shown in Fig. 1. Deuterochloroform for spectroscopic studies was obtained from Merk Co. and CEA, France.

Methods

All the ^{1}H-NMR spectra were recorded with a Bruker WP80SY at 311 K. Chemical shifts were measured in deuterochloroform with respect to tetramethylsilane (TMS) as internal reference. Calculation of the self-association constants of barbiturates and association constants with DPPC were carried out according to the previous reports[9,15].

Infrared spectra were measured in organic solvents such as $CHCl_3$ and $CDCl_3$ on a Perkin-Elmer, model 1710 spectrophotometer, using 0.5 mm KBr cell in the regions of hydroxyl group streching and carbonxyl group streching.

The lipid mixtures for the calorimetry measurements were prepared by combination of chloroform solvents containing appropriate amounts of DPPC, with or without cholesterol in a round bottomed flask. After the remove of solvent by a rotary evaporator, the samples were then further desiccated under vacuum during 2-3 hr, to remove the last trace of solvent. After the addition of aqueous solution containing phenobarbital and 10 mM tris buffer, pH 7.44, multibilayer vesicles were formed by mixing, using a vortex mixer. Lipid pellets were obtained by centrifugation at 45,000 rpm for 15 min. These pellets were then sealed in small aluminum pan and scanned in a Du Pont instruments 990 thermal analyzer using an empty pan reference. The heating rate was 5°C/min and the range of temperature was from 20°C to 60°C.

RESULTS AND DISCUSSION

Differential scanning calorimetry (DSC) measurements

It has been reported that barbiturates could lower

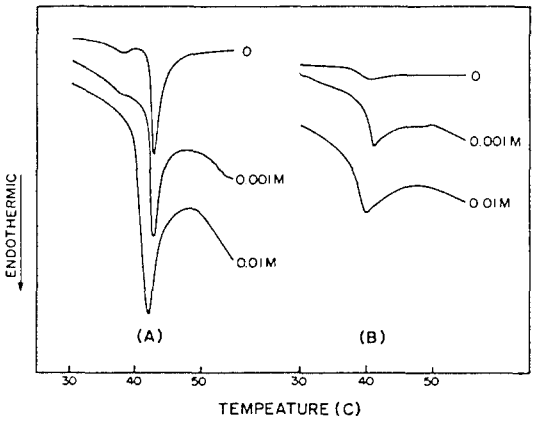


Fig. 2 DSC calorimetric curves for the DPPC multibilayer vesicles with or without cholesterol as the concentration of phenobarbital solution contained increased, pH 7.44. The concentration of drug solution is indicated on the curves.
(A) only DPPC (B) DPPC + 15 mol%z cholesterol

the temperature of the phase transition of lipid multibilayer vesicles made of phosphatidylcholine[26]. To study the properties of lipid vesicles, previous investigators used fluorescence polarzation or electron spin resonance spectroscopy which need probes. However, the effects of membrane active agent such as anesthetics on the lipid vesicles could not be investigated exactly by these methods, because probes could influence the properties of vesicles. On the contrary, DSC is nonperturbing method becasue of not using the probes[10]. We could investigated the exact effect of barbiturate on the lipid multibilayer vesicles by this method.

By DSC measurements, phenobarbital affected the phase transitions of two types of lipid vesicles prepared. For the vesicles made of only DPPC, the pretransition appeared at 37.5°C and the main transition did at 42.7°C (Fig. 2). With the incorporation of cholesterol in the DPPC vesicles, the pretransition disappeared, and the main transition was broadened. And, phenobarbital lowered the temperature of main transition by 1.0°C at 0.01 M for both samples.

This results hinted that barbiturates could perturb the mammalian membrane directly.

Neclear magnetic resonance spectroscopy studies

Proton magnetic resonance of certain barbiturates in deuterochloroform have been reported previously[11]. Fig. 3 shows the effect of DPPC on ^{1}H-NMR spectrum of allobarbital in deuterochloroform at 311 K. The N-H proton of allobarbital gave rise to a singlet at 8.3 ppm. Addition of DPPC produced sig-

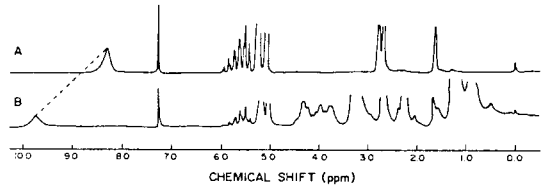


Fig. 3. Effect of DPPC on ^{1}H-NMR spectra of allobartial in deuterochoroform.

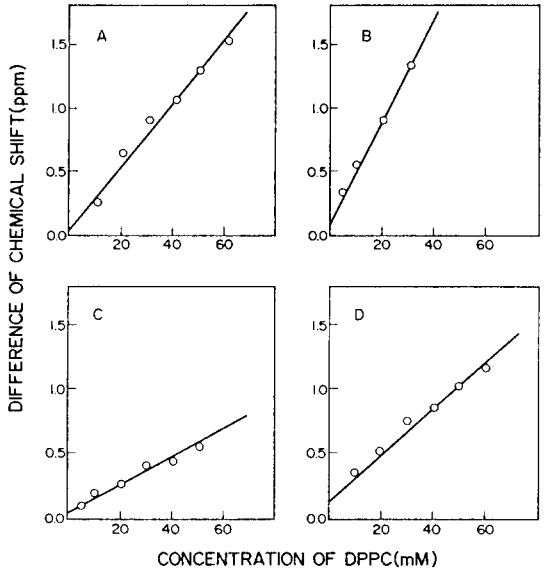


Fig. 4. Changes in N-H proton chemical shifts of barbiturates as a function of increasing concentration of DPPC. Concentration of barbiturates used are indicated below.
(A) amobarbital, 60 mM (B) allobarbital, 40 mM
(C) thiopental, 50 mM (D) hexobarbital, 60 mM

nificant line broadening and a considerable downfield shift in the N-H peak of allobarbital with no change in the remainder of the spectrum. Novak *et al.* identified hydrogen bond formation involving N-H protons of barbiturates and orthophosphate moiety of phospholipid as the driving force of N-H proton shift[5,6].

Plots of the changes in chemical shift of the N-H peak of four barbiturates against the concentration of DPPC are given in Fig. 4. With the increase of the concentration of DPPC, N-H peaks were shifted to downfield. Therefore, we could verify again the result obtained by previous reporters.

The chemical shift of signal corresponding to the protons attached to the nitrogen atoms of the ring with concentration. Fig. 5 shows the concentration dependence of N-H proton chemical shift of barbiturates

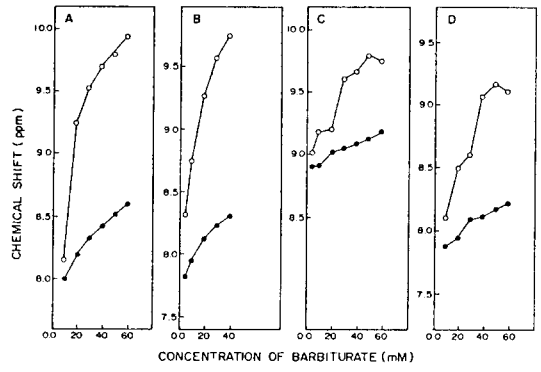


Fig. 5. Concentration dependence of N-H proton chemical shifts of barbiturates in each barbiturate solution (●) and in barbiturate-DPPC mixtures (○).

Table I. Self-association constants of barbiturates in deuterochloroform.

Barbiturate	K (M^{-1})	δ_b (ppm)
Amobarbital	6.2	10.3
Allobarbital	23.6	9.1
Thiopental	8.0	9.8
Hexobarbital	7.6	9.1

* δ_b is the limiting chemical shift value for the fully self-associated species.

Table II. Association constants of barbiturates with DPPC in deuterochloroform.

System	K (M^{-1})	δ_{bb} (ppm)
Amobarbital-DPPC	54.7	11.5
Allobarbital-DPPC	48.7	12.0
Thiopental-DPPC	24.6	11.3
Hexobarbital-DPPC	4.9	16.7

* δ_{bb} is the limiting chemical shift value for the fully compexed species.

in the barbiturates solution and in the barbiturate-DPPC 1:1 mixtures. From these data, we calculated the self-association constants of four barbiturates and the association constants with DPPC (Table I. II). Association constants were in order of allobarbital, amobarbital, thiopental, hexobarbital.

By the calculation of association constants, we

could compare the force of interaction between barbiturate and DPPC as phospholipid. As a result, oxybarbiturates such as allobarbital and phenobarbital interacted with DPPC more forcefully than thiopental did, and thiopental than hexobarbital. These results are probably related to the fact that sulfur is less electronegative than oxygen, and that hexobarbital have one proton attached to the nitrogen atom of the ring, while oxybarbiturate and thiopental have two. Above explanation is based on the fact that association constants we calculated were apparent association constants which considered the population of every bond between N-H groups of barbiturates and phosphate group of DPPC[9,27].

These results may explain the activity of barbiturates in part, but complete explanation of activity of barbiturate was not achieved by these results[22]. Therefore, other factors may well be considered.

Infrared spectroscopy studies

We took notice of cholesterol as another important factor to explain the pharmacological action of barbiturates. In fact, mammalian membrane contained considerable amount of cholesterol and its role, especially the control of membrane fluidity, seems very important. Also, cholesterol has important structural feature; it has 3 β -hydroxyl group whih can interact with other biomolecules through hydrogen bond[25,28-30]. In these backgrouds[12-14], we investigated the direct molecular interaction between barbiturates and cholesterol.

The infrared spectra of cholesterol[16-18] and certain barbiturates in organic solvent[19-21] have been reported previously. Fig. 6 shows O-H streching region of the spectra of choleterol and cholesterol-barbiturate mixture in deuterochloroform. From Fig. 6 free O-H streching band of cholesterol is centered at 3690 cm^{-1}. Hydrogen bounded O-H band which is centered at 3450 cm^{-1} is shown partly in the Fig. 6 With the increase in the concentration of drug, the intensity of the free band decreased gradually. On the other hand, the intensity of the broad hydrogen hydroxyl band increased slightly.

Next, we have observed the C=O streching absorption band of four barbiturates in deuterochloroform. In this regain, oxybarbiturates which have oxygen at 2-C position showed three absorption bands, but thiopental have two. These bands were assigned previously[24]. Two of four barbiturates were not shown because they were similar to the case of phenobarbital. Fig. 7 shows the spectra of the mixtures which contained great excess of cholesterol ; 0.2 M of cholesterol with 0.01 M barbiturate. From Fig. 7, the dotted line is the theoretical sum of the spectrum of

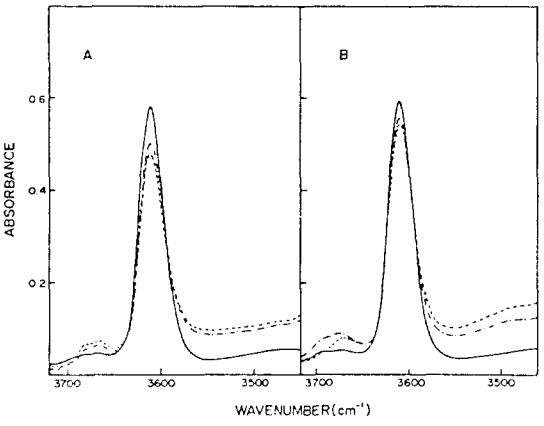


Fig. 6. Infrared spectra of barbiturates-cholesterol system in deuterochloroform.

(A) thiopental-cholesterol
——— ; cholesterol (0.08 M) only
— — — ; cholesterol + thiopental (0.08 M)
------- ; cholesterol + thiopental (0.10 M)
(B) phenobarbital-cholesterol
——— ; cholesterol (0.08 M) only
— — — ; cholesterol + phenobarbital (0.04 M)
- - - - - ; cholesterol + phnobarbital (0.06 M)

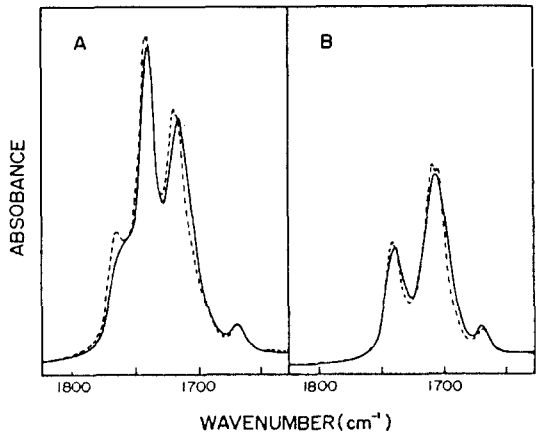


Fig. 7. Infrared spectra of the mixture of barbiturate and cholesterol in chloroform. Path lengh of KBr cell was 0.1 cm.

The solid shows the observed spectrum and the dotted line the calculasted spectrum (the sum of the cholesterol spectrum and barbiturate spectrum) which would be obtained if the molecules not interact. The concentration of barbiturate is 0.01 M and cholesterol 0.02 M.
(A) phenobarbital (B) thiopental

cholesterol and barbiturate. These spectra would be obtained if barbiturates and cholesterol did not interacted. However, the calculated spectra differed from the observed spectra of mixtures. The intensity of the C=O streching bands decreased.

Hydrogen bonding of the band for unassociated species and the appearance at lower energy of a broader band for the associated species[23]. By this explanation, we could charaterized the intermolecular hydrogen bonds between the hydroxyl group of cholesterol and the carbonyl groups of barbiturates.

The characterized interaction would contribute the explanation of the mechanism of action of barbiturates, in that cholesterol might play the critical role on the control of membrane fluidity. For this conclusion, however, further studies on the cholesterol in the membrane must be carried out.

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