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# FLUORESCENCE POLARIZATION STUDY OF THE INTERACTION BETWEEN PHENOTHIAZINE AND LIPOSOMES

Miss Witinee Theeravit

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การตรวจสอบปฏิกิริยาต่อกันระหว่างยากล่อมประสาทฟีโนไทอาซีนกับลิโปโซม ซึ่งใช้เป็นแบบอย่างของแผ่นเนื้อเยื่อโดยใช้เทคนิคฟลูออเรสเซนโพลาไรเซชัน คลอโพรมาซีนและไทโอริดาซีนได้ถูกเลือกเป็นยาตัวอย่าง และใช้ฟลูออเรสเซนโพรบ 1,6-ไดฟีนิล-1,3,5-เฮกซะไตรอีนเป็นตัวตรวจวัดความเป็นระเบียบและการเคลื่อนไหวภายในบริเวณเส้นเอซิลของผนังลิโปโซม พบว่าฟีโนไทอาซีนลดอุณหภูมิซึ่งใช้บ่งถึงการเปลี่ยนสถานะของสภาวะ (อุณหภูมิเปลี่ยนสภาวะ) และทำให้ช่วงอุณหภูมิดังกล่าวกว้างขึ้นในลิโปโซมซึ่งประกอบด้วยไดไมริสโทอิลฟอสฟาทิดิลโคลีนและในลิโปโซมของไดสเตียโรอิลฟอสฟาทิดิลโคลีน ยาเพิ่มสภาวะเหลวที่อุณหภูมิต่ำกว่าอุณหภูมิเปลี่ยนสภาวะและเพิ่มความเป็นระเบียบของเส้นเอซิลของฟอสฟอลิปิดที่อุณหภูมิสูงกว่าอุณหภูมิเปลี่ยนสภาวะ สำหรับลิโปโซมซึ่งมีส่วนประกอบเป็นฟอสฟอลิปิดที่ได้จากธรรมชาติคือฟอสฟาทิดิลโคลีนจากไข่แดงพบว่าฟีโนไทอาซีนให้ผลเฉพาะเพิ่มความเป็นระเบียบของเส้นเอซิลเนื่องจากแผ่นเนื้อเยื่อของลิโปโซมอยู่ในสภาวะเหลวตลอดช่วงอุณหภูมิที่ศึกษา ยาแสดงปฏิกิริยาต่อลิโปโซมซึ่งมีฟอสฟาทิดิลโคลีนกับคอเรสเตอรอลเป็นองค์ประกอบในลักษณะเดียวกันแต่ให้ผลน้อยกว่าซึ่งเกิดจากการเพิ่มความหนาแน่นในการเรียงตัวของลิปิดจึงอาจทำให้ลดการแทรกตัวของยาเข้าไปภายในแผ่นเนื้อเยื่อ ฟีโนไทอาซีนให้ผลมากที่สุดต่อลิโปโซมซึ่งมีประจุลบตามด้วยไม่มีประจุและประจุบวกซึ่งอาจอธิบายได้จากการดึงดูดเข้าหากันทางไฟฟ้าระหว่างประจุบวกบนฟีโนไทอาซีนและลิโปโซมซึ่งมีประจุลบจึงเพิ่มการแทรกตัวของยาเข้าไปภายในชั้นของลิปิด ขณะที่การผลักกันทางไฟฟ้าที่เกิดขึ้นกับลิโปโซมซึ่งมีประจุบวกให้ผลตรงกันข้าม การเกิดปฏิกิริยาของฟีโนไทอาซีนต่อลิโปโซมที่ใช้ในการศึกษาครั้งนี้ขึ้นกับความเข้มข้นของยา ผลของการวิจัยแสดงว่าฟีโนไทอาซีนแสดงปฏิกิริยาต่อแผ่นเนื้อเยื่อและผลของยาขึ้นกับส่วนประกอบของแผ่นเนื้อเยื่อ

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WITINEE THEERAVIT : FLUORESCENCE POLARIZATION STUDY OF THE INTERACTION BETWEEN PHENOTHIAZINE AND LIPOSOMES.

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The interaction between, tranquilizer, phenothiazines and liposomes, a model membrane, was examined by using fluorescence polarization technique. Chlorpromazine (CPZ) and thioridazine (TRZ) were selected as drugs of choice and a fluorescent probe, 1-6-diphenyl-1, 3, 5-hexatriene (DPH) was used to monitor the order and dynamic within the acyl chain region of liposomal bilayer. These phenothiazines were found to lower and broaden thermotropic phase transition temperature ($T_m$) of liposomes reconstituted with dimyristoylphosphatidylcholine (DMPC) and also in distearoylphosphatidylcholine (DSPC). They increased membrane fluidity (fluidizing effect) at temperature below $T_m$ and ordered the acyl chains of phospholipid (condensing effect) at temperature above $T_m$. For liposomes composed of natural phospholipid, egg yolk phosphatidylcholine (EPC), only condensing effect of phenothiazines was observed since EPC liposomes were in fluid phase overall the temperature studies. Same phenomena were also observed in phosphatidylcholine liposomes containing cholesterol but in a less extent due to an increase in lipid packing that might reduce penetration of drugs. Phenothiazines exhibited greatest effect on negatively charged liposomes, following by neutral and positively charge liposomes, respectively. These might explained by electrostatic attraction between positive charged on phenothiazines and negatively charged liposomes which enhanced more penetration of drugs into lipid bilayer while electrostatic repulsion was found in positively charged liposomes, thus the opposite effect was obtained. The activities of phenothiazines on all kinds of liposomes presented here were concentration dependence. The research suggested that the phenothiazines played their action on membrane and their effects depended on membrane composition.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percent |
| °C | Degree Celcius |
| μmol | Micromole |
| CPZ | Chlorpromazine |
| DCP | Dicetyl phosphate |
| DMPC | Dimyristoylphosphatidylcholine |
| DPH | 1,6-diphenyl-1,3,5-hexatriene |
| DSPC | Distearoylphosphatidylcholine |
| EPC | Egg yolk phosphatidylcholine |
| g | Gram |
| hr | Hour |
| LUVs | Large unilamellar vesicles |
| mg | Milligram |
| min | Minute |
| mM | Millimolar |
| Mol% | Mole percent |
| nm | Nanometer |
| P | Fluorescence polarization |
| PC | Phosphatidylcholine |
| r | Fluorescence anisotropy |
| REVs | Reversed phase evaporation vesicles |
| S | Lipid order parameter |
| S.D. | Standard deviation |
| SL | Stearylamine |
| $T_m$ | Thermotropic phase transition temperature |

| | |
|---|---|
| TRZ | Thioridazine |
| THF | Tetrahydrofuran |
| Vol | Volume |

# CHAPTER I

# INTRODUCTION

## Liposomes

Liposomes were discovered in the early 1960s by the British scientist, Alec Bangham. In the course of his research on the effect of phospholipids on the clotting of blood, Bangham would create reagents by adding water to a phospholipid film. He soon recognized that the phospholipid film formed closed spherical structures that encapsulated part of the liquid medium in their interior. These are so-called liposomes which are also known as lipid vesicles [Lasic, 1992].

Liposomes are simply vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). They form spontaneously when these lipids are dispersed in aqueous media, given rise to a population of vesicles. They can be constructed so that they entrap quantities of materials both within their aqueous compartment and within the membrane. The value of liposomes as model membrane systems derives from the fact that liposomes can be constructed of natural constituents such that the liposomal membrane forms a bilayer structure which is in principle identical to the lipid portion of natural cell membranes [New, 1990].

Due to biological membranes are a complex mixture of different types of lipid and protein molecules, therefore artificial bilayer membranes, liposomes are in widespread use as models for biological membrane [Wang et al., 1991] to investigate the molecular interactions and barrier functions of isolated and selected membrane

constituents because other components such as cholesterol, fatty acids as well as proteins can be used and incorporated easily to lipid bilayers in exact ratio.

Phospholipids are the major structural components of biological membranes as illustrated in Figure 1.

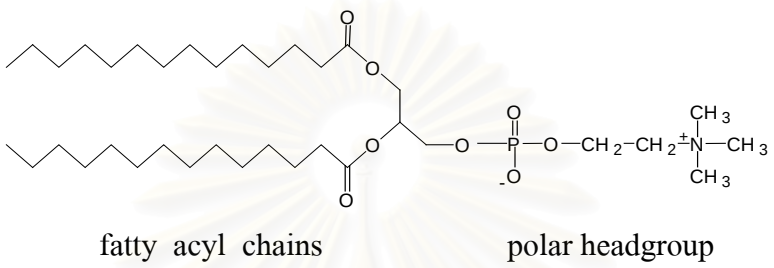
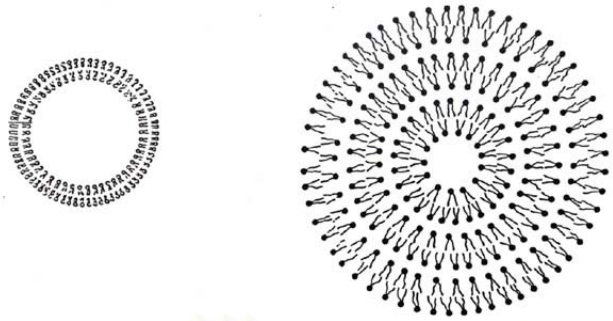



Figure 1 Chemical structure of phosphatidylcholine.

The most common phospholipid is phosphatidylcholine (PC) molecule, amphiphatic molecules in which a glycerol bridge link a pair of hydrophobic acyl hydrocarbon chains, with a hydrophilic polar headgroup, phosphocholine. Molecules of PC are not soluble in water in the accepted sense but in aqueous media they align themselves closely in a planar bilayer sheets in order to minimize the unfavourable interactions between the bulk aqueous phase and the long hydrocarbon fatty acyl chains. Such interactions are completely eliminated when the sheets fold on themselves to form vesicles which are called liposomes as shown in figure 2.



(A) (B)

Figure 2 Liposomes; (A) unilamellar liposome, (B) multilamellar liposome.

Phosphatidylcholine, also known as 'lecithin', can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean. Forming as they do the major phospholipid component of many cell membranes, they are often used as the principle phospholipid in liposomes for a wide range of applications because of their neutral charge and chemical inertness. Lecithin from natural sources is in fact a mixture of phosphatidylcholines, each with chains of different length and varying degrees of unsaturation. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contains a higher proportion of fully saturated chains.

Lipid bilayer is a dynamic structure and most of its components can move laterally in the membrane plane or rotationally. The extent of these movements, reflecting phase structure, rate and steric hindrance, are referred to as 'fluidity'. The membrane fluidity is therefore a composite of a dynamic component and an order component, and their ratio varies under different conditions [Borenstain and Barenholz, 1993; Lentz, 1993].

Current views on biomembranes emphasize coupling between their organization and function [Kinnunen, 1991; 2000] which are further strongly connected to the physical properties of the lipid bilayer [Mouristen and Kinnunen, 1996]. Accordingly, changes in the organization and dynamics of the membrane can be lead to alterations in the functions of membrane proteins as shown for p-glycoprotein [Romsicki and Sharom, 1999], phopholipase $A_2$ [Burack et al., 1997] and opioid receptor [Lazar and Medzihradsky, 1992], for example. The ligand affinity of the latter has been shown to be sensitive to the changes in the 'fluidity' in the interfacial region, but insensitive to change in the hydrocarbon core [Lazar and Medzihradsky, 1992]. A model involving coupling of the membrane lateral pressure profile to the conformation and function of integral membrane proteins has been recently mentioned by Cantor (1997) and could provide a mechanistic basis for the effects of membrane composition on opioid receptor function, for instance. While the above findings demonstrated the importance of the lipid environment to the function of proteins, they also revealed the importance of drug induced changes in membrane organization, dynamics and function, and further suggested that these properties could be considered as potential drug targets [Jutila, Rytomaa and Kinnunen, 1998].

**Cholesterol**

Sterols are important components of most natural membranes. In mammals, the predominant sterol is cholesterol as illustrated in Figure 3, and yet its content varies from one membrane to another, for example, it ranges from 40 mole% in myelin to 6 mole% in mitochondria. The exact function of cholesterol in the membrane is still unknown, but biophysical studies on model membrane show that one role of cholesterol in membranes is to regulate the degree of order and mobility of the acyl chains of lipids, i.e., above the transition temperature of the phospholipid,

cholesterol reduces the mobility of the acyl chain while below the transition temperature, it has the opposite effect. Cholesterol thus acts as a modulator of the packing of the acyl chains of phospholipids [Pang and Miller, 1978].

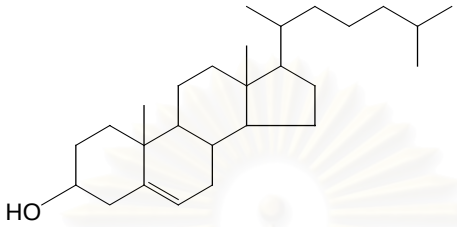



Figure 3 Chemical structure of cholesterol.

Cholesterol does not by itself form bilayer structures but it can be incorporated into phospholipid membranes in very high concentrations up to 1:1 or even 2:1 molar ratios of cholesterol to phospholipid. In natural membranes, the molar ratio varies from 0.1–1, depending upon the anatomical and cellular location [Gregoriadis, 1986].

Being an amphiphatic molecule, cholesterol inserts into the membrane with its hydroxyl group orients towards the aqueous surface, and the aliphatic chain aligns parallel to the acyl chains in the center of the bilayer. The 3β-hydroxyl group is positioned in level with the carboxyl residues of the ester linkages in the phospholipids, with very little vertical freedom of movement. The presence of the rigid steroid nucleus alongside the first ten carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining carbons towards the terminal end of the chain [Forrest and Mattai, 1983].

Although cholesterol has a marked effect on membrane organization. It has been demonstrated by a variety of physical techniques that cholesterol has a small fluidizing effect below the phase transition temperature (gel phase) of phospholipids and a large ordering effect above this temperature (liquid–crystalline phase) [Marsh and Smith,1973; Blitterswijk, Van Hoeven and Van der meer, 1981; Forrest et al., 1983].

**DPH as a Fluorescent Probe**

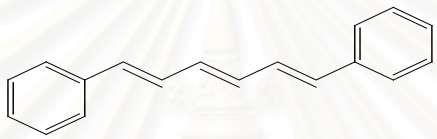


Figure 4 Chemical structure of 1,6–diphenyl–1,3,5–hexatriene.

DPH is one of the most useful fluorescent probes for monitoring fatty acyl chain movement or fluidity in membrane interior, hydrocarbon region [Palmeira and Oliveira, 1992]. From its chemical structure (Figure 4), it belongs to linear class. All of probes in this class have long rigid molecules of roughly the shape and size of fully extended phospholipid acyl chains. These fluorophores have excitation and emission dipoles (fluorescence dipoles) that are roughly colinear with their long molecular axis. For this reason, rotations about the long molecular axis make essentially no contribution to the depolarization of polarized fluorescence, while rotations about the two remaining rotational axes (mutually orthogonal and orthogonal to the long axis) are equally capable of depolarizing fluorescence. The depolarization of their fluorescence is monitored as a mean of estimating membrane fluidity [Lentz, 1993].

From several reports, steady-state fluorescence depolarization theory can be used to characterize depolarization of this probe in membranes, although this theory corresponds rigorously to motional randomization of the orientation of only one axis (the axis of fluorescence polarization) during the lifetime of the corresponding excited electronic state, it is generally assumed that this loss of initial orientation is correlated with overall reorientations of the probe molecules which in turn will reflect the fluidity of membranes [Lentz, 1993].

On steady-state theory, the ground and excited state electronic distributions of any fluorophores define directions within the molecule in which the probabilities of absorption or emission of a photon are greatest. These are the directions of the excitation and emission dipole moments, the magnitudes of which determine the maximal probabilities of absorption or emission when polarized exciting light (Figure 5) is directed toward a chromophore molecule whose excitation dipole moment is aligned with the electric vector of the exciting light, the chromophore will preferentially absorb this light. Since the absorption process is so much faster than molecular rotation, the use of oriented exciting light creates a population of preferentially oriented excited fluorophore. This is refered to as photoselective. Since the emission of a photon by the exited fluorophore requires a much longer time (the excited state lifetime, $\tau$) than does absorption, the fluorophore can often reorient before emission occurs as illustrated in Figure 5. If such a situation occurs (i.e., the rotational correlation time of the excited molecule is less than or on the order of the excited state lifetime), the emitted photon will no longer be polarized parallel to the exciting photon, even if the molecular excitation and emission dipoles are parallel within the fluorophore. The resulting polarization of fluorophore is often defined in terms of the steady-state fluorescence polarization. The degree of florescence polarization, fluorescence anisotropy and order parameter were

calculated according to Shinitzky and Barenholz (1974) from the equations as shown below :

$$\text{Fluorescence polarization (P)} = \frac{(I_{ll} - GI_{\perp})}{(I_{ll} + GI_{\perp})} \quad (1)$$

$$\text{Fluorescence anisotropy (r)} = \frac{(I_{ll} - GI_{\perp})}{(I_{ll} + 2\,GI_{\perp})} = \frac{2P}{(3 - P)} \quad (2)$$

$$\text{Lipid order parameter (S) ; } S^2 = \frac{[(4r/3) - 0.1]}{r_o} \quad (3)$$

Fluorescence polarization and anisotropy are determined from the emission intensities through an analyzer oriented parallel ($I_{ll}$) and perpendicular ($I_{\perp}$) to the direction of polarization of excitation light. G is a factor used to correct the instrument's polarization and is given by the ratio of vertically to horizontally polarized emission components when the excitation light is polarized in the horizontal direction [Palmeira and Oliveira, 1992]. $r_0$ is the maximal fluorescence anisotropy value in the absence of any rotational motion (take as 0.40 for the case of DPH) [Balasubramanian, Straubinger and Morris, 1997].

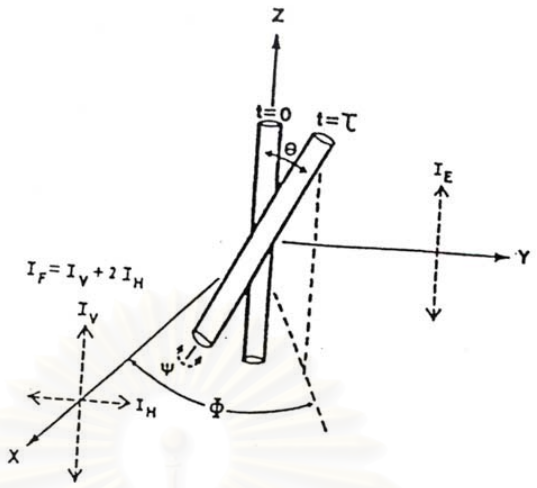



Figure 5 Fluorescence anisotropy of a cylindrically – symmetric fluorophore.

Figure 5 shows fluorescence anisotropy of a cylindrically symmetric fluorophore whose excitation and emission dipoles are parallel to the symmetric axis. Vertically polarized light ($I_E$) from the Y-axis vertical polarizer (not shown) selectively excites (photoselective) a population of fluorophores whose excitation dipoles are assumed to be symmetrically distributed about the z-axis polarizer (not shown). Rotation of the probe during the excited state lifetime, $\tau$, diminishes the vertical component ($I_{\parallel}$) and enhances the horizontal component ($I_{\perp}$) of analyzed light in a way described by time-dependence of fluorescence anisotropy ([r(t)]) [Lentz, 1989; 1993].

DPH is often the fluorescent probe of choice for studies of the structure and dynamic properties of membranes and has been used extensively to estimate membrane fluidity and/or order. Because of its advantageous structural and spectral properties [Wang et al., 1991; Balasubramanian et al., 1997] such as:

1. Its rigid uncharged rod-like linear shape which is about 13 $A^{\circ}$ long [Heyn, 1979; Palmeira and Oliveira, 1992], allows DPH to pack well with fatty acyl chains of the membrane bilayer [Kaiser and London, 1988].
2. According to its shape, DPH may exhibit a wobbling motion confined to a double hard-cone [Kim et al., 1993]. This motion is highly sensitive to change in fluidity as demonstrated by appreciable changes in the degree of fluorescence polarization or anisotropy which follow small changes in temperature [Shinitzky and Barenholz, 1974; Kaiser and London, 1988; Palmeira and Oliveira, 1992; Kim et al., 1993].
3. The high extinction coefficient (80,000 $M^{-1}cm^{-1}$) and fluorescence quantum yield in nonaqueous solvent enable us to use a smaller ratio of probe to lipid which minimizes the membrane perturbation [Wang et al., 1991; Borenstain and Barenholz, 1993].
4. The limiting anisotropy values remain constant for a wide range of excitation wavelengths (320 – 380 nm) [Wang et al., 1991].
5. DPH is a small lipophilic molecule which has been shown to partition into lipid bilayer and exhibits a strong fluorescence while it has very low fluorescence intensity in the aqueous phase due to its aggregation as crystals or micelles [Borenstain and Barenholz, 1993]. Thus free DPH in the aqueous phase will not interfere the experiments [Andrich and Vanderkooi, 1976; Wang et al., 1991; Borenstain and Barenholz, 1993; Balasubramanian et al., 1997].

6. Very high lipid to aqueous phase partition coefficient ($K_p$) facilitates a rapid means of introducing this probe into the lipid bilayer, which is of a particular advantage in studies of living cell membranes [Borenstain and Barenholz, 1993].

Since the thickness of rod-shaped DPH approximates one acyl chain of lipid molecule, each probe molecule presumably replaces one acyl chain in the lipid bilayer structure. Since the hypothetical cone around DPH would be filled with lipid acyl chains, the tumbling of the DPH rod occurs as a neighboring acyl chains wobbles out of the cone. Thus, the restricted rotation of DPH directly reflects the thermal motion of lipid acyl chains [Kawato, Kinosita and Ikegami, 1978].

The use of DPH as a fluorescent probe has, however, the limitation that should be considered. This relates to reversible decrease in fluorescence intensity upon prolonged excitation, which presumably originates from a reversible photoisomerization. Therefore, the DPH-labeled sample should be exposed to the excitation beam for as short periods as possible [Shinitzky and Barenholz, 1974].

**Liposomal Membrane Thermotropic Phase Transition**

At different temperatures, phospholipid membranes can exist in different phase. The temperature which they change from one phase to another is called thermotropic phase transition temperature, $T_m$ .

When a phospholipid bilayer goes from the gel phase to the liquid-crystalline phase upon increase in temperature, the transition is accompanied by several structural changes in the lipid molecules. Below the phase transition temperature, the phospholipid molecules in the gel phase are packed in a highly ordered array with the hydrocarbon chains of the fatty acids in an all-*trans* conformation,

exhibiting a strongly restricted motional freedom. But at the transition to the liquid-crystalline phase, the hydrocarbon chains gain more motional freedom, some C-C bonds adapt *gauche* conformations, as depicted in Figure 6, the thickness of the bilayer decreases and the bilayer expands, since each hydrocarbon chain requires additional lateral space. Accordingly, the packing density in the region of the polar headgroups is thought to be loosened [Hanpft and Mohr, 1985], as demonstrated in Figure 7.

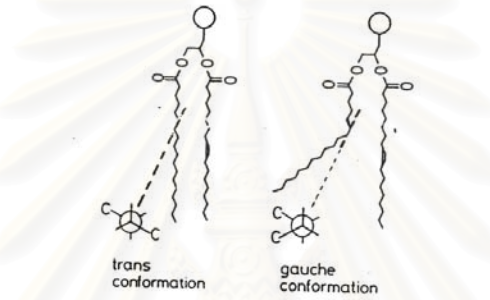



Figure 6 The difference in configuration of hydrocarbon chains of phospholipid.

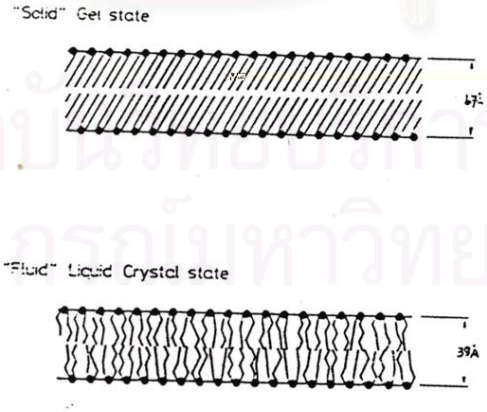



Figure 7 Appearance of the structure and elimination of chain tilt of membrane in the gel phase and liquid-crystalline phase.

**Determining Thermotropic Phase Transition of Liposomal Membrane Using DPH Fluorescence Polarization**

The most widely used technique to determine liposomal membrane phase transition is the measurement of fluorescence polarization in which the emission polarization (or anisotropy) of membrane bound fluorophore such as DPH is used as a measure of the extent of probe wobbling motion during its excited lifetime. In that this wobbling motion is limited by the motion properties and degree of ordering of the surrounding hydrocarbon chain, therefore the probe obviously provides a measure of hydrocarbon order [Palmeira and Oliveira, 1992]. The more disordered the membrane environment, the greater is the motional freedom of the fluorophore and hence the lower the observed fluorescence polarization or anisotropy [Florine-Casteel, 1990] which exhibited an abrupt decrease of fluorescence polarization (or anisotropy) at phase transition temperature ($T_m$) [Gregoriadis, 1986; Florine-Casteel, 1990; Zolese, Gratton and Curatola, 1990] as shown in Figure 8.

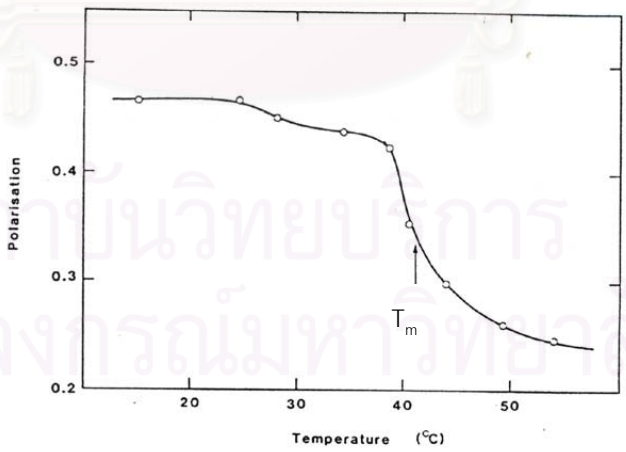



Figure 8 The effects of a thermotropic phase transition of phospholipid liposomes upon the fluorescence polarization of the membrane-bound DPH.

**Phenothiazine**

Phenothiazine is a group of drugs which are generally used as antipsychotic and neuroleptic agents [Lin and Chen, 2001; Lin et al., 2002]. They present the common characteristic of being amphiphilic cationic, small molecule having a tricyclic hydrophobic (phenothiazine) ring bound to a short chain hydrocarbon with a charged amino group [Carey, Hirom and Small, 1976; Ahyahauch and Bennouna, 1999; Jutila et al., 2001] as depicted in Figure 9.

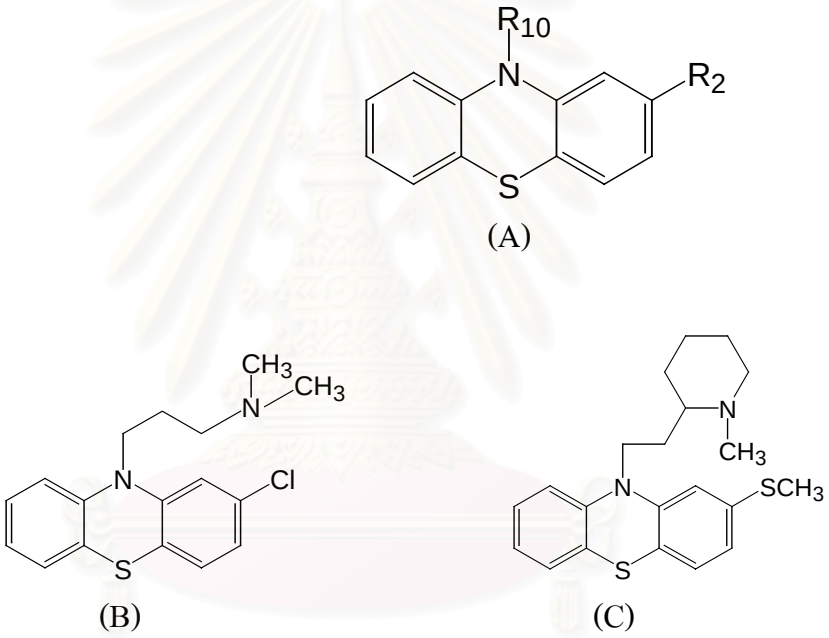



Figure 9 Chemical Structures of (A) phenothiazines, (B) chlorpromazine and (C) thioridazine.

Potentially useful phenothiazine derivatives have different substituents attached at the 2-position ($R_2$) and 10-position of the phenothiazine ring ($R_{10}$) with an aliphatic side chain containing an amino group or an alkyl piperazine group [Delgado and Remers, 1998] such as chlorpromazine and thioridazine, respectively.

Chlorpromazine and thioridazine are widely used to treat many psychotic disorders, particularly those which involve hyperactivity and anxious excitement. These pharmacological agents are major tranquilizers, and act to suppress hallucinations in conditions such as schizophrenia, probably by interfering with the dopaminergic transmission in the limbic system and are non-specific dopamine antagonists which bind to all kinds of dopamine receptors (D1-D5), as well as to receptors for histamine [Ahyahauch and Bennouna, 1999; Bymaster et al., 1999]. The effects of the various neuroleptic drugs further depend on the differences between affinities towards the different receptor subtypes [Ahyahauch and Bennouna, 1999].

Because schizophrenia is a common psychiatric disorder, many theories have been proposed for the pathogenesis of this disorder, including dysfunction of dopaminergic, serotonergic and glutaminergic systems, maldevelopment, and membrane dysfunctions [Kandel, 1991; Horrobin, Glen and Vaddadi, 1994; Ahyahauch and Bennouna, 1999]. Interestingly, changes in the cell membrane phospholipid compositions in the brain of schizophrenic patients have been related to the onset of clinical symptoms[Petter and Minshew, 1992]. Alteration of membrane properties and an effect to the modulation on signal transduction were suggested as the mechanism of action of these drugs [Jutila et al., 2001].

The focus of this study was therefore to investigate the interactions between phenothiazines and liposomal membranes using fluorescence polarization technique with DPH as a fluorescent probe to detect changes in fluidity accompanying the gel to liquid crystalline phase transition of liposomal membranes. Two phenothiazines, chlopromazine and thioridazine were chosen as drug of chioce because of their widely application and no fluorescence interference on DPH measurement. Afterthat, modifications of liposomal membrane component with various molar

ratios of cholesterol and charged amphiphiles have performed to observe the influence of these components to the drug membrane interactions since cell membrane compositions vary in different organs.

# CHAPTER II

# EXPERIMENTS

## Instruments

1. Ultraviolet-visible spectrophotometer. (Milton-Roy Spectronic 3000 Array)
2. Spectrofluorometer. (Jasco FP-777) equipped with thermostated controller (Eyela Cool ACE CA 1100)
3. Thermostat shaker bath (Heto Model TB SH 02)
4. pH meter (Consort C231)
5. Bath type-sonicator (Bransonic 321)
6. Scanning Electron Microscope (JSM-5410LV scanning microscope, JEOL)
7. Light microscope (Olympus)

## Materials

1. Phospholipids
   1.1 Synthetic phospholipids

Dimyristoylphosphatidylcholine (DMPC, C14:0) and distearoyl-phosphatidylcholine (DSPC, C 18:0) were purchased from Sigma Chemical Co., St. Louis. Thin-layer chromatography of all lipids on silica gel in a chloroform : methanol : water (65 : 25 : 4, by vol) with iodine vapor as a detecting agent showed a single spot, thus they were used without further purification.

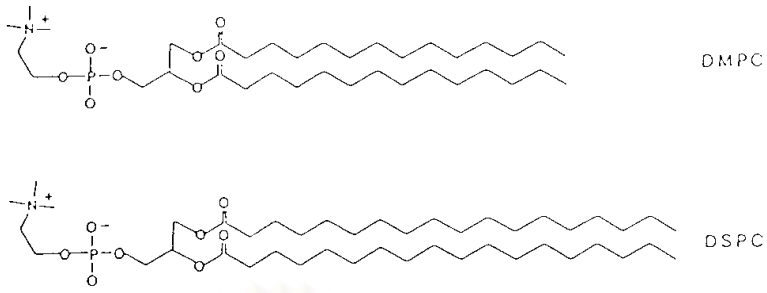



Figure 10 Schematic representation of phospholipids illustrating their relative lengths and position in the bilayer.

1.2 Egg yolk phosphatidylcholine (EPC)

EPC was obtained from hen egg yolks by method of Faure (1950) and liposomes prepared of EPC were used to imitate biological membrane which are composed of mixed various acyl chain length phospholipids.

2. Cholesterol

Cholesterol was obtained from Fluka AG, Switzerland and purified by recrystallization from hot methanol for three times. Chemical structure of cholesterol is shown in Figure 3.

3. Charged amphiphiles

Dicetylphosphate (DCP) and stearylamine (SL) were purchased from Sigma Chemical Co., St. Louis and used as negatively and positively charged amphiphiles, respectively.

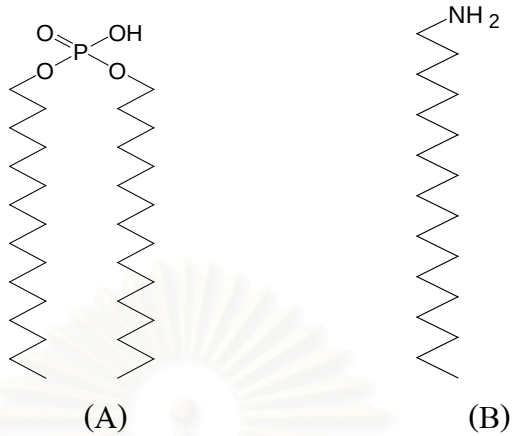



Figure 11 Chemical structure of charged amphiphiles;
(A) dicetylphosphate (B) stearylamine.

4. Phenothiazines

Two phenothiazines were used as follow

Chlorpromazine Hydrochloride [CPZ, 3-(2-Chlorophenothiazine-10-yl) – N, N-dimethyl propylamine monohydrochloride] was a gift from Pharmasant Laboratories Co., Ltd.

1 mM. Stock solution of CPZ was prepared in distilled water, protected from light and kept in refrigerator until used.

Thioridazine Hydrochloride {TRZ, 10- [2-(Methyl-2-piperidyl) ethyl] –2-(methylthio) phenothiazine monohydrochloride} was a gift from Atlantic Laboratories Co., Ltd.

1 mM stock solution of TRZ was prepared in distilled water, protected from light and kept in refrigerator until used. The chemical structures of these phenothiazines were shown in Figure 9.

5. 1, 6-Diphenyl–1, 3, 5–hexatriene (DPH).

1, 6-Diphenyl–1, 3, 5–hexatriene, a fluorescent probe for monitoring movement of fatty acyl chain of phospholipid in hydrocarbon region, was purchased from Sigma Chemical Co., St. Louis. Stock solution of DPH was prepared by dissolving in tetrahydrofuran to a final concentration of 1 mM and kept in refrigerator, protected from light until used. The chemical structure of DPH was shown in Figure 4.

6. Tris buffer ; 10 mM Tris-HCl pH 7.0

Dissolved 1.2125 g of Tris-HCl [Tris-(Hydroxymethyl)–aminomethane] in 1,000 ml. of distilled water and adjusted to pH 7.0.

Other reagents used in the experiments were of reagent grade or better.

**Methods**

1. Preparation of Large Unilamellar Liposomes

Large unilamellar liposomes (LUVs) were prepared by reversed-phase evaporation (REVs) method [Glagasigij, Sato and Suzuki, 1988]. 20 μmole thin dried lipid film was prepared from synthetic phosphatidylcholine or egg yolk phosphatidylcholine (EPC) or its mixture with other components. Then, mixture of 3 ml. of isopropylether (washed three times with water just prior to use in order to eliminate any peroxides) and 1.8 ml. of chloroform was added, followed by 1 ml. of Tris buffer and the mixture was vigorously vortexed and followed by sonication in a bath type sonicator. This procedure was repeated until good emulsion was obtained. The organic solvent was evaporated off under reduced pressure until a clear suspension was obtained. Trace of organic solvent was eliminated by continuing evaporation under highly reduced pressure for another 2 hr. All of the experiments

were performed at temperature above the transition temperature of each phospholipid eg., -15/-7 °C for egg yolk phosphatidylcholine, 23 °C for DMPC, and 54 °C for DSPC. In order to characterize these prepared liposomes, it was performed by electron micrographic technique. The phospholipid content of each preparation was determined by method of Barlett (1969).

All of the following experiments were performed with three preparations of liposomes which each of these was repeated for three determinations.

2. Fluorescence Polarization Spectroscopy

For 3 ml. DPH-labelling liposome suspensions, fluorescence polarization (P) and fluorescence anisotropy (r) were measured with excitation and emission wavelengths at 360 and 430 nm, respectively. P and r were determined from the emission intensities through an analyzer oriented parallel ($I_{ll}$) and perpendicular ($I_{\perp}$) to the direction of polarization of the excitation light. The background intensities were subtracted from $I_{ll}$ and $I_{\perp}$ before calculations were performed using equations given in (1), (2) and (3).

The controls were composed of liposomes without fluorescent probe.

3. Effect of Incubation Time and Probe Concentration on Fluorescence Polarization of Probe Labelling Liposomes.

Liposomes were prepared from phosphatidylcholine (DMPC or DSPC or EPC) with Tris buffer. Fluorescent probe (3 μl of 2 mM DPH in tetrahydrofuran) was added directly to each liposomal suspension with various concentrations to obtain desired probe-to-lipid molar ratio (1:250 to 1:1000). Controlled liposomes, without probe, were added with THF in the equal amount of every set of samples. After that liposomes with and without probes were vortexing for 1 min and then incubated in

thermostat shaker bath above their phase transition temperature of each phospholipid (30 °C for DMPC, 60 °C for DSPC and 30 °C for EPC) in various period of time (from 0.25 to 6 hr) in order to allow equlibration of probes in liposomal membrane. Afterthat liposomal suspension was adjusted to final volume of 3 ml with Tris buffer and fluorescence polarization measurements were then performed at 30 °C using spectrofluorometer equipped with thermostated controller. Finally, fluorescence polarization and fluorescence anisotropy were calculated as mentioned earlier.

4. Determination of Phase Transition of Liposomes Prepared from Various Acyl Chain Length Phospholipids.

Because of biological membranes are a complex mixture of various types of phospholipid, in order to mimic this diversity, liposomes with various acyl chain length phospholipid e.g. DMPC (C14:0), DSPC (C18:0) were prepared by using optimal probe concentration and incubation time obtaining from step 3. Fluorescence polarization and anisotropy were measured at various temperature (15 to 30 °C for DMPC; 45 to 65 °C for DSPC) in order to determine thermotropic phase transition of liposomes and compared with liposomes prepared from natural phosphatidylcholine (egg yolk phosphatidylcholine).

5. Influence of Cholesterol on Thermotropic Phase Transition of Liposomes.

Cholesterol is one of the major components in biological membrane and its role is to regulate the organization and fluidity of lipids in membrane. To study this, liposomes were prepared from pholphatidylcholine (DMPC or DSPC or EPC) and cholesterol in molar ratios of 100 : 10, 100 : 25, 100 : 50 and 100 : 75 with Tris buffer. Liposomal suspension (150 μl, 10 mM phospholipid) were labeled with DPH by using optimal condition obtained from step 3. Fluorescence polarization was measured at various temperatures in order to determine their thermotropic phase transitions.

6. Influence of Charged Amphiphiles on Thermotropic Phase Transition of Liposomes.

Charged amphiphiles were also components of cell membrane in some tissues, then to imitate this, liposomes were prepared from phosphatidylcholine (DMPC or DSPC or EPC), cholesterol and charged amphiphile in molar ratios of 100 : 10 : 5 and 100 : 10 : 10 with Tris buffer. Dicetylphosphate (DCP) was used for providing negative charged while stearylamine (SL) was a positive one. Liposomes (150 μl, 10 mM phospholipid) were labelled with DPH by using optimal conditions obtained from step 3. Fluorescence polarization was measured at various temperatures in order to determine their thermotropic phase transitions.

7. Influence of Incubation Time on Phenothiazines Penetration into Liposomes.

Liposomes were prepared from phosphatidylcholine (DMPC or DSPC or EPC) with Tris buffer. Liposomes (150 μl, 10 mM phospholipid) were labelled with DPH by using optimal conditions obtained from step 3. Afterthat desired amount of chlorpromazine (CPZ) or thioridazine (TRZ) (1 mM in $H_2O$) were added to obtain desired drug to lipid molar ratios (e.g., 1 : 25, 1 : 50, 1 : 75 etc. depended on their detectable effects on thermotropic phase transition of liposomes). The mixtures were then vortexing for 1 min and incubated in a thermostat shaker bath above their phase transition temperature of each pholphatidylcholine in a various period of time (0 to 6 hr.) in order to allow equilibration of drugs within liposomes. At the end of each incubation time, liposomes were adjusted with Tris buffer to 3 ml and fluorescence polarization was measured at 30 °C.

8. Effect of Phenothiazine Concentration on Thermotropic Phase Transition of Liposomes.

Liposomes were prepared from phosphatidylcholine, binary mixture of phosphatidylcholine cholesterol (molar ratios 100 : 10, 100 : 25, 100 : 50 and

100 : 75) and tertiary mixture of phosphatidylcholine : cholesterol : charged amphiphiles (molar ratio of 100 : 10 : 5 and 100 : 10 : 10) with Tris buffer and then labelled with DPH. Afterthat CPZ or TRZ (1 mM in $H_2O$) was added to liposomes to obtain desired drug to lipid molar ratios (e.g. 1 : 25, 1 : 50, 1 : 75, etc. depending on their detectable effects on thermotropic phase transition of liposomes). The mixtures were then vortexed for 1 min and incubated in a thermostat shaker bath above their phase transition temperature of each phosphatidylcholine at a suitable time obtained from step 7. At the end of incubation time, liposomes were adjusted to 3 ml with Tris buffer and fluorescence polarization was measured at various temperatures to determine their thermotropic phase transitions.

9. Effect of Experimental Condition on Stability of Phenothiazines.

To ensure that phenothiazines were stable last long in some experimental conditions without significant degradation, the experiments were performed by spectroscopic mean as follows. Phenothiazine were prepared in Tris buffer at various concentrations as described in 8, then they were incubated in thermostat shaker bath at 65 °C, the highest temperature in this study, for 1.5 hr. Afterthat, the absorbances were measured at wavelength of maximum absorption ($\lambda_{max}$) of each phenothiazine (CPZ, $\lambda_{max}$ 253 nm ; TRZ, $\lambda_{max}$ 261 nm)

# CHAPTER III

# RESULTS AND DISCUSSION

## Characterization of Prepared Liposomes.

To characterize bilayer structure of liposomes, electron micrographs were taken and the results showed that the preparations mainly existed of unilamellar liposomes with a fairly homogeneous size distribution. Figure 12 showed unilamellar structure of REV liposomes prepared from EPC.

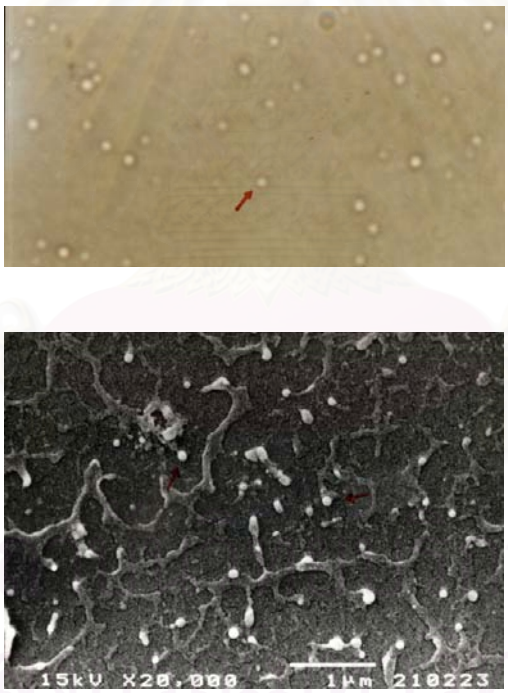



Figure 12 Electron micrograph of REV liposomes prepared from EPC , showing their 'unilamellar structure'

(A) from light microscope (*1000)

(B) Scanning electron micrograph, bar : 1 μm

**DPH Fluorescence Spectroscopy.**

DPH was determined by fluorescence spectroscopy. Figure 13 showed fluorescence spectra of DPH in tetrahydrofuran (THF) between 300 and 600 nm. The maximum excitation and emission wavelengths were 360 and 430 nm, respectively, in accordance with fluorescence spectra of DPH in liposomal membrane. These two spectra were not exactly identical as a consequence of the difference in polarity of DPH environment [Ambrosini et al., 1991].

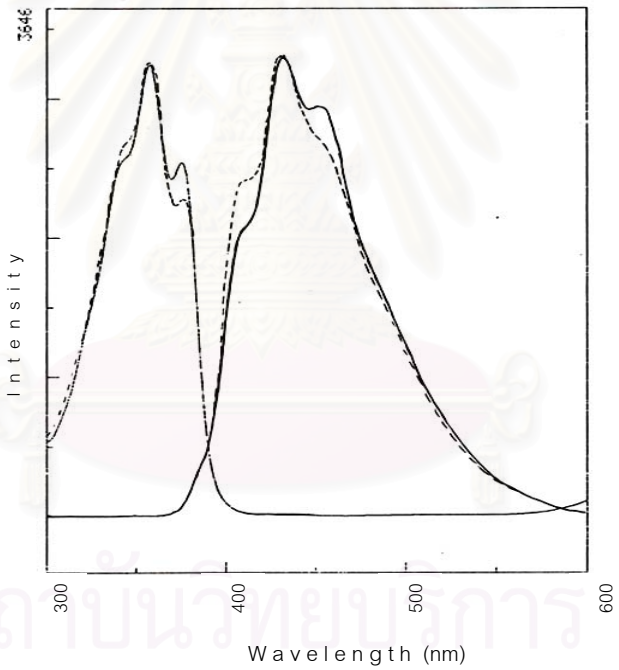



Figure 13 Fluorescence spectra of DPH in THF (——) and in liposomal membrane (– – – – –).

No fluorescence was observed in liposomes containing phenothiazine alone (CPZ and TRZ), thus DPH can be used effectively as a fluorescent detecting probe to investigate alteration in liposomal membrane properties caused by phenothiazines.

Control fluorescence polarization measurements done without probe did not give any appreciable fluorescence polarization values suggesting that there was no light scattering problem at the vesicle concentration used.

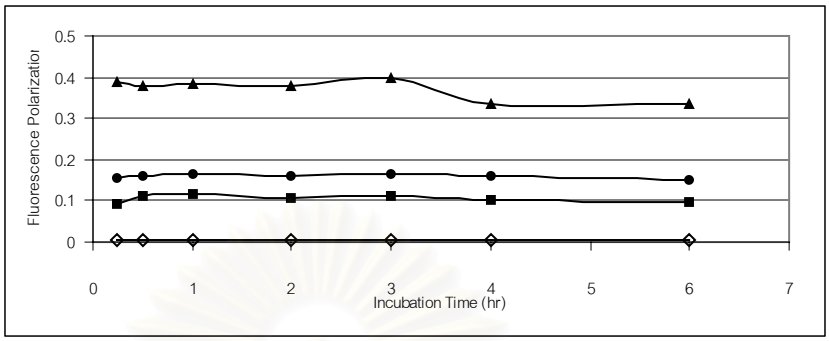
**Effect of Incubation Time and DPH Probe Concentration on Fluorescence Polarization of Liposomes.**

Thorough investigations such as incubation time and DPH probe concentration were studied to find optimal conditions suitable as a standard procedure for measurement of alteration in fluorescence polarization caused by phenothiazine in DPH labelling liposomes.

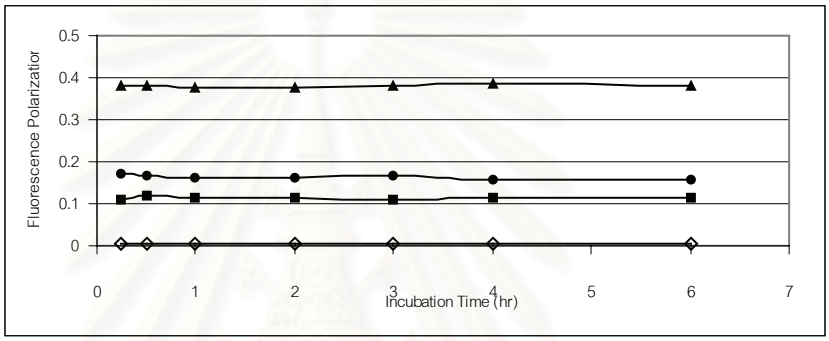
As illustrated in Figure 14, fluorescence polarization occurred suddenly and reached a plateau in all types of lipid used (EPC, DMPC and DSPC liposomes), suggesting spontaneously penetration of DPH from aqueous into liposomal membranes as shown by strongly enhancement in fluorescence polarization value comparing with no significant fluorescence polarization observed in DPH alone in Tris buffer. No increase in fluorescence polarization was observed on longer incubation time, consequently, a 30 min incubation period was selected for subsequent experiments.

Amount of DPH probe in liposomal membrane is also an important factor since high concentration of fluorescent probe will exhibit quenching effect, follow by declining of fluorescence value. Figure 14 and 15 demonstrated that in EPC and DMPC liposomes, fluorescence polarization remained constant upon DPH concentration used (1:250 to 1:1000 probe-to-lipid molar ratio). However, DSPC liposomes showed an increment of fluorescence polarization from 0.32 to 0.38 when DPH concentration was increased from 1:1000 to 1:750 (probe to lipid molar ratio). Further increase in DPH concentration to 1:250 exhibited unchange in fluorescence polarization, thus DPH concentration 1:500 (probe to lipid molar ratio) was selected as the concentration of choice in following experiments since it was sufficiently concentration and no quenching effect was observed.

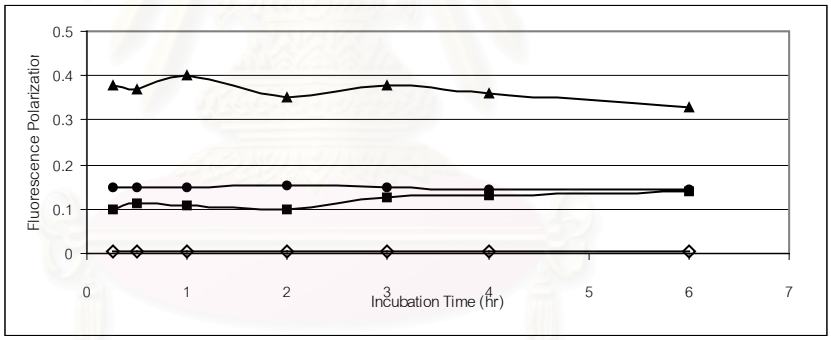
A.




B.




C.




D.

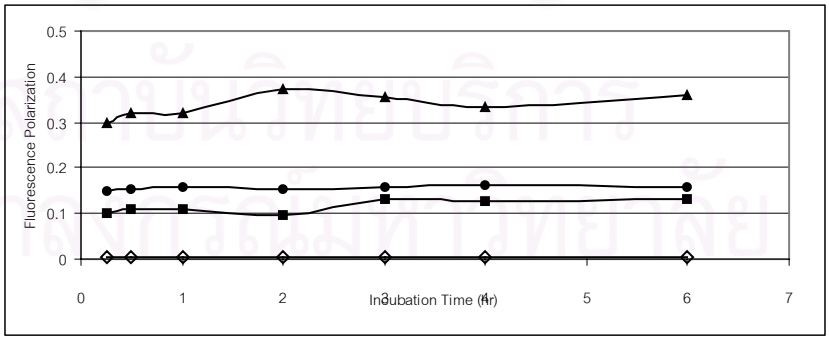



Figure 14. Effect of incubation time and DPH probe concentration (probe to lipid molar ratio) on fluorescence polarization of liposomes. A) 1:250, B) 1:500, C) 1:750 and D) 1:1000

◇, Tris buffer ; ■, EPC ; ●, DMPC ; ▲, DSPC

Each point represents the mean of at least three samples±S.D.

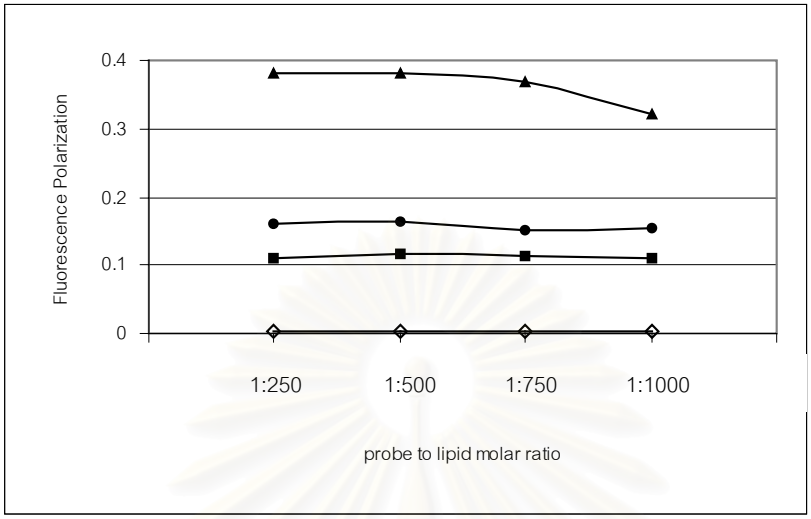



Figure 15. Dependence of DPH probe concentration (probe to lipid molar ratio) on fluorescence polarization of liposomes.

◇, Tris buffer ; ■, EPC ; ●, DMPC ; ▲, DSPC

Each point represents the mean of at least three samples±S.D.

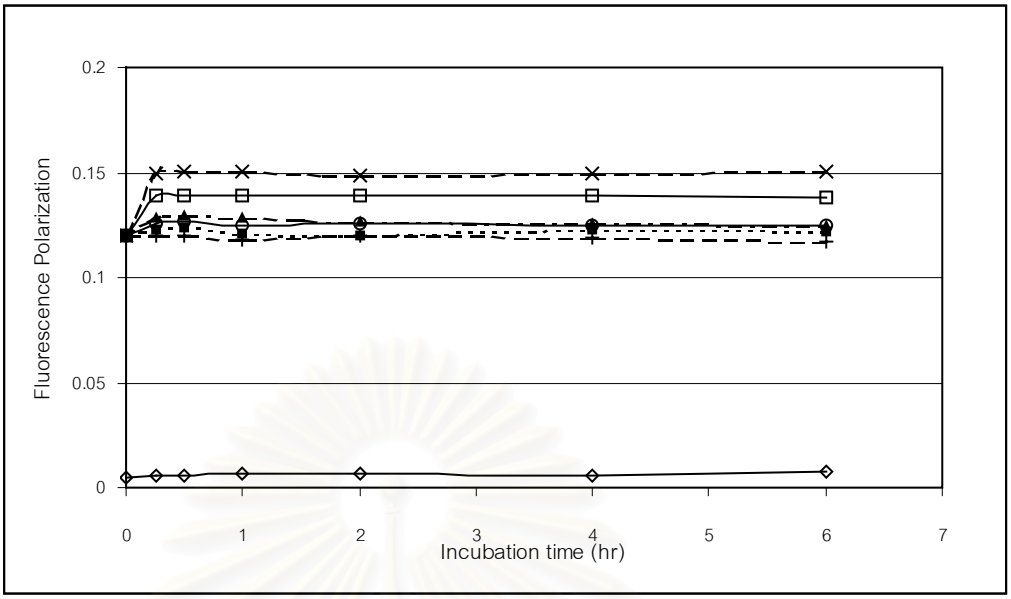
**Influence of Incubation Time on Phenothiazine Penetration into Liposomes.**

In order to find out optimal incubation time for phenothiazine penetration into liposomes, the experiments were then performed using DPH labelled liposomes at optimal probe to lipid molar ratio (1:500) and afterthat incubated with desired amount of each phenothiazine at various incubation periods (30 °C). The fluorescence polarization was detected at the end of each incubation time.

As depicted in Figure16-18, fluorescence polarization changed suddenly and reached a plateau in all types of lipids and all phenothiazine concentrations used, suggesting spontaneously penetration of chlorpromazine and thioridazine from aqueous into liposomal membranes. According to phenothiazine's structure, phenothiazine are amphiphilic molecules, then they could spontaneously penetrate into lipid bilayer with high partition coefficient [Welti et al., 1984; Luxnat and Galla, 1986] as shown by instantaneously altering in fluorescence polarization. No significant enhancement in fluorescence polarization was observed in liposome without phenothiazines. Fluorescence polarization remained constant upon the period time studied (6 hours), both in liposomes with and without drug, thus an appropriate time (30 minutes) was selected as an experimental incubation time.

Since fluorescence may decay upon long standing time, therefore stability of fluorescence value throughout period of investigation is necessary and important. As described earlier in the topic "Effect of Incubation Time and DPH Probe Concentration on Fluorescence Polarization of Liposomes", fluorescence of DPH in liposomes with and without phenothiazine remained unchange along 6 hours, thus alteration in fluorescence measurement should reflect behavior caused by verious experimental factors not by fluorescent probe per se.

A.




B.

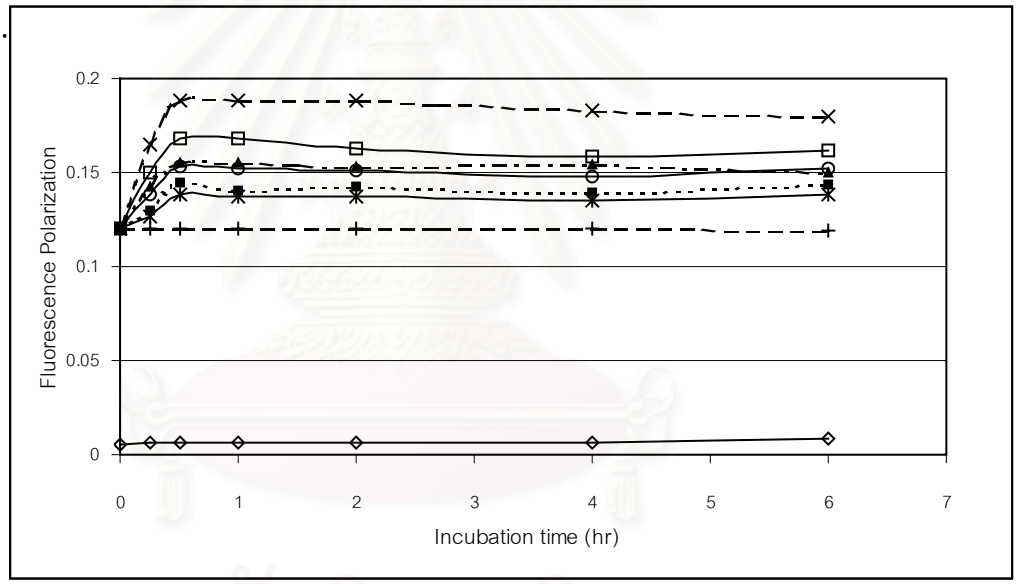


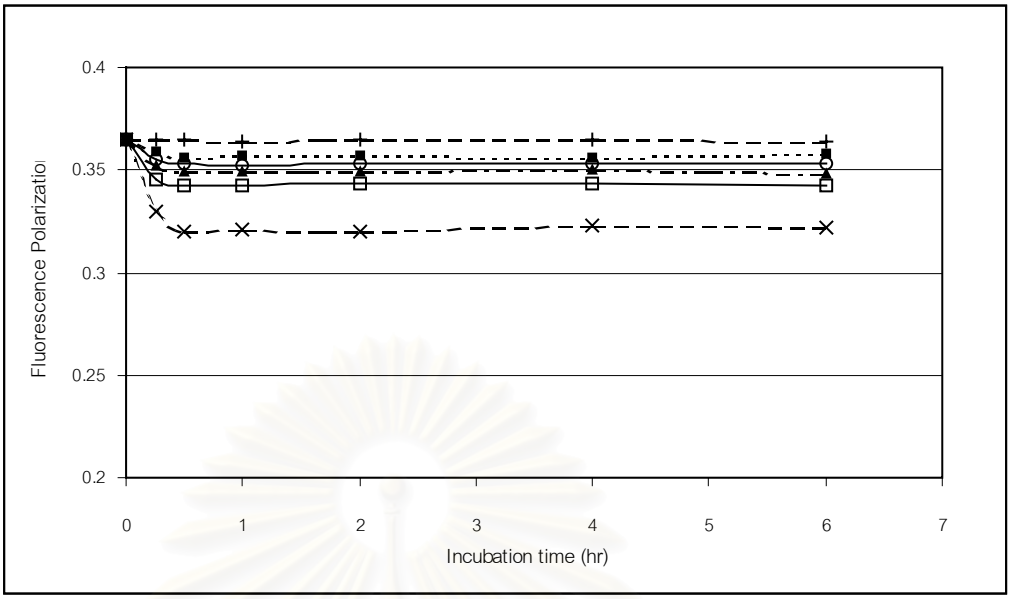

Figure 16 Effect of incubation time and drug concentration on fluorescence polarization of DPH in DMPC liposomes.

(A) CPZ (B) TRZ

Lipid to drug molar ratio: ◊ , no liposome ; + , without drug ; ✶ , 150:1 ; ■ , 75:1 ; ○ , 50:1 ; ▲ , 25:1 ; □ , 15:1 and × , 7.5:1

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

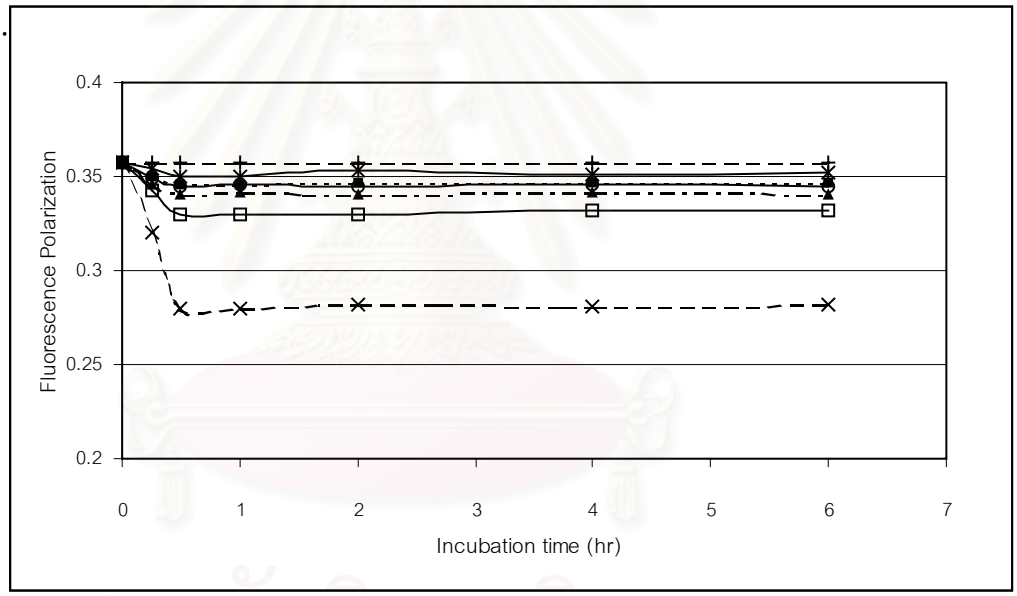



Figure 17 Effect of incubation time and drug concentration on fluorescence polarization of DPH in DSPC liposomes.

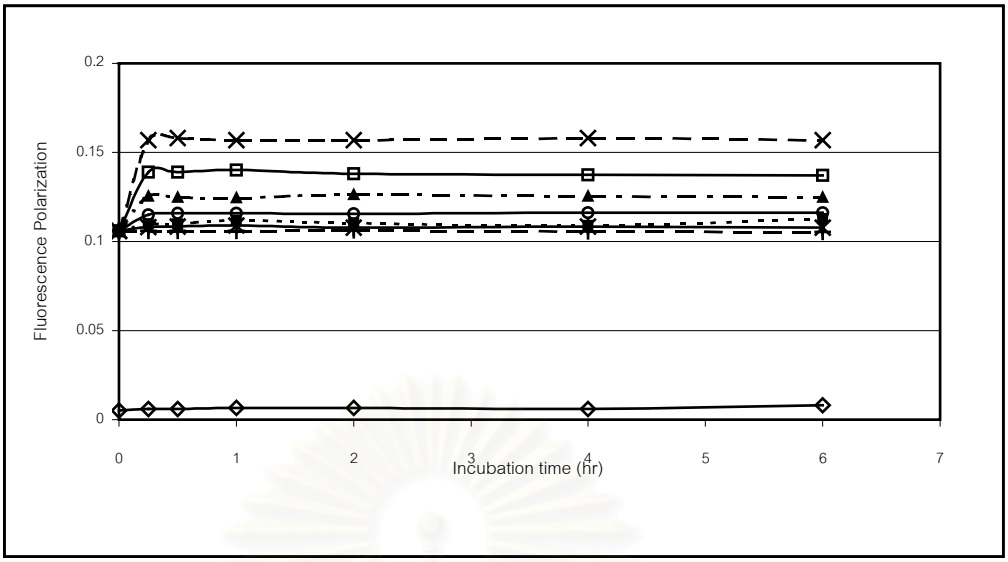
(A) CPZ (B) TRZ

Lipid to drug molar ratio: + , without drug ; ✶ , 150:1 ;

■ , 75:1 ; ○ , 50:1 ; ▲ , 25:1 ; □ , 15:1 and × , 7.5:1

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

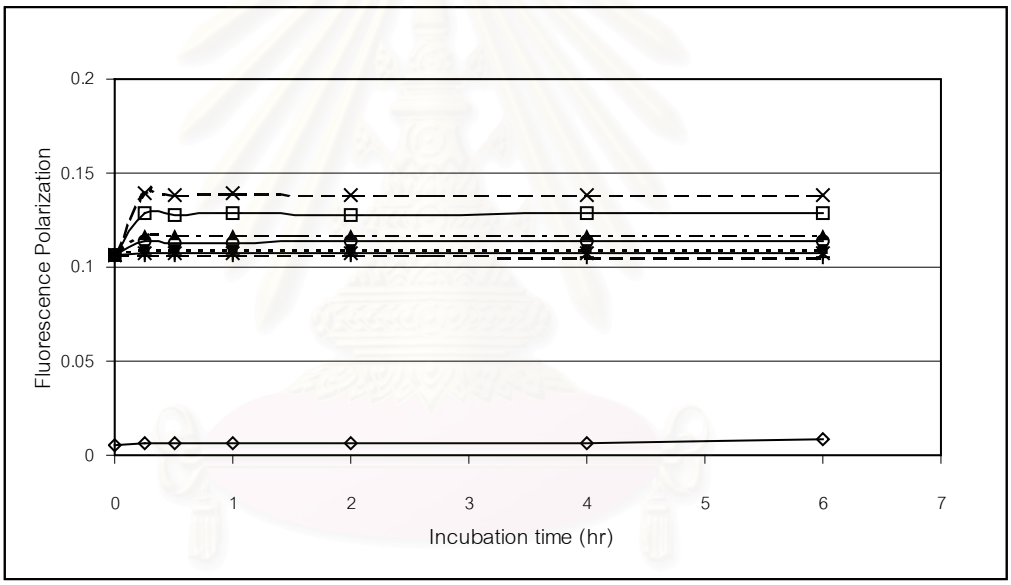



Figure 18 Effect of incubation time and drug concentration on fluorescence polarization of DPH in EPC liposomes.

(A) CPZ (B) TRZ

Lipid to drug molar ratio: ◊ , no liposome ; + , without drug ; ✶ , 150:1 ; ■ , 75:1 ; ○ , 50:1 ; ▲ , 25:1 ; □ , 15:1 and × , 7.5:1

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

**Effect of Experimental Conditions on Stability of Phenothiazines.**

Due to some experimental conditions of this study was executed at quite high temperature (maximum at 65°C), then it was important to ensure that these phenothiazines were stable last long in experimental conditions, without significant degradation.

Fortunately, both phenothiazines (CPZ and TRZ) could absorb and exhibit absorption spectrum in Tris buffer in the range of ultraviolet region as demonstrated in Figure 19 and 20. In agreement with previous reports, CPZ absorption spectrum showed maxima at 253 nm, and 309 nm with a minima at 280 nm [Letterrier, Mendyk and Viret, 1976; Romer and Bickel, 1979]. TRZ spectrum was similar to CPZ with maxima at 261 nm and 311 nm and a minima at 290 nm.

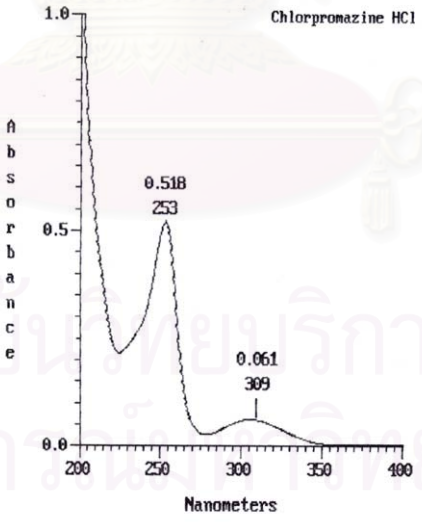



Figure 19 Absorption spectrum of CPZ in Tris buffer.

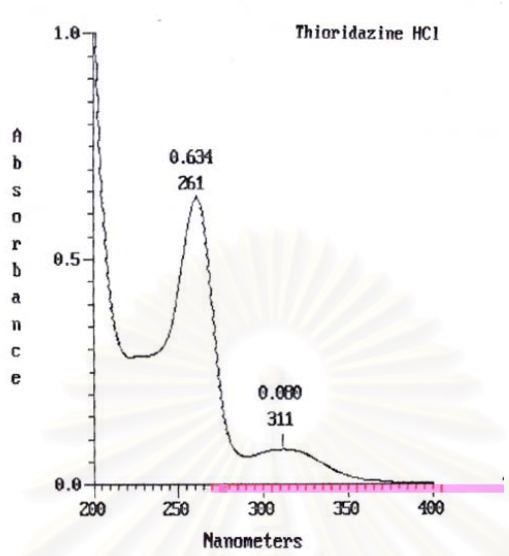



Figure 20 Absorption spectrum of TRZ in Tris buffer.

Base on spectroscopic theory and other reports [Letterrier, Mendyk and Viret, 1976], absorbance of the substances should be related to the amount of substance. Therefore absorption of drug under investigation should remain constant in experimental period to make sure that no alteration in drug properties beyond interested studies happened.

The experiment was then performed by preparing each phenothiazine in Tris buffer at various concentration used in the fluorescence polarization studies and they were incubated in thermostat shaker bath at 65°C for 1.5 hr.

Afterthat, the absorbances were measured at wavelength of maximum absorption ($\lambda_{max}$) of each phenothiazine (CPZ, $\lambda_{max}$ 253 nm; TRZ, $\lambda_{max}$ 261 nm).

The result of this experiment was shown in Figure 21, it was found that there was no different in absorption of drug, upon incubation at 65°C for 1.5 hr. This evident showed that both phenothiazines were stable enough at high temperature condition, as a consequence, the results from fluorescence polarization experiment were reliable.

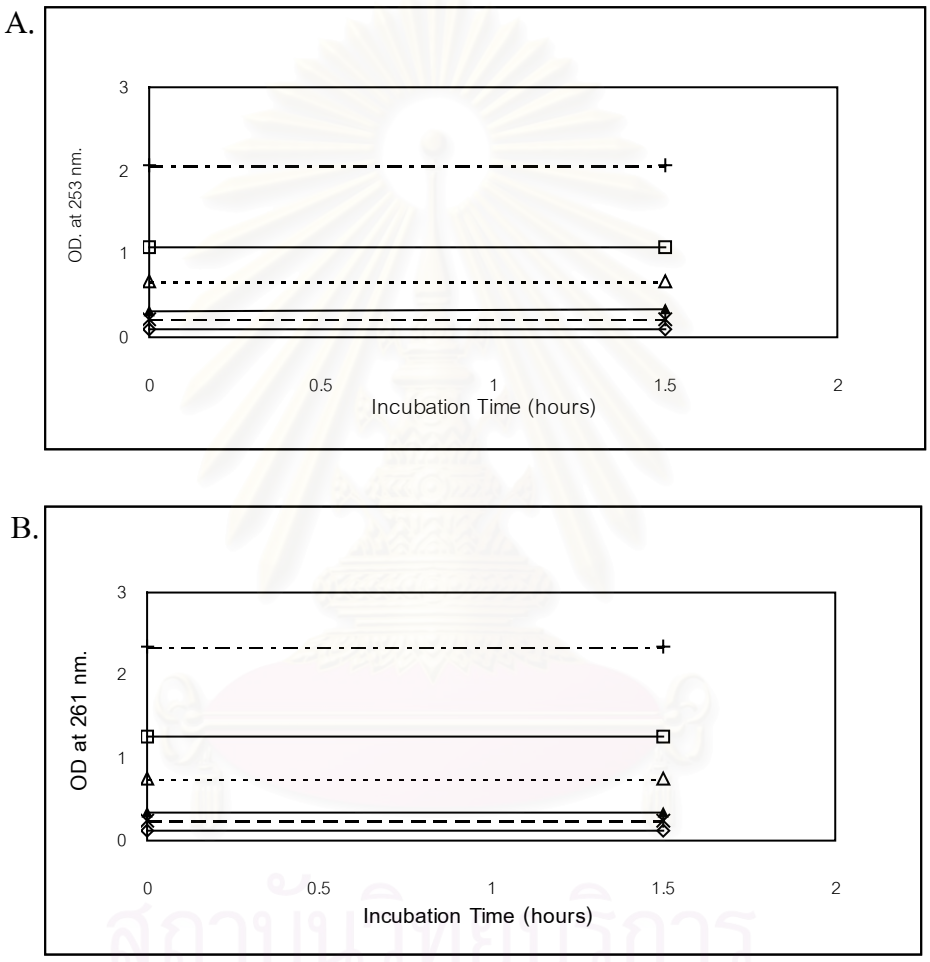



Figure 21 Effect of incubation time on optical density of phenothiazine.
Phenothiazine was incubated at 65°C at various concentrations.
(A) CPZ (253 nm) (B) TRZ (261 nm)
◊ , 0.66 mol% ; × , 1.33 mol% ; ▲ , 2 mol%
Δ , 4 mol% ; □ , 6.66 mol% and ＋, 13.33 mol%.
Each point represents the mean of at least three samples±S.D.,
bar was omitted for clarity.

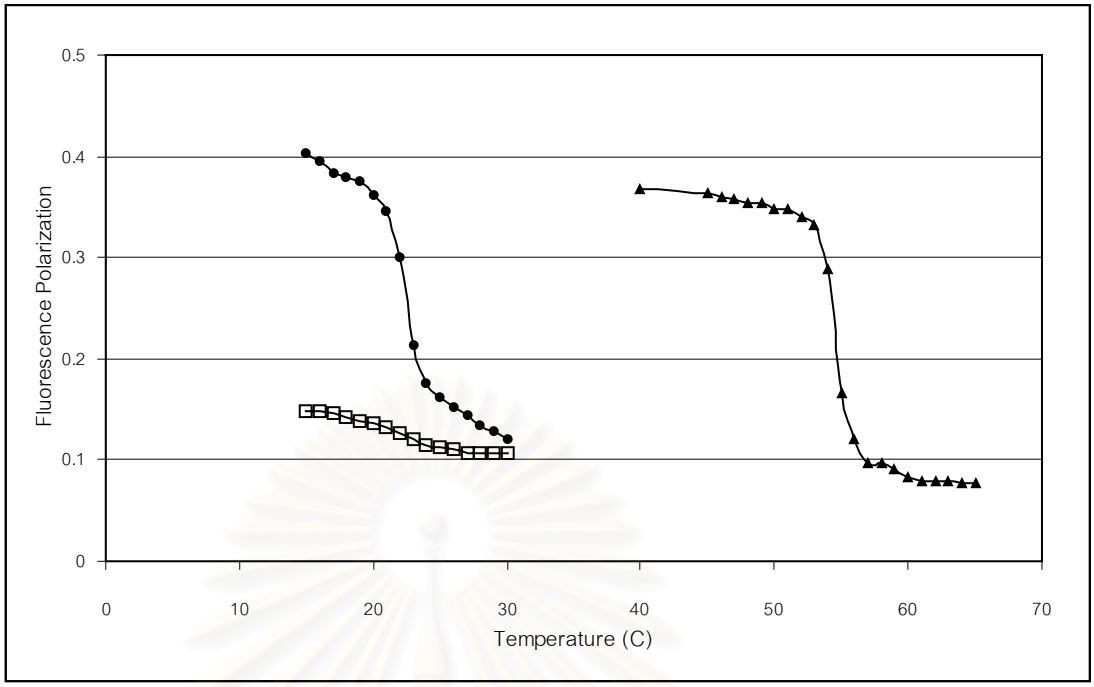
**Determination of Thermotropic Phase Transition of Liposomes.**

Fluorescence polarization (anisotropy) was performed using DPH fluorescent probe which was located in the hydrocarbon core of the lipid bilayer and provided information in this region [Lentz, Barenholz and Thomson, 1976]. The fluorescence polarization (anisotropy) of DPH in liposomes prepared from various acyl chain length phosphatidylcholines, e. g. DMPC, DSPC and EPC as a function of temperatures were shown in Figure 22. Abrupt changes in fluorescence polarization (anisotropy) value were observed at their thermotropic phase transition and thermotropic gel to liquid-crystalline phase transitions were occurred at 22.5 °C and 54 °C for DMPC and DSPC liposomes, respectively, coinciding with the reported values of about 23 °C for DMPC and 54 °C for DSPC [Lentz et al., 1974; Moya-quiles, Munoz-Delgado and Vidal, 1996]. Due to the fact that DSPC has longer fatty acyl chains (C18:0) than DMPC (C14:0), then increasing chain length of fatty acyl chains enhanced Van der Waals force between the acyl chains, thus higher energy was needed to pass from gel phase to liquid-crystalline phase leading to increase in thermotropic phase transition temperature ($T_m$) above DMPC [Lentz et al., 1974].

From fluorescence polarization studies, (Figure 22), high value of fluorescence polarization (anisotropy) indicated high rigidity in hydrocarbon region where fatty acyl chains were restricted motion. In contrast, low value referred to more fluidity, consequently by increasing probe mobility. The explanation might be above $T_m$ (liquid-crystalline phase), where the lipid interior was fluid in nature, DPH showed only a small degree of preferential orientation and its fluorescence was decayed. Nevertheless, below $T_m$ (gel phase), it illustrated a high degree of orientation.

In the case of EPC liposomes, there was no abrupt change in fluorescence polarization (anisotropy) because it remained in the liquid-crytalline phase overall the temperature studied since its phase transition range is –15/-7 °C [Gregoriadis, 1984]. EPC composes of various types of phosphatidylcholine as described previously in Chapter I thus, it has a wide range of phase transition temperature.

A.




B.

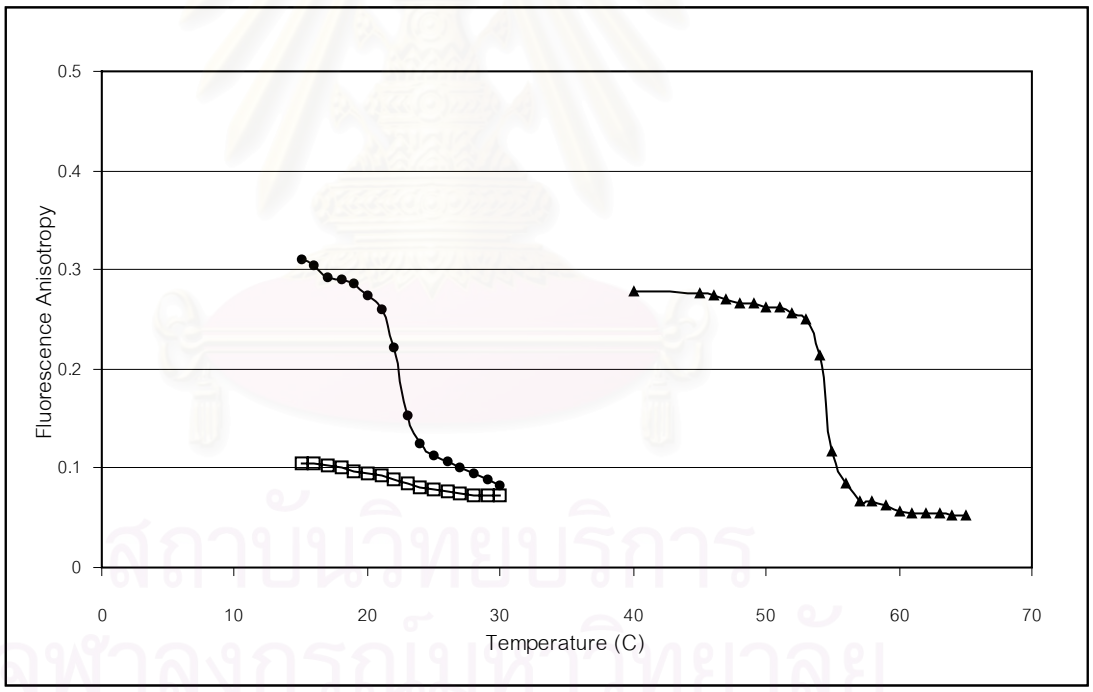



Figure 22 Fluorescence polarization (A) anisotropy (B) of DPH in phosphatidylcholine liposomes.

□ , EPC ; ● , DMPC ; ▲ , DSPC

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

The lipid order parameter of membrane lipids was related to the degree of molecular packing [Blitterswijk, Van Hoeven, and Van der meer, 1981]. Lipid order parameter of all liposomes in the gel phase was higher than in liquid-crystalline phase according to the fact that liposomal membrane in the gel phase has highly ordered structure than in the liquid-crystalline phase. A high degree of polarization represented a high lipid order parameter or low membrane fluidity [Shimooka, Shibata, and Terada, 1992]. As illustrated in Table 1, DSPC liposomes ($T_m$ 54°C) showed high order parameter (0.82502) in gel phase at 45°C compared with low order parameter (0.27602) in liquid-crystalline phase at 65°C. The same phenomenon was also observed in DMPC liposome ($T_m$ 23°C). Since EPC liposomes have low $T_m$ value (–15/-7°C), thus they were in liquid-crystalline phase at studied temperatures (15°C to 30°C), however, at 15°C, EPC liposomes showed higher order parameter than at 30°C. The order parameter data of liposomes prepared from various types of phospholipids correlated well with fluorescence polarization data.

Table 1 Lipid order parameter (S) of liposomal membrane composed of various phosphatidylcholines at different temperatures.
Data represents the mean of at least three samples ± S.D.

| Type of Lipid | S | | | |
|---|---|---|---|---|
| | 15°C | 30°C | 45°C | 65°C |
| EPC | 0.31036±0.23 | 0.07782±0.32 | - | - |
| DMPC | 0.88555±0.14 | 0.16739±0.20 | - | - |
| DSPC | - | - | 0.82502±0.44 | 0.27602±0.52 |

**Influence of Phenothiazines on Thermotropic Phase Transition of Liposomes.**

The effects of phenothiazines, CPZ and TRZ on the fluidity of DMPC, DSPC and EPC bilayers were accessed by measuring the changes in fluorescence polarization (anisotropy) of fluorescent probe, DPH, which was preferentially located near the center of the bilayer (hydrophobic core). Thus, the alteration in fluorescence polarization (anisotropy) values of this probe would reflect the perturbations induced by CPZ and TRZ in the bilayer structure.

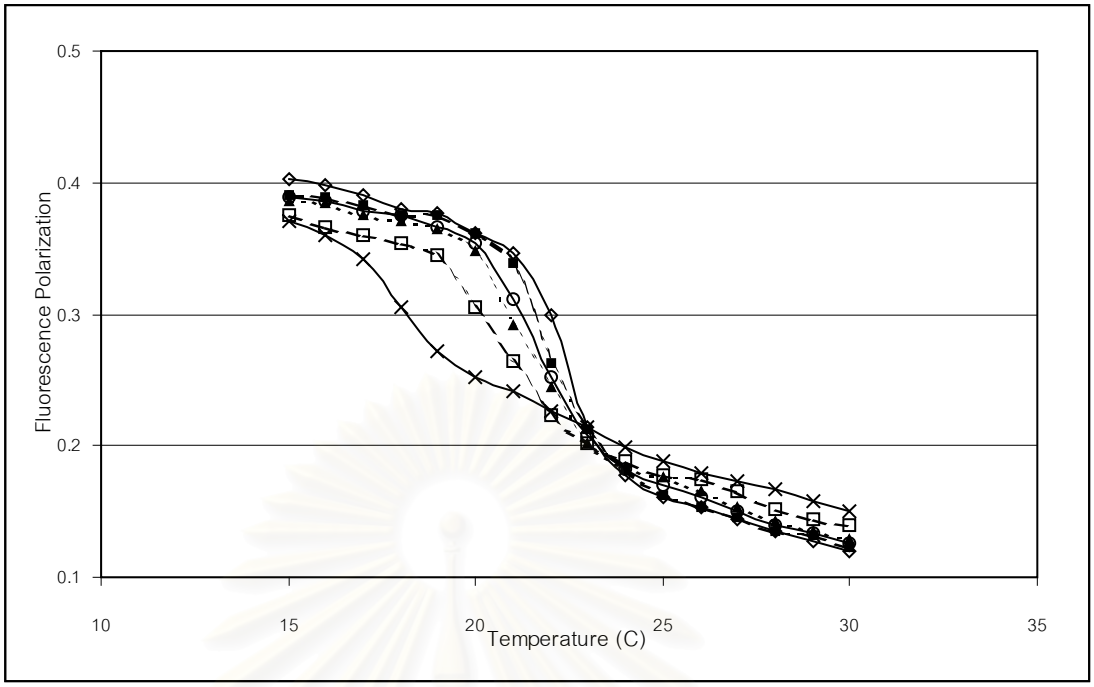
The effects of CPZ and TRZ on thermotropic phase transition of DMPC bilayers were demonstrated in Figure 23 and 24, respectively. These phenothiazines broadened the transition profile, i. e., expanded the temperature range at which fluid and gel domains coexisted and shifted the phase transition temperature ($T_m$) to lower temperatures. Both effects were phenothiazine concentration dependence. When the molar ratio of DMPC to phenothiazines was up to 7.5 : 1 in both CPZ and TRZ, the thermotropic phase transition of DMPC was abolished, these results suggested that liposomal membranes might be greatly perturbed by these phenothiazines at high concentration leading to loss their thermotropic properties.

The results showed that the dominant effect of both phenothiazines on the thermotropic phase transition of DMPC depended on phase state of liposomes. In gel phase liposomes, the fluidization of DMPC membranes was demonstrated by decreasing in $T_m$ of DMPC followed by the fluidizing of the gel phase since a decreased in fluorescence polarization (anisotropy) values was observed. On the contrary, the ordering effect in the liquid-crystalline phase liposomes was apparently observed by enhancement in fluorescence polarization (anisotropy) values. The results concerning DPH fluorescence polarization (anisotopy) indicated that CPZ

and TRZ have dual effects on liposomal membrane and perturbed the packing order of the hydrophobic core of the membrane in the same manner.

In gel phase state where two acyl chains of DMPC arranged themselves in *trans* form, phenothiazines might perturb their ordered structure, consequently by changing their conformation to *trans* and *gauche* forms. Thus the disordering phenomenon was obtained. However, when DMPC liposomes were in liquid-crystalline phase, the acyl chains wobbled freely. Then, phenothiazines might intercalated between the acyl chains of the lipid molecules, thus ordering of acyl chains was observed.

A.




B.

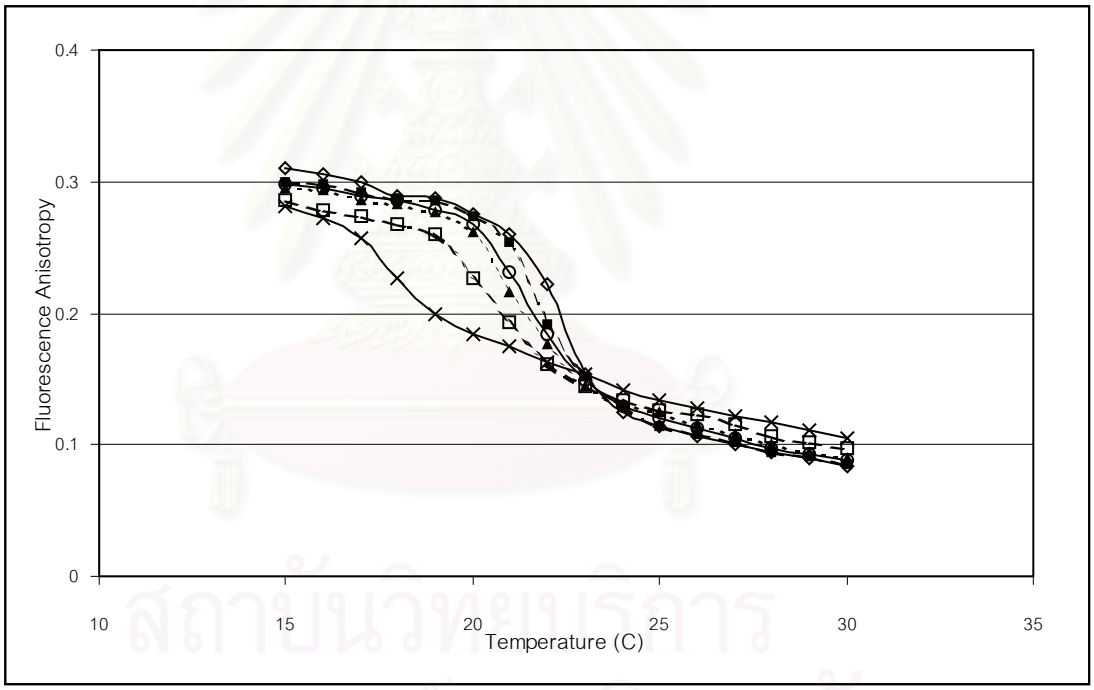


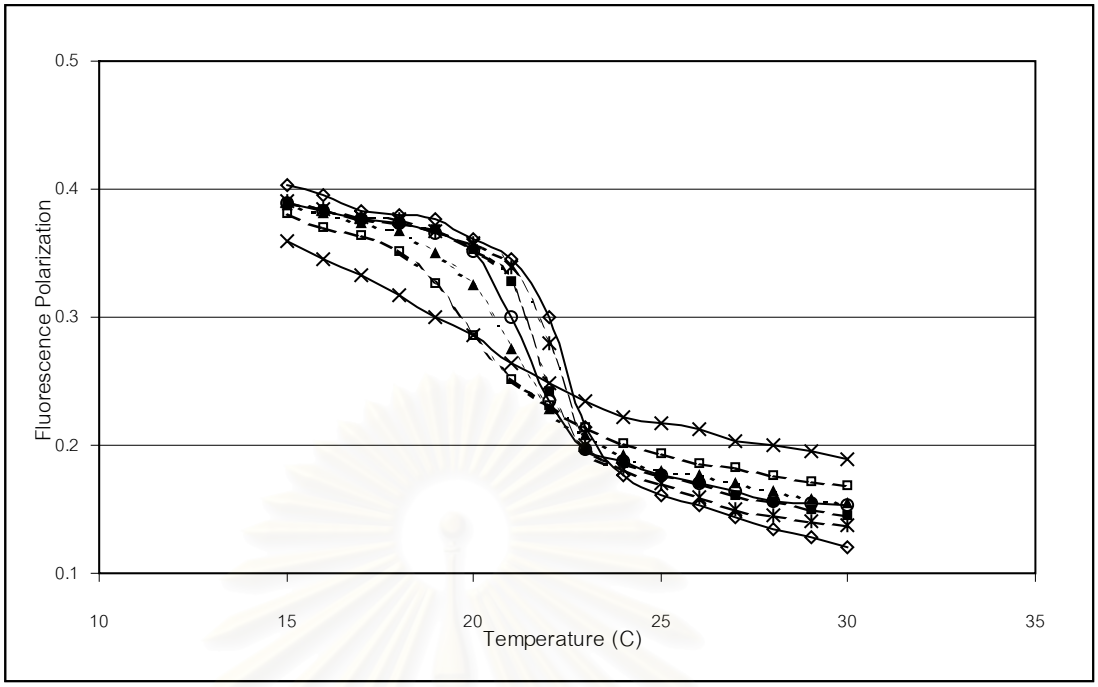

Figure 23 Effect of CPZ on thermotopic phase transition of DMPC liposomes (A) fluorescence polarization (B) anisotropy.

Concentration of DMPC to CPZ, by mole

◇ , without CPZ ; ■ , 75:1 ; ○ , 50:1 ; ▲ , 25:1 ; □ , 15:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

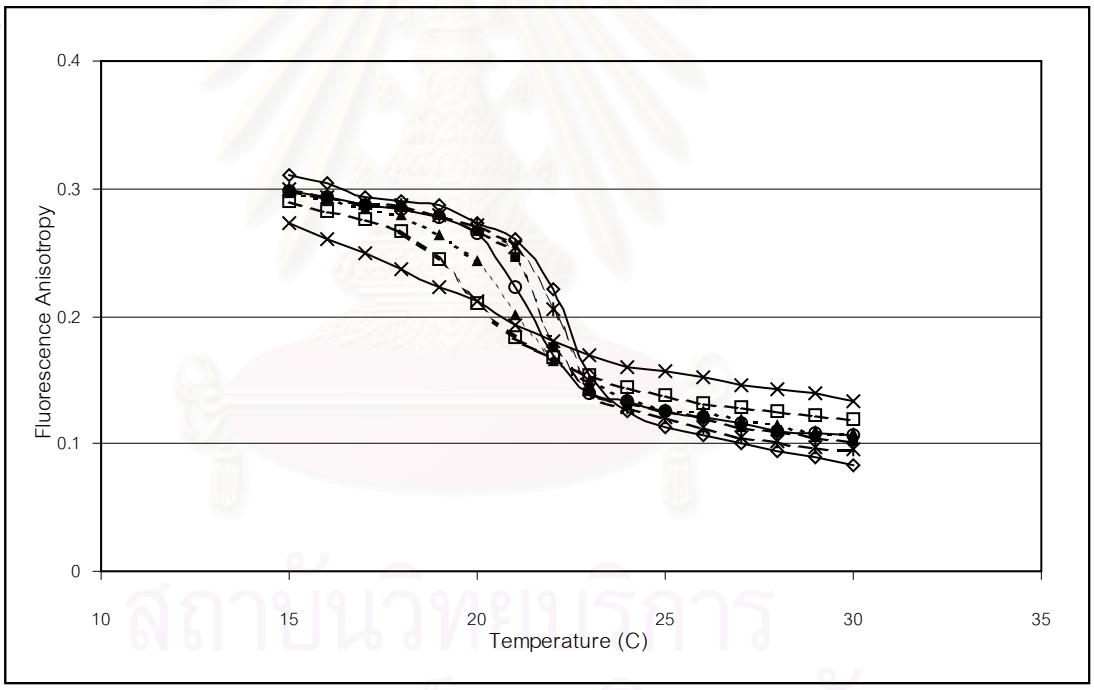



Figure 24 Effect of TRZ on thermotopic phase transition of DMPC liposomes (A) fluorescence polarization (B) anisotropy.

Concentration of DMPC to TRZ, by mole

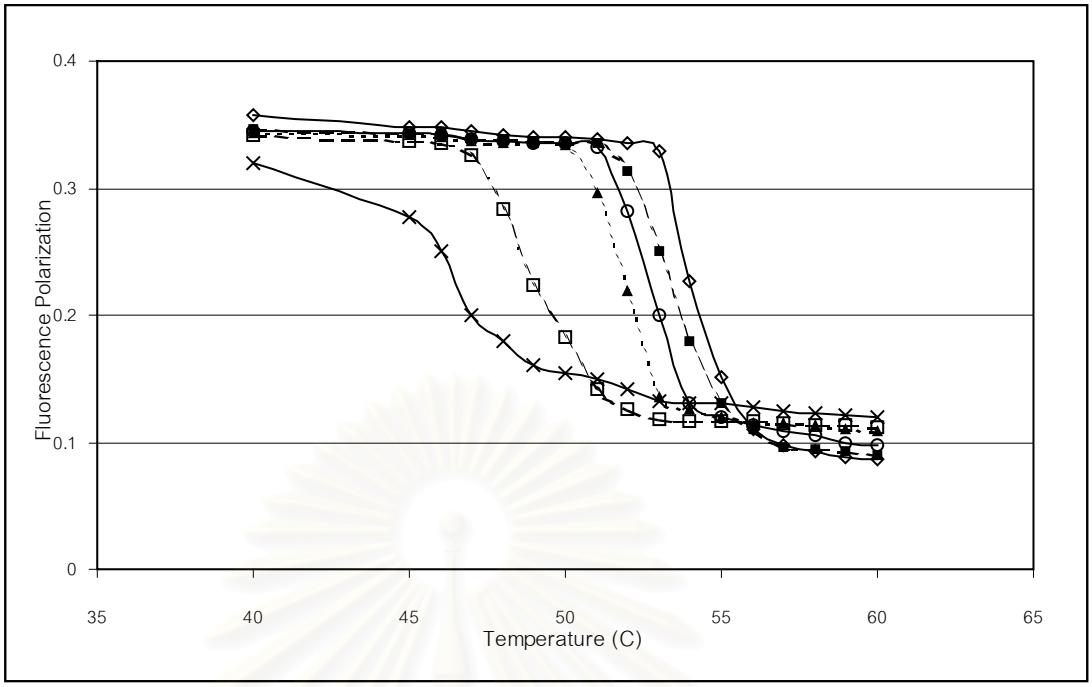
◇ , without CPZ ; ■ , 75:1 ; ○ , 50:1 ; ▲ , 25:1 ; □ , 15:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

The effects of CPZ and TRZ on thermotropic properties of DSPC bilayers were also investigated (Figure 25 and 26). Same evidence was achieved as in the case of DMPC, both drugs decreased the phase transition temperature ($T_m$) and broadened the transition range. These effects were also drug concentration dependence. When the molar ratio of DSPC to phenothiazines were reached 7.5 : 1 in both CPZ and TRZ, the phase transitions were disappeared due to greatly membrane structure perturbation as previously explained in DMPC liposomes.

Phenothiazines also exerted fluidization effect on DSPC liposomes below $T_m$ (54°C) and condensation effect above $T_m$ as described earlier in DMPC liposomes.

A.




B.

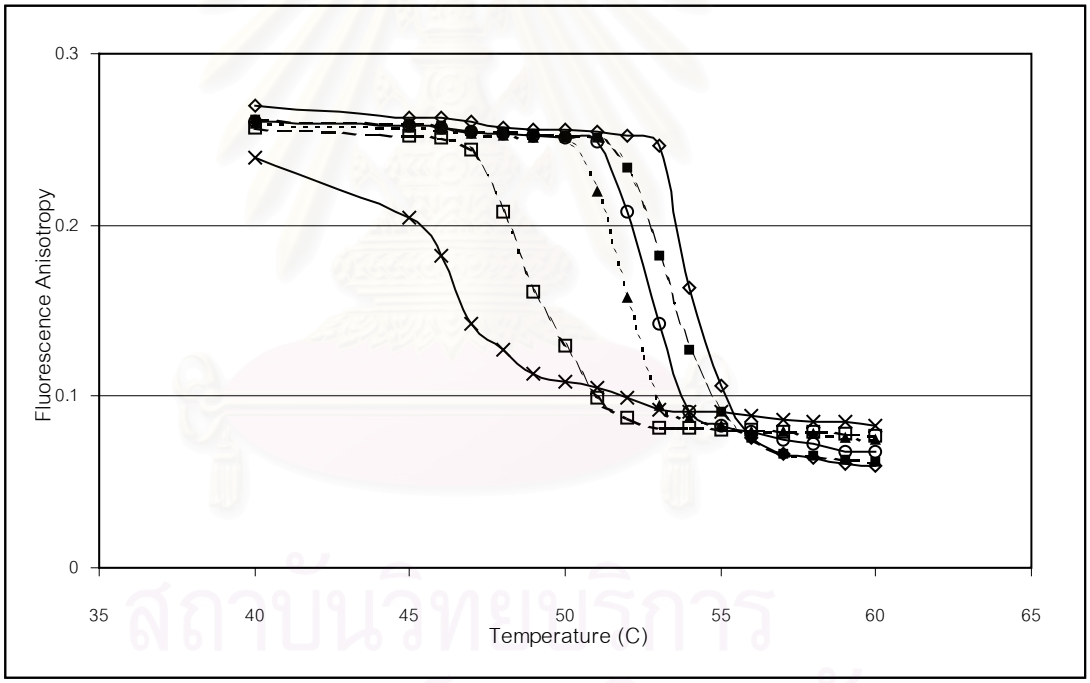


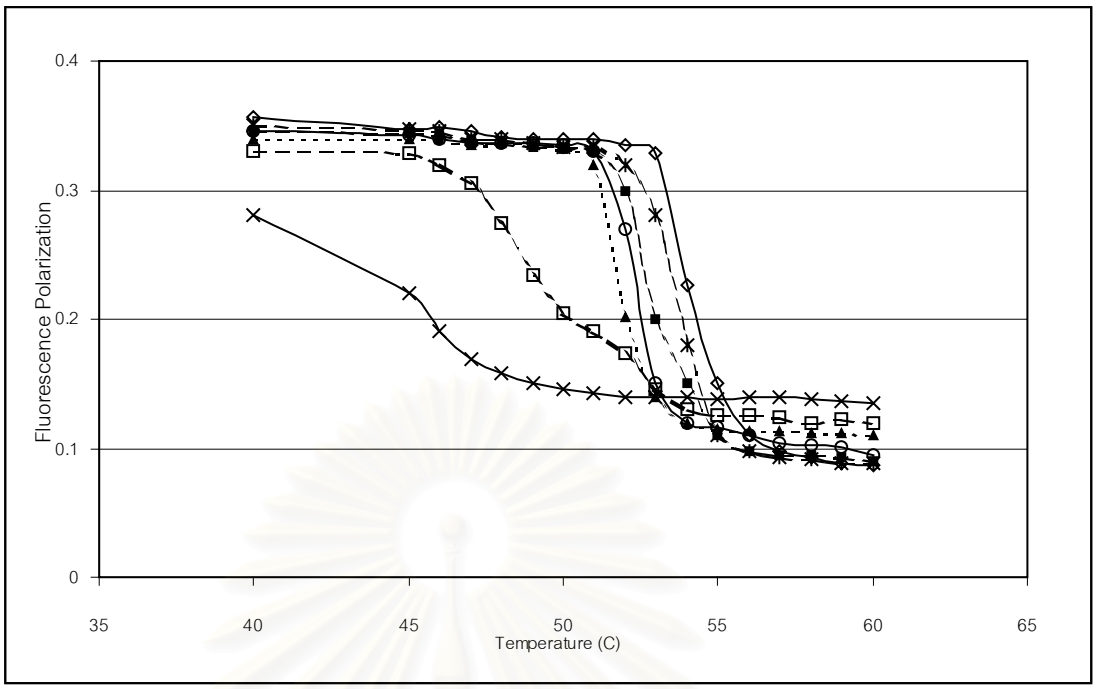

Figure 25 Effect of CPZ on thermotopic phase transition of DSPC liposomes (A) fluorescence polarization (B) anisotropy.

Concentration of DSPC to CPZ, by mole

◇, without CPZ ; ■, 75:1 ; ○, 50:1 ; ▲, 25:1 ; □, 15:1 and ×, 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

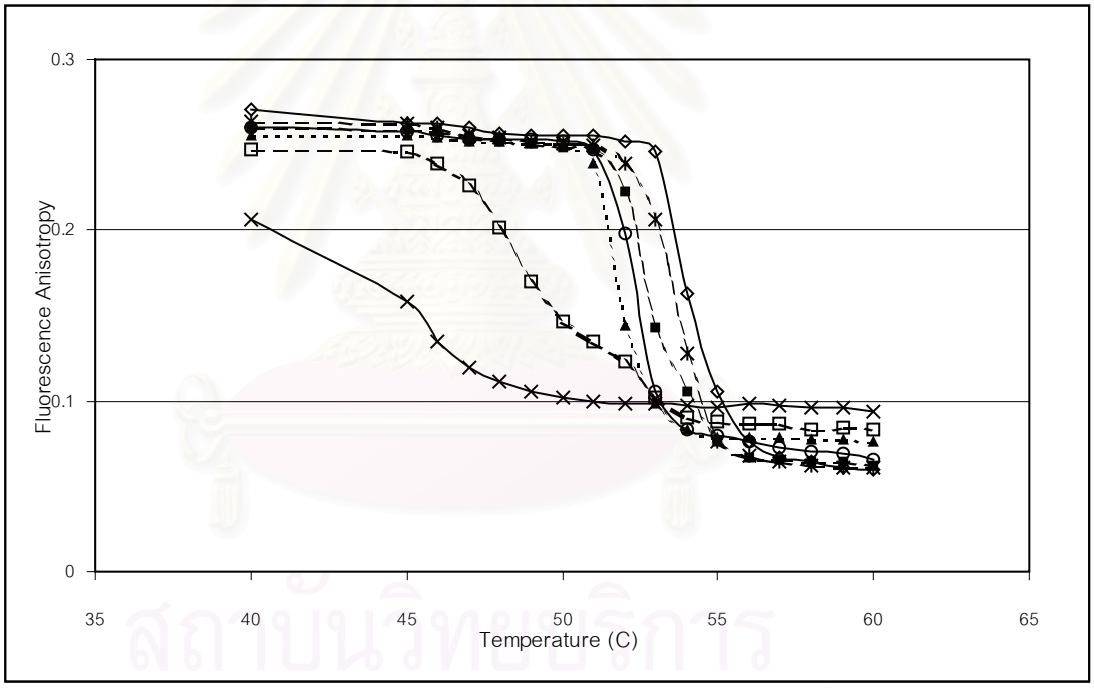


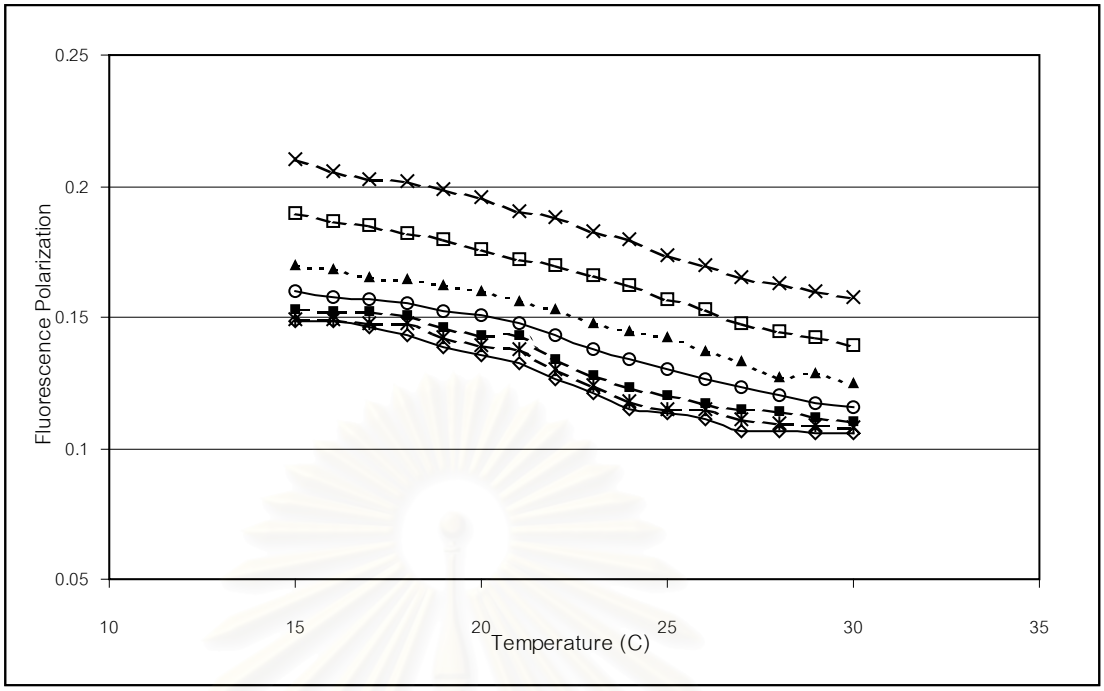

Figure 26 Effect of TRZ on thermotopic phase transition of DSPC liposomes (A) fluorescence polarization (B) anisotropy.

◇, without CPZ ; ∗, 150:1 ; ■, 75:1 ; ○, 50:1 ; ▲, 25:1 ; □, 15:1 and ×, 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

Because biological membranes are a complex mixture of various types of phospholipids, in order to mimic this diversity, egg yolk phosphatidylcholine (EPC), a natural phospholipid, was used to prepare liposomes and studied the interaction between phenothiazines and membranes. Since EPC composes of various types of phospholipid with saturated and unsaturated fatty acyl chains, consequently its $T_m$ is very low and broad (-15 to -7 °C) [Gregoriadis, 1984.], thus EPC liposomes were in liquid-crystalline state overall the temperature range studied (15 to 30°C). As shown in Figure 27 and 28, membrane fluidity was decreased as could be seen from increasing of fluorescence polarization (anisotropy) upon increasing amount of phenothiazines. CPZ showed little stronger effect on EPC liposomal membrane than TRZ since steeper slope was obtained (Figure 27 and 28), however, both drugs exhibited direct relationship between drug concentration and their condensation effect on liposomal membrane and the effect was more pronounced at low temperature more than high temperature.

A.




B.

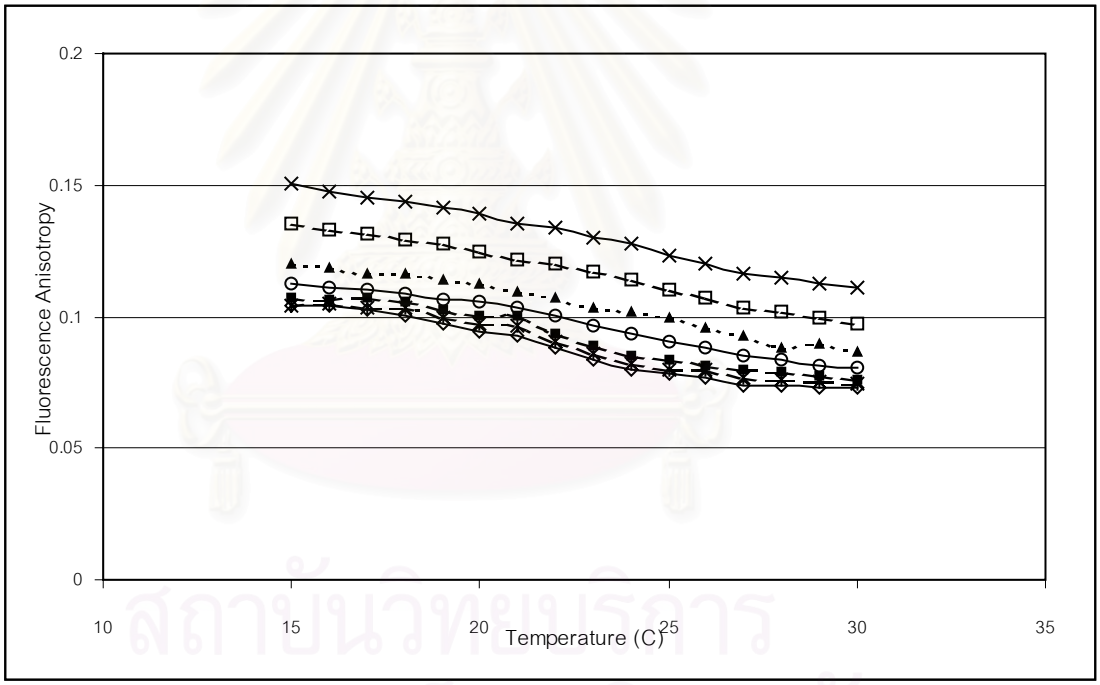


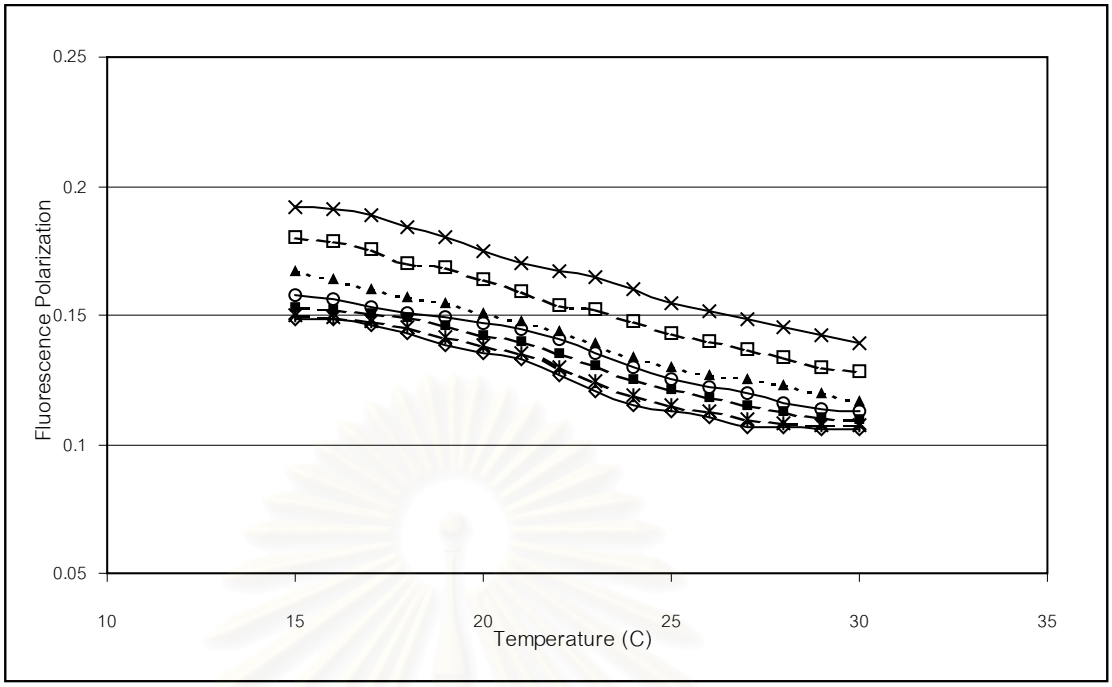

Figure 27 Effect of CPZ on thermotopic phase transition of EPC liposomes (A) fluorescence polarization (B) anisotropy.

Concentration of EPC to CPZ, by mole

◇, without CPZ ; ∗, 150:1 ; ■ , 75:1 ; ○ , 50:1 ; ▲ , 25:1 ; □ , 15:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

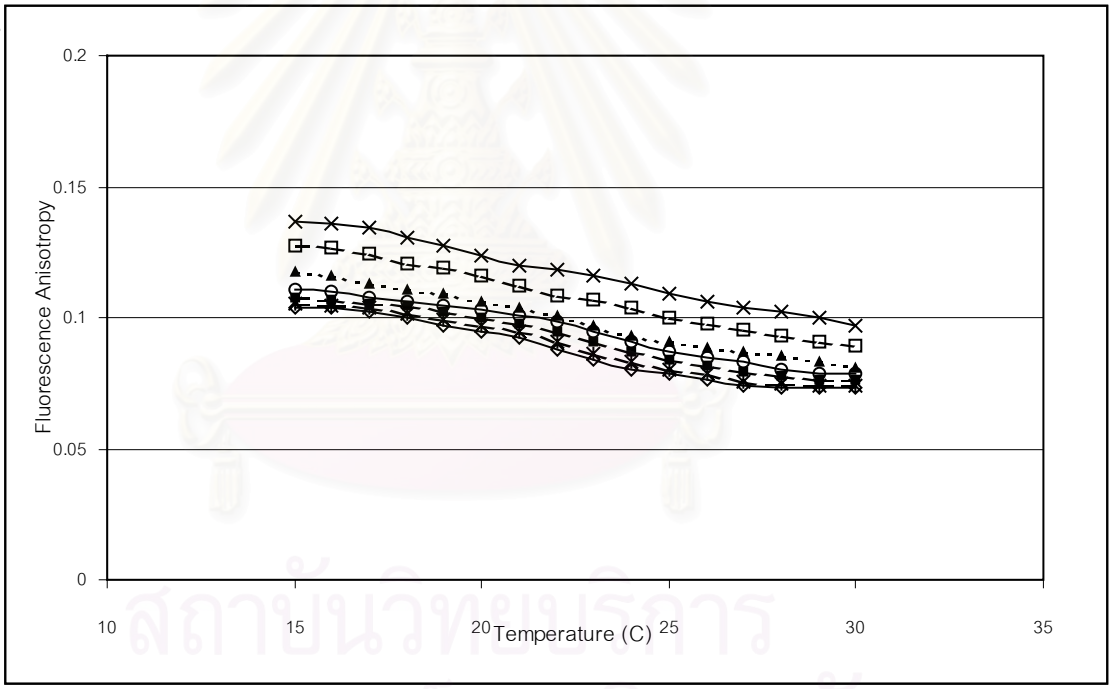



Figure 28 Effect of TRZ on thermotopic phase transition of EPC liposomes (A) fluorescence polarization (B) anisotropy.

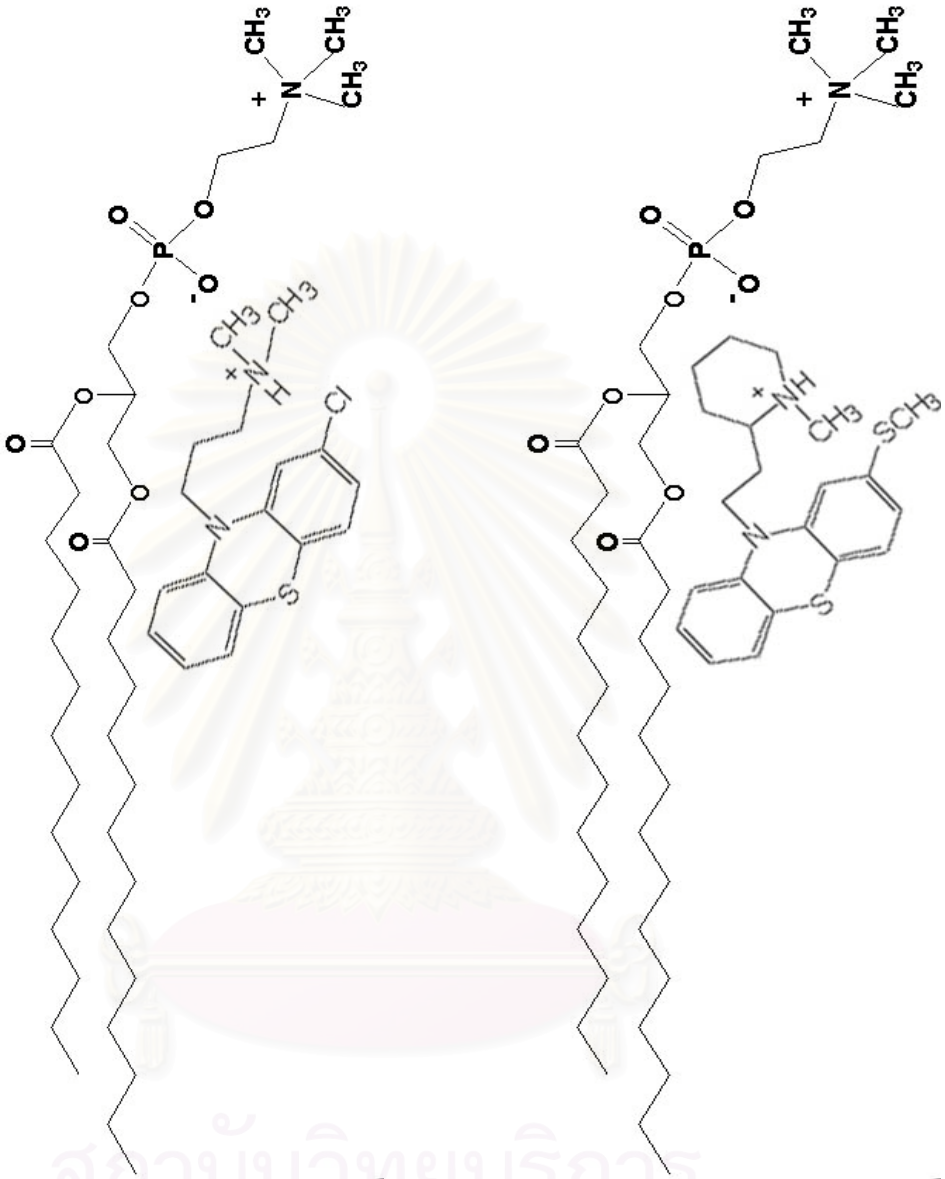
◇, without CPZ ; ∗, 150:1 ; ■ , 75:1 ; ○ , 50:1 ; ▲ , 25:1 ; □ , 15:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

Phenothiazines present the common characteristic being amphiphilic cationic, small molecule having a tricyclic hydrophobic (phenothiazine) ring bound to a short chain hydrocarbon with a polar tertiary amino group [Carey et al., 1976; Ahyahauch and Bennouna, 1999; Jutila et al., 2001]. Amphiphiles are known to perturb the structure of artificial and natural membranes [Luxnat and Galla, 1986]. Since the region of the polar headgroups of phospholipids provided a favorable environment for cationic amphiphilic molecules, they intercalated and thus influenced the phase transition temperature of phospholipid bilayers. The intercalated drug molecules might interrupt hydrogen bonds spanned between adjacent headgroups and thus destroyed the specific structural arrangement of the bilayers [Hanpft and Mohr, 1985]. In order to visualize phenothiazines interaction with phospholipid bilayers, experimental results can be interpreted on the basis of the following model of the interaction of CPZ and TRZ with model membrane. Because their pKa values (9.3 for CPZ and 9.5 for TRZ), both phenothiazines exist essentially in their charged forms at experimental pH (7.0) with a negligible amount of neutral forms. From their common characteristics, phenothiazine molecules might attach to the bilayer in such a way that the protonated amino might intercalate between phosphatidylcholine headgroups while aromatic rings were pointed directly to hydrophobic part of the phospholipid bilayer due to hydrophobic interaction [Kursh, Lullmann,and Mohr, 1983; Suwalky et al., 1983; Maher and Singer, 1984] as illustrated in Figure 29.

Since the headgroup of phenothiazines are bulky, therefore it might create space between phosphatidylcholine headgroups. As a consequence, less energy was required for the transition from gel phase to liquid-crystalline phase, then $T_m$ decreased due to the perturbation of headgroup orientation of phosphatidylcholine. In the ordered gel phase, bulky of phenothiazine ring disrupted the packing of phospholipid acyl chains, allowing greater conformation freedom of acyl chains as

illustrated by decreasing in fluorescence polarization (anisotropy), Figure 23-26. However, in the liquid-crystalline phase, intercalated phenothiazine ring inhibited swinging motion of the fatty acyl chains closed to glycerol moiety, providing more ordered state. As a result, the cone angle and the wobbling motion of DPH, a fluorescent probe, might decrease thus enhancement of fluorescence polarization (anisotropy) values was observed (Figure 23-28). Therefore, phenothiazines exhibited dual effect on liposomal membranes depending on there phase.



(A) (B)

Figure 29 Proposed interaction between phenothiazines and phospholipids (A) CPZ (B) TRZ.

**Influence of Cholesterol on Thermotropic Phase Transition of Liposome.**

Cholesterol is a major lipid component of many biological membranes, then understanding of its interaction with phospholipids is essential for the description of the hydrophobicity environment in which various membrane processes occur.

Cholesterol has been reported to have a "dual effect on fluidity" of phospholipid bilayers. The ordered array of lipid acyl chains in the gel phase was fluidized by the addition of cholesterol, whereas in the liquid-crystalline phase, cholesterol reduced the fluidity [Lippert and Peticolas, 1971; Kawato, Kinosita and Ikegami, 1978; Pang and Miller, 1978; Blitterswijk et al., 1981]. At sufficiently high concentrations, cholesterol abolished the phase transition [Oldfield and Chapman, 1971]. Therefore, its action was on changing of membrane fluidity.

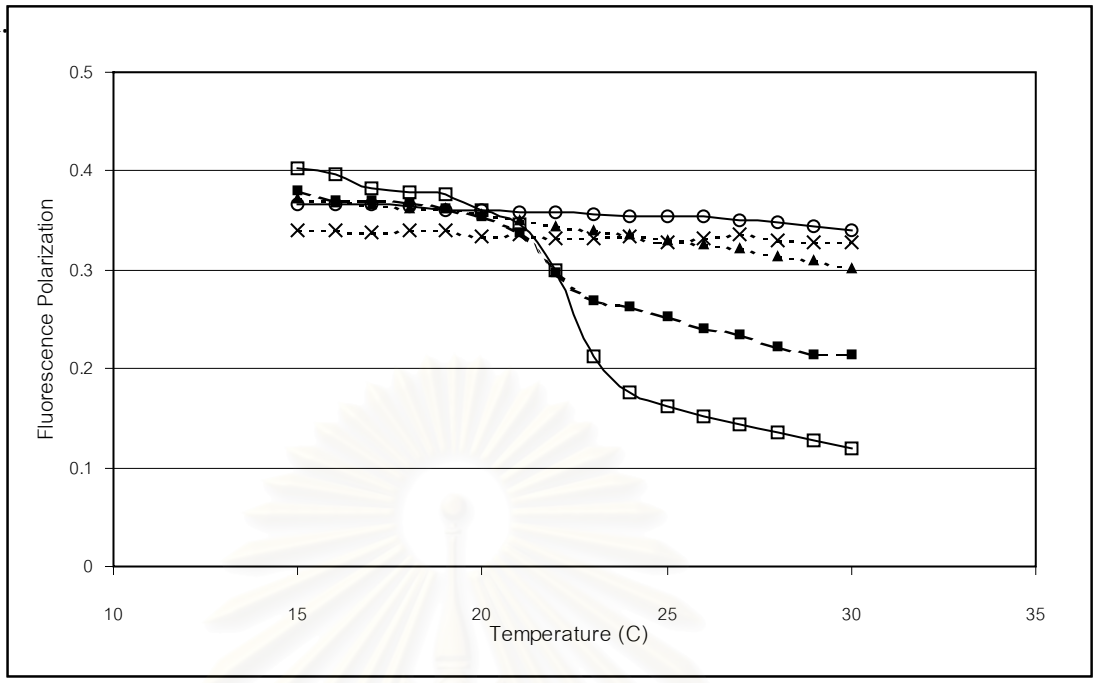
To understand more clearly about the state of membrane, cholesterol was incorporated as one of liposomal membrane components, in the various molar ratios of phospholipid:cholesterol such as 100:10, 100:25, 100:50 and 100:75 compared with pure phospholipid.

As illustrated in Figure 30-32 and Table 2-4, the fluorescence polarization (anisotropy) of DPH in DMPC-cholesterol, DSPC-cholesterol and EPC-cholesterol liposomes were measured as a function of temperature compared with pure phosphatidylcholine. As discussed previously, cholesterol was found to have a condensing effect on phosphatidylcholine liposomes in their liquid-crystalline phase and have a fluidizing effect on the gel phase one. From the fluorescence polarization experiments of DMPC and DSPC liposomes studies here, they were shown that incorporation of cholesterol made membrane in liquid-crystalline phase more rigid compared to pure lipid as indicated by higher values of fluorescence polarization

(anisotropy) and lipid order parameter. Enhancement of membrane rigidity was direct proportional to amount of cholesterol added. In the other hand, cholesterol produced fluidizing effect on gel phase membranes by lowering values of fluorescence polarization (anisotropy) and lipid order parameter compared to pure lipid. Further addition of cholesterol resulted in increment of fluorescence polarization (anisotropy) overall the range of temperatures studied which might be due to restricted motion of phospholipid acyl chains at high cholesterol content.

When focused on thermotropic phase transition temparature ($T_m$) of phospholipid-cholesterol liposomes, it was found that cholesterol had little effect on position of $T_m$, however, it provided pronounced effect on broadening transition range. At low molar ratios of phospholipid to cholesterol (100:10, 100:25), $T_m$ of both DMPC and DSPC were broadened until molar ratio of phospholipid:cholesterol reached 100:50, $T_m$ was abolished in both DMPC and DSPC due to stiffness of membrane caused by high cholesterol content as mentioned above.

A.




B.

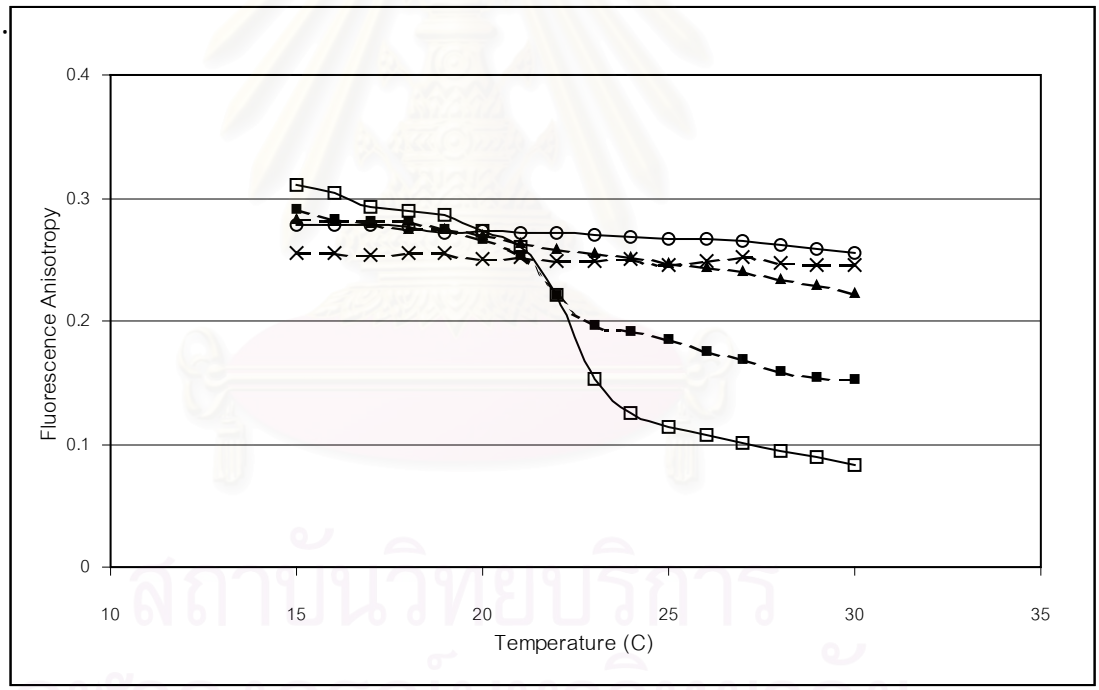



Figure 30 Effect of cholesterol on thermotropic phase transition of DMPC liposomes (A) fluorescence polarization (B) anisotropy.

Concentration of DMPC to cholesterol, by mole.

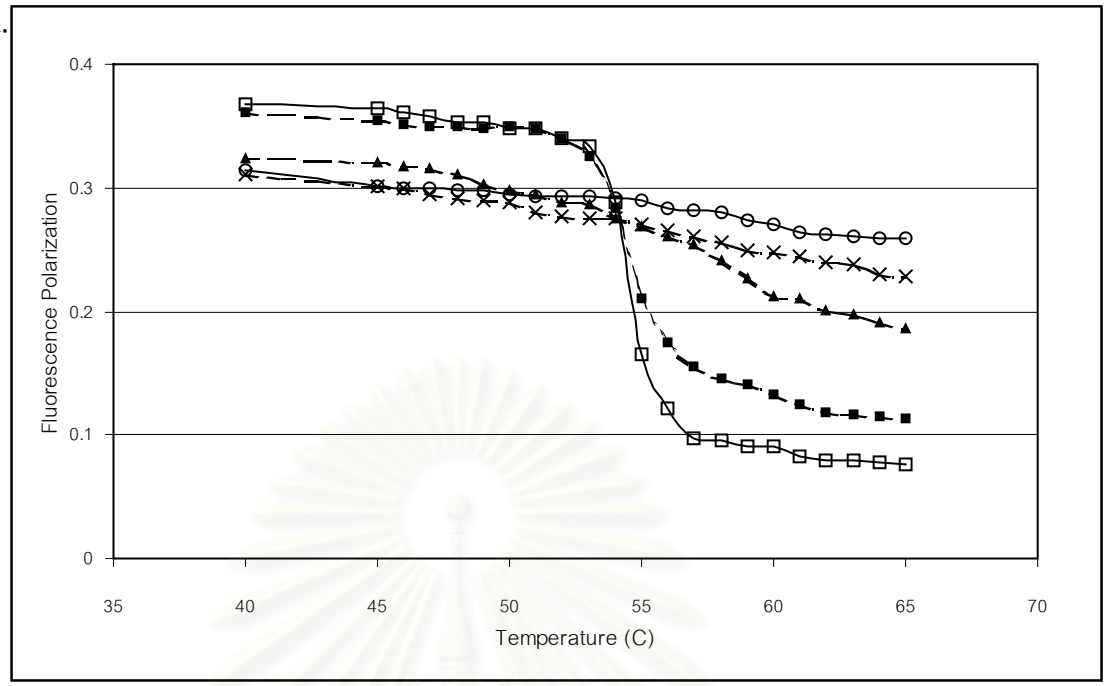
□ , without cholesterol ; ■ , 100:10 ; ▲ , 100:25 ; × , 100:50 and ○ , 100:75

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

Table 2 Lipid order parameter (S) of the liposomal membrane composed of DMPC and Cholesterol in various molar Ratios.
Data represents the mean of at least three samples±S.D.

| DMPC:Cholesterol (molar ratio) | Lipid order parameter (S) | |
|---|---|---|
| | Gel phase (15°C) | Liquid crystalline phase (30°C) |
| 100:0 | 0.88555±0.23 | 0.16739±0.76 |
| 100:10 | 0.84761±0.18 | 0.51101±0.63 |
| 100:25 | 0.83343±0.32 | 0.70342±0.45 |
| 100:50 | 0.77622±0.55 | 0.75396±0.32 |
| 100:75 | 0.82401±0.41 | 0.77661±0.39 |

A.




B.

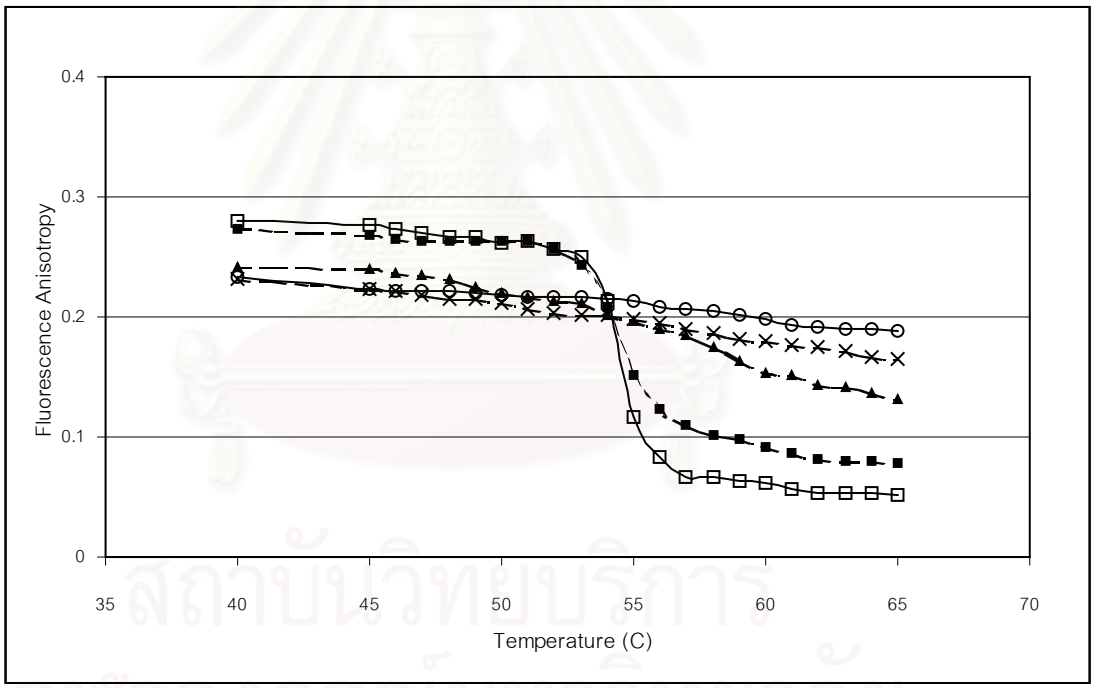



Figure 31 Effect of cholesterol on thermotropic phase transition of DSPC liposomes (A) fluorescence polarization (B) anisotropy.

Concentration of DSPC to cholesterol, by mole.

□ , without cholesterol ; ■ , 100:10 ; ▲ , 100:25 ; × , 100:50 and ○ , 100:75

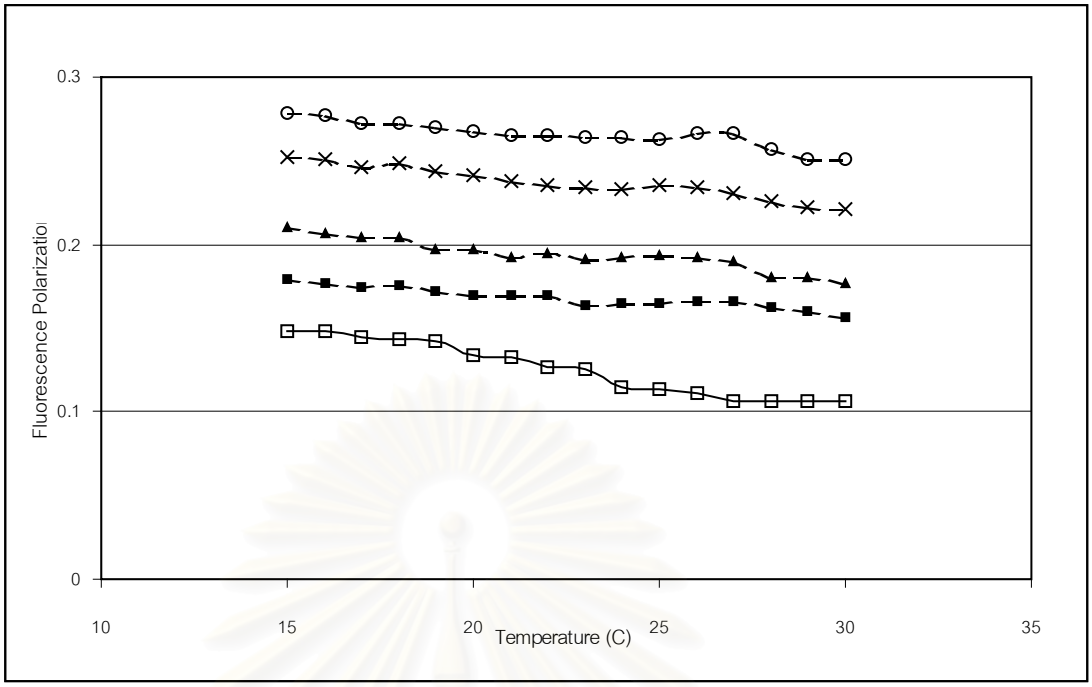
Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

Table 3 Lipid Order parameter (S) of the liposomal membrane composed of DSPC and Cholesterol in various molar ratios.
Data represents the mean of at least three samples±S.D.

| DSPC:Cholesterol (molar ratio) | Lipid order parameter (S) | |
|---|---|---|
| | Gel phase (45°C) | Liquid-crystalline phase (60°C) |
| 100:0 | 0.82502±0.36 | 0.27602±0.88 |
| 100:10 | 0.80291±0.25 | 0.31116±0.73 |
| 100:25 | 0.74054±0.79 | 0.43589±0.56 |
| 100:50 | 0.72078±0.44 | 0.54697±0.68 |
| 100:75 | 0.72672±0.60 | 0.61641±0.43 |

Incorporation of cholesterol into EPC liposomes which membrane presented in liquid-crystalline phase overall the temperature studied resulted in enhancement of fluorescence polarization (anisotropy) and lipid order parameter as shown in Figure 32 and Table 4. Thus, cholesterol provided rigidity to liposomal membrane and its condensation effect was also cholesterol concentration dependent.

A.




B.

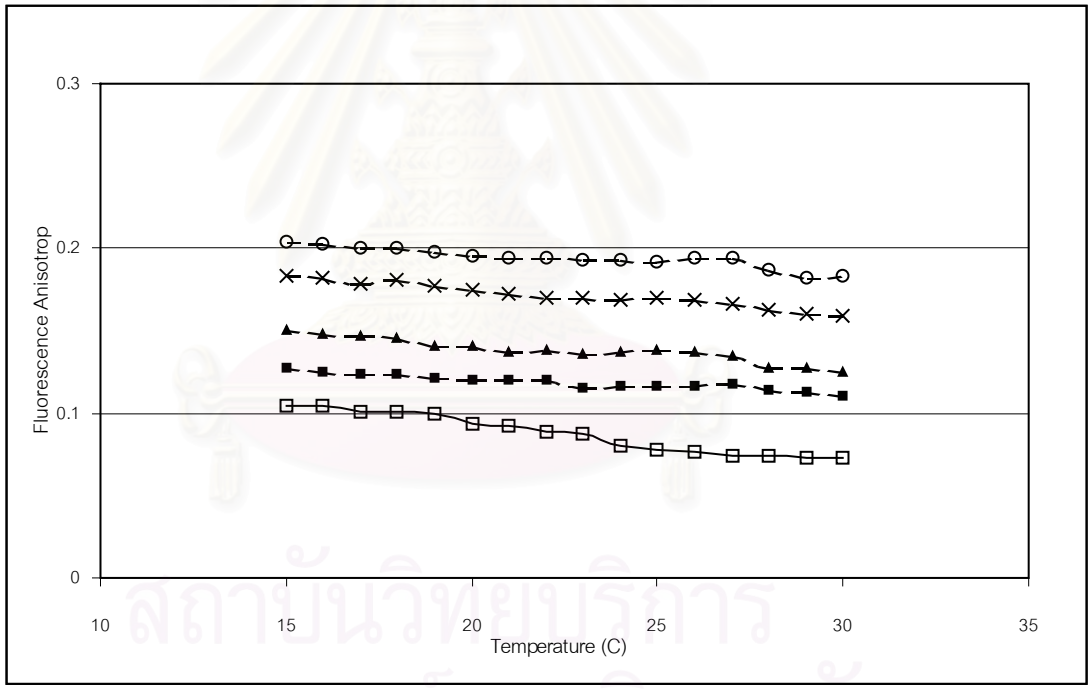



Figure 32 Effect of cholesterol on thermotropic phase transition of EPC liposomes (A) fluorescence polarization (B) anisotropy.

Concentration of EPC to cholesterol, by mole.

□ , without cholesterol ; ■ , 100:10 ; ▲ , 100:25 ; × , 100:50 and ○ , 100:75

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

Table 4 Lipid order parameter (S) of the liposomal membrane composed of EPC and Cholesterol in various molar ratios.
Data represents the mean of at least three samples±S.D.

| EPC:Cholesterol (molar ratio) | Lipid order parameter (S) | | |
|---|---|---|---|
| | Gel phase | Liquid-crystalline phase | |
| | | 15 °C | 30 °C |
| 100:0 | - | 0.31036±0.55 | 0.07782±0.97 |
| 100:10 | - | 0.41643±0.63 | 0.34268±0.69 |
| 100:25 | - | 0.50258±0.89 | 0.40880±0.54 |
| 100:50 | - | 0.60093±0.74 | 0.53090±0.66 |
| 100:75 | - | 0.65694±0.96 | 0.59989±0.75 |

However, the accurate picture of the dual effect on fluidity of lipids appears to be complicated. The effect of cholesterol on the cone angle would be called the "dual effect", the incorporation of cholesterol greatly decreased cone angle ($\theta$, Figure 5) in the liquid-crystalline phase but slightly increased it in the gel phase. Therefore, the reported changes in the fluidity in the presence of cholesterol mainly reflected the change of $\theta$.

The differential effect of cholesterol on $\theta$ might be explained by a difference in hydrophobic regions of cholesterol molecule which composes of a bulky rigid steroid nucleus and a relatively narrow flexible acyl chain. As shown in Figure 33, cholesterol molecule is largely immersed in the hydrocarbon core of the bilayer where its methyl chain terminus is located near the core of the bilayer and its small hydrophilic hydroxyl group at the level of the glycerol backbone (near the water interface) [Franks, 1976] thus, the methyl terminal of the cholesterol acyl would be allowed greater conformational freedom than bulky rigid steroid nucleus. The difference in motion between these two regions of cholesterol molecule might create the differential effect upon the movement of fatty acyl chains of phospholipid. In the gel phase, the addition of cholesterol disturbed the packing of phospholipid acyl chains, and might chiefly increase the conformation freedom and the tumbing rate of lower half of chains which are adjacent to the narrow tails of cholesterol. As a result of these changes of the acyl chains of phospholipid, the cone angle and the wobbling motion of DPH might increase below the phase transition thus declination in fluorescence polarization was obtained (Figure 30-31). In the liquid-crystalline phase, the range of swinging motion of the upper half of fatty acyl chains will be suppressed by the bulky rigid steroid nuclei of cholesterol. Therefore, the cone angle and the wobbling motion of DPH, as demonstrated by enhancement of fluorescence polarization, decreased. No decrease of the tumbling rate of DPH may reflect that the wobbling rate to DPH strongly depends on the tumbling motion of

lower half of lipid acyl chains which is much less hindered by the addition of cholesterol. At high cholesterol level, these dual effects were balanced, subsequently by abolishing phospholipid phase transition. Therefore, the environment of the probe appeared to be changed by cholesterol.

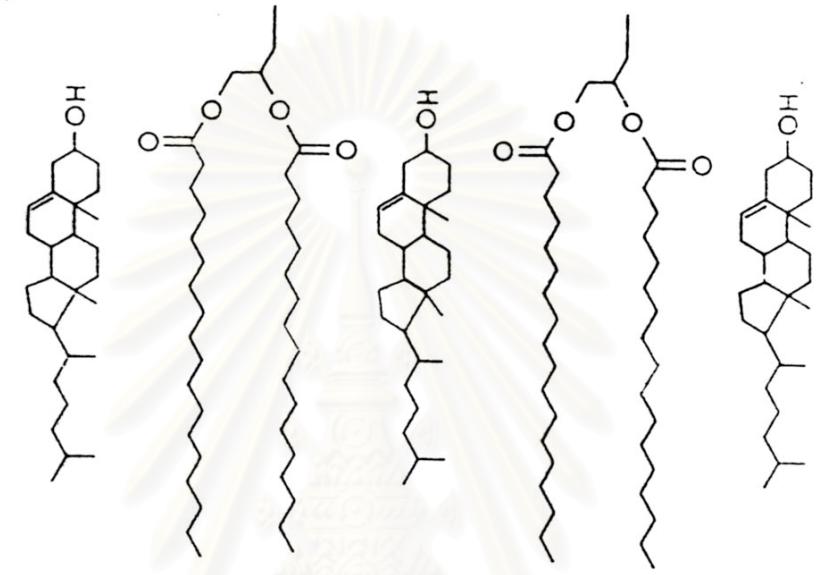


Figure 33 Position occupied by cholesterol in the phospholipid bilayers.

**Influence of Phenothiazines on Thermotropic Phase Transition of Phosphatidylcholine-Cholesterol Liposomes.**

As described earlier, cholesterol had fluidizing effect below the phase transition temperature of phospholipids and condensing effect above these temperatures [Ladbrooke, Williams and Chapman, 1968; McIntosh, 1978]. Therefore, cholesterol plays an important role is to regulate the organization and fluidity of lipids in membrane. Investigation of drug interaction with phosphatidylcholine membrane containing cholesterol was then performed to examine the role of cholesterol in membrane where perturbation might be occurred. The experiments were executed by varying cholesterol content in molar ratios from 100:10 to 100:75 (phosphatidylcholine to cholesterol) while fixing concentration of phosphatidylcholine to drug in molar ratios of 75:1, 25:1 and 7.5:1 in order to observe the influence of cholesterol content to the interaction between drugs and membranes comparing with membrane composed of pure phosphatidylcholine alone in the previous section. These molar ratios of phosphatidylcholine-to-drug were chosen since their effects on liposomes were detected clearly and easily.

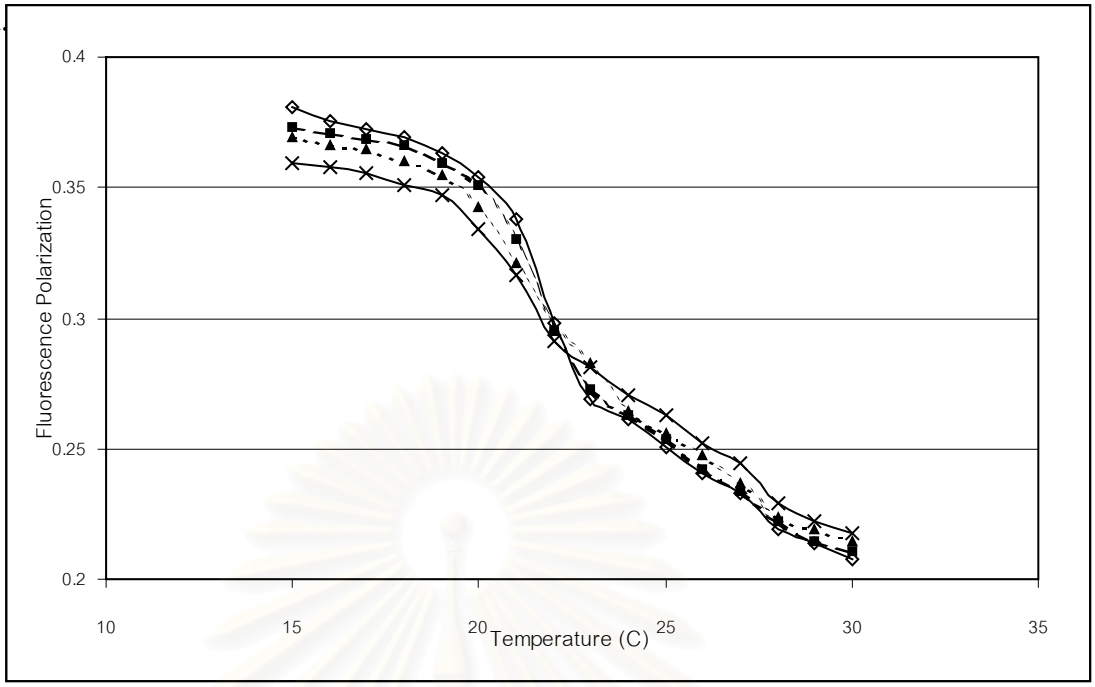
The previous studies showed that the main roles of phenothiazines in DMPC and DSPC phospholipid membranes were the condensing effect on the fluid phase lipid and the fluidizing effect on gel phase lipid, accompanying by the shift of thermotropic phase transition temperature to the lower temperature. For EPC liposomes, phenothiazines provided the condensation effect on liposomal membrane since they were in fluid phase overall the temperature range studies (10 to 30°C).

Effect of phenothiazines on DMPC and DSPC liposomes containing various amounts of cholesterol were demonstrated in Figure 34-41 and Figure 42-49, respectively. It was found that at low cholesterol contents (up to phospholipid :

cholesterol = 100:25, by mole), phenothiazines exhibited dual effects (fluidizing effect on gel phase and condensing effect on liquid-crystalline phase) similar to DMPC liposomes (Figure 23-24) and DSPC liposomes ( Figure 25-26) but in a less extent. These might be owing to enhancement of lipid components in phospholipid bilayer that generated dense packing of lipid consistuents, consequently by decreasing penetration of phenothiazines into liposomal membranes.

At high level of cholesterol contents (phospholipid:cholesterol, by mole 100:50 to 100:75) abolishment of thermotropic phase transition was obtained due to the balance in dual effect and phenothiazines provided only condensing effect which was also concentration dependence along the studied temperature range (Figure 36-37, 40-41, for DMPC:Cholesterol liposomes and Figure 44-45, 48-49 for DSPC:Cholesterol liposomes). This evidence clearly supported the role of cholesterol in regulation of fluidity and organization of membrane components.

A.




B.

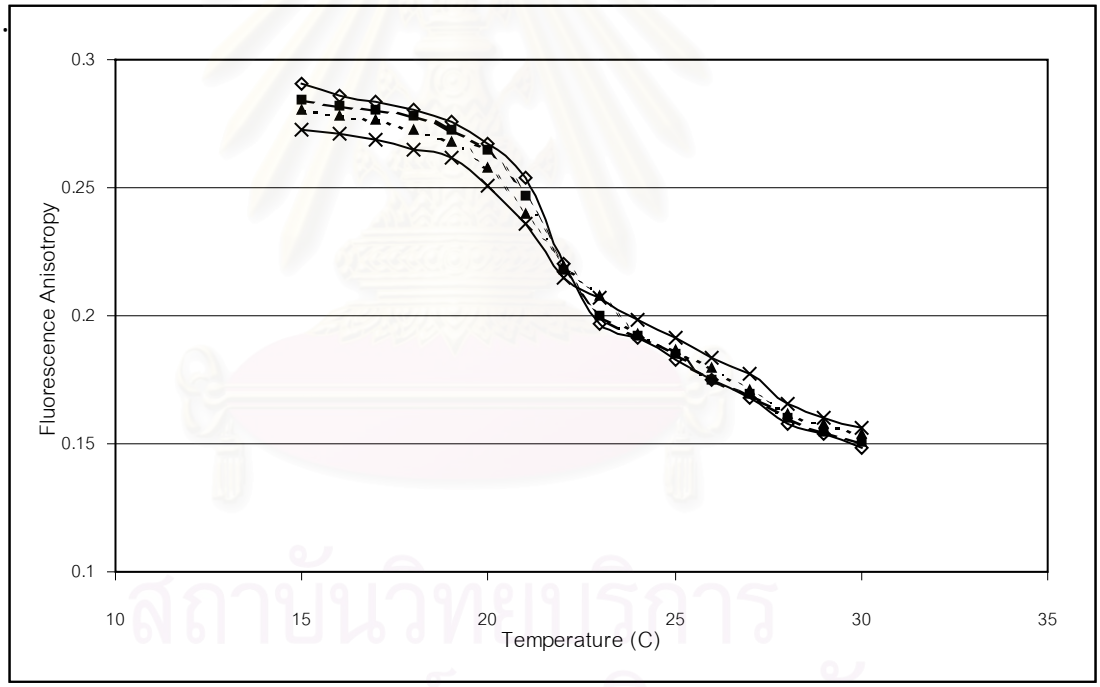
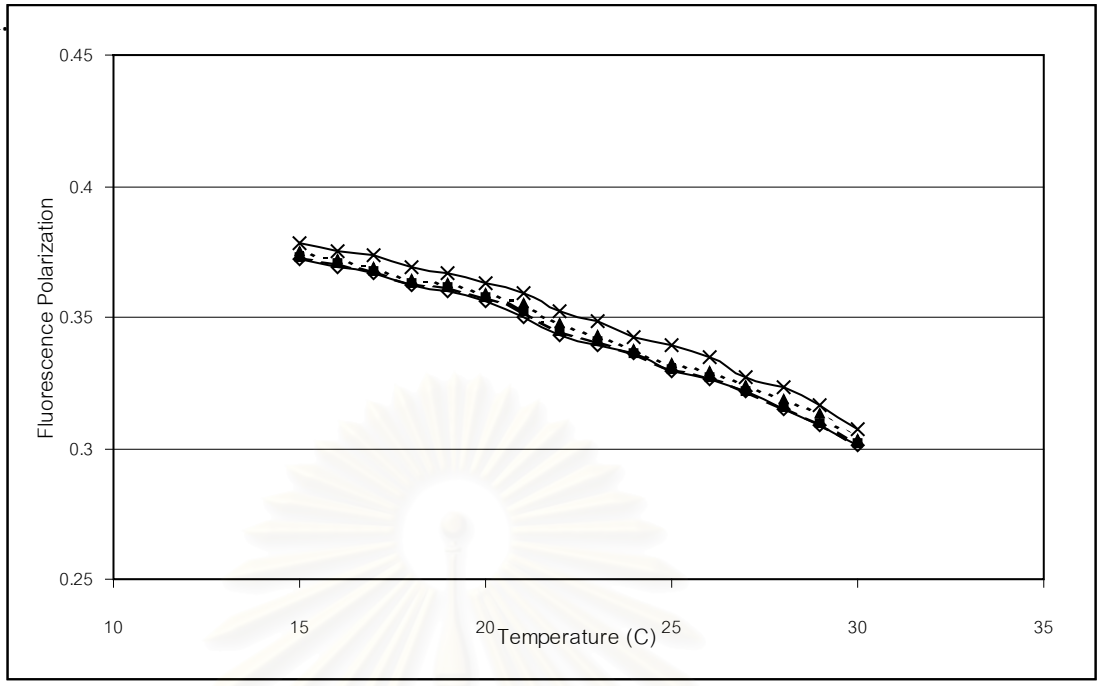



Figure 34 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:10, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

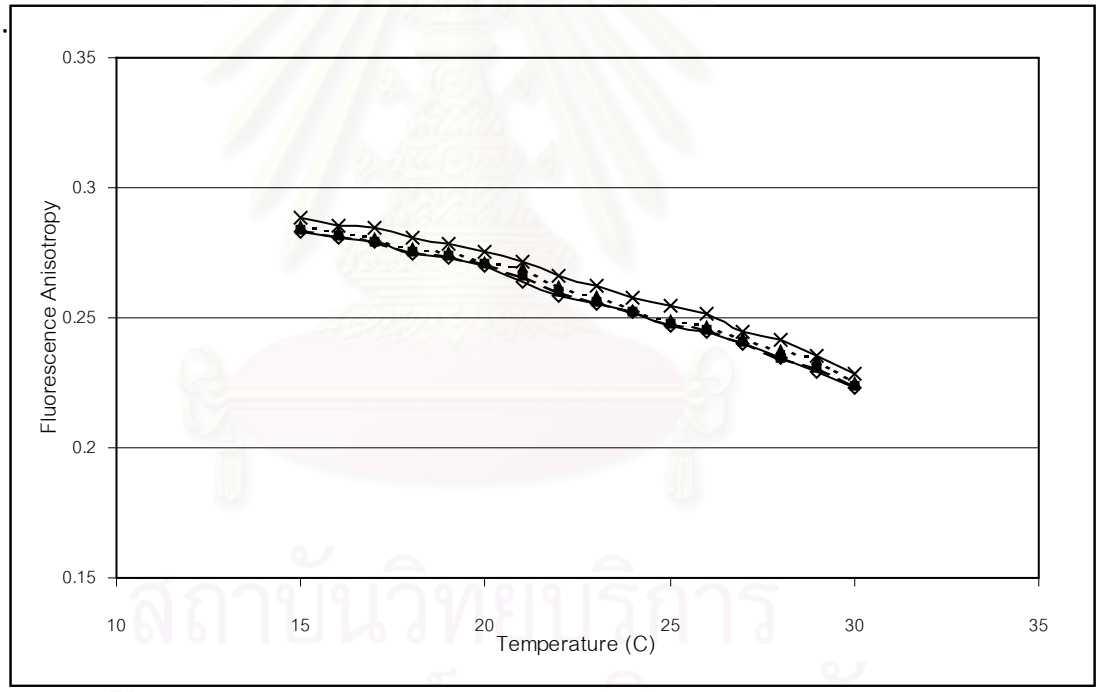
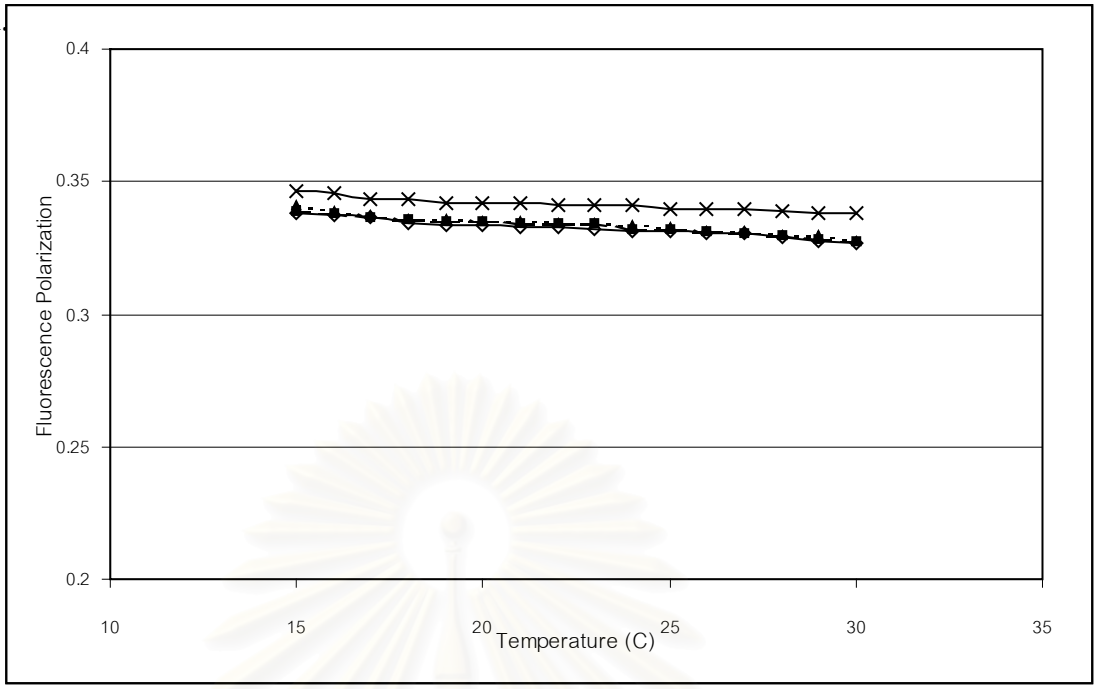



Figure 35 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:25, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

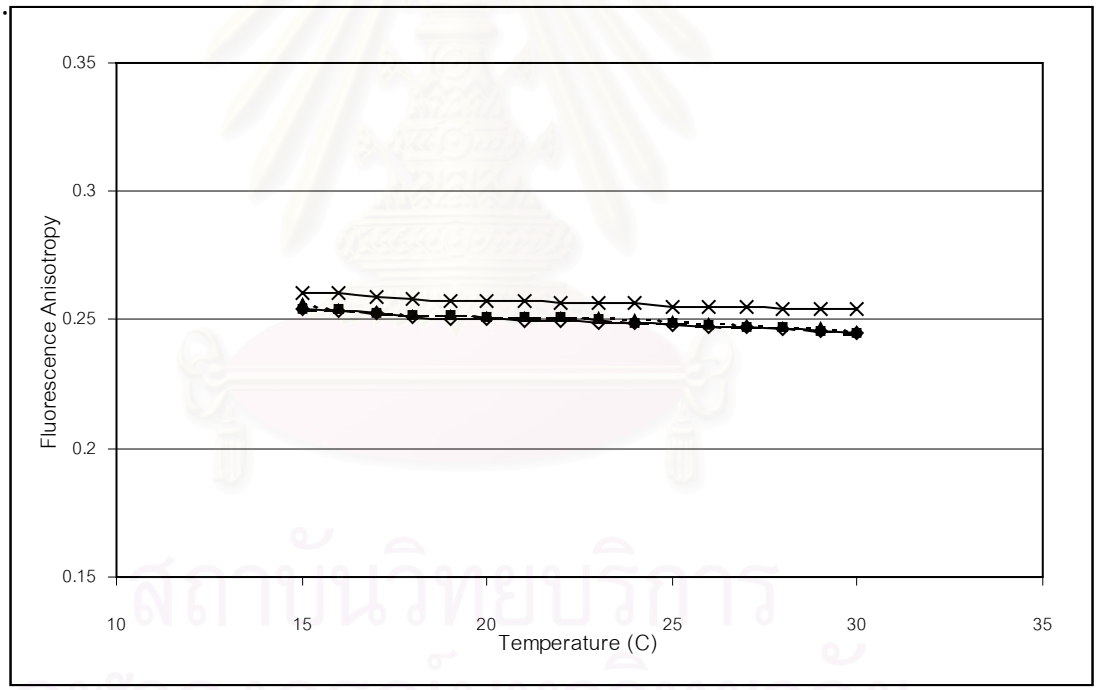
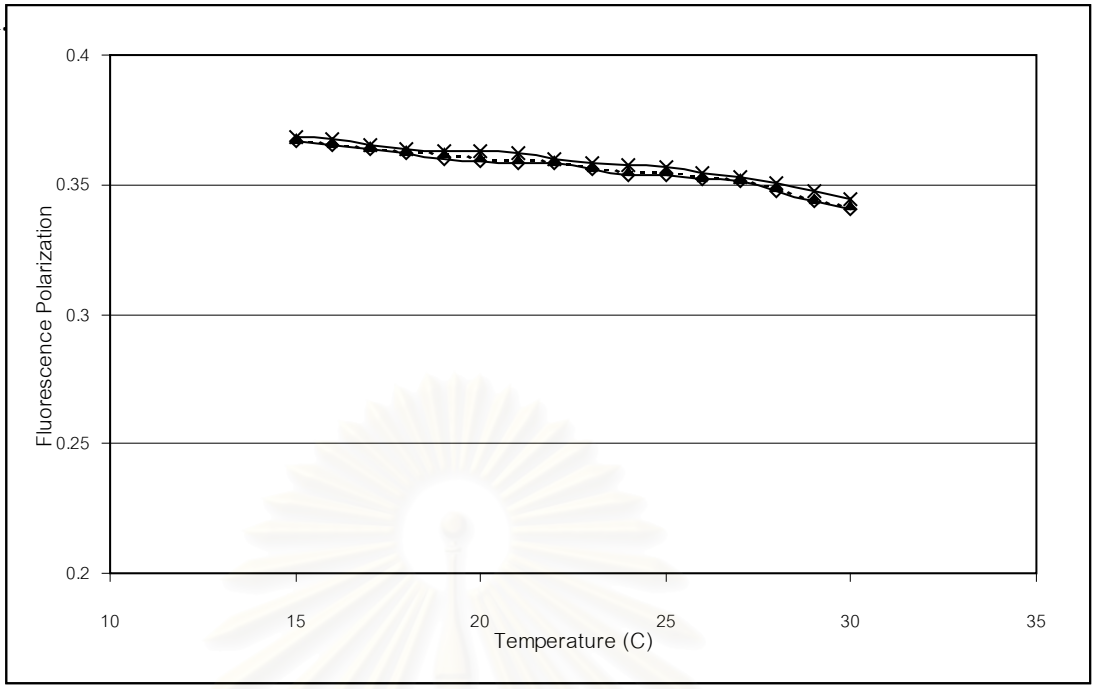



Figure 36 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:50, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

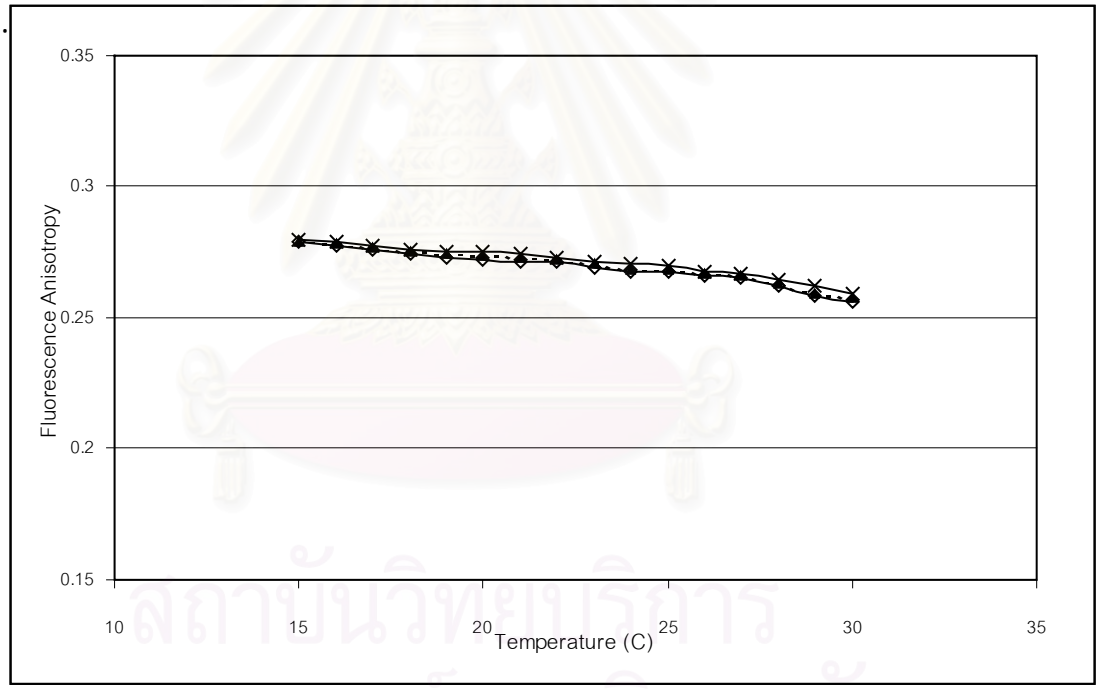
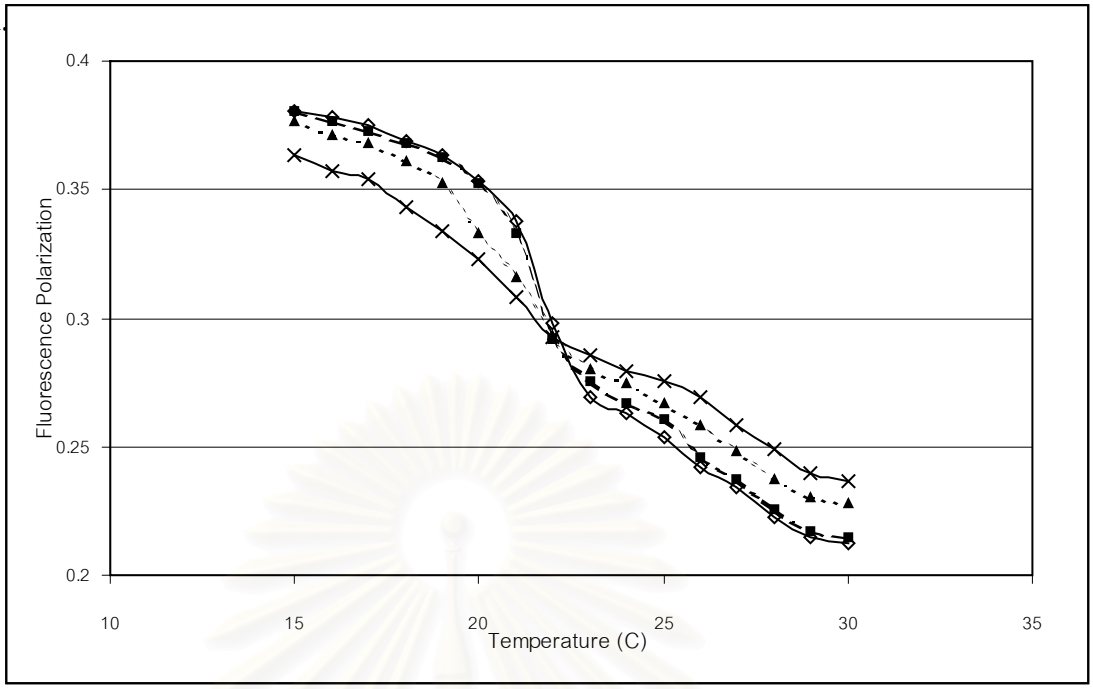



Figure 37 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:75, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

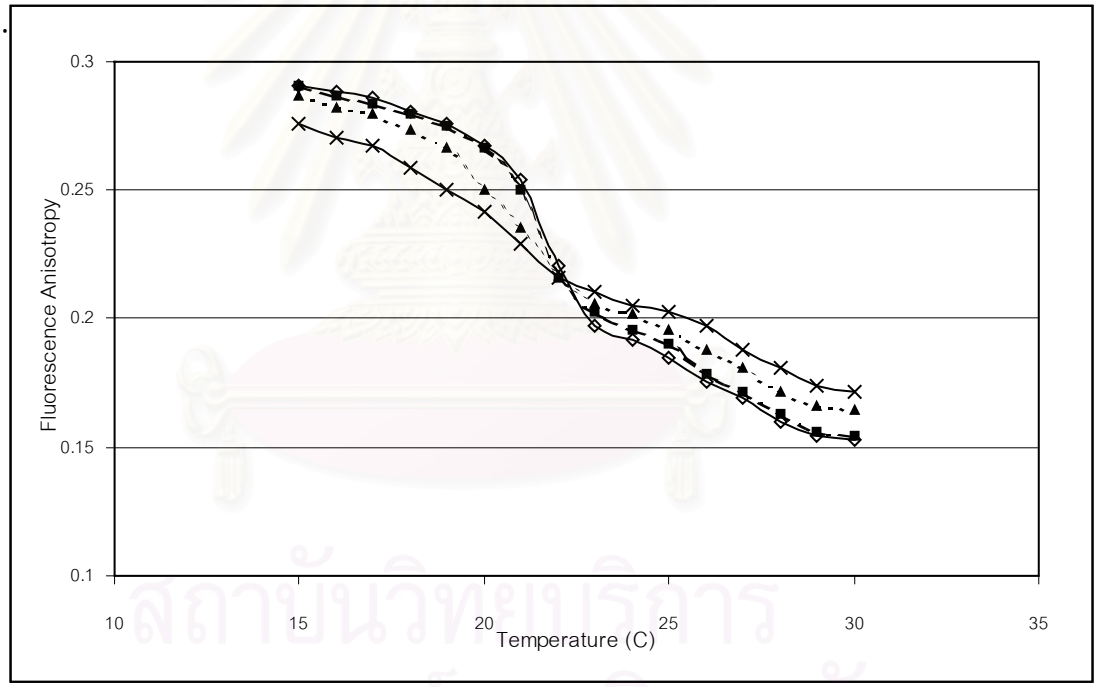
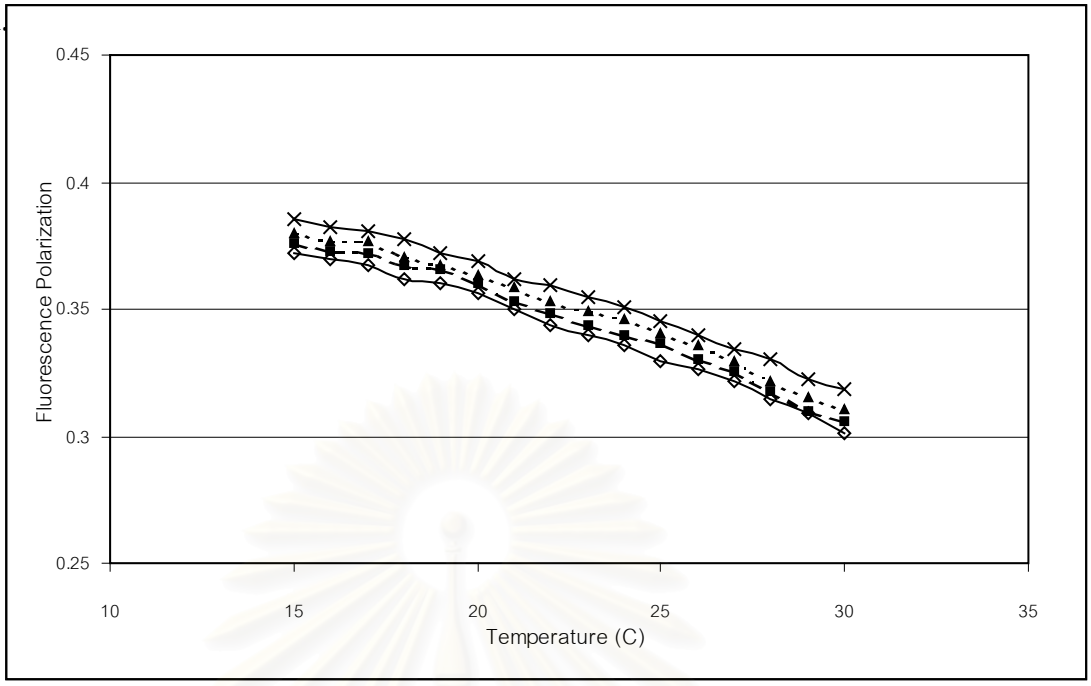



Figure 38 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:10, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

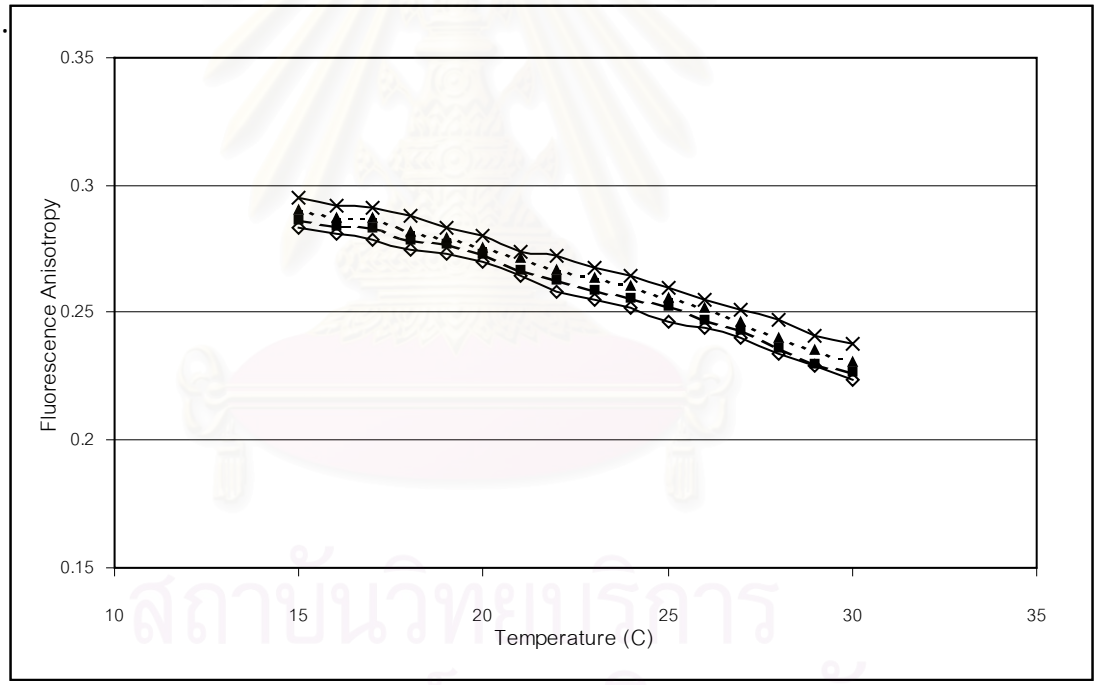
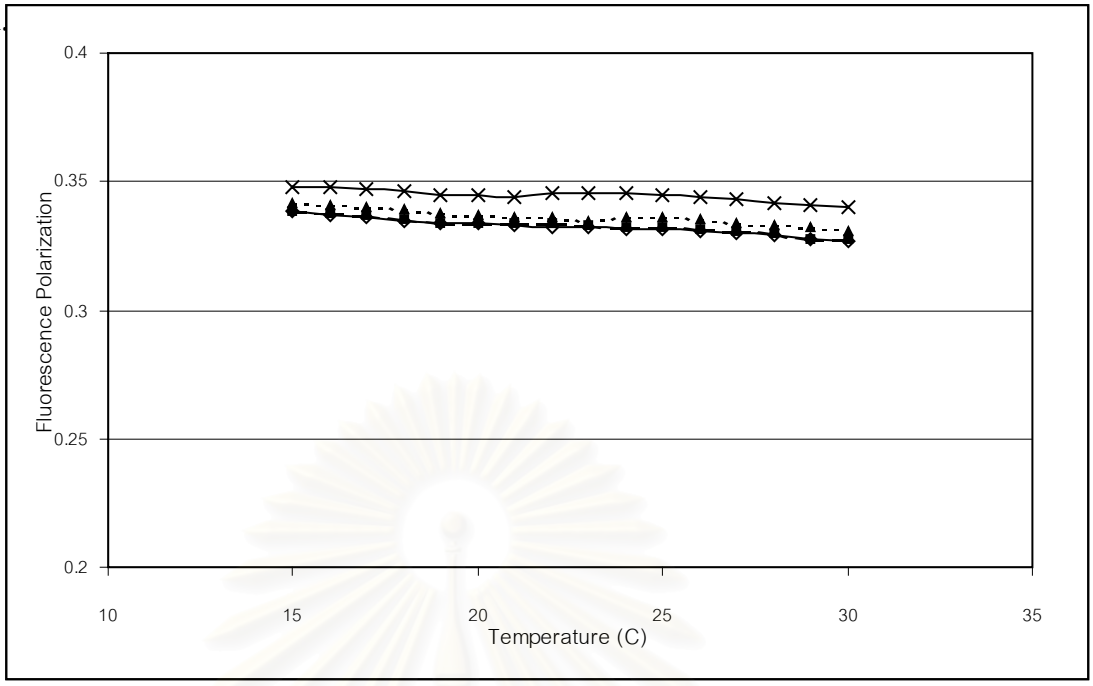



Figure 39 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:25, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

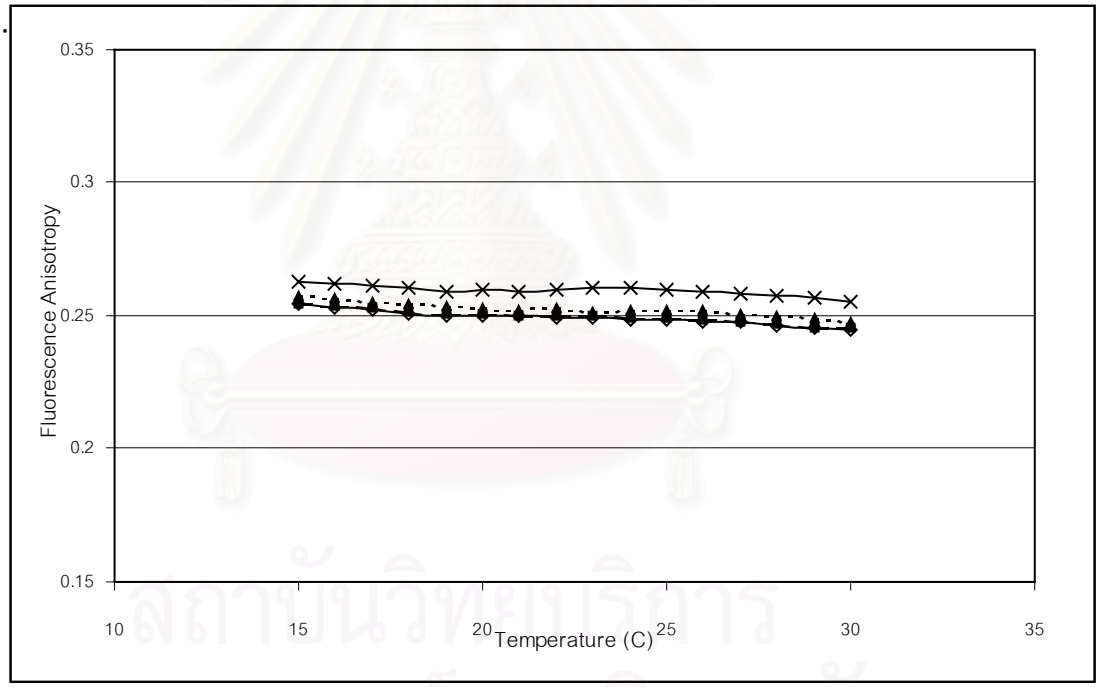
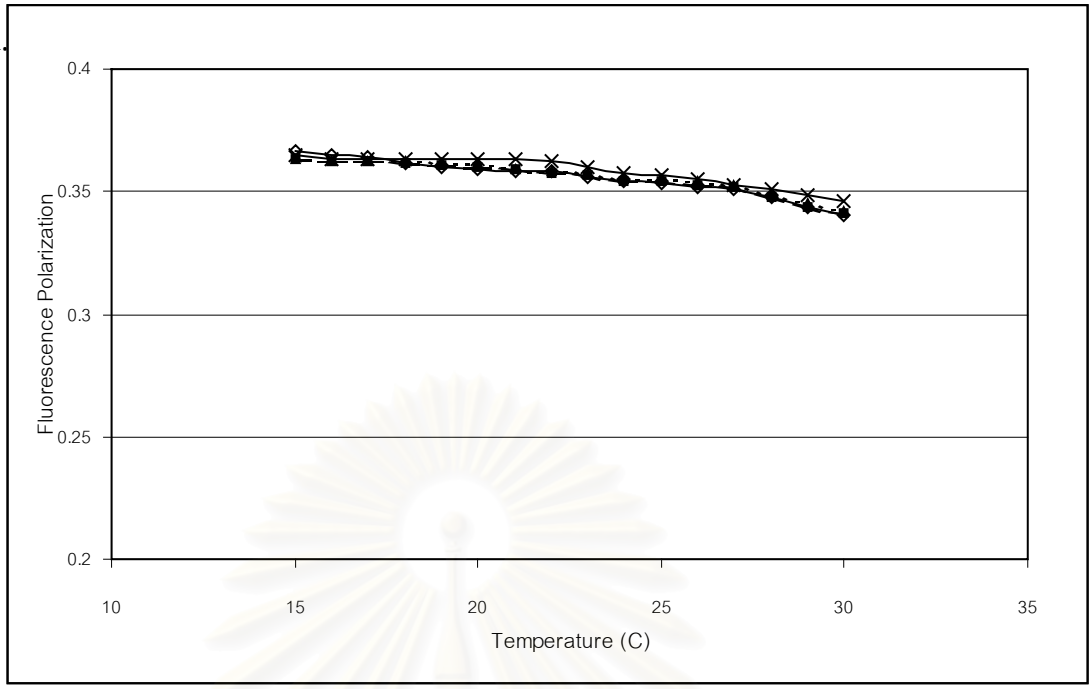



Figure 40 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:50, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

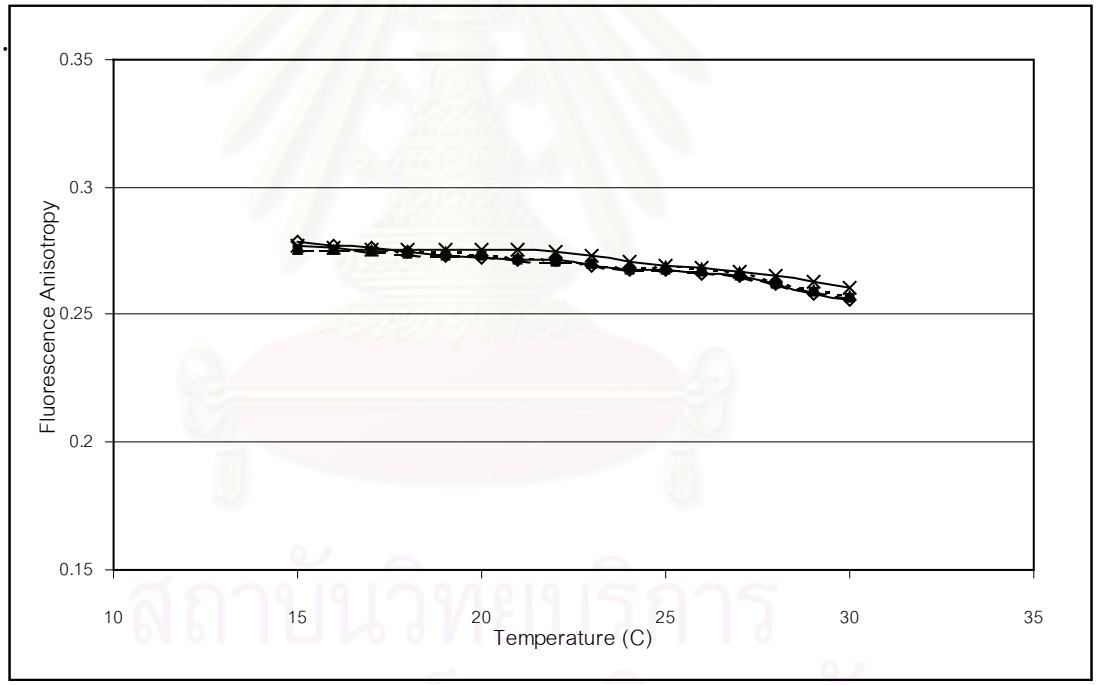



Figure 41 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol (100:75, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DMPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

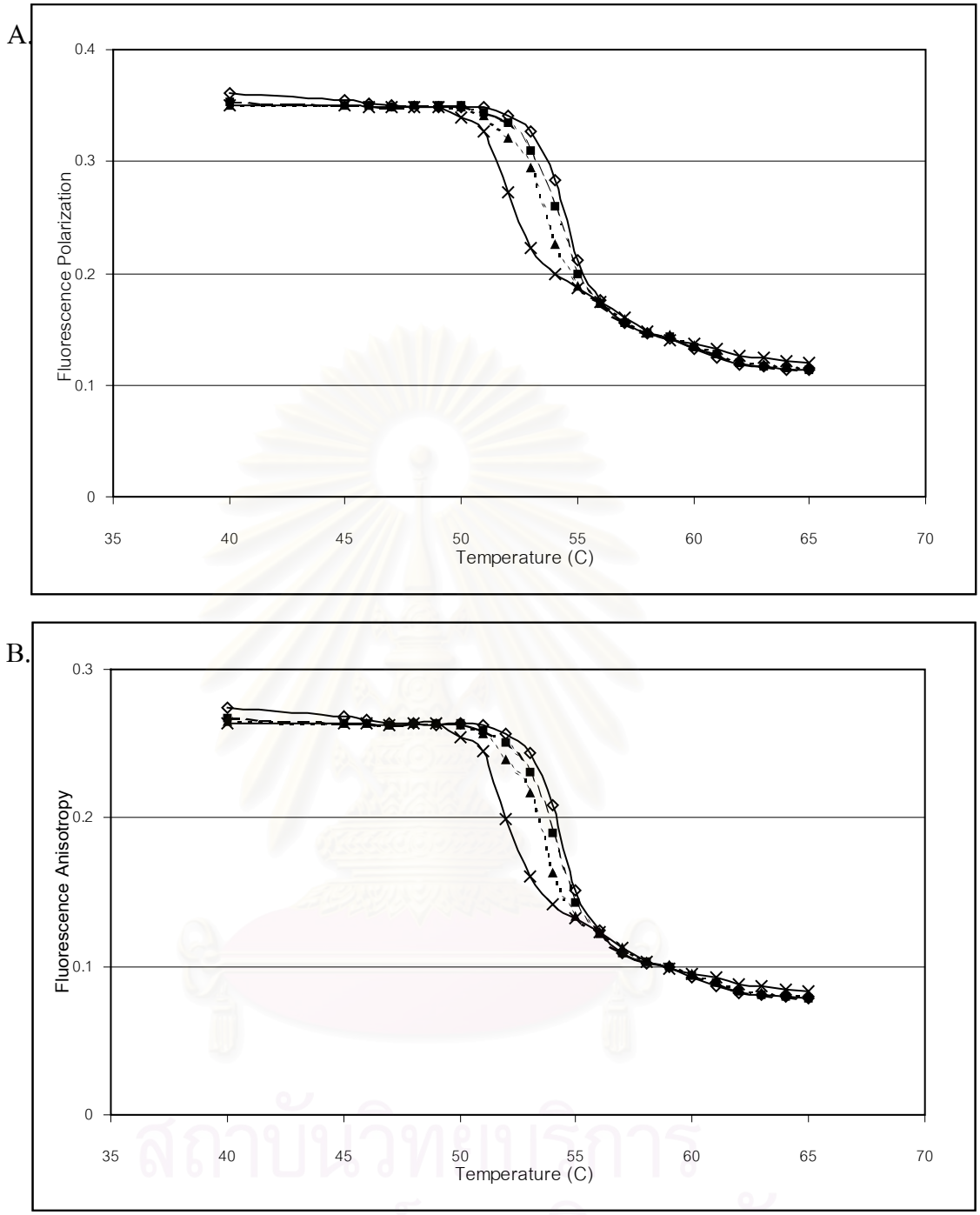
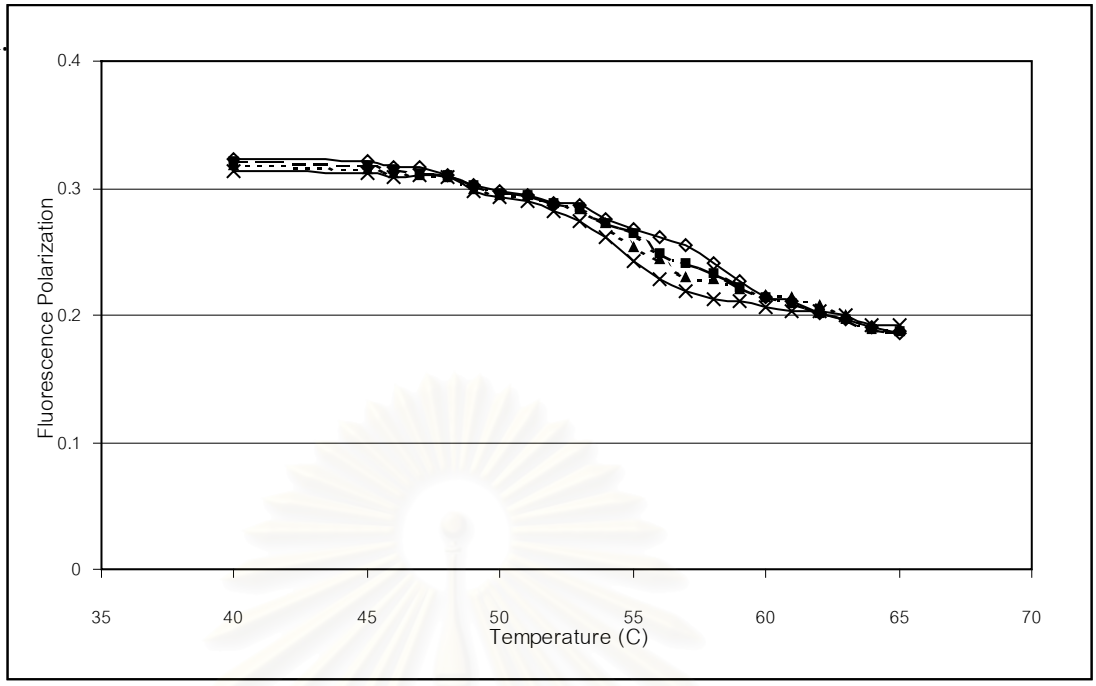



Figure 42 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:10, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

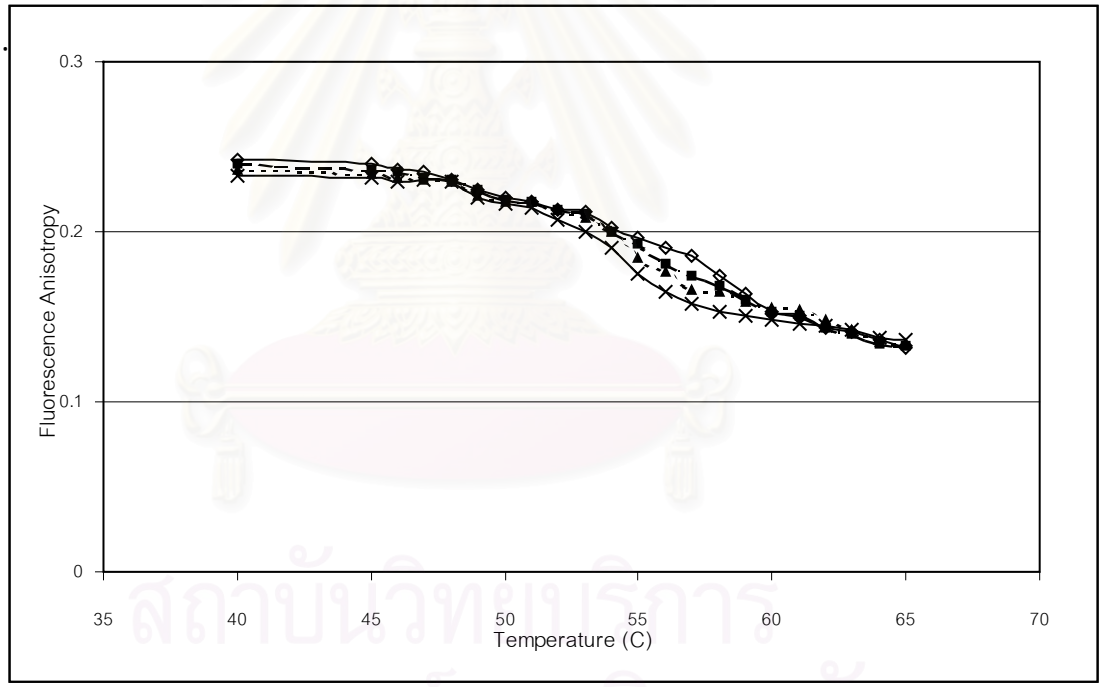
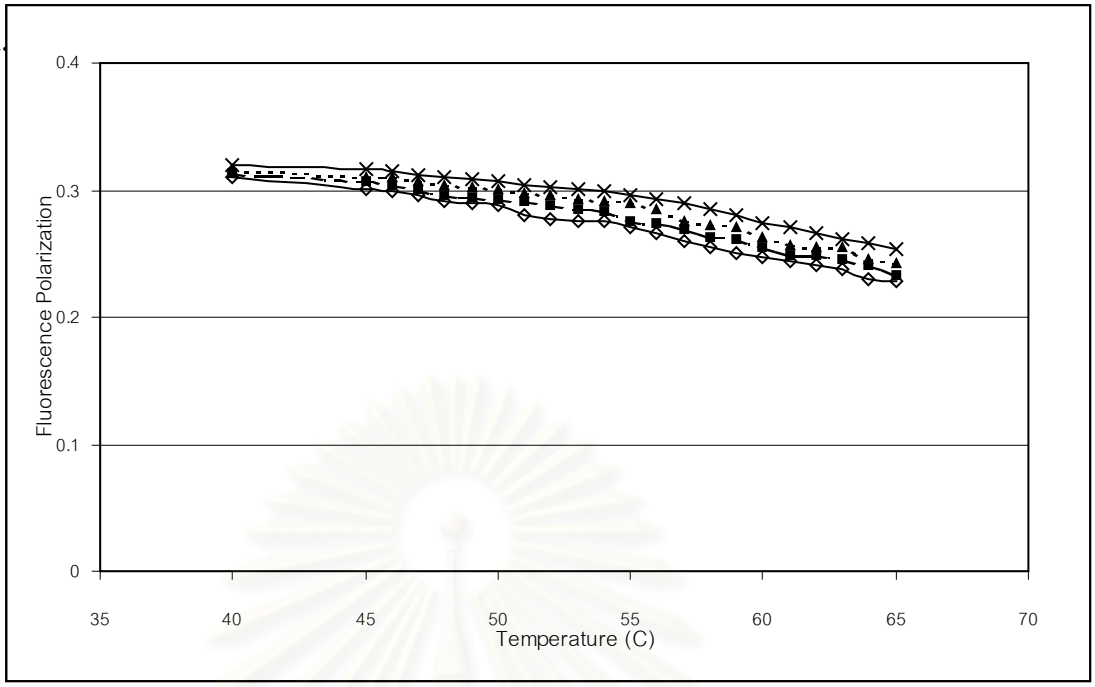



Figure 43 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:25, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

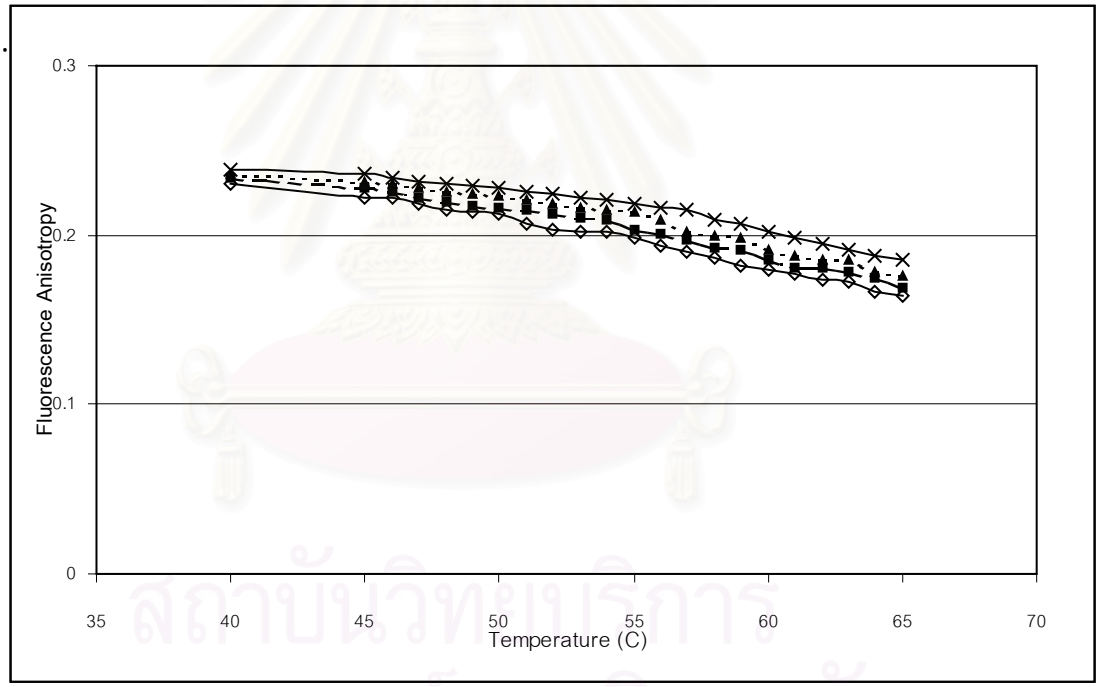
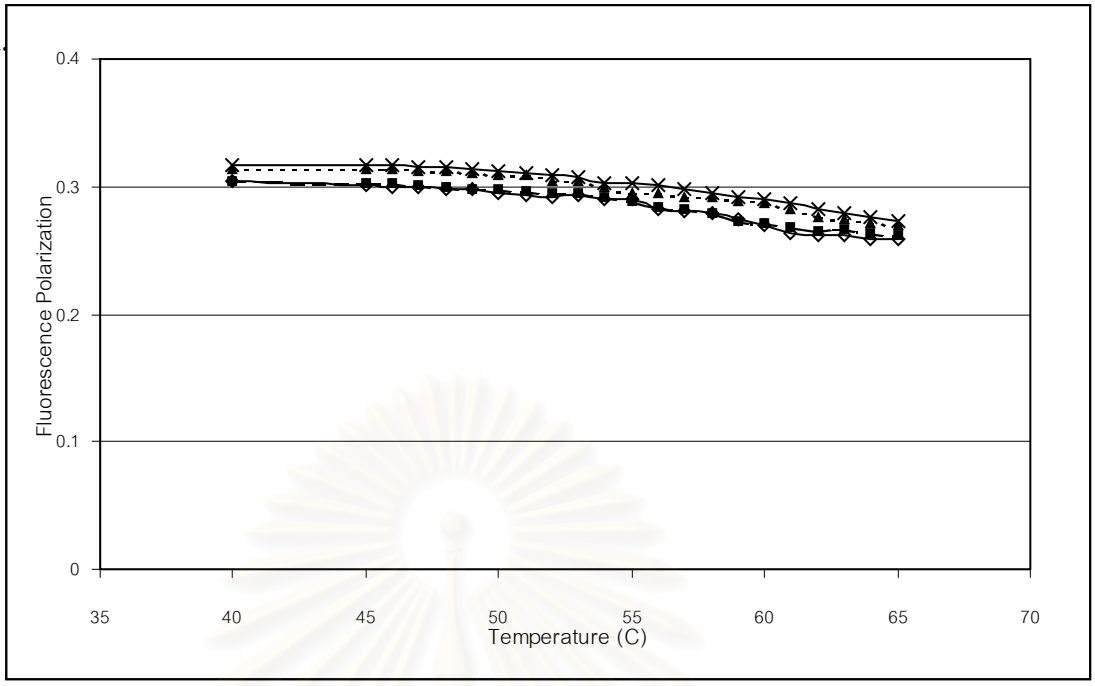



Figure 44 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:50, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

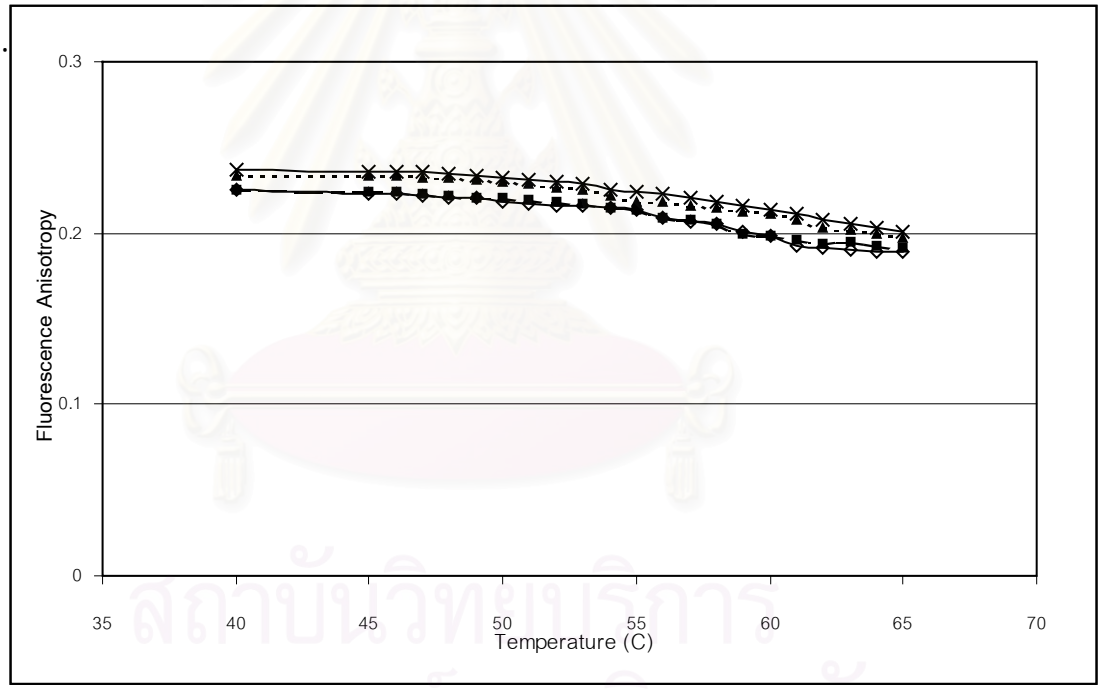
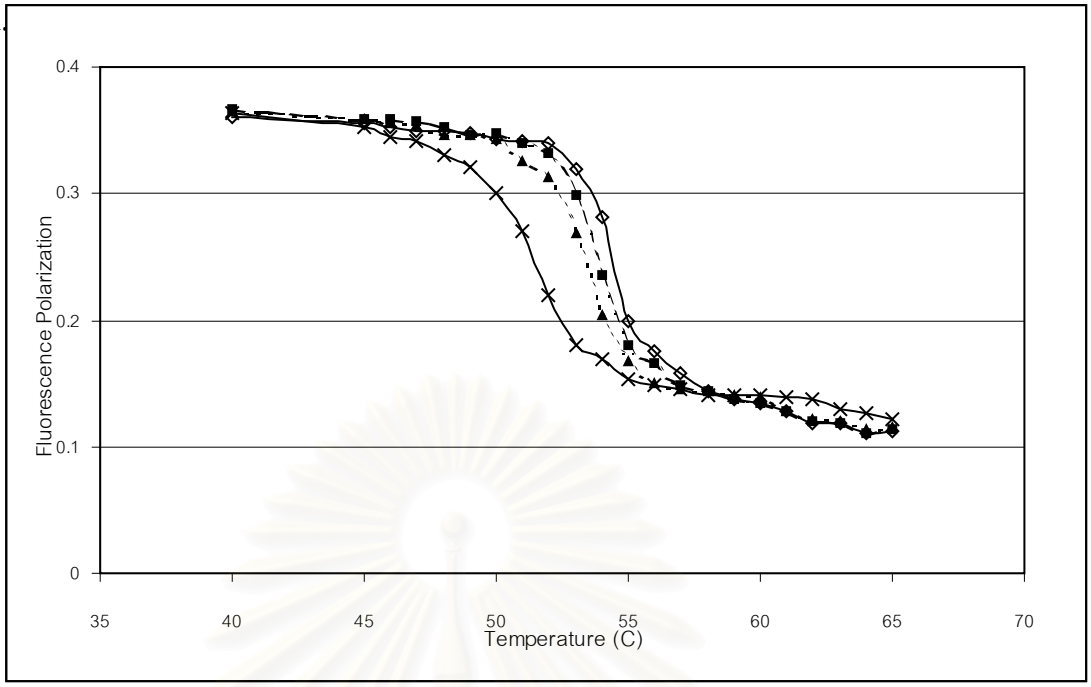



Figure 45 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:75, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

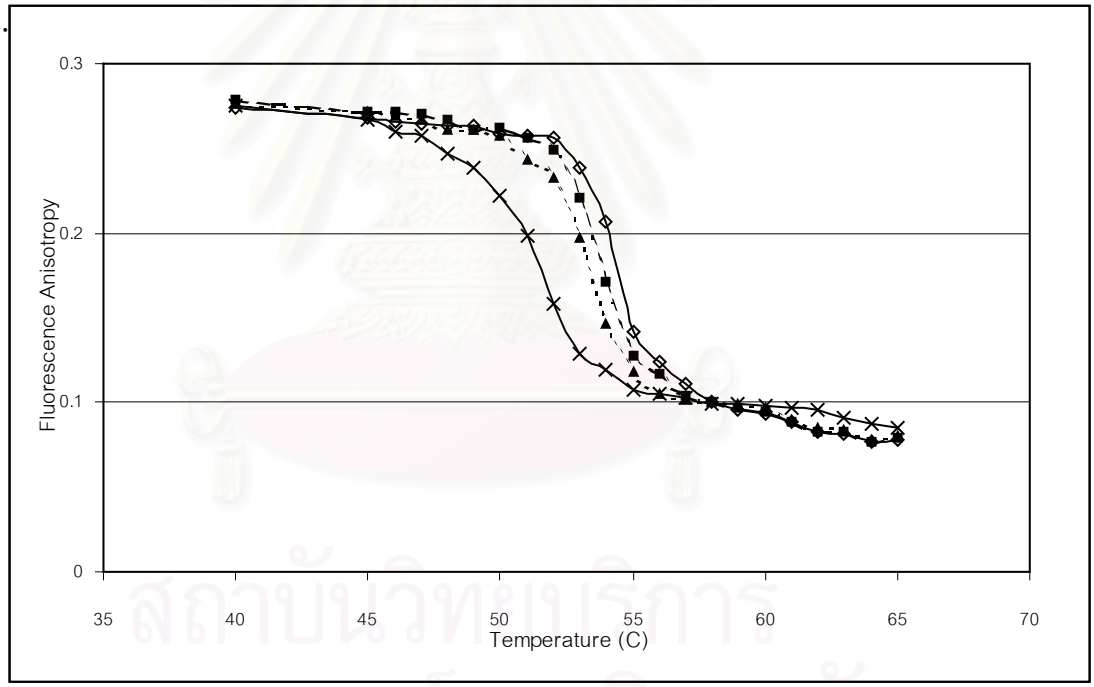
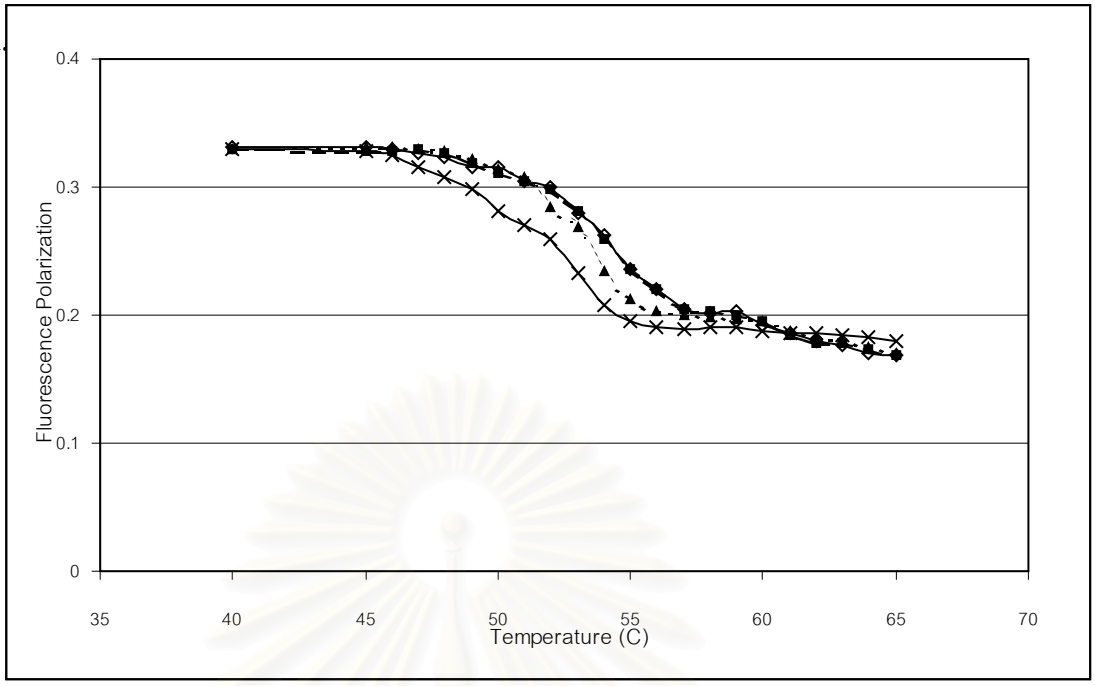



Figure 46 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:10, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

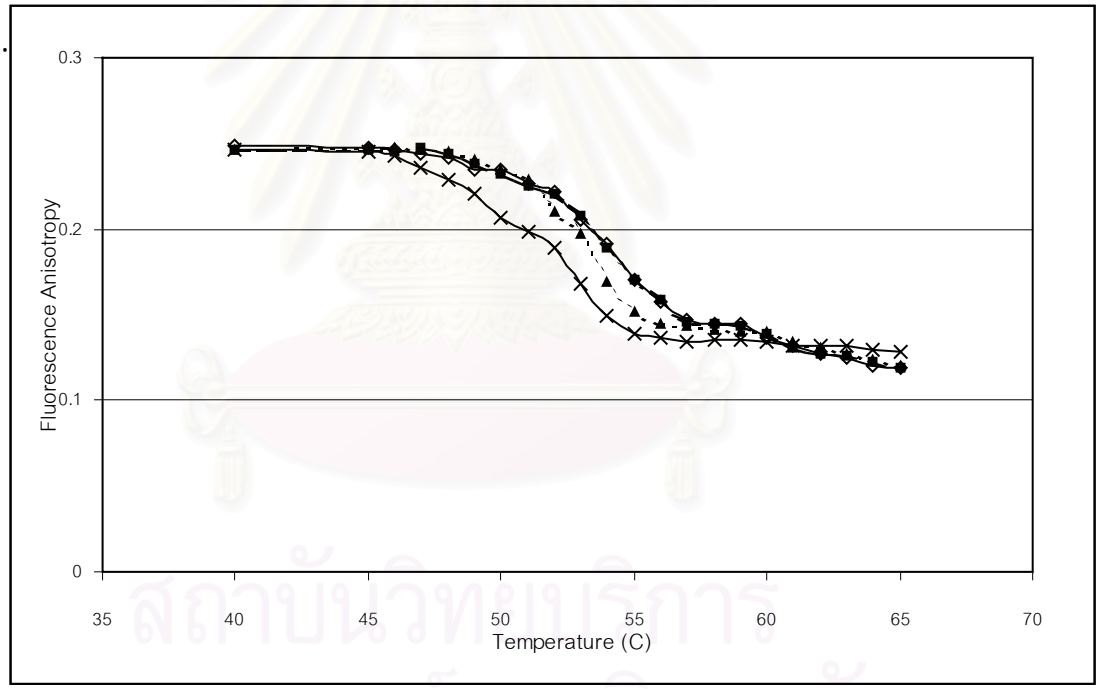



Figure 47 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:25, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

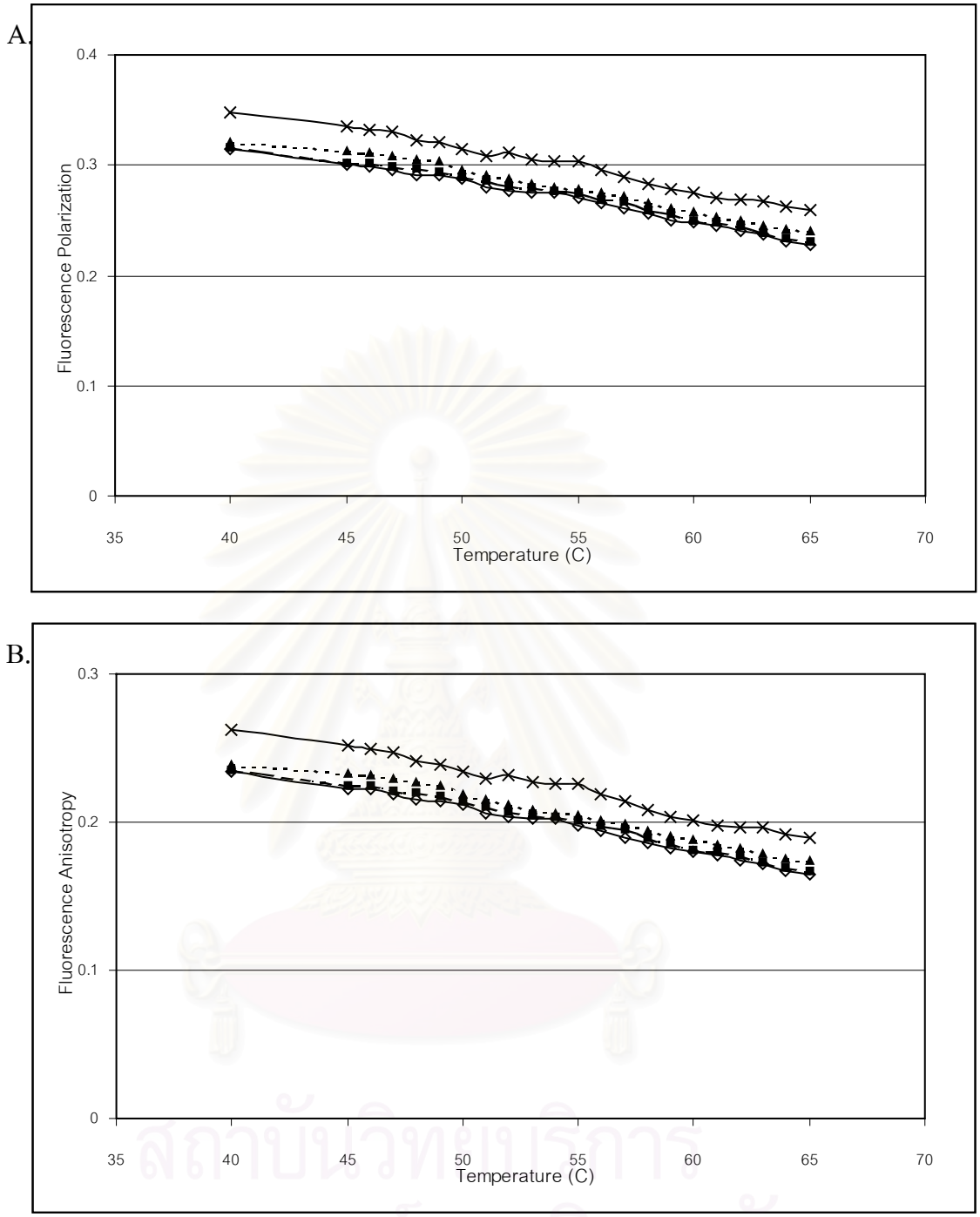
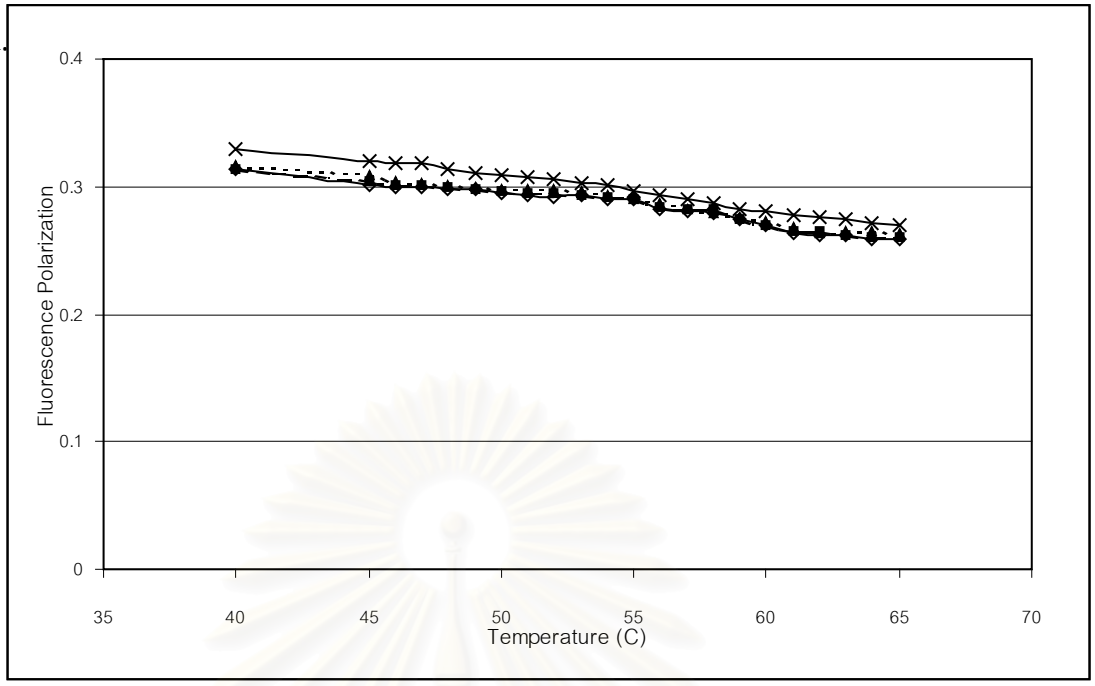



Figure 48 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:50, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

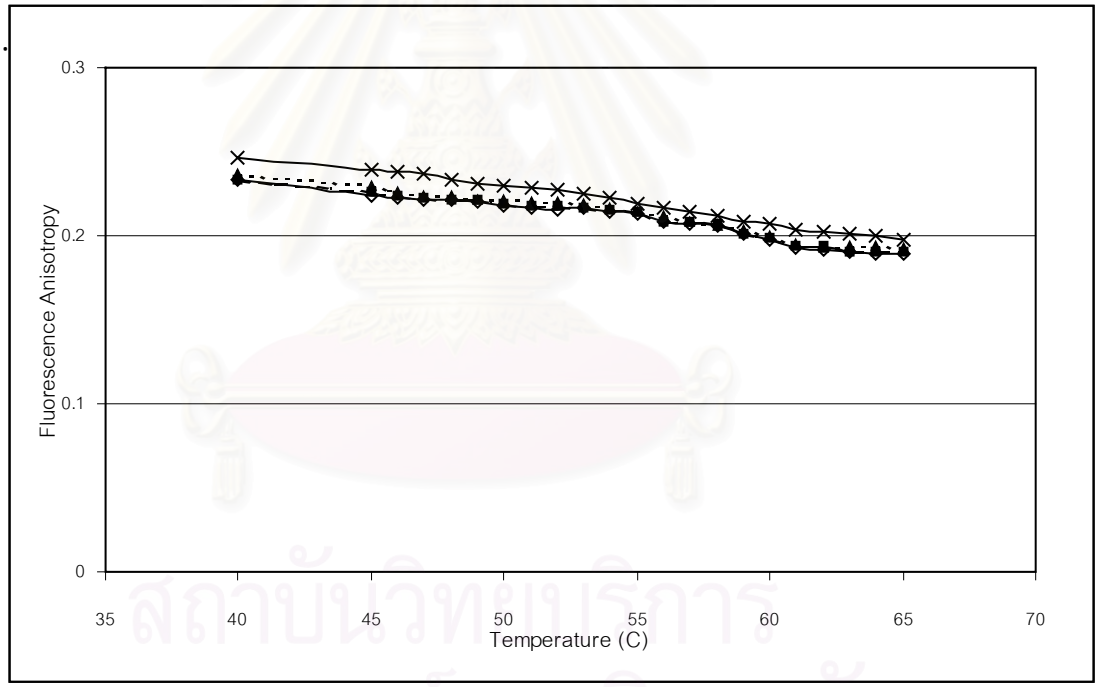


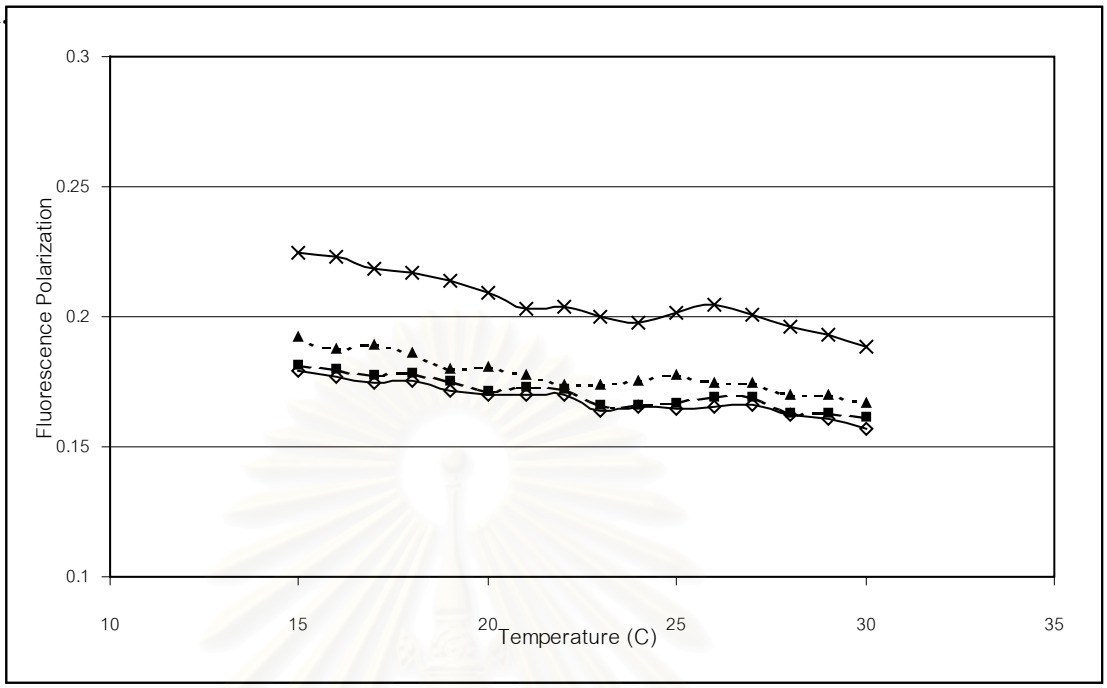

Figure 49 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol (100:75, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of DSPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

Phenothiazines provided condensing effect on EPC incorporating cholesterol liposomes as illustrated in Figure 50-57 since liposomes were in liquid-crystalline phase overall the range of temperature studied (Figure 27-28) and their action was also drug concentration dependence. At low concentration of cholesterol content (EPC: cholesterol = 100:10, by mole), TRZ exhibited a slightly condensing effect than CPZ (Figure 50 and 54) but no significant difference was observed at higher concentration. The condensing power of phenothiazines decreased as increasing amount of cholesterol content in liposomal membrane. The reasons might be the same as previously described in DMPC and DSPC incorporating cholesterol liposome.

A.




B.

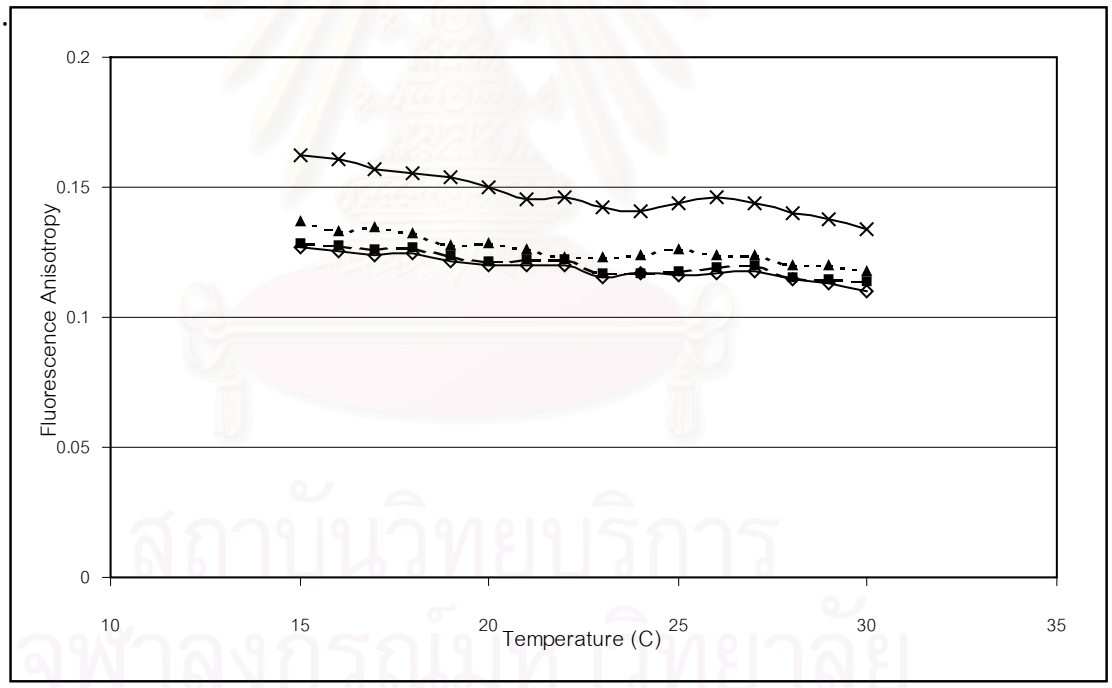
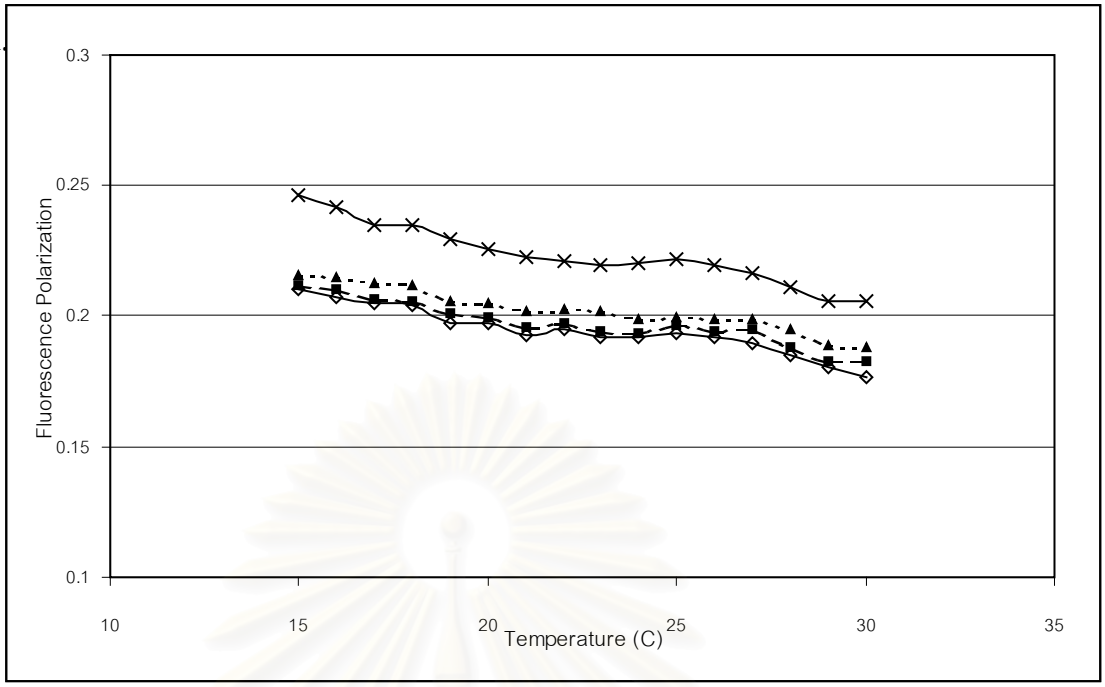



Figure 50 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:10, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of EPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

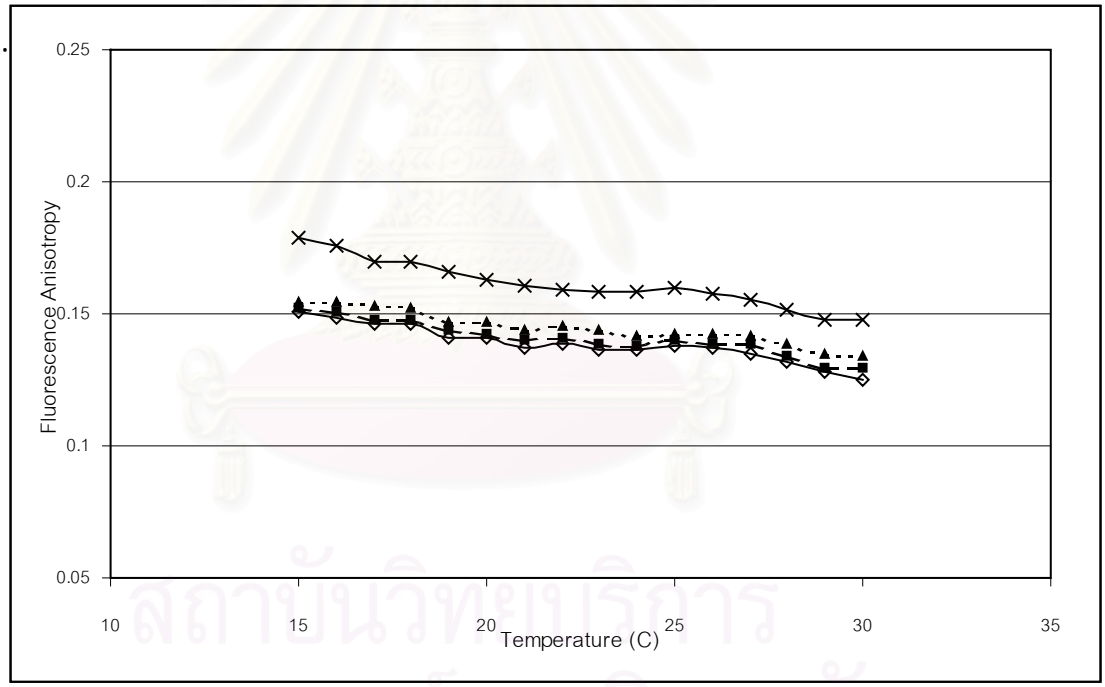
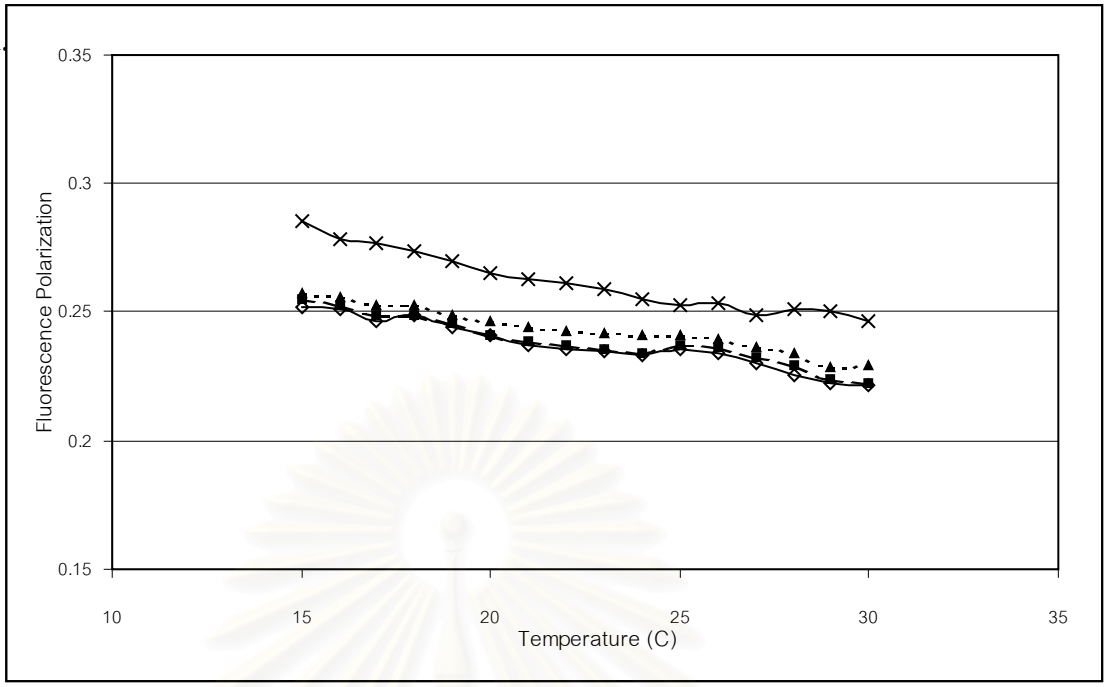



Figure 51 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:25, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of EPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

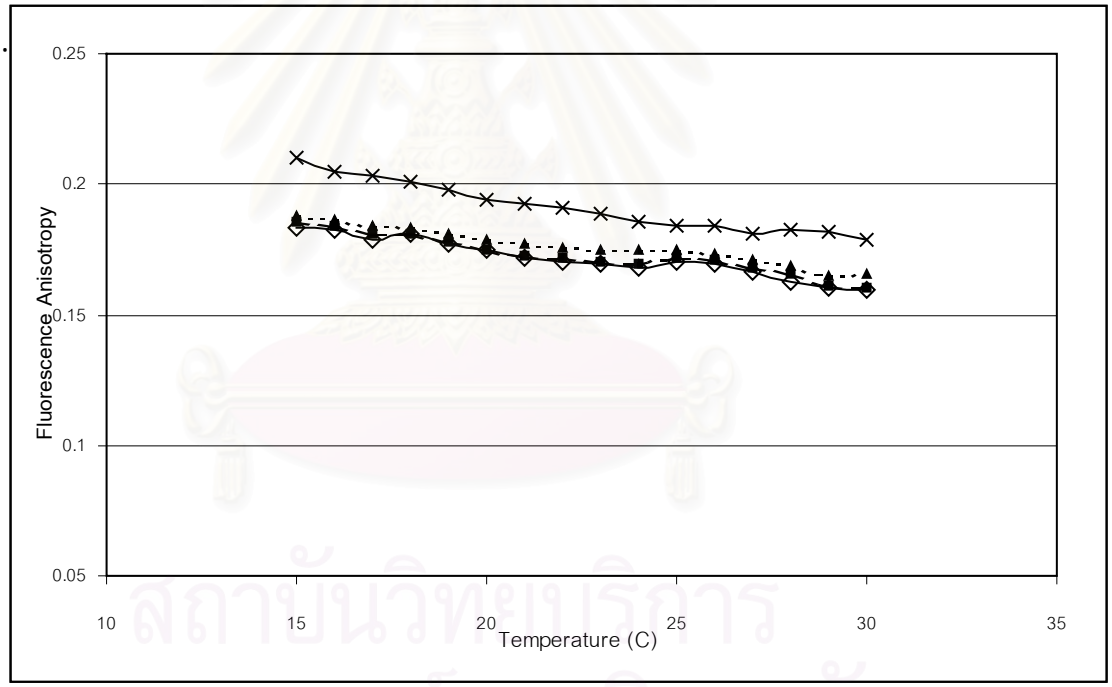
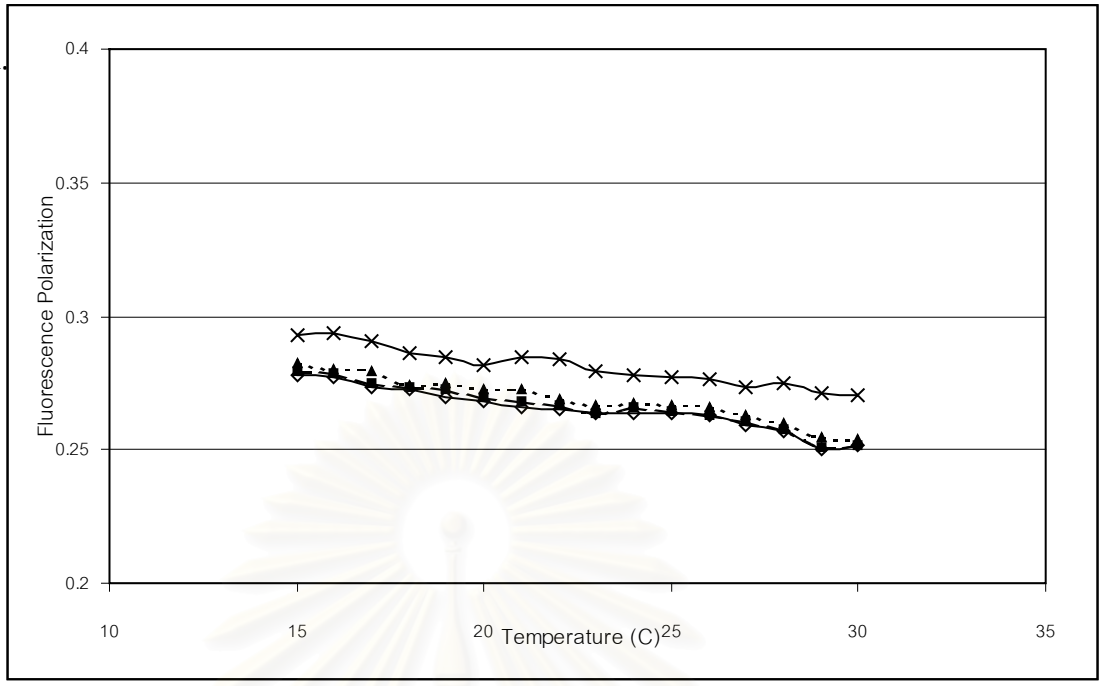



Figure 52 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:50, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of EPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

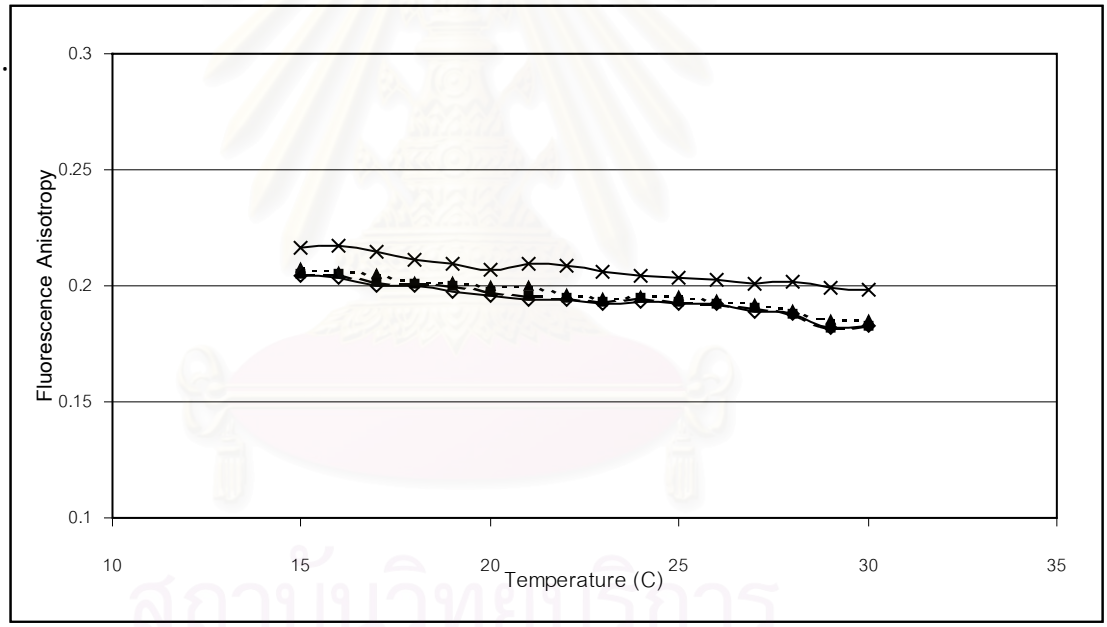
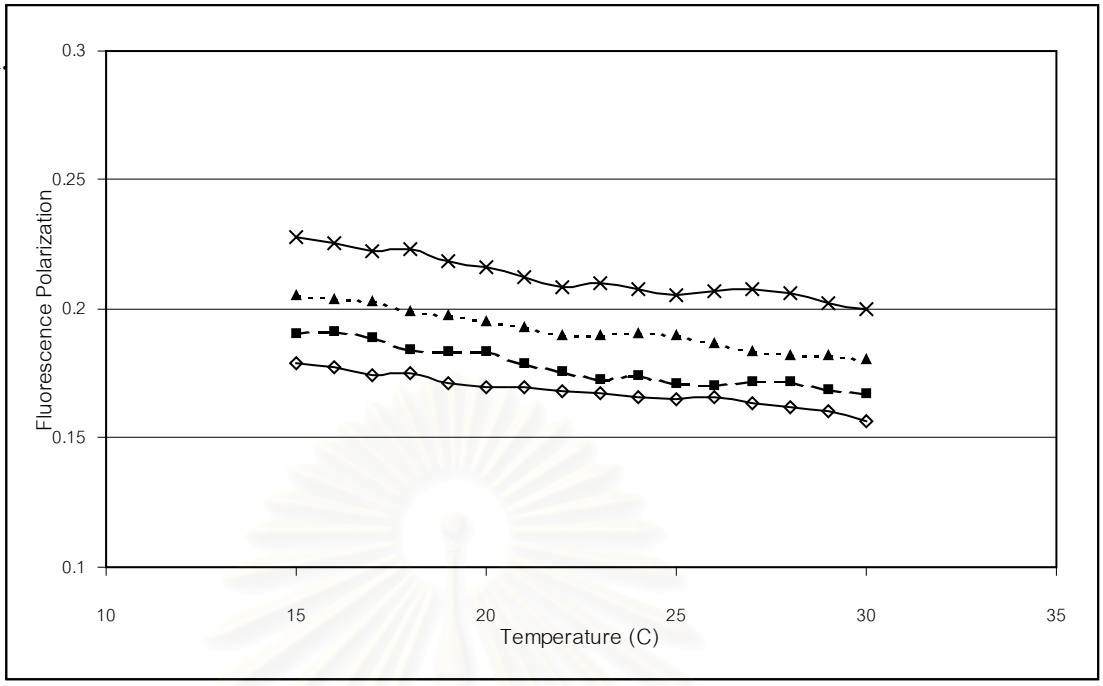



Figure 53 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:75, by mole). (A) fliorescence polarization (B) anisotropy. Concentration of EPC to CPZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

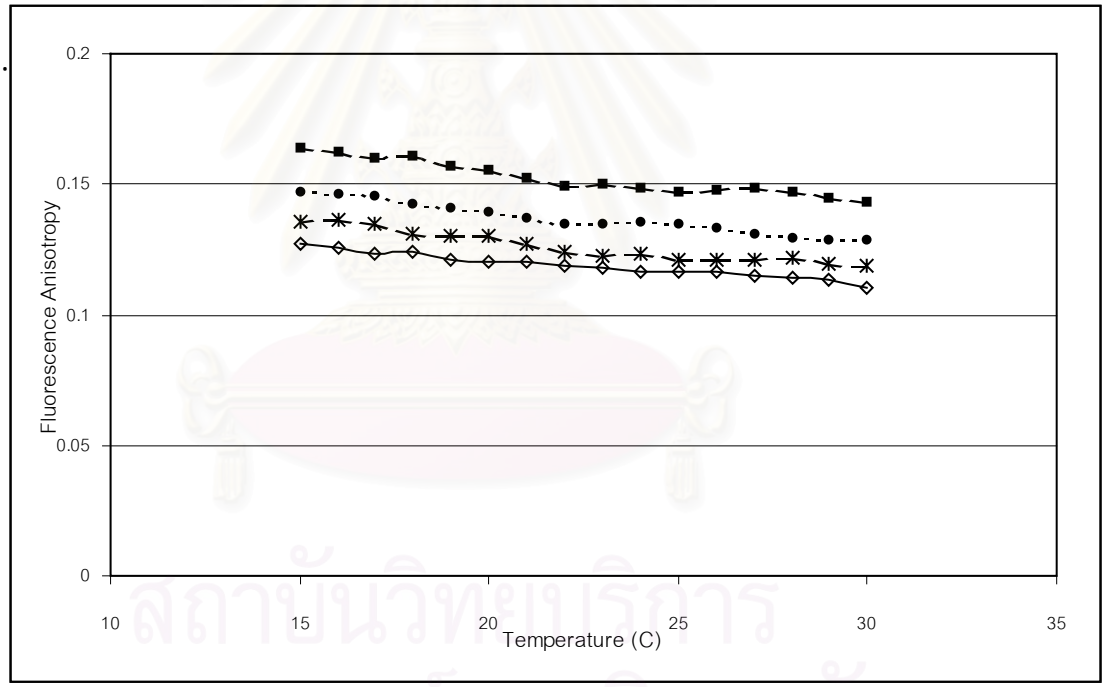
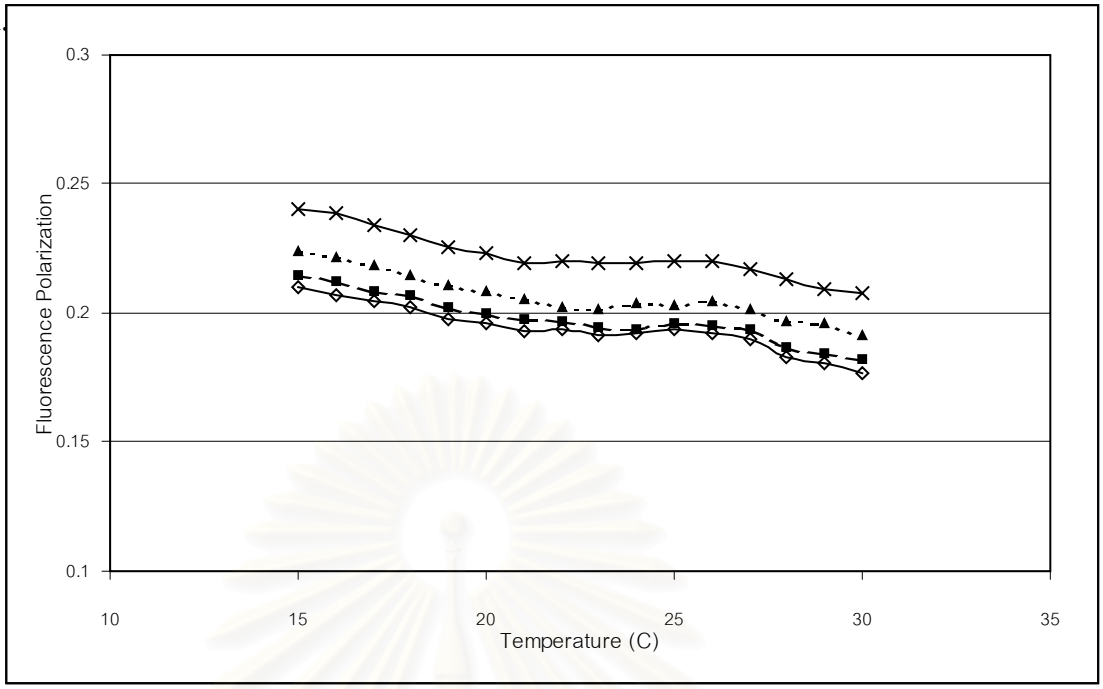



Figure 54 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:10, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of EPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

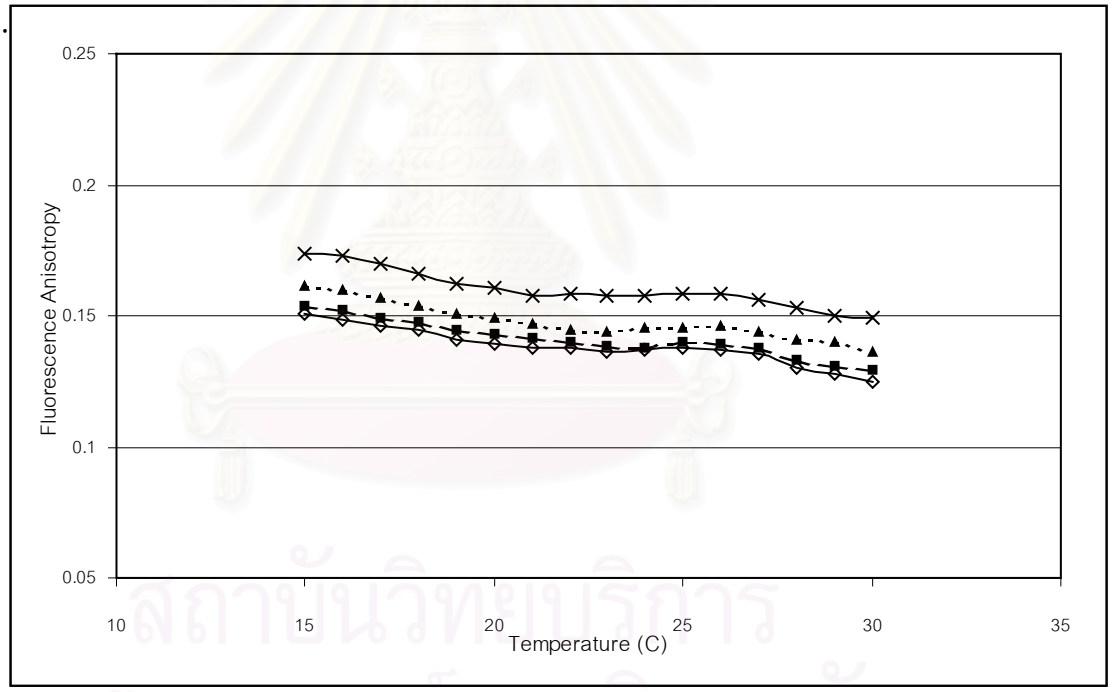
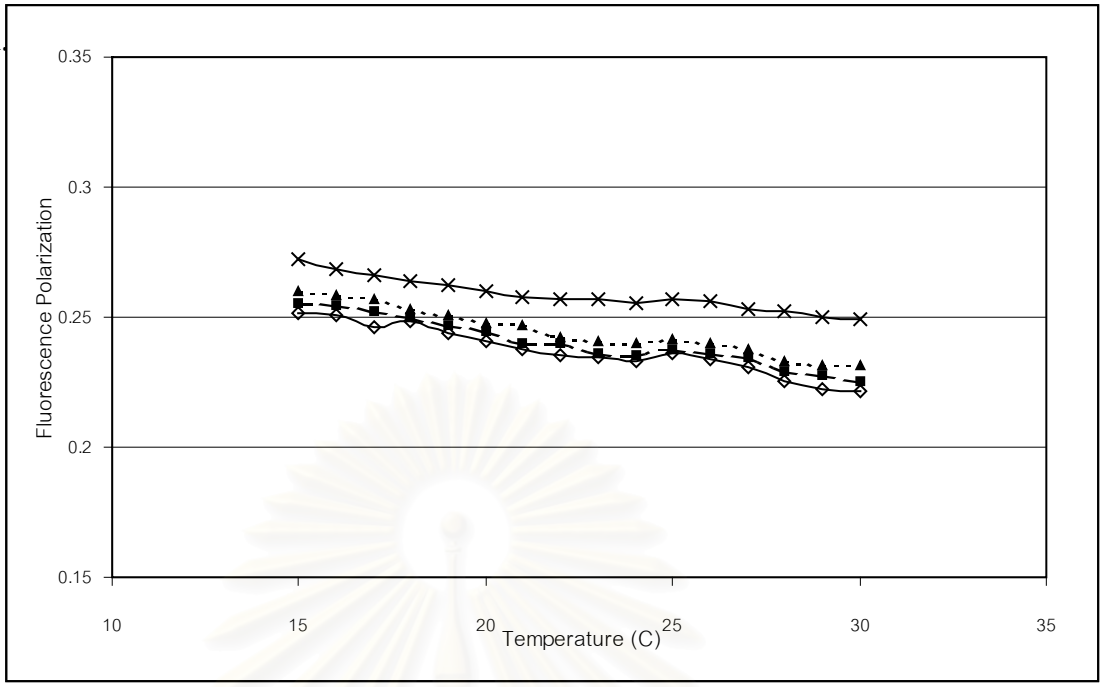



Figure 55 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:25, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of EPC to TRZ, by mole.

◇ , no CPZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

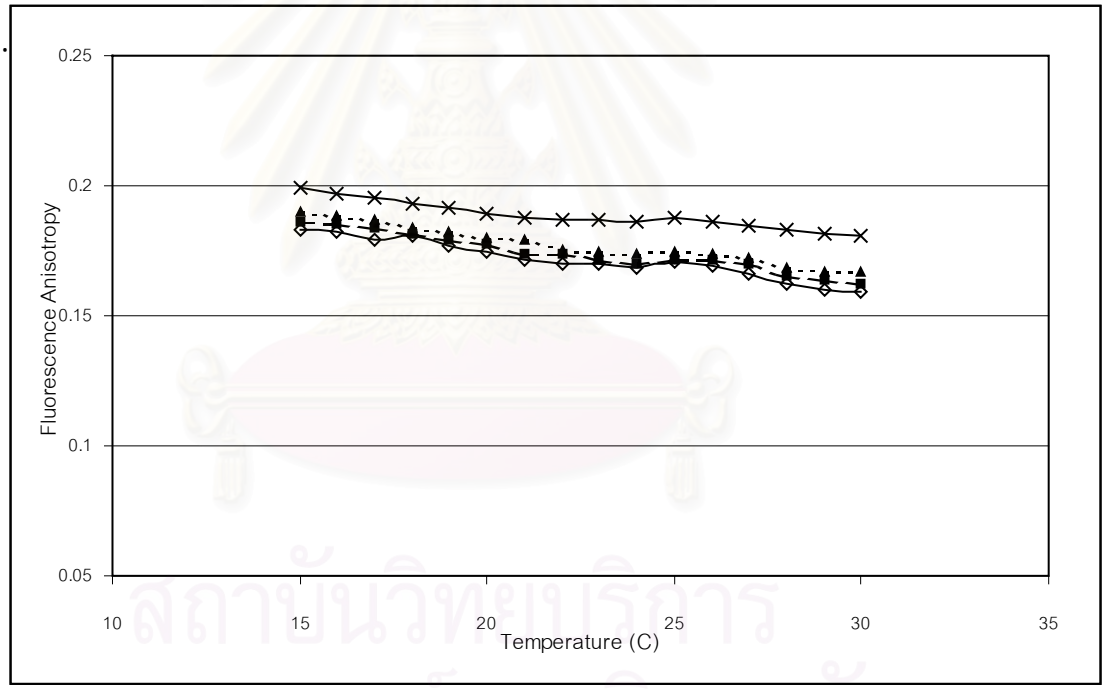
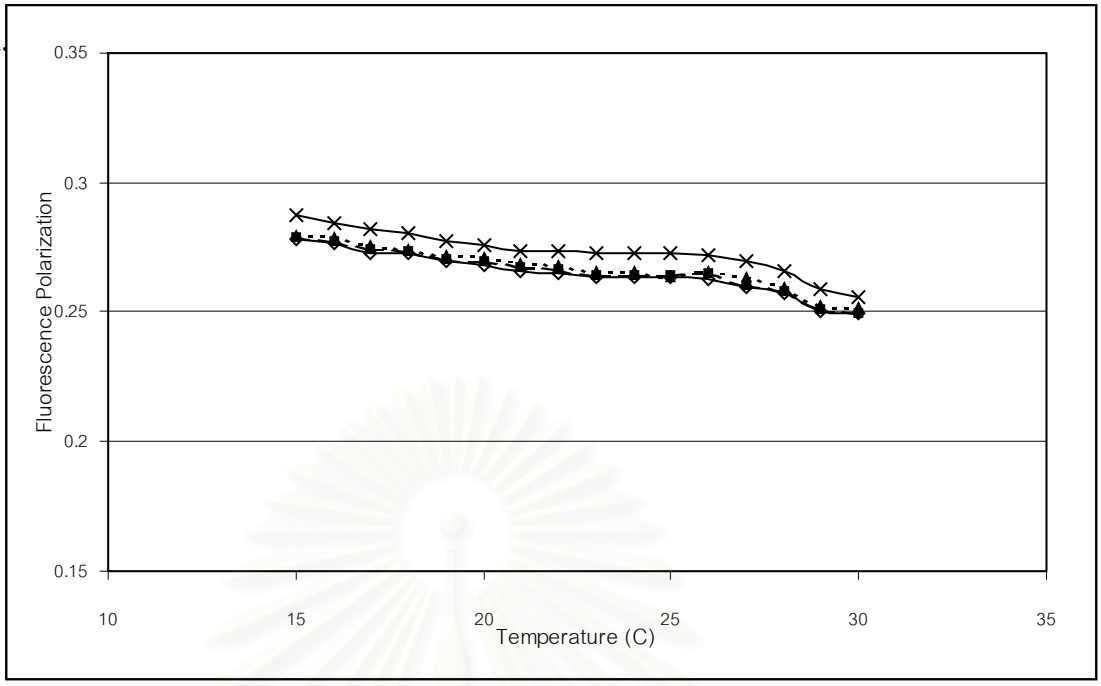



Figure 56 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:50, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of EPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

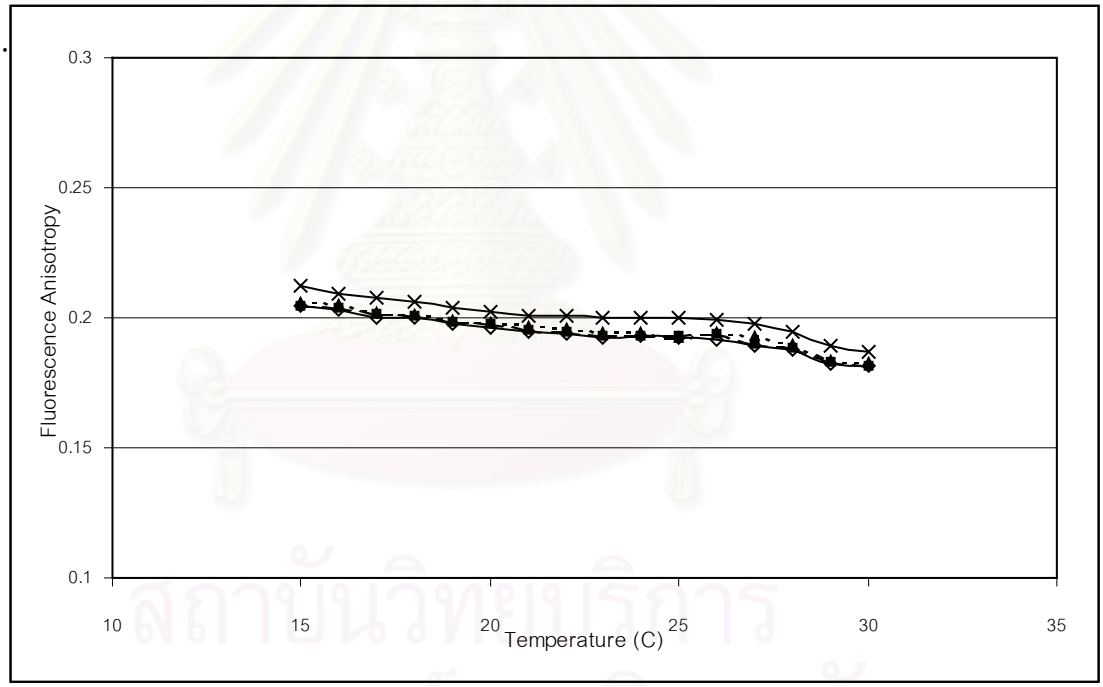



Figure 57 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol (100:75, by mole). (A) fluorescence polarization (B) anisotropy. Concentration of EPC to TRZ, by mole.

◇ , no TRZ ; ■ , 75:1 ; ▲ , 25:1 ; × , 7.5:1.

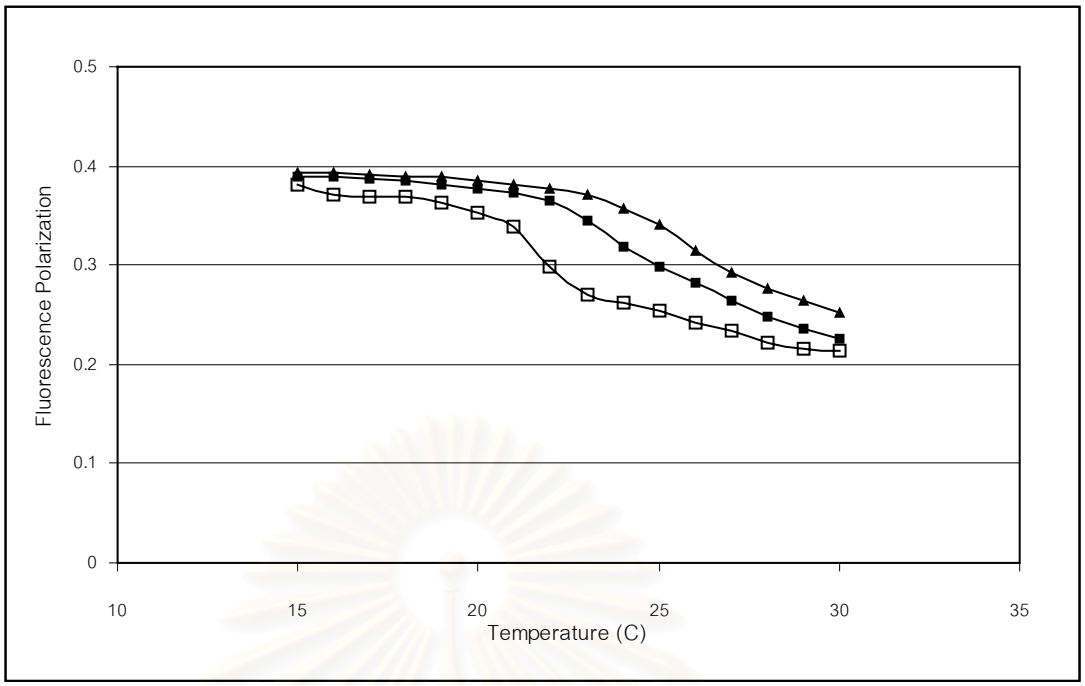
Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

**Influence of Charged Amphiphiles on Thermotropic Phase Transition of Liposomes.**

Cell membranes in some organs or some physiological conditions (e.g., abscess) possess surface charge that influences the properties of cell membrane, e. g., thermotropic phase transition. Thus experiment was performed by incorporation of charged amphiphile, for example dicetylphosphate (DCP) as a negative charge or stearylamine (SL) as a positive charge into bilayer of phosphatidylcholine:cholesterol (molar ratio of 100:10) to make molar ratio of 100:10:5 and 100:10:10. This molar ratio of phosphatidylcholine:cholesterol (100:10) was selected because it could provide clearly detection in alteration of liposomal membrane organization caused by charged amphiphiles.

As illustrated in Figure 58 for DMPC:Cholesterol liposomes, incorporation of DCP resulted in shifting of $T_m$ to higher temperature and also broadening transition range. Moreover an increase in fluorescence polarization (anisotropy) values was observed throughout temperature studied with more pronounced at high temperatures and at high concentration of DCP. The same results were also obtained in DSPC:cholesterol liposomes (Figure 59) but in a less extent.

A.




B.

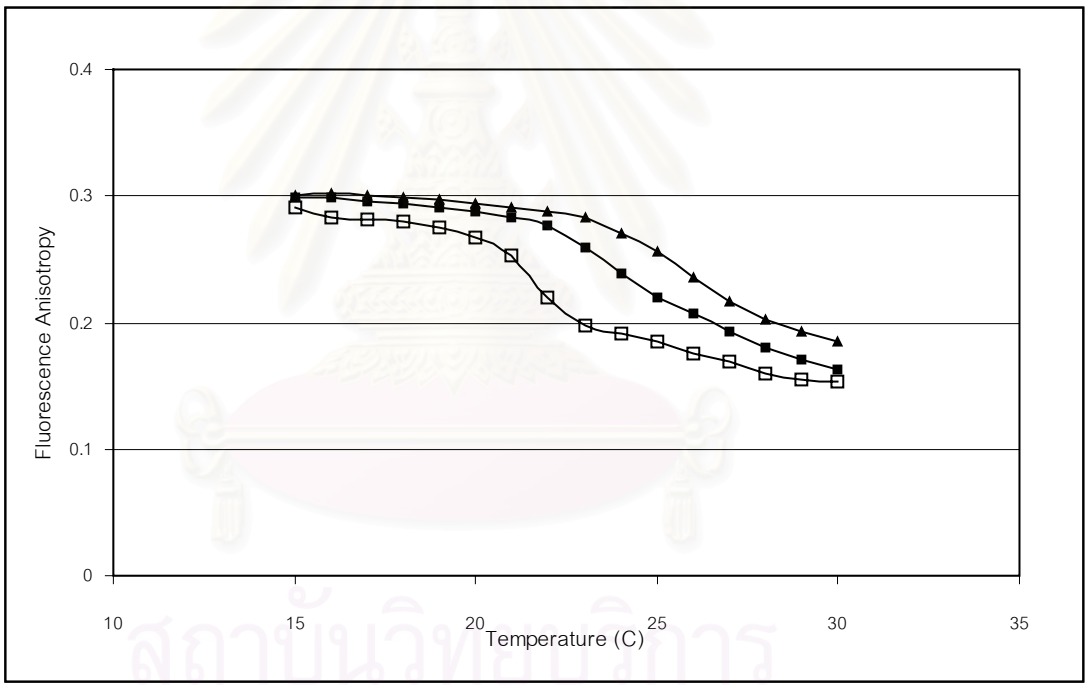


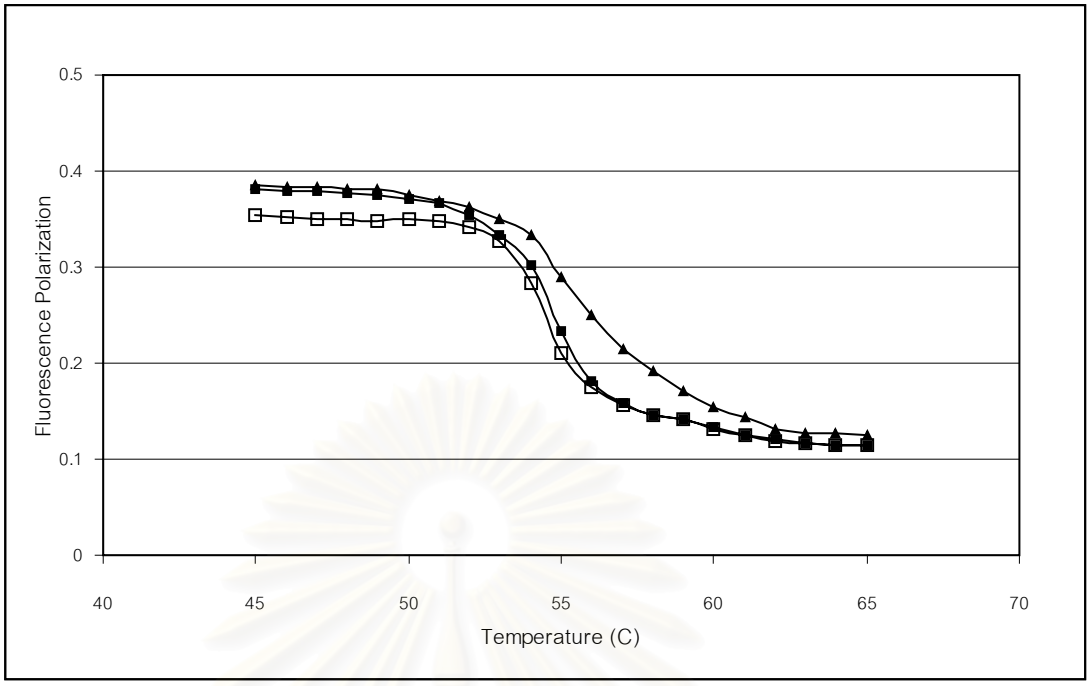

Figure 58 Effect of DCP on thermotropic phase transition of DMPC liposomes containing cholesterol.

(A) fluorescence polarization (B) anisotropy

Liposomes composed of DMPC:Cholesterol:DCP (100:10:C, by mole). □ , C = 0 ; ■ , C = 5 ; ▲ , C = 10.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

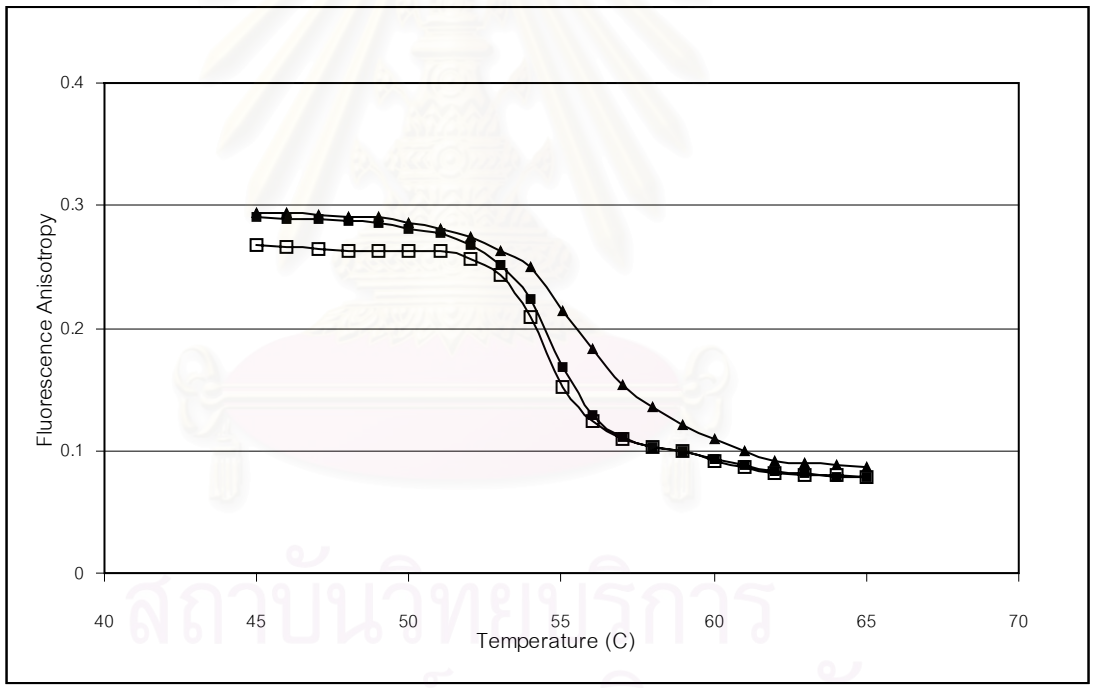


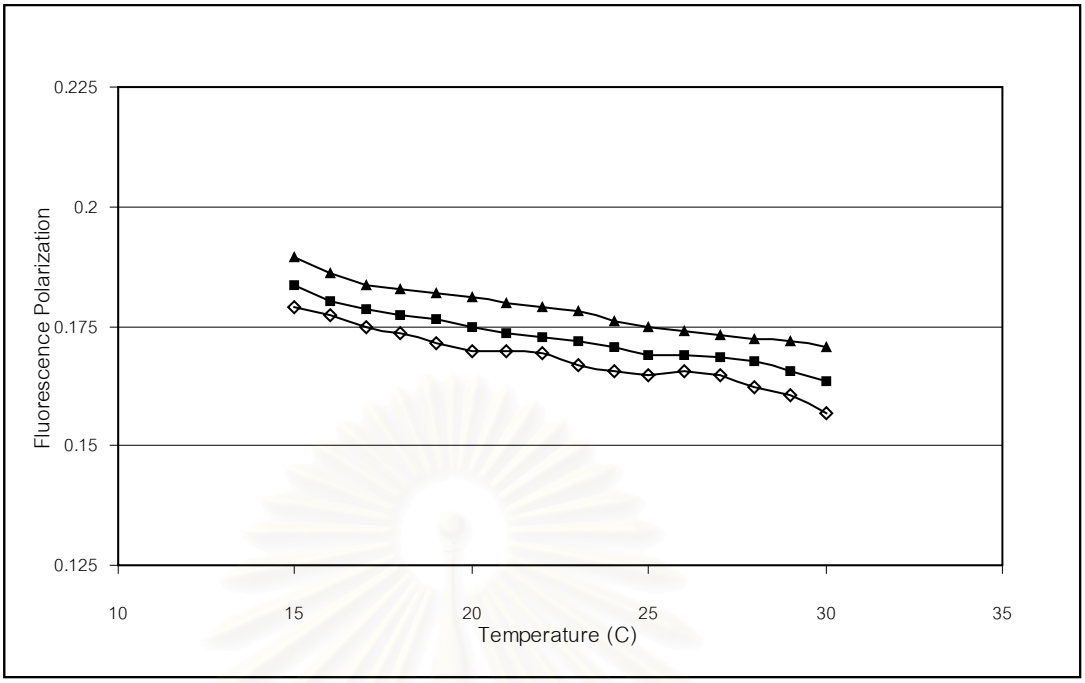

Figure 59 Effect of DCP on thermotropic phase transition of DSPC liposomes containing cholesterol.

(A) fluorescence polarization (B) anisotropy

Liposomes composed of DSPC:Cholesterol:DCP (100:10:C, by mole). □ , C = 0 ; ■ , C = 5 ; ▲ , C = 10. Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

When DCP was incorporated in EPC-cholesterol liposomes, it produced a little ordering effect as detected by increasing in values of fluorescence polarization (anisotropy) (Figure 60).

A.




B.

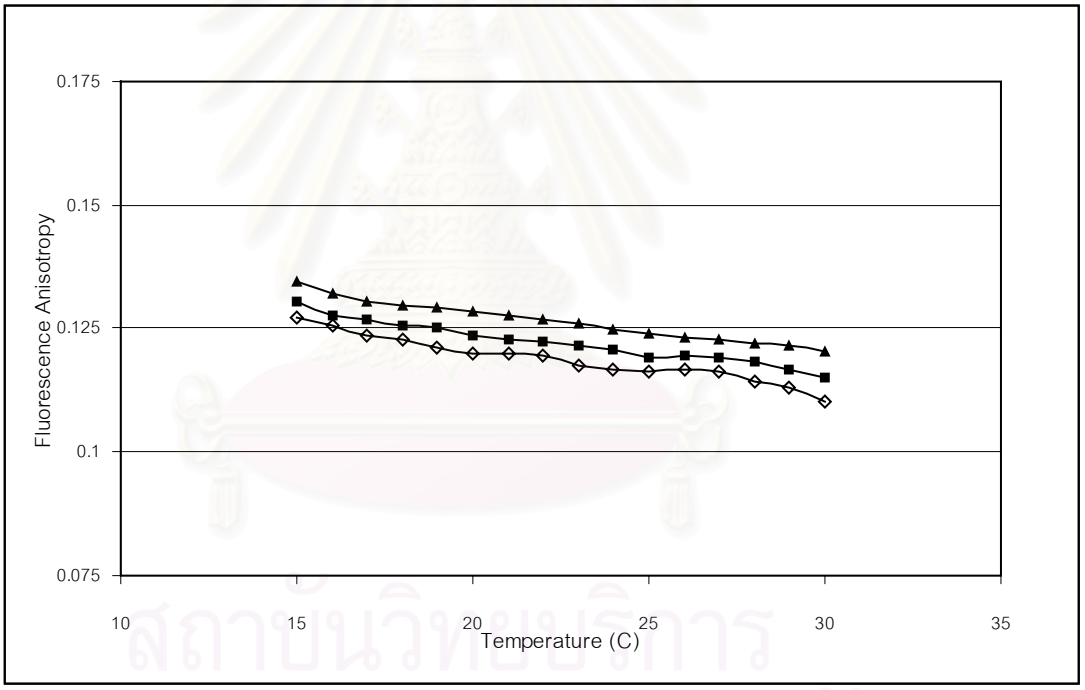



Figure 60 Effect of DCP on thermotropic phase transition of EPC liposomes containing cholesterol.

(A) fluorescence polarization (B) anisotropy

Liposomes composed of EPC:Cholesterol:DCP

(100:10:C, by mole). □ , C = 0 ; ■ , C = 5 ; ▲ , C = 10.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

All these investigations suggested that the incorporation of DCP could produce structural change in liposomal membrane. It might be explained from the fact that DCP molecule would be fully ionized since its pKa is 4.5-4.7 [Zuidam and Crommelin, 1995] at the experimental pH (7.0) thus they contained negative charge. When it embedded into the bilayer composed of phosphatidylcholine and cholesterol (Figure 58-60), electrostatic interaction between phosphocholine headgroup of phospholipid and phosphate headgroup of occurred and lateral expansion of membrane was obtained, leading to broaden transition range, consistently with a report by Kogure et al. (1999).

DCP possesses two C16:0 acyl chains which are different from two acyl chains of DMPC (C14:0) and DSPC (C18:0). The difference in lengths of acyl chains between DCP and DMPC (or DSPC) might cause mismatch at the hydrocarbon termini at the center of the bilayer that leaded to a partially interdigitated structure. However, in lipid mixtures, lipid molecules are flexible such that they can undergo length alterations to produce a homogeneous lipid bilayer of a particular phase type. Thus, in principle, lipids with a small number of carbons per chain can increase their effective length by increasing the proportion of *trans* conformers while those with a large number of carbons per chain can decrease their length by decreasing the *trans* conformer population to reach an equilibrium value. These processes are energetically favorable, therefore lateral expansion might be obtained as observed by increment of $T_m$ and broadening phase transition range. This phenomena was also described by Sankaram and Thompson(1992) on studying phase equilibria in binary phospholipid mixed bilayers.

In the other way, phase transition temperature of DCP is 76-80°C which is quite different from DMPC ($T_m$ 23°C) and DSPC ($T_m$ 54°C), thus coexistence of gel phase (DCP or mixture of DCP and DMPC) and liquid-crystalline phase (DMPC) at

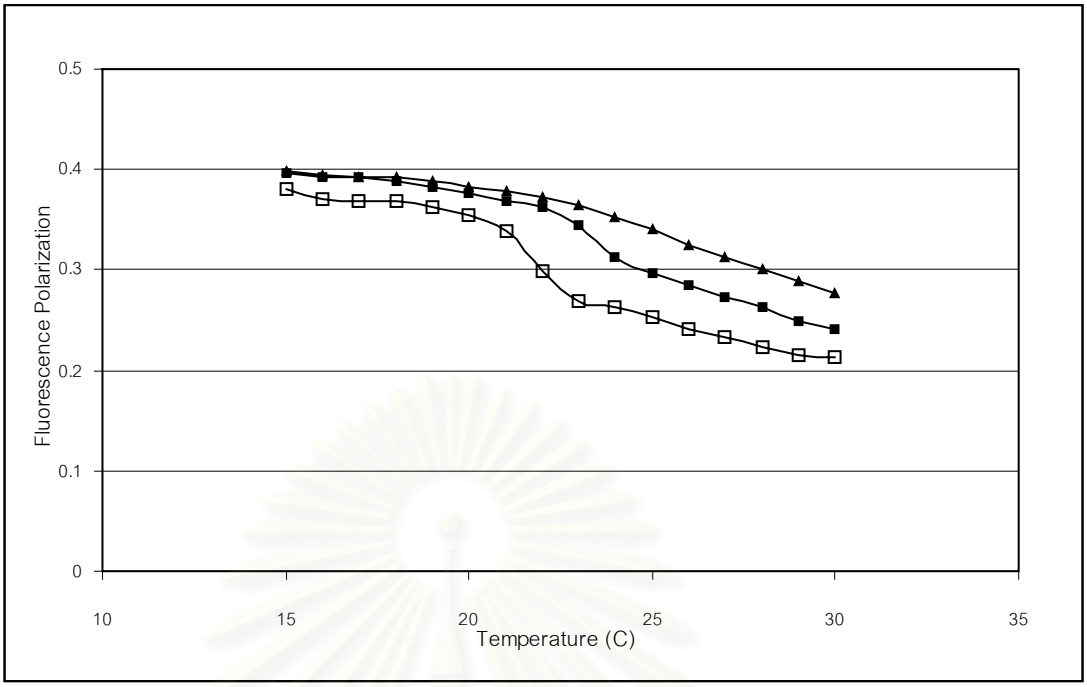
high concentration of DCP might be occurred, as could be seen in broadening of phase transition temperature, distinctly observed in DMPC-Cholesterol-DCP liposomes (Figure 58) with diminution effect in DSPC-cholesterol-DCP liposomes (Figure 59). Although, electrostatic repulsion between phosphate headgroup of phospholipid and phosphate headgroup of DCP and also between headgroup of DCP themselves might occur and this tend to expand the area occupied by the phosphocholine headgroups, leading to loosening of headgroup area. As a consequence, $T_m$ should be decreased. However, phase properties of lipids are complex and depend on both headgroup interaction and fatty acyl chain packing, then in lipid system studied here, fatty acyl chain packing and coexistence of gel and liquid-crystalline phases might become dominant and diminish electrostatic repulsion effect as shown by enhancement of $T_m$.

Increasing in fluorescence polarization (anisotropy) values was observed throughout the temperature studied (Figure 58-59). This might be achieved by intercalation of two acyl chains of DCP between two acyl chains of phospholipids, consequently by tightly packing in hydrophobic region of liposomal membrane. Since two alkyl chains of DCP are not large like steroid nucleus in cholesterol, thus they provided just only stiffness or ordering effect on membrane both in gel and liquid-crystalline phases as revealed by increasing in fluorescence polarization (anisotropy) values.

The explanation for increasing in fluorescence polarization (anisotropy) values for EPC-cholesterol-DCP liposomes was the same as described above.

For study the effect of positively charged amphiphile, identical procedure was performed by using stearylamine (SL). The effect of SL on thermotropic phase transition of DMPC and DSPC liposomes incorporated cholesterol was illustrated in Figure 61 and 62, respectively. The results demonstrated that when SL molecules were embedded into the membrane, the enhancement in fluorescence polarization (anisotropy) values above and below transition temperature was obtained concomitance with shifted $T_m$ to higher temperature and broadened in thermotropic phase transition range. However, slightly greater degree in increasing and broadening $T_m$ was observed in DMPC-cholesterol liposomes when using SL compared with DCP and controversial effect was found in DSPC-cholesterol liposomes.

A.




B.

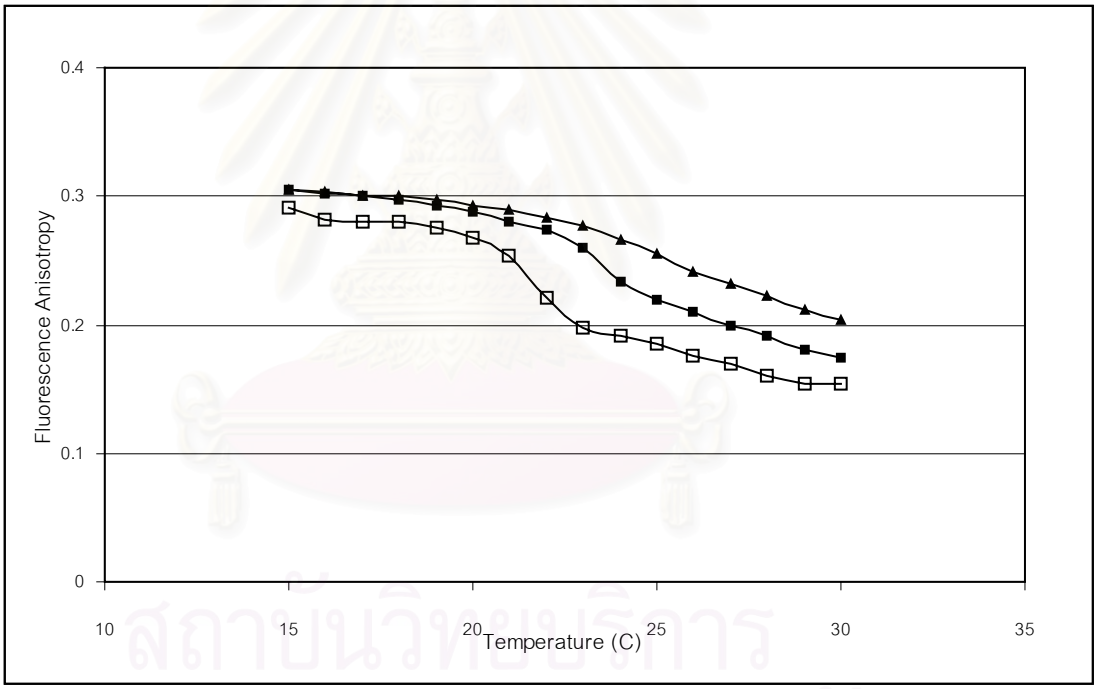



Figure 61 Effect of SL on thermotropic phase transition of DMPC liposomes containing cholesterol.

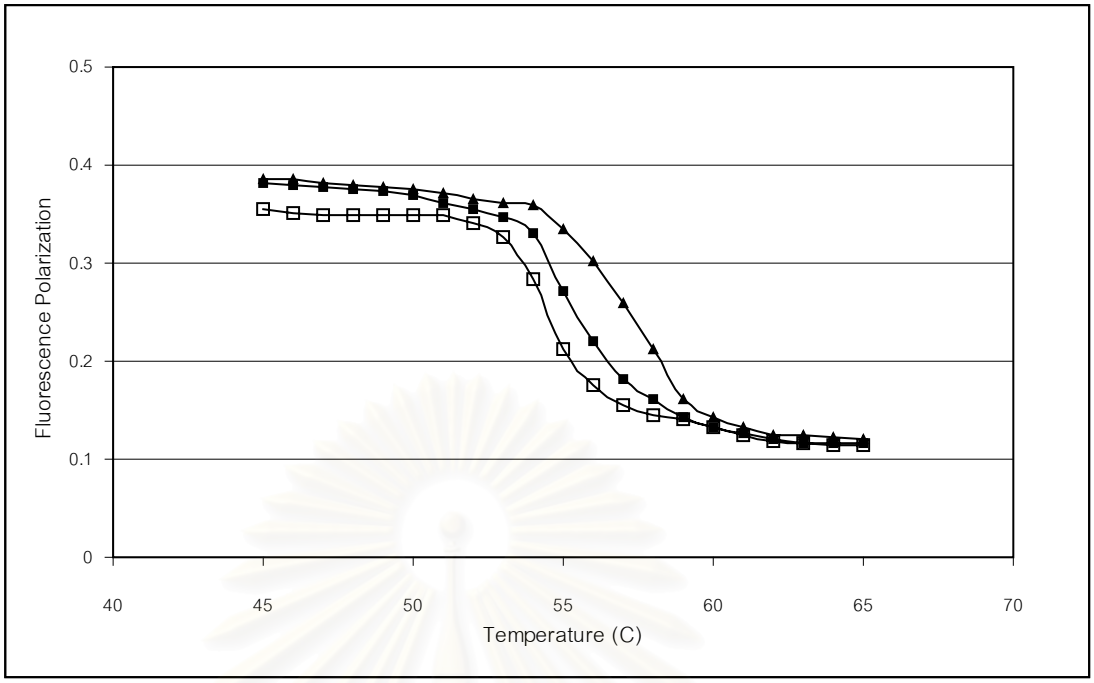
(A) fluorescence polarization (B) anisotropy

Liposomes composed of DMPC:Cholesterol:SL

(100:10:C, by mole). □ , C = 0 ; ■ , C = 5 ; ▲ , C = 10.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

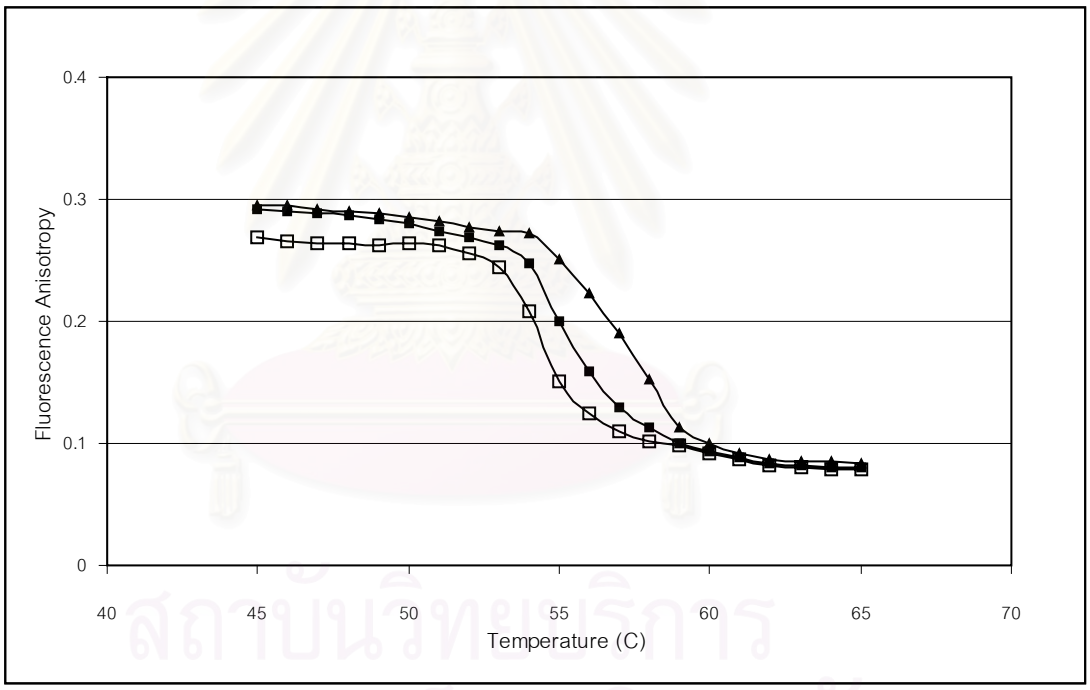



Figure 62 Effect of SL on thermotropic phase transition of DSPC liposomes containing cholesterol.

(A) fluorescence polarization (B) anisotropy

Liposomes composed of DSPC:Cholesterol:SL

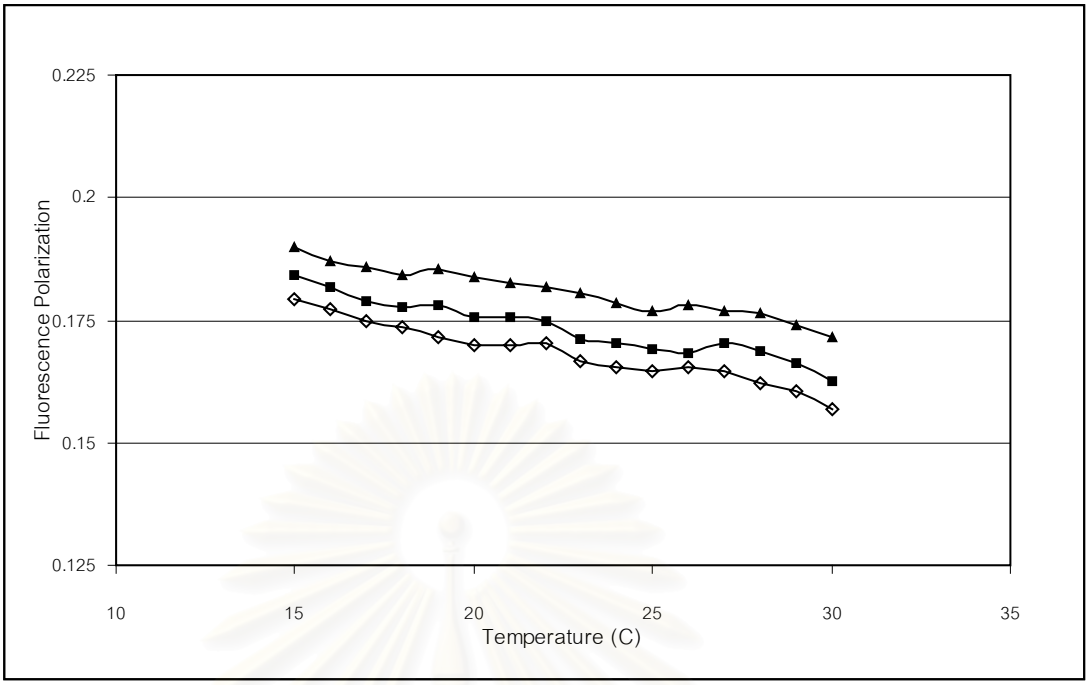
(100:10:C, by mole). □ , C = 0 ; ■ , C = 5 ; ▲ , C = 10.

Each point represents the mean of at least three samples±S.D.,

bar was omitted for clarity.

For EPC liposome incorporated cholesterol, same result as DCP was obtained when SL was used as a positively charged amphiphile. Enhancement of fluorescence polarization (anisotropy) values was achieved throughout the studied temperatures (Figure 63) with no significant difference observed compared with DCP (Figure 60).

A.




B.

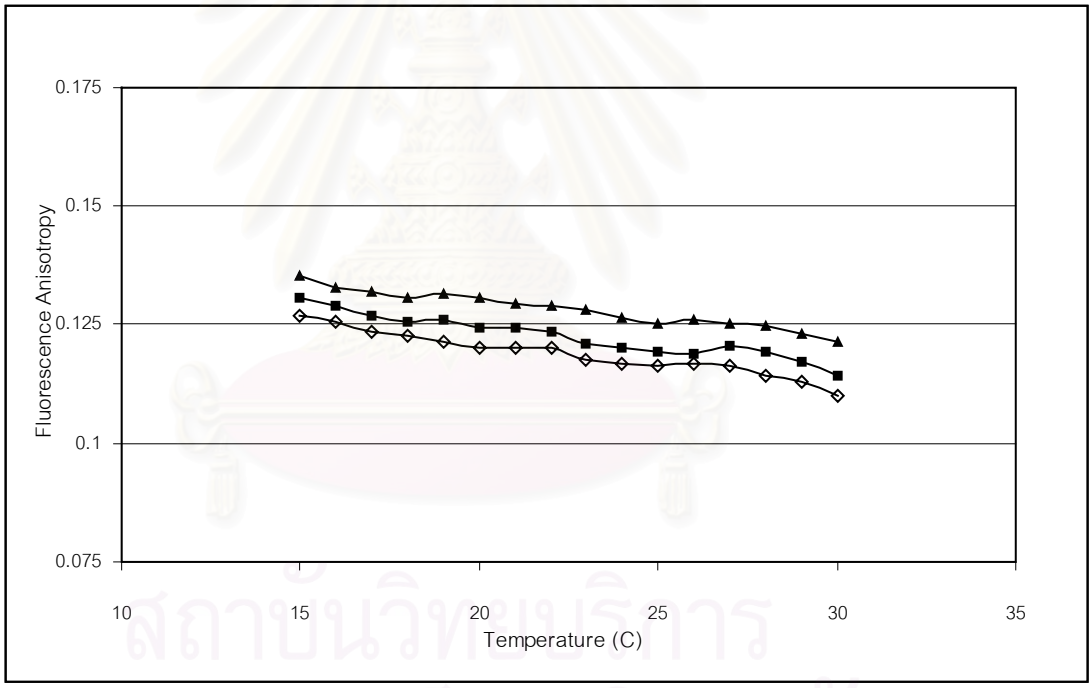



Figure 63 Effect of SL on thermotropic phase transition of EPC liposomes containing cholesterol.

(A) fluorescence polarization (B) anisotropy

Liposomes composed of EPC:Cholesterol:SL

(100:10:C, by mole). □ , C = 0 ; ■ , C = 5 ; ▲ , C = 10.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

All three possible forces regulating headgroup interactions and hence phase transition properties of bilayer membranes are electrostatic charge, steric hindrance and hydrogen bonding. Stearylamine was a weak base (with a pKa∼9 for amino group), it was protonated at the experimental pH and positive charge was obtained. Nevertheless, incorporation of SL into the liposomal membrane might also produced lateral expansion due to the charged repulsion between protonated amino groups themselves or between SL and choline group of phosphatidylcholine which could push the headgroup apart, as a consequence, the reduction of $T_m$ and broadening phase transition range should be observed, comparing with liposomes without SL.

Stearylamine molecule composes of an amino headgroup with one alkyl chain (C18:0) that the molecules appear to be aligned with the glycerol backbone approximately perpendicular to the plane of the bilayer. Since headgroup of stearylamine is so small, then it reduces steric hindrance between headgroups, therefore, it could bring the chains of adjacent molecules into closer proximity, to maximize Van der Waals interactions. Therefore, packing density of hydrocarbon region was increased, and as expected, raising the transition temperature and also fluorescence polarization (anisotropy) values (Figure 61-63).

Stearylamine headgroup (amino group) is able to take part in hydrogen bonding interactions with its neighbours in the membrane which have to be broken before phase transition occurs, resulting in an elevated $T_m$.

In the experimental studies with SL, it was found that steric hindrance and hydrogen bonding circumvented electrostatic charge (repulsion), as revealed by raising in $T_m$ (Figure 61-62).

SL provided a greater effect on DMPC-cholesterol liposomes (Figure 61) and DSPC-cholesterol liposome (Figure 62) comparing with DCP (Figure 58 and 59). This might be due to larger difference beetween acyl chain of SL (C18:0) with DMPC (C14:0), then a great mismatch at the hydrocarbon termini was obtained, as a consequence much more broadening in thermotropic phase transition was demonstrated in DMPC-cholesterol-SL liposomes.

Mismatch of acyl chain lengths is not obtained from liposomes composed of DSPC and SL since they possess same acyl chain length (C18:0), thus it did not provide any effect on liposomes composed of DSPC-Cholesterol-SL. SL has $T_m$ about 50-53°C which is closer to DSPC ($T_m$ 54°C) than DMPC ($T_m$ 23°C), therefore coexistence of gel and liquid-crystalline phase should be obtained with DMPC, accompanying by distortion of liposomal bilayer and finally phase seperation might be achieved. Thus alteration in thermotropic phase transition in DSPC-Cholesterol-SL liposomes was affected only by headgroup interaction. However, SL provide a greater effect on DSPC-cholesterol-SL liposomes, comparing with DSPC-cholesterol-DCP liposomes, suggesting stronger headgroup interaction.

**Influence of Phenothiazines on Thermotropic Phase Transition of Liposomes composed of Phospholipid-Cholesterol-Charged Amphiphile.**

Since the region of the polar headgroups provided a favorable environment for charged amphiphilic molecules, they intercalated and thus influenced the thermotropic phase transition of phospholipid bilayers even when bilayers composed of cholesterol as seen in the previous experiments.

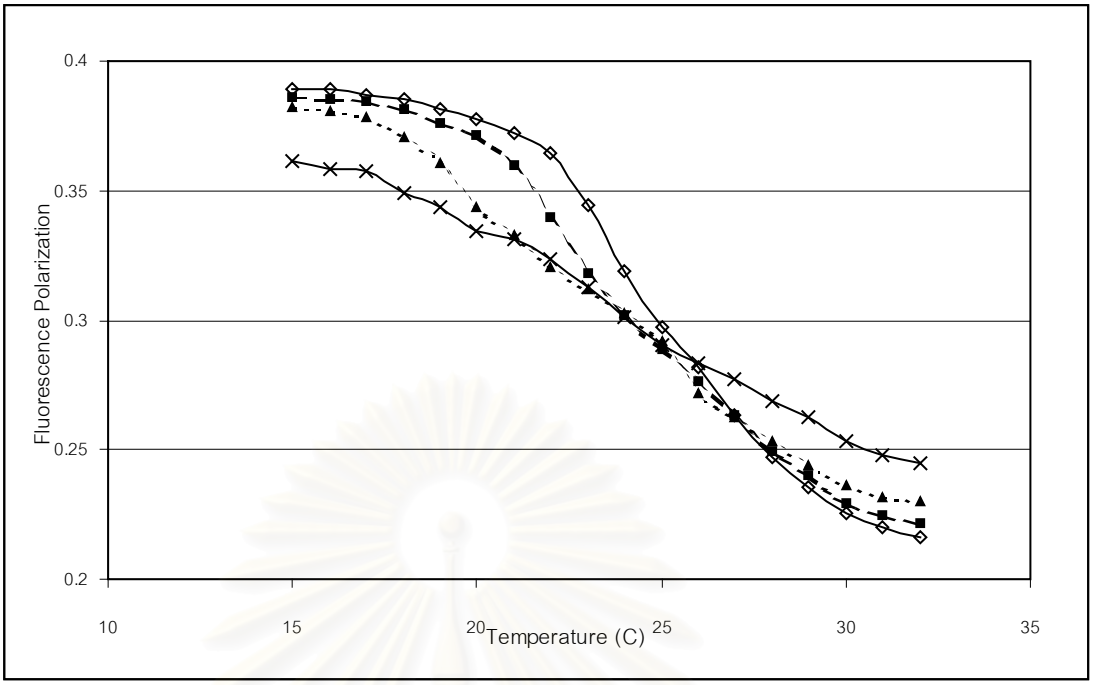
In this study, charged ampiphiles were used to modify surface charge of liposomal membrane. Dicetylphosphate (DCP) and stearylamine (SL) were used as negatively and positively charged amphiphiles, respectively. Investigation of drug interaction with liposomal membrane containing cholesterol and charged amphiphile was also examined to provide insights into the structure and dynamics of bilayers that have a direct effect on biological processes. The experiments were performed with liposomes composed of phospholipid-cholesterol-charged amphiphile in molar ratio of 100:10:5 and 100:10:10 while varying concentration of phospholipid to drug in molar ratios of 75:1, 25:1 and 7.5:1 comparing with liposomes without charged amphiphile.

The influence of phenothiazines on fluorescence polarization (anisotropy) of DPH as a function of temperature in DMPC liposomes containing cholesterol and DCP were reported in Figure 64-65 for CPZ and Figure 68-69 for TRZ or SL as charged amphiphile in Figure 66-67 for CPZ and Figure 70-71 for TRZ. It was found that the transition temperature ($T_m$) was shifted to lower temperature and the transition range became broader, moreover, the fluorescence polarization (anisotropy) value was lower below the transition and got higher value above the transition comparing with the corresponding liposomes but without drug. (Figure 58 for DCP and Figure 60 for SL). The magnitude of the phenothiazine effect was also

concentration dependence. These finding confirmed the dual effects of phenothiazine on liposomal membrane even in liposomes composed of charged amphiphiles. However, these dual effects of phenothiazines appeared to be high in the case of negatively charged amphiphile, DCP and less in the case of positively charged amphiphile, SL as comparing with their effects on liposomes composed of the same molar ratio of DMPC and cholesterol (Figure 34 for CPZ and Figure 38 for TRZ). Furthermore, the effect of phenothiazines on liposomal membrane increased with increasing amount of DCP in contrast with SL.

In the case of DSPC liposomes, dual effects of phenothiazines were obtained in both DCP (Figure 72-73 for CPZ and Figure 76-77 for TRZ) and SL (Figure 74-75 for CPZ and Figure 78-79 for TRZ). However, CPZ exhibited more dominant effect on DMPC liposomes more than DSPC while TRZ acted in the opposite way.

A.




B.

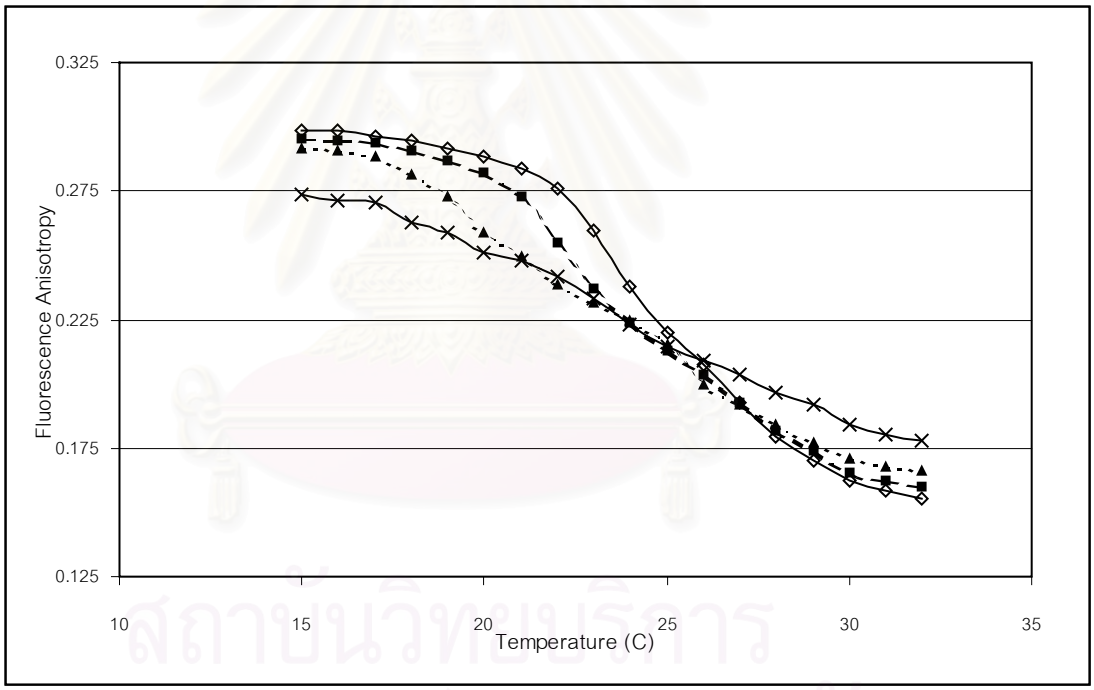



Figure 64 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol and DCP (100:10:5, by mole).

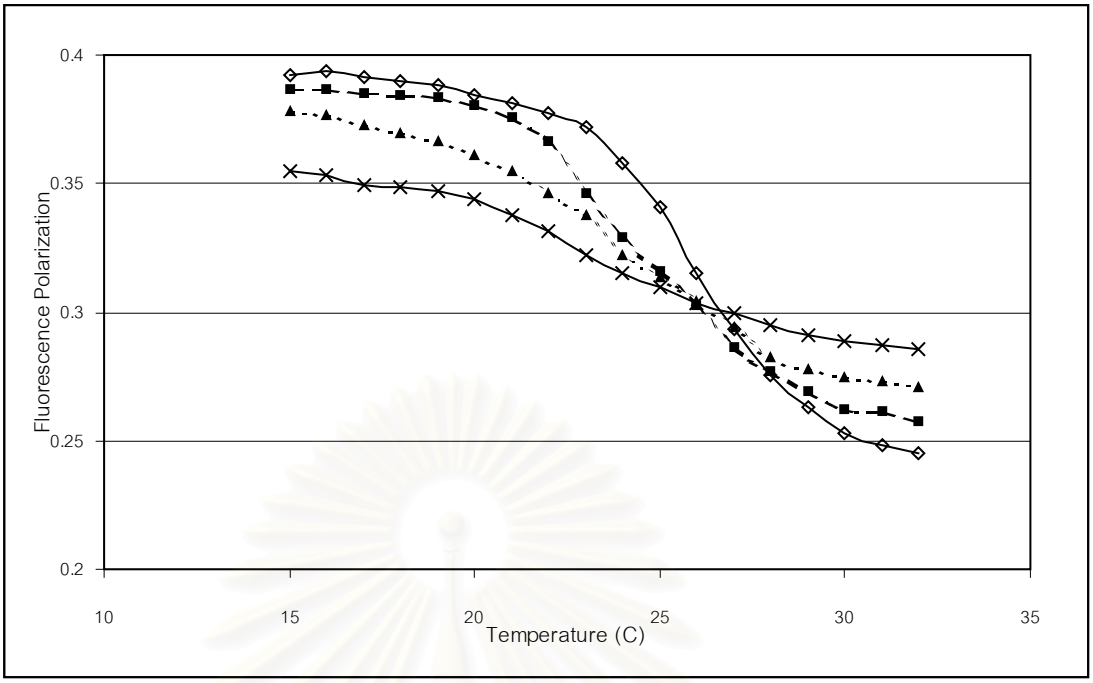
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

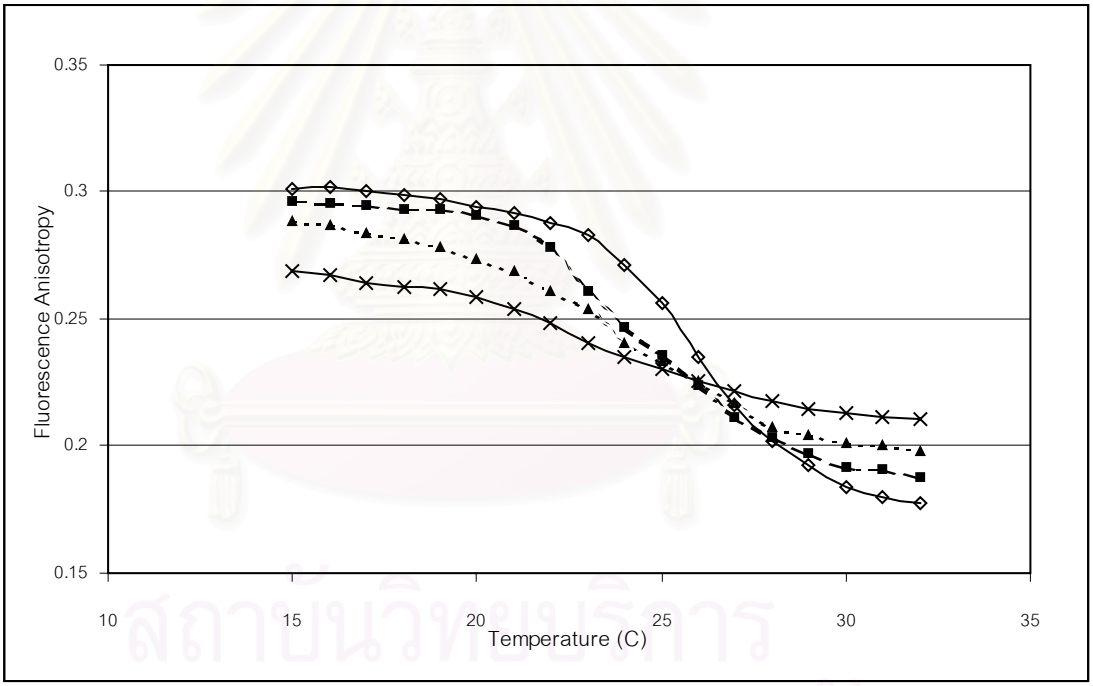



Figure 65 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol and DCP (100:10:10, by mole).

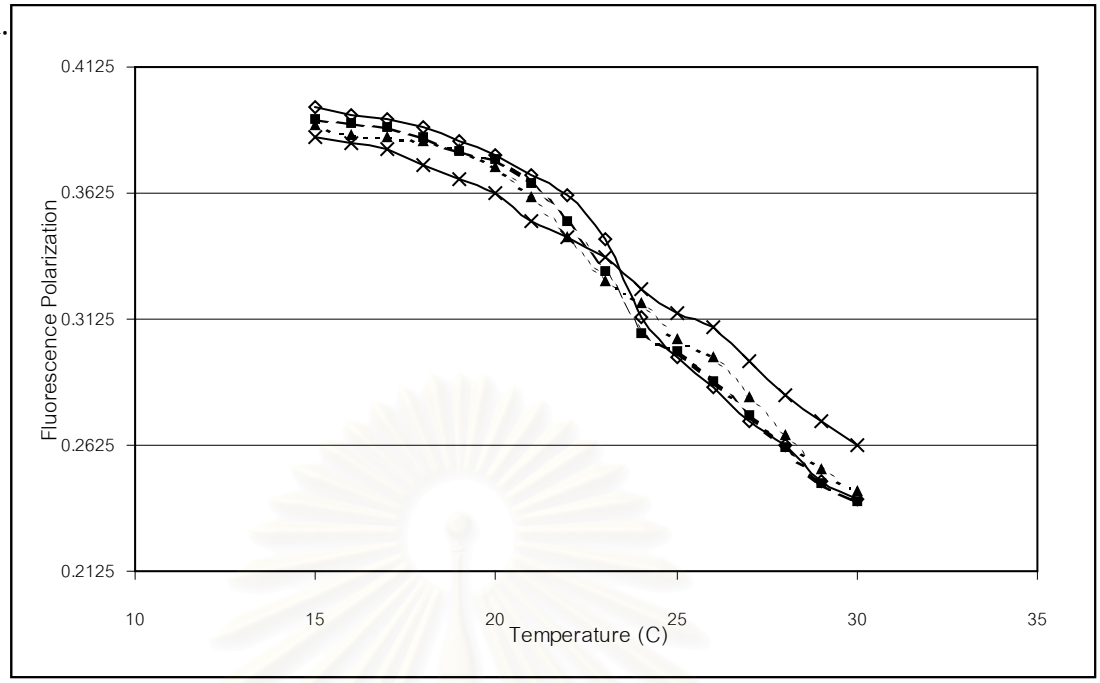
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

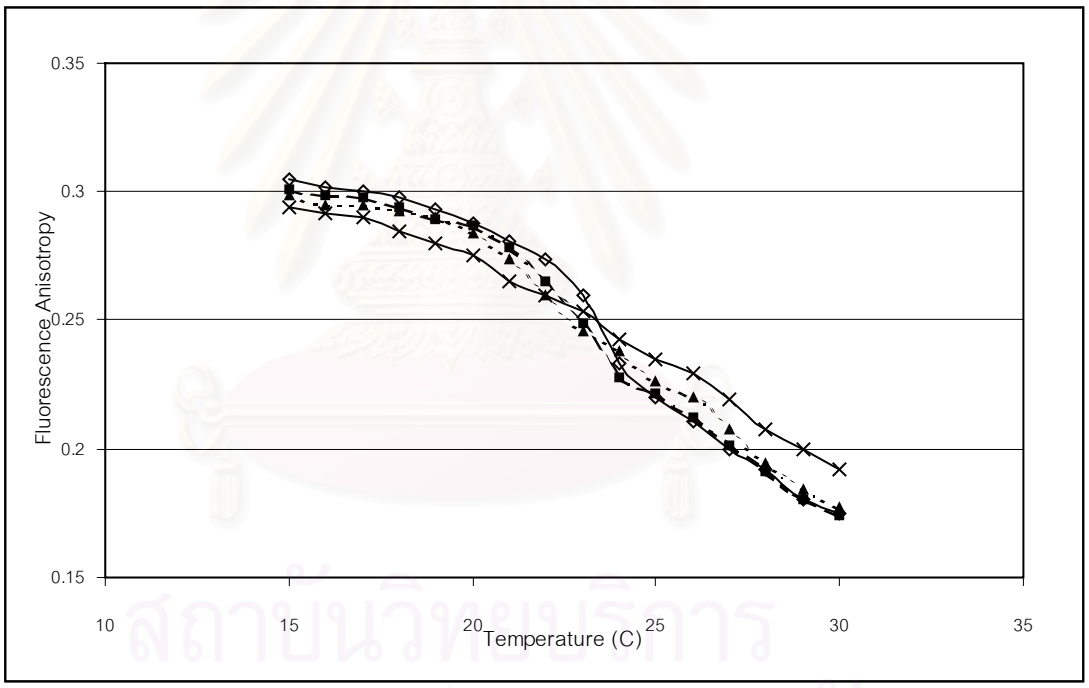



Figure 66 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol and SL (100:10:5, by mole).

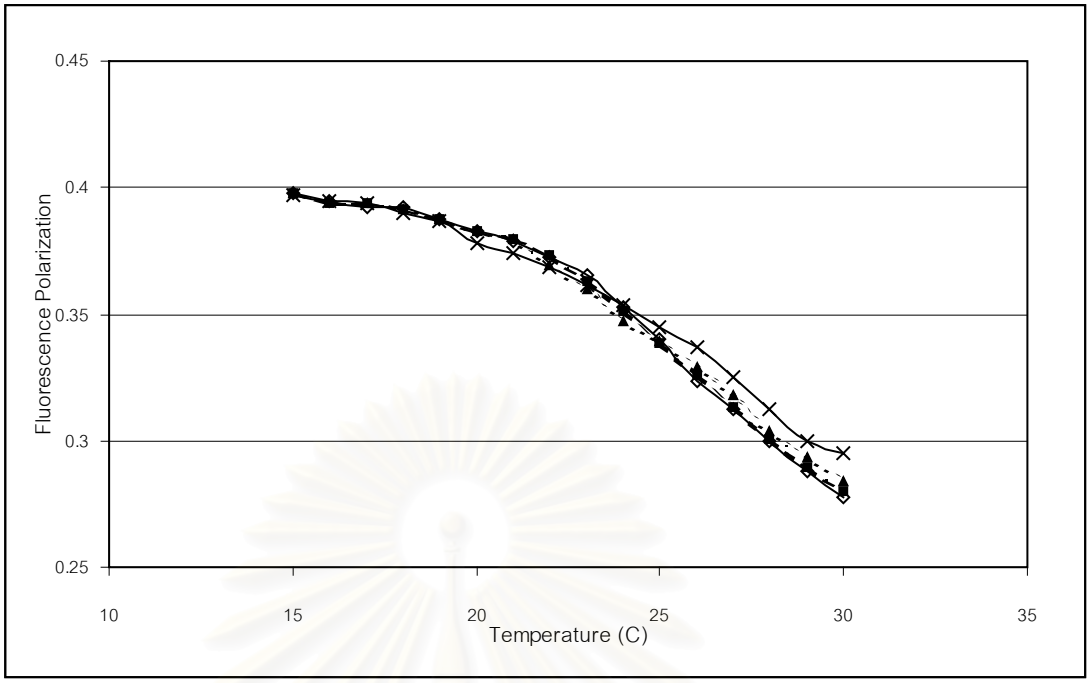
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

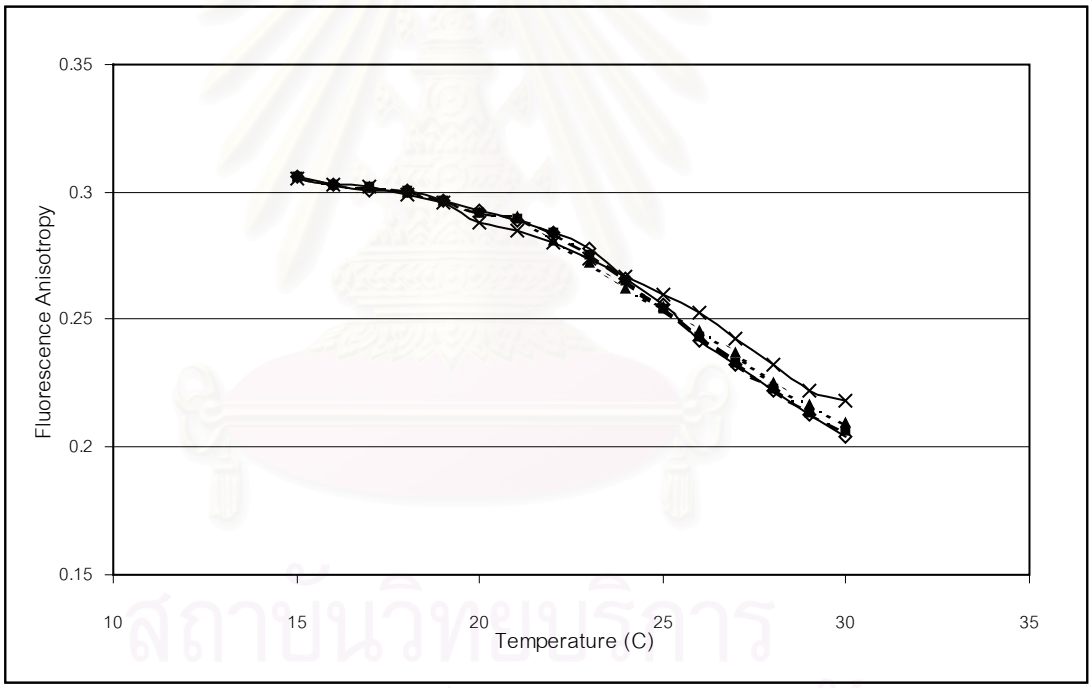



Figure 67 Effect of CPZ on thermotropic phase transition of DMPC liposomes containing cholesterol and SL (100:10:10, by mole).

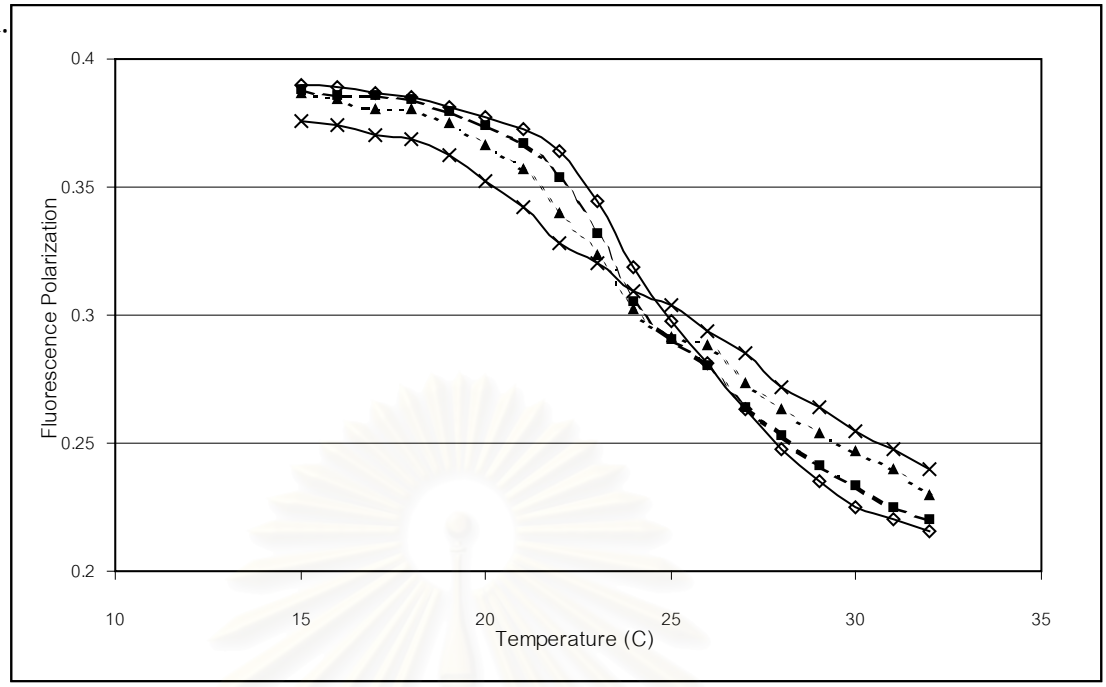
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

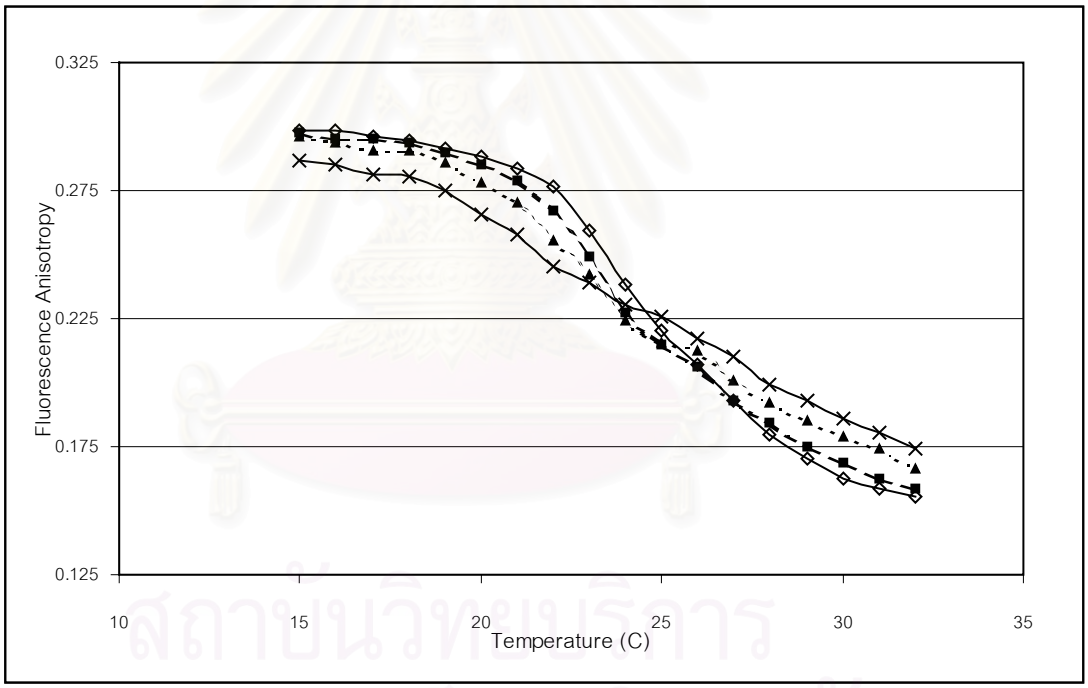



Figure 68 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol and DCP (100:10:5, by mole).

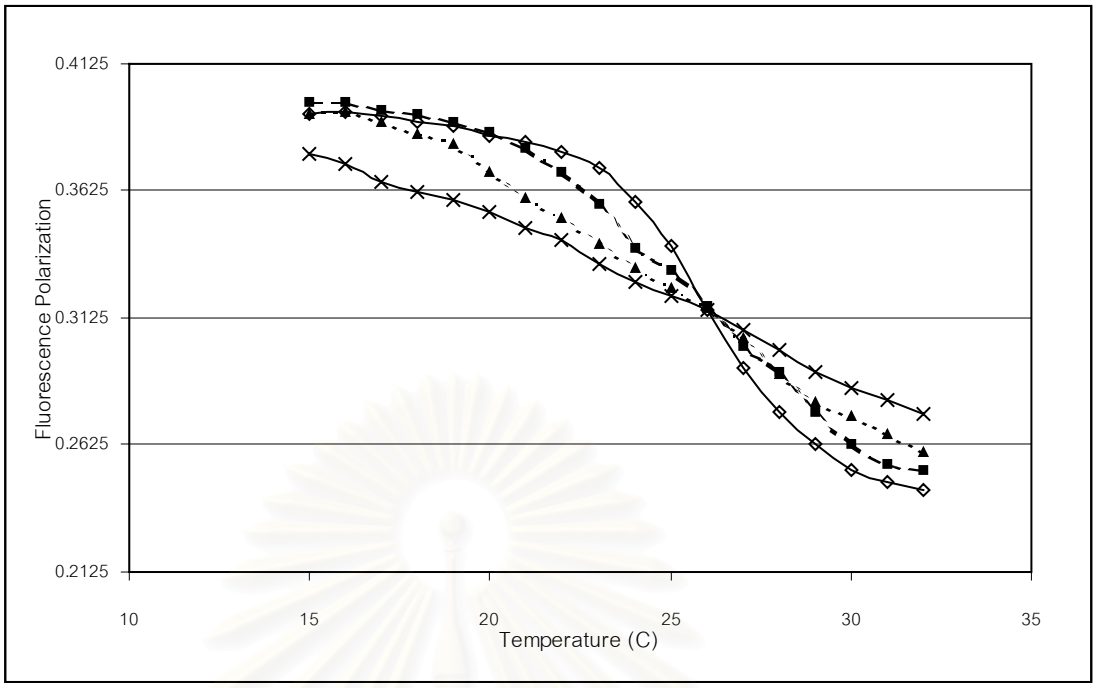
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.

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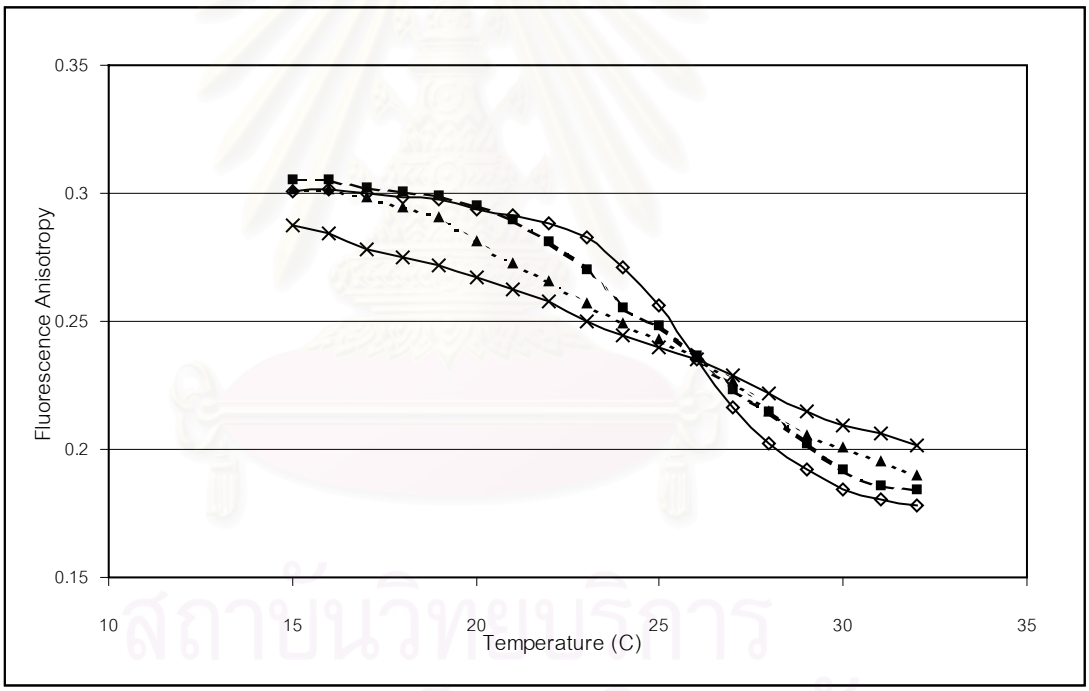



Figure 69 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol and DCP (100:10:10, by mole).

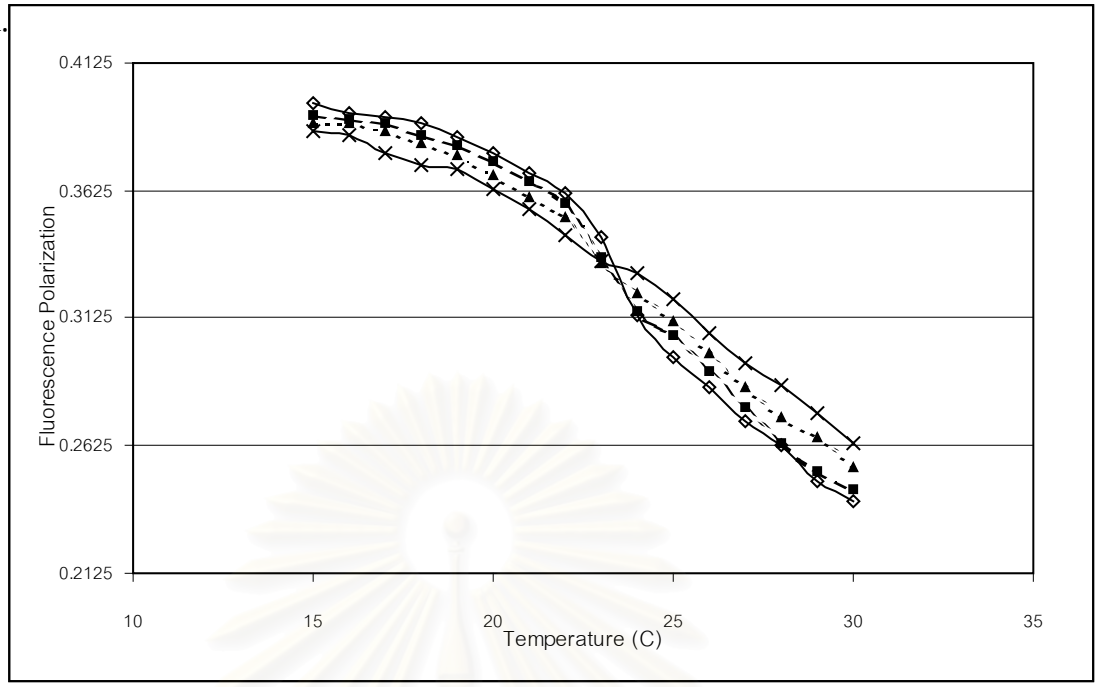
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.

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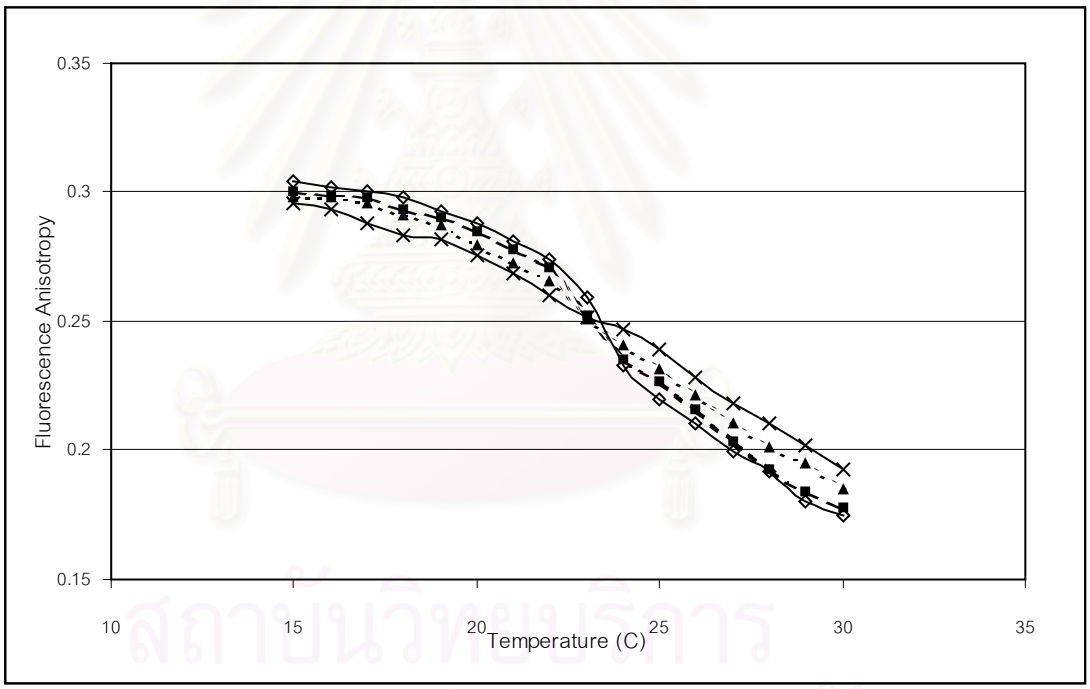



Figure 70 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol and SL (100:10:5, by mole).

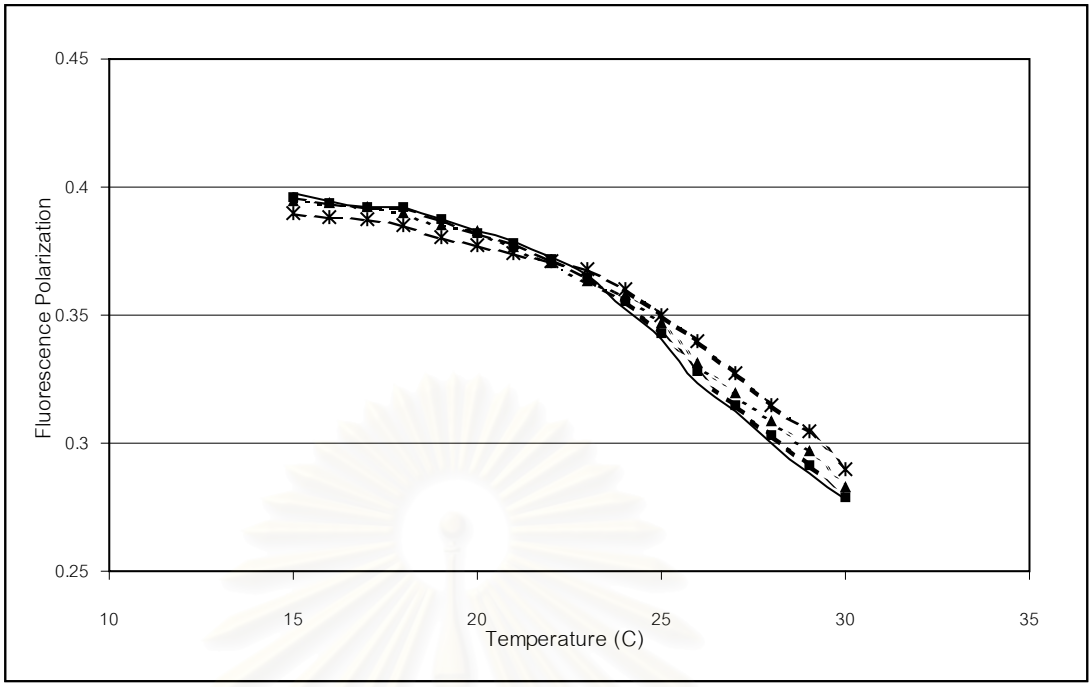
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

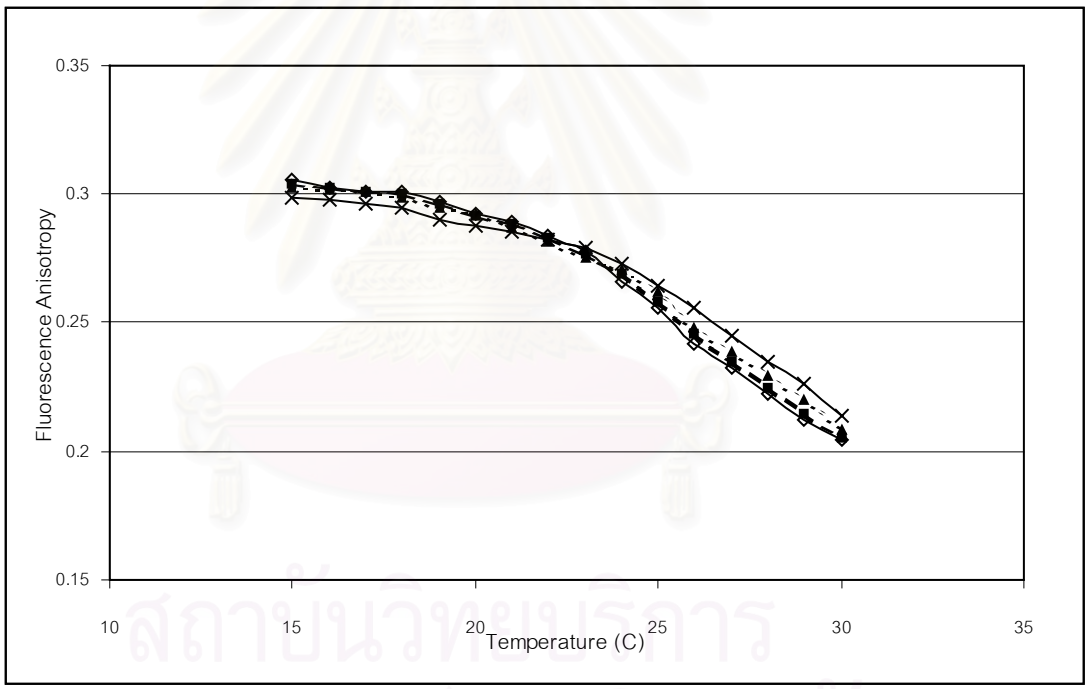



Figure 71 Effect of TRZ on thermotropic phase transition of DMPC liposomes containing cholesterol and SL (100:10:10, by mole).

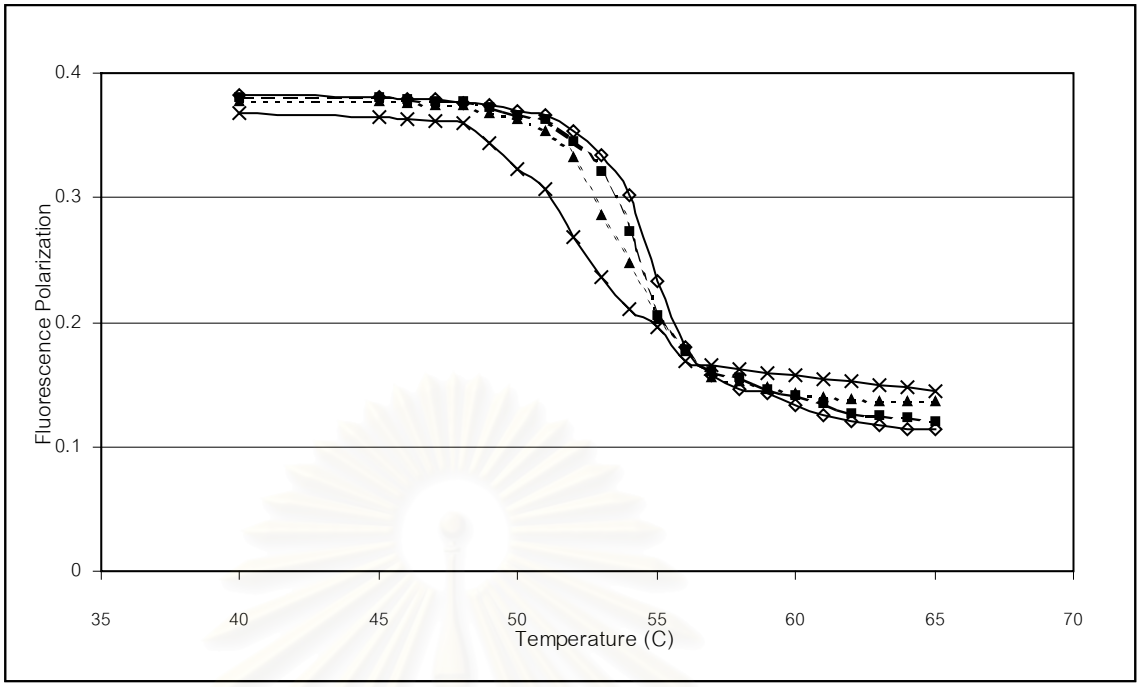
(A) fluorescence polarization (B) anisotropy

Concentration of DMPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

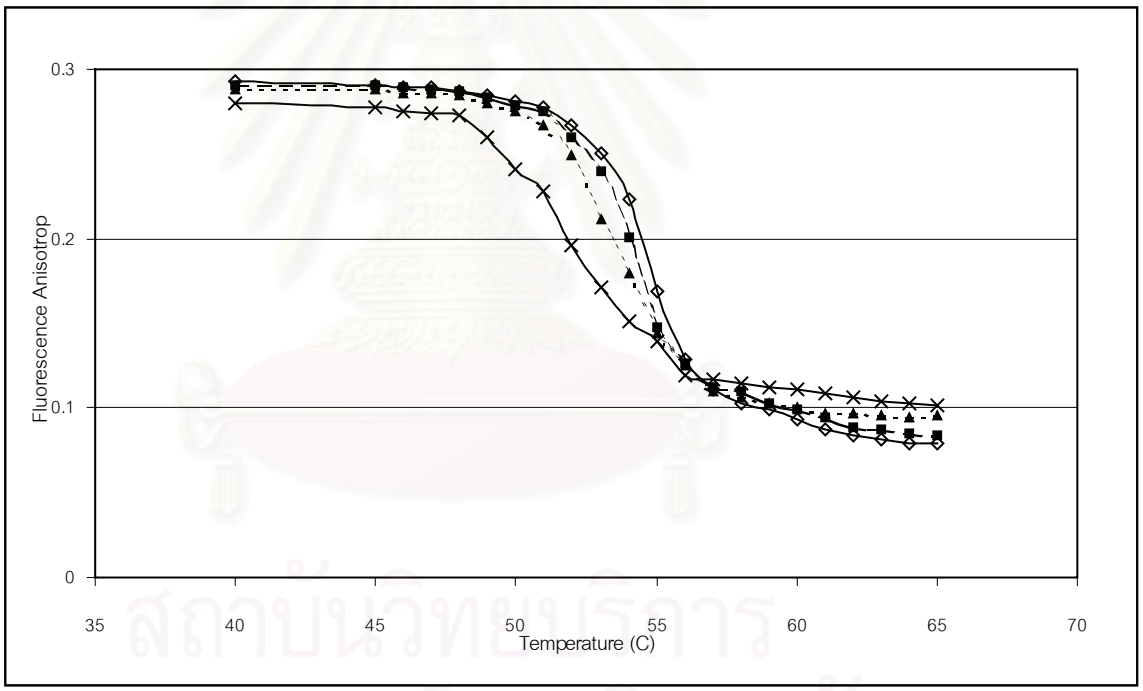



Figure 72 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol and DCP (100:10:5, by mole).

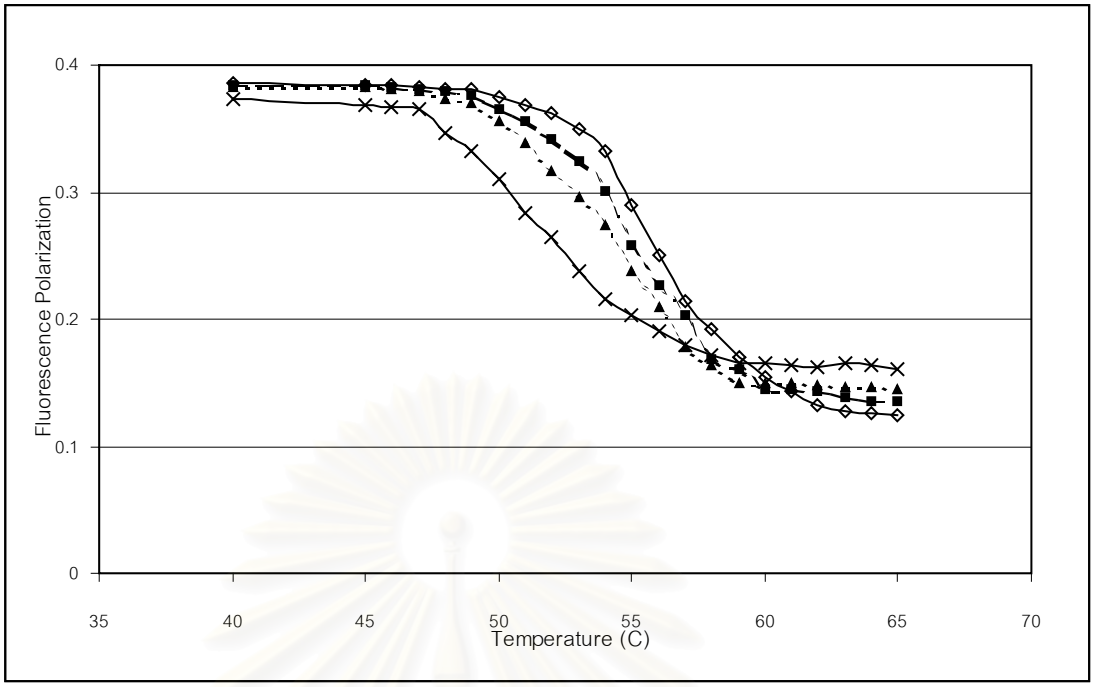
(A) fluorescence polarization (B) anisotropy

Concentration of DSPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

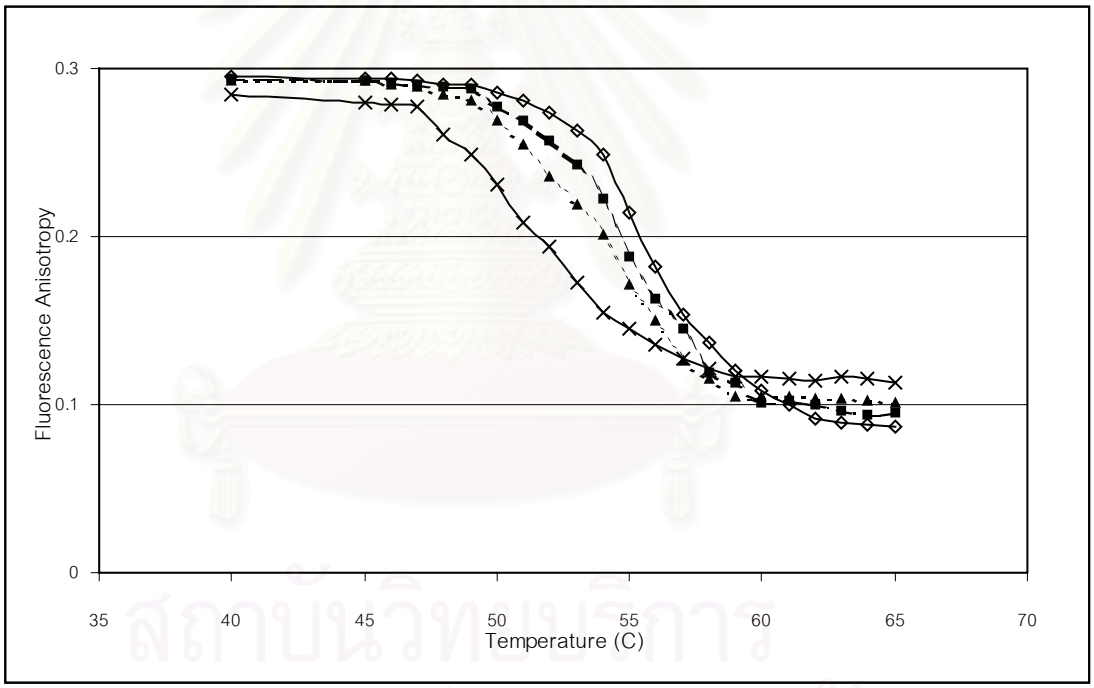



Figure 73 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol and DCP (100:10:10, by mole).

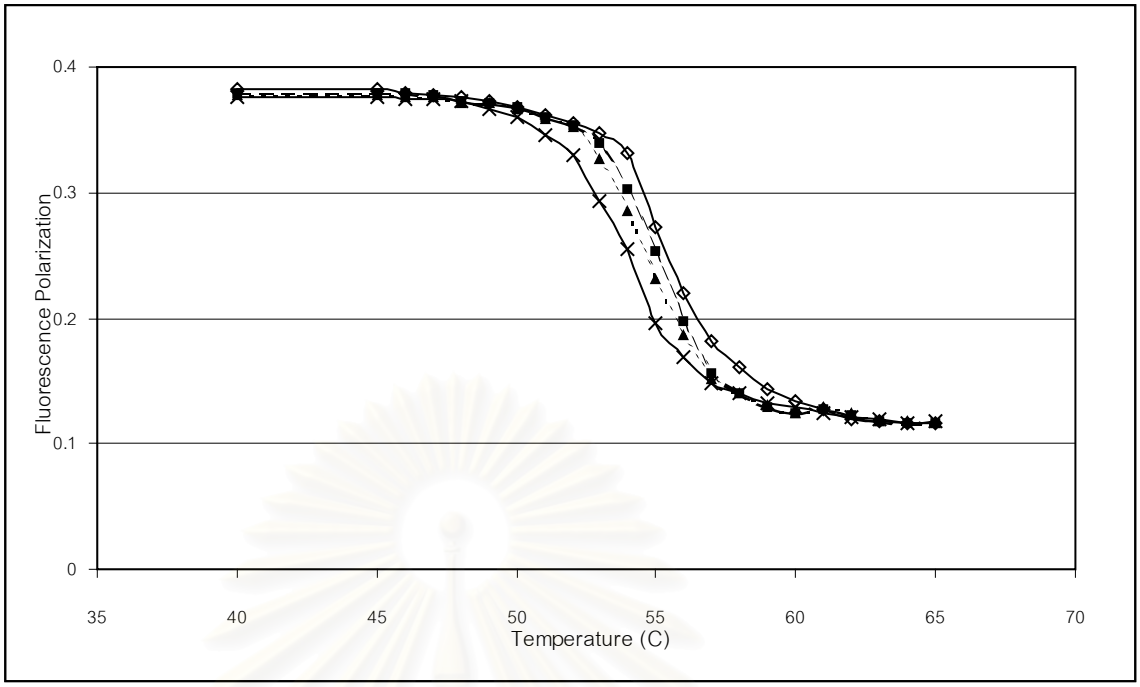
(A) fluorescence polarization (B) anisotropy

Concentration of DSPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

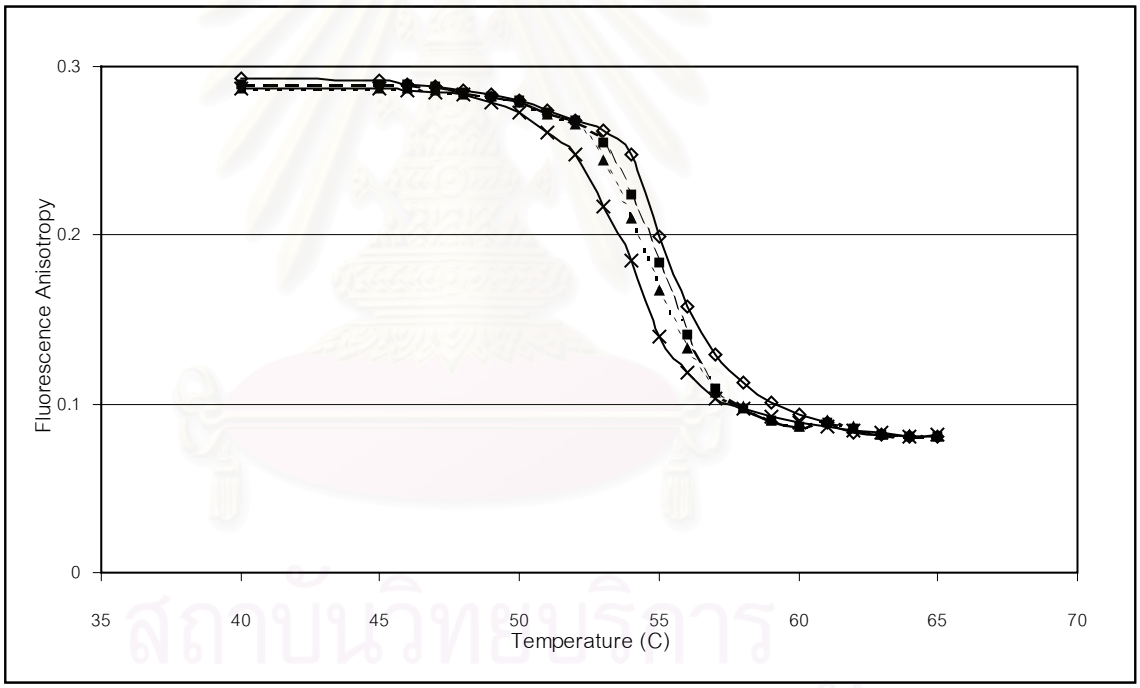



Figure 74 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol and SL (100:10:5, by mole).

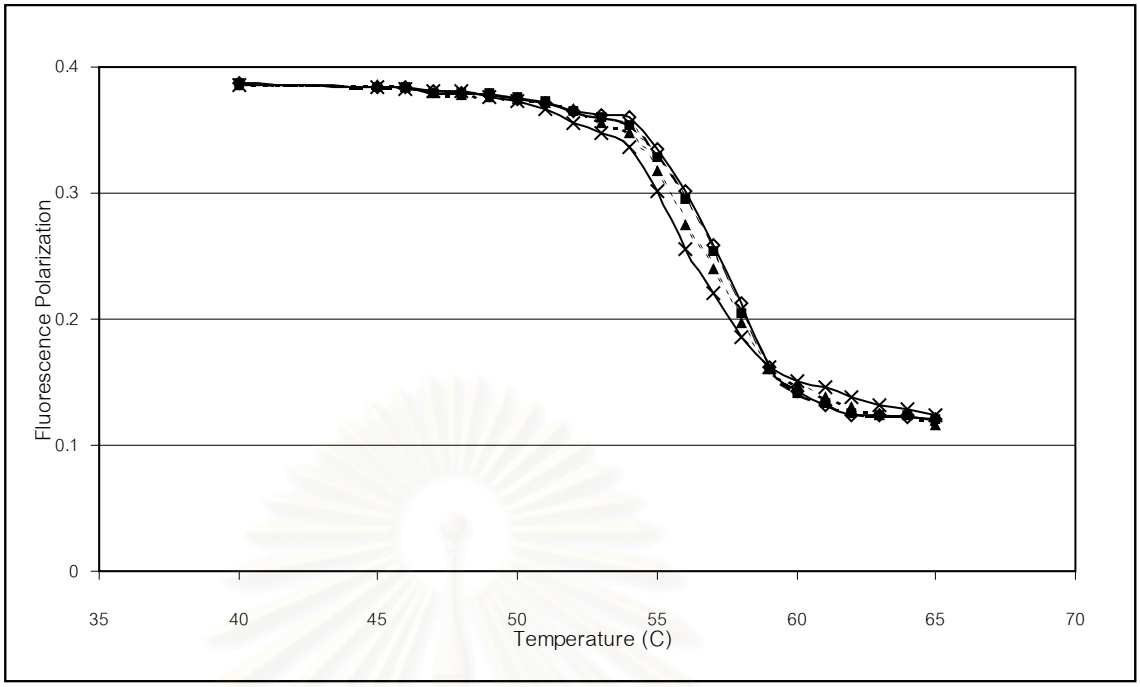
(A) fluorescence polarization (B) anisotropy

Concentration of DSPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

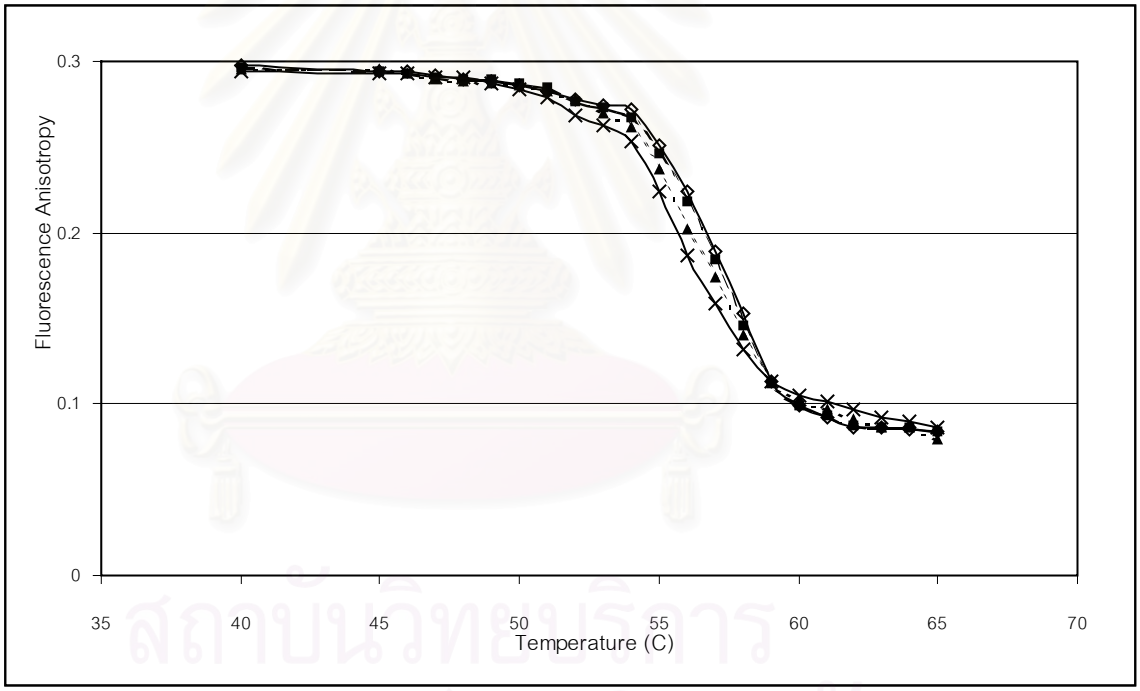



Figure 75 Effect of CPZ on thermotropic phase transition of DSPC liposomes containing cholesterol and SL (100:10:10, by mole).

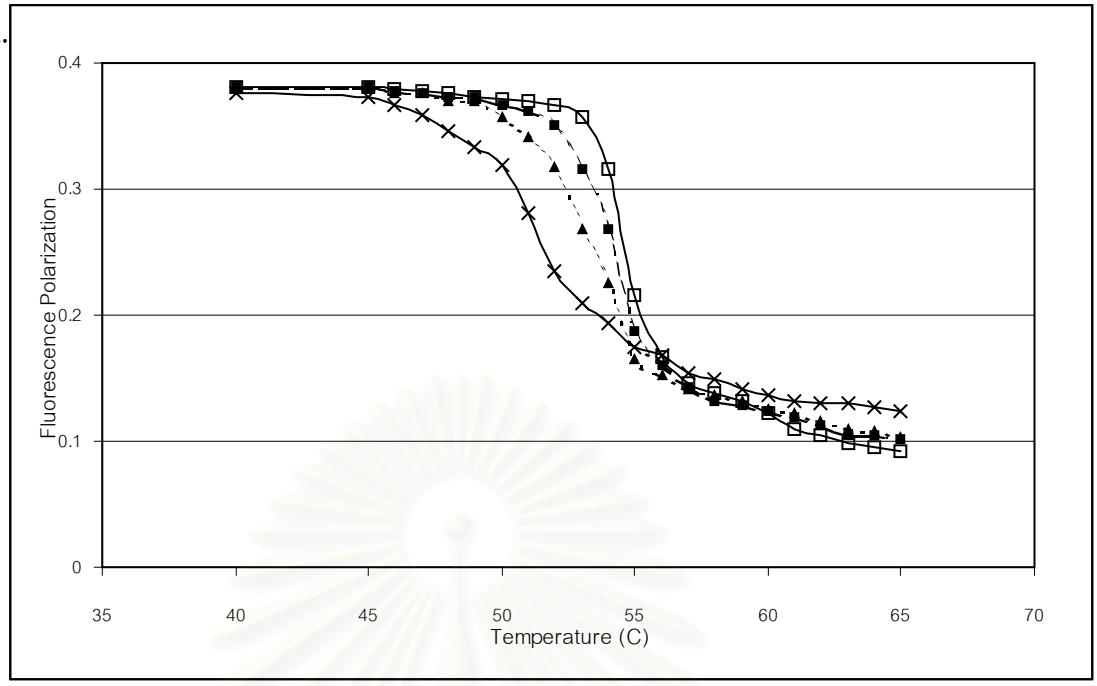
(A) fluorescence polarization (B) anisotropy

Concentration of DSPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.

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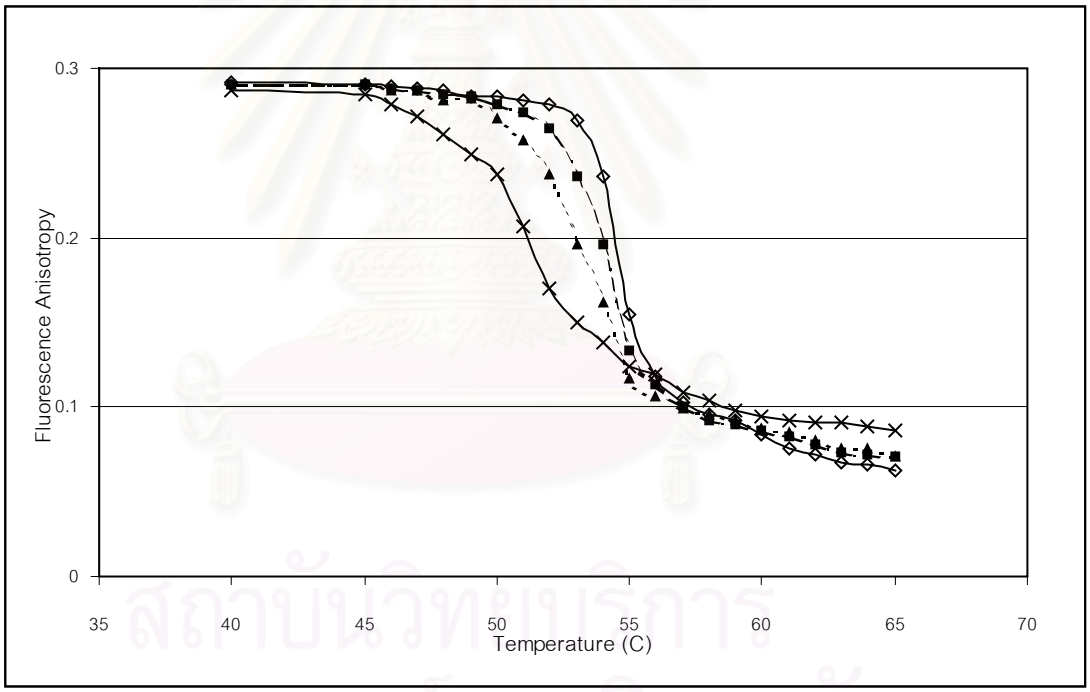



Figure 76 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol and DCP (100:10:5, by mole).

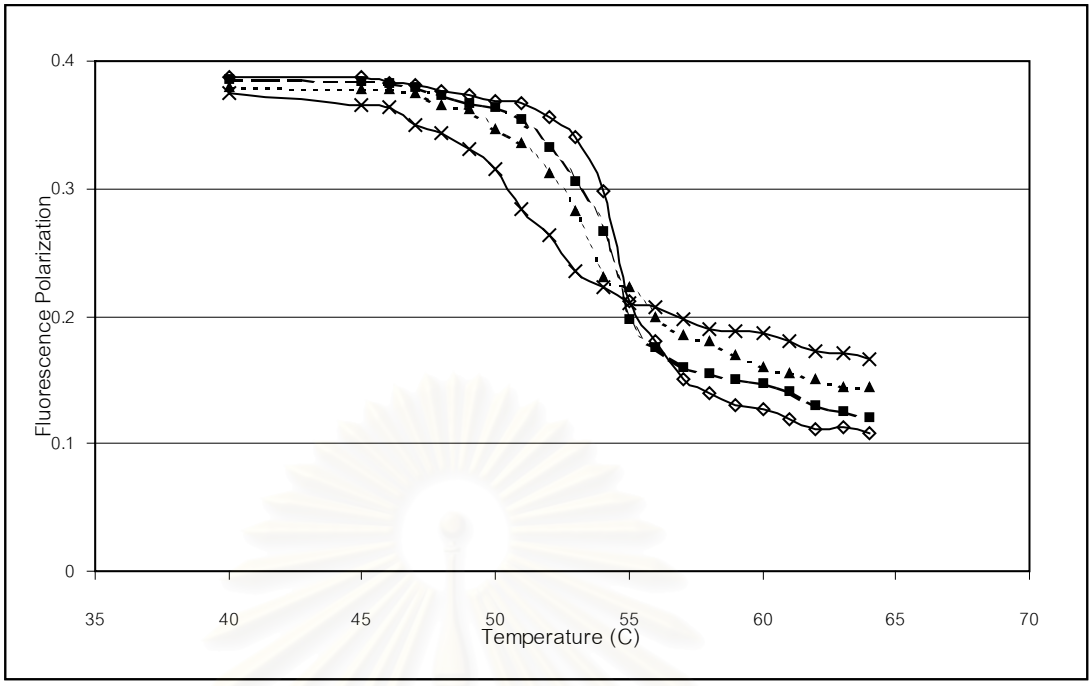
(A) fluorescence polarization (B) anisotropy

Concentration of DSPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.

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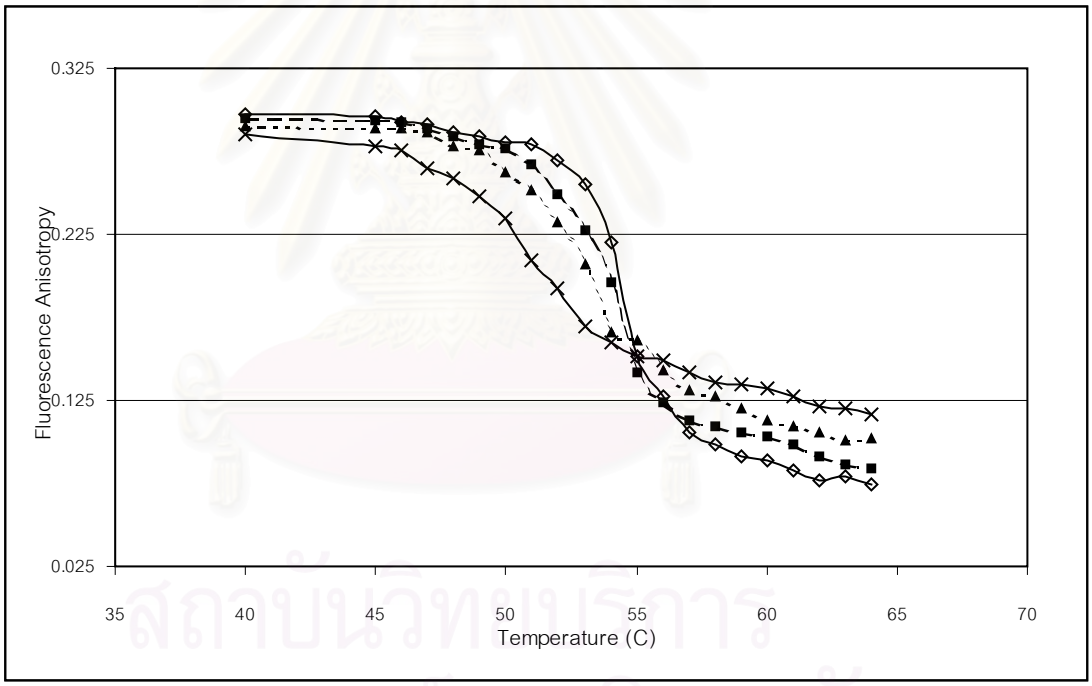



Figure 77 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol and DCP (100:10:10, by mole).

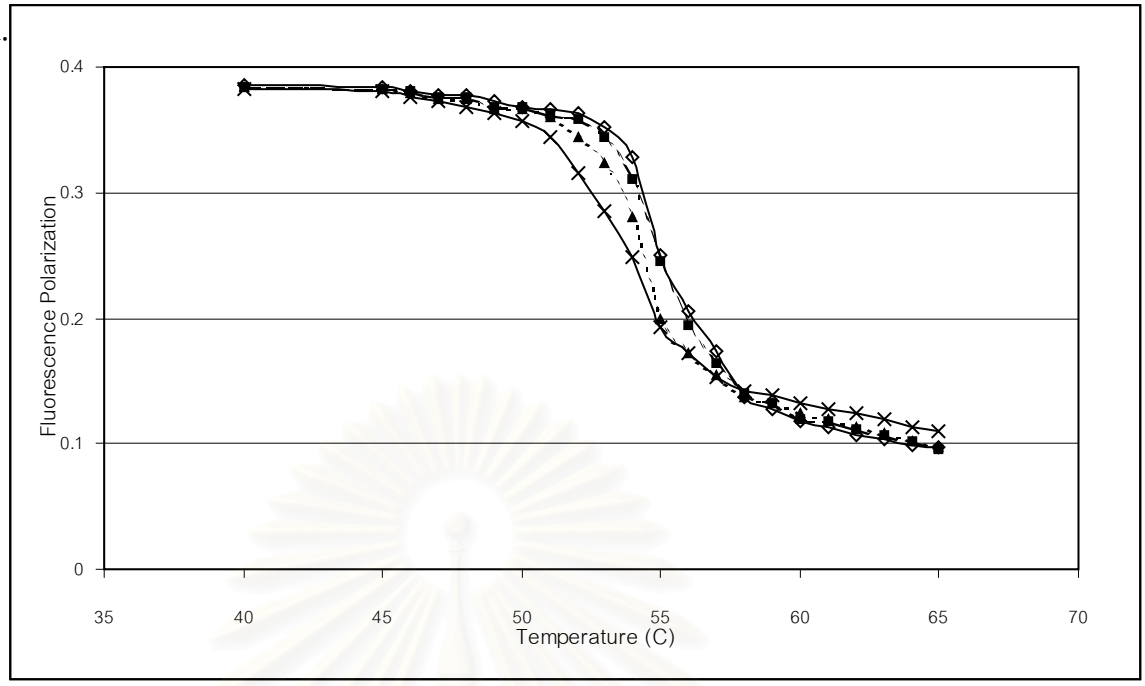
(A) fluorescence polarization (B) anisotropy

Concentration of DSPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

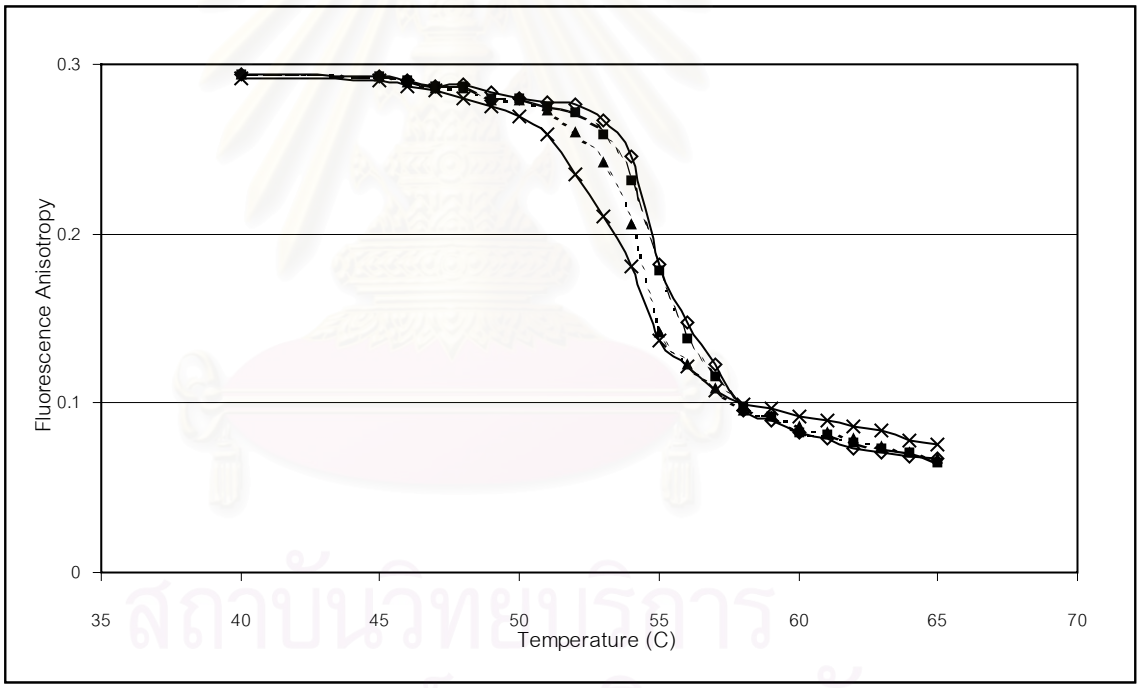



Figure 78 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol and SL (100:10:5, by mole).

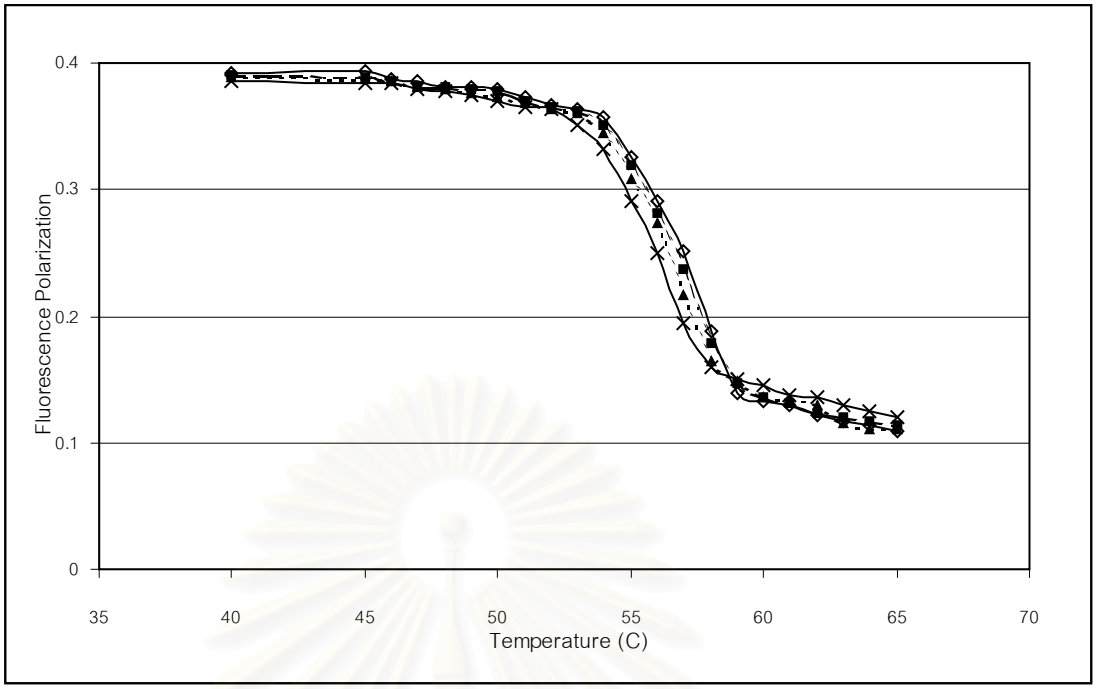
(A) fluorescence polarization (B) anisotropy

Concentration of DSPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

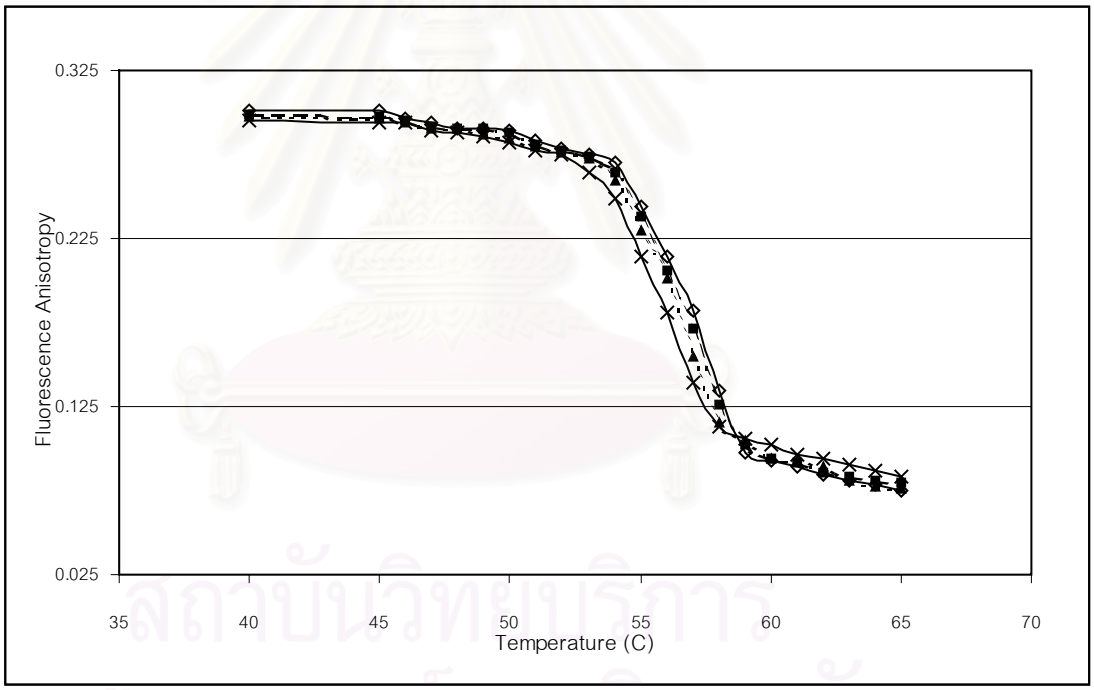



Figure 79 Effect of TRZ on thermotropic phase transition of DSPC liposomes containing cholesterol and SL (100:10:10, by mole).

(A) fluorescence polarization (B) anisotropy

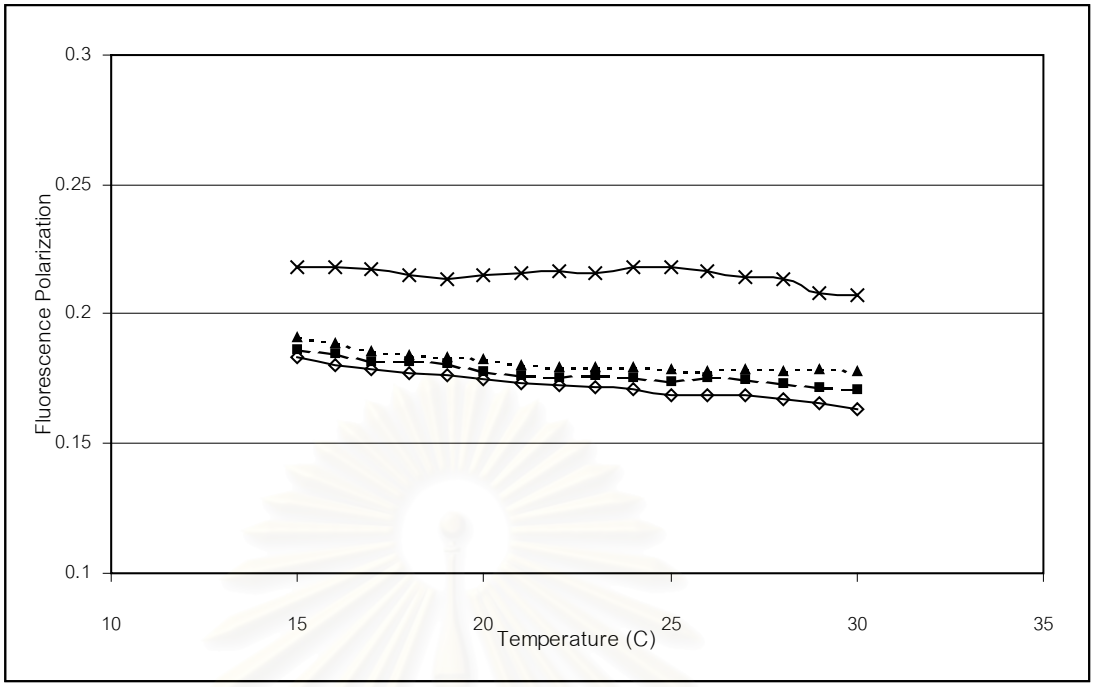
Concentration of DSPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

Phenothiazines still provided condensing effect on EPC incorporating cholesterol and charged amphiphiles as illustrated in Figure 80-87. This condensing effect was influenced by species and content of charged amphiphile, e.g., the effect was relatively high in the presence of negatively charged amphiphile (DCP) and also the higher content of DCP, the stronger effect on membrane. In general, TRZ exhibited stronger effect than CPZ.

A.




B.

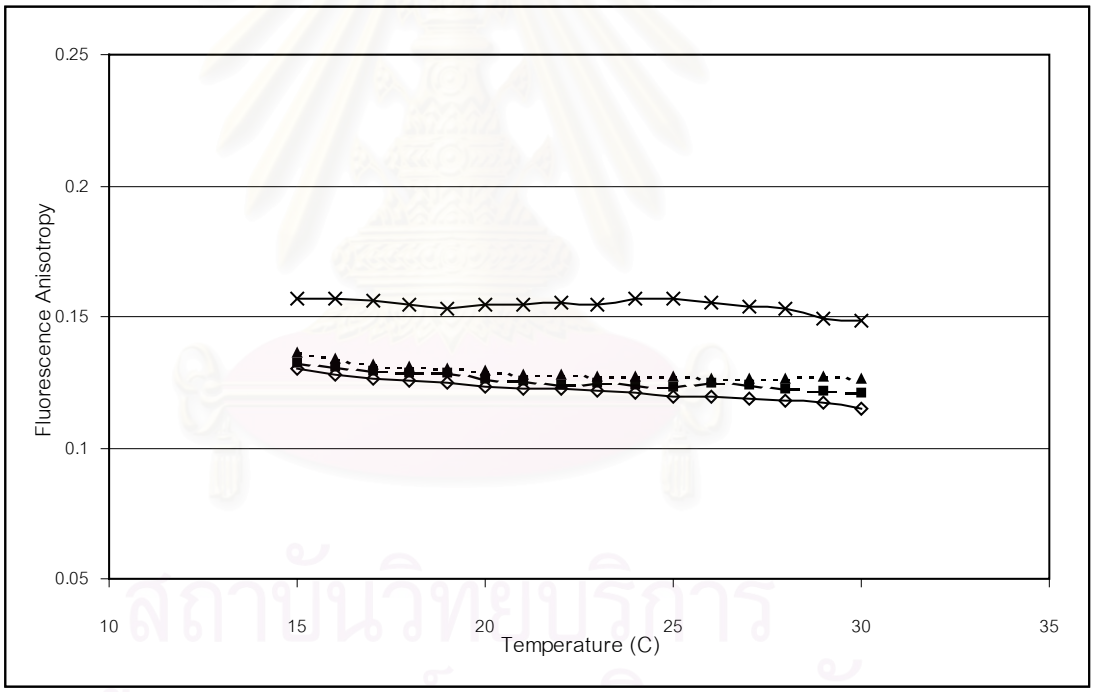



Figure 80 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol and DCP (100:10:5, by mole).

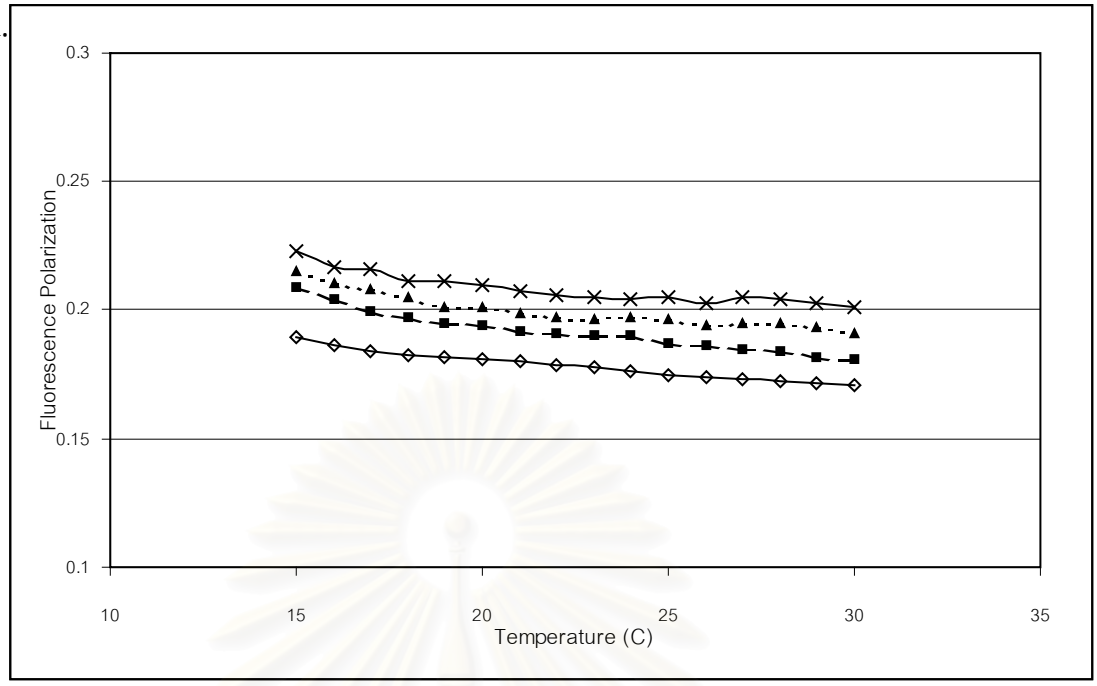
(A) fluorescence polarization (B) anisotropy

Concentration of EPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

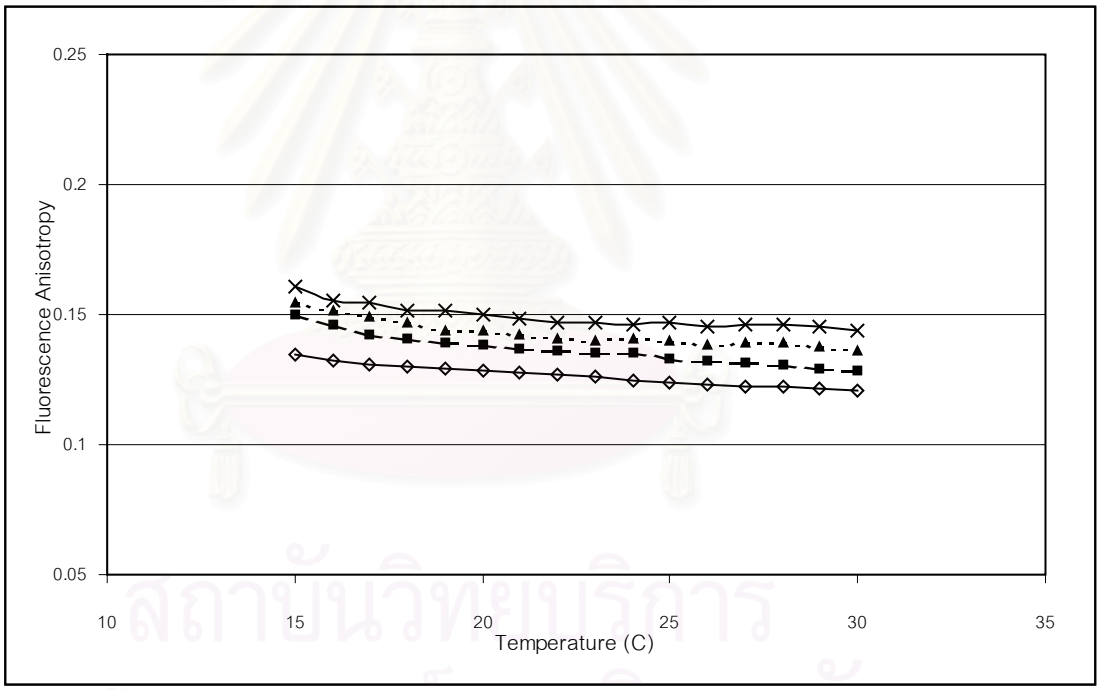



Figure 81 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol and DCP (100:10:10, by mole).

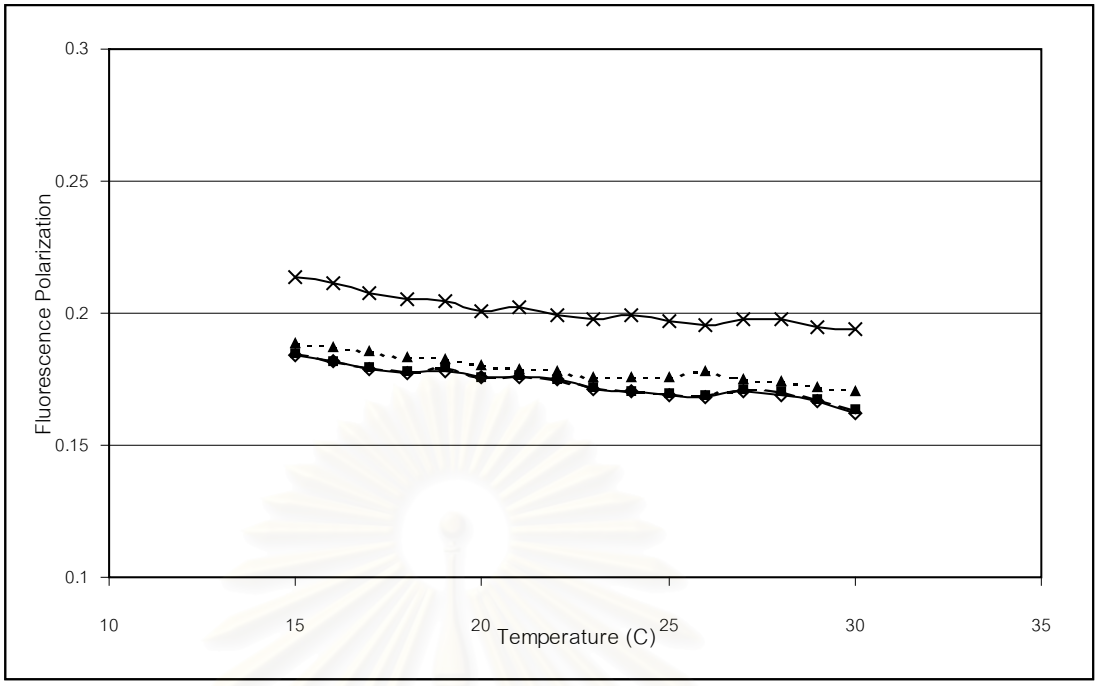
(A) fluorescence polarization (B) anisotropy

Concentration of EPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

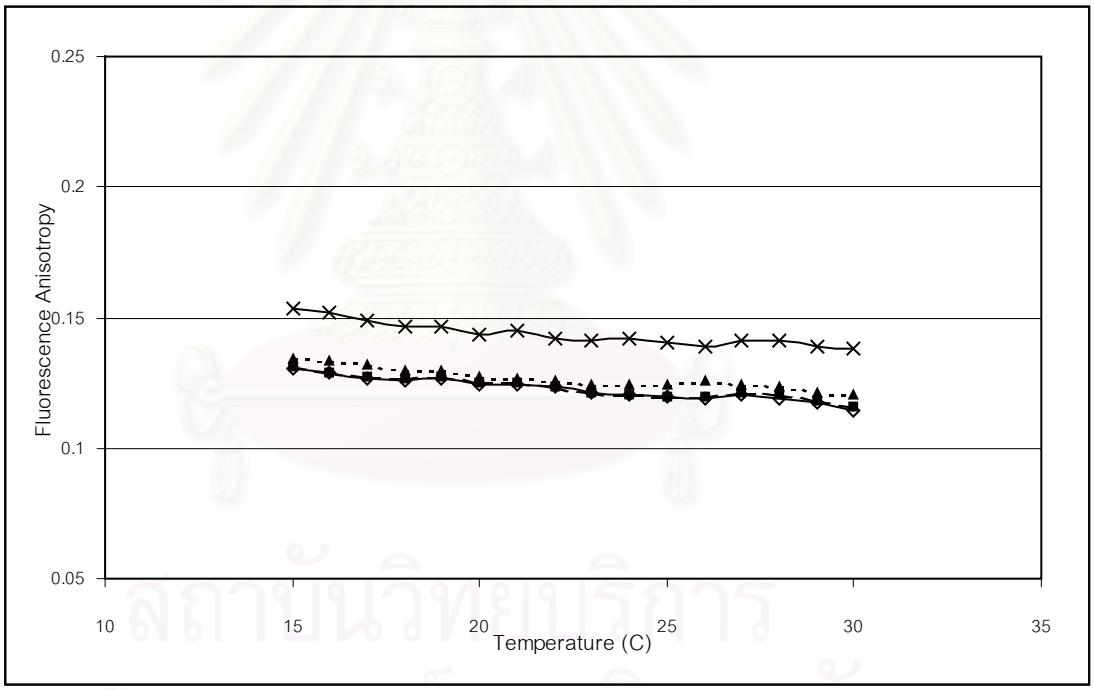



Figure 82 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol and SL (100:10:5, by mole).

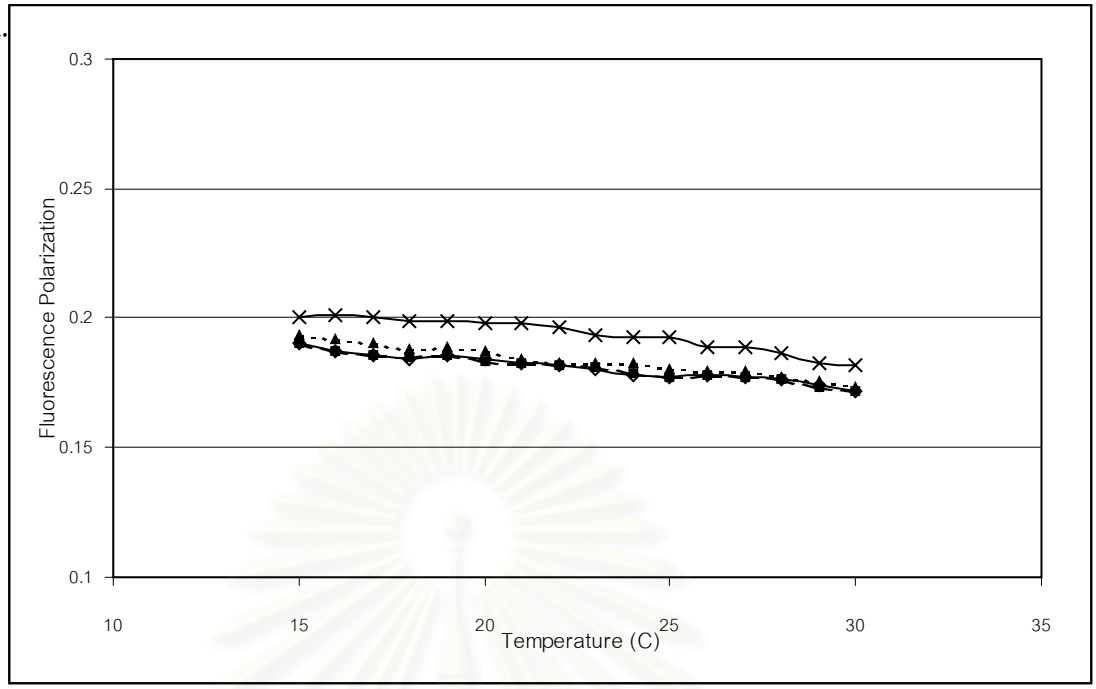
(A) fluorescence polarization (B) anisotropy

Concentration of EPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

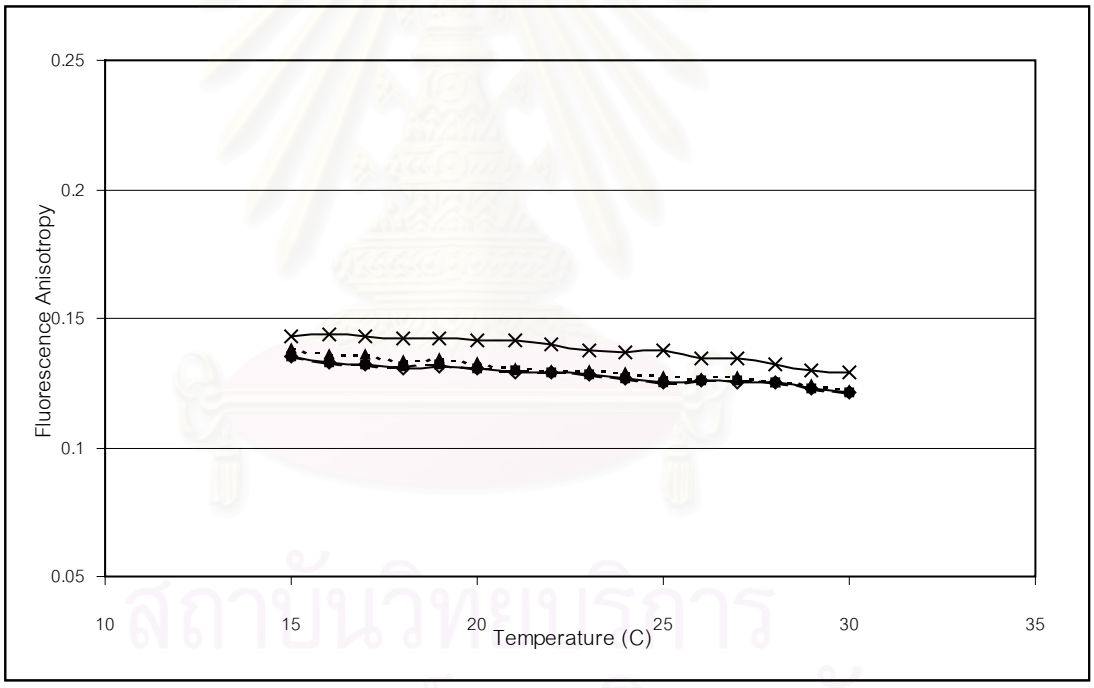



Figure 83 Effect of CPZ on thermotropic phase transition of EPC liposomes containing cholesterol and SL (100:10:10, by mole).

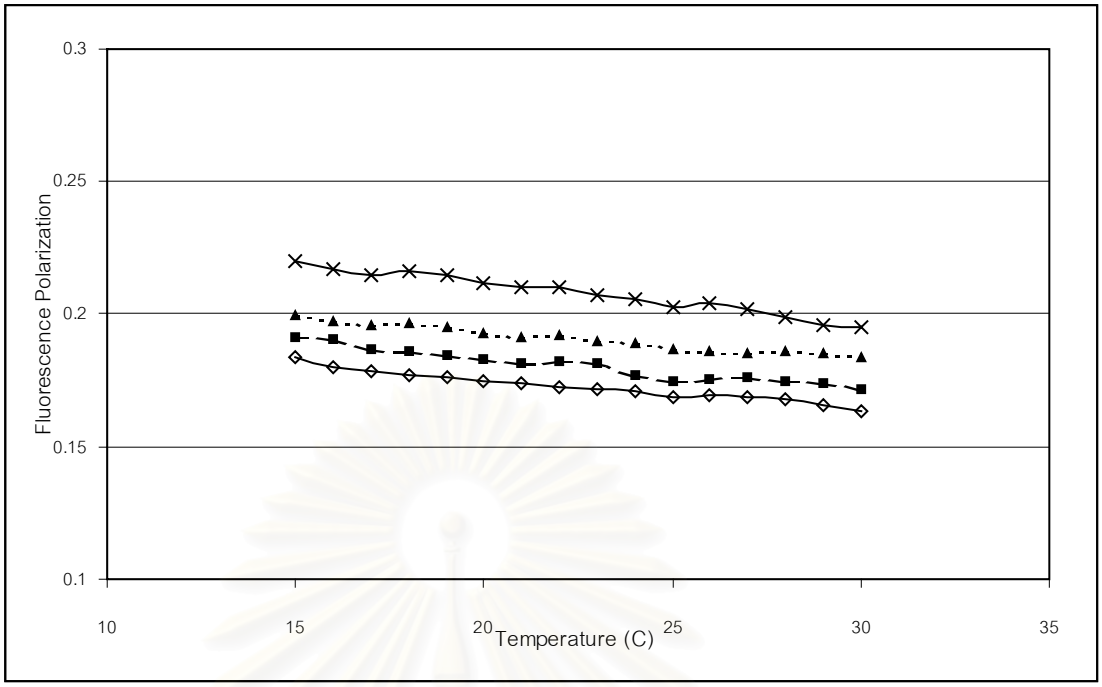
(A) fluorescence polarization (B) anisotropy

Concentration of EPC to CPZ, by mole:

◇ , without CPZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

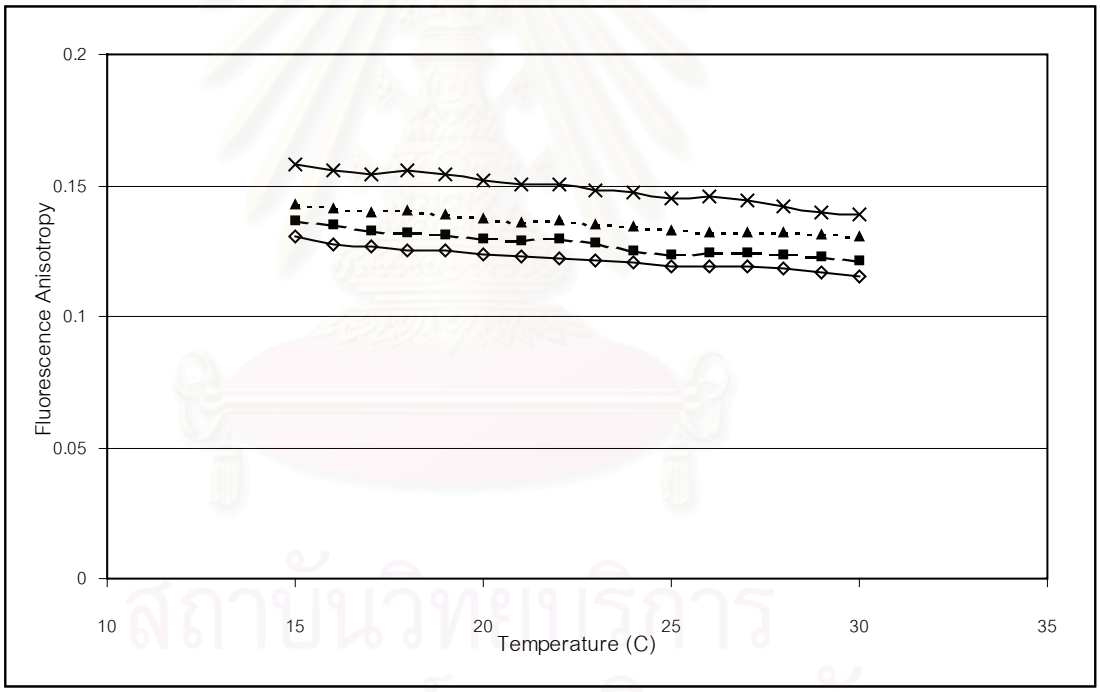



Figure 84 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol and DCP (100:10:5, by mole).

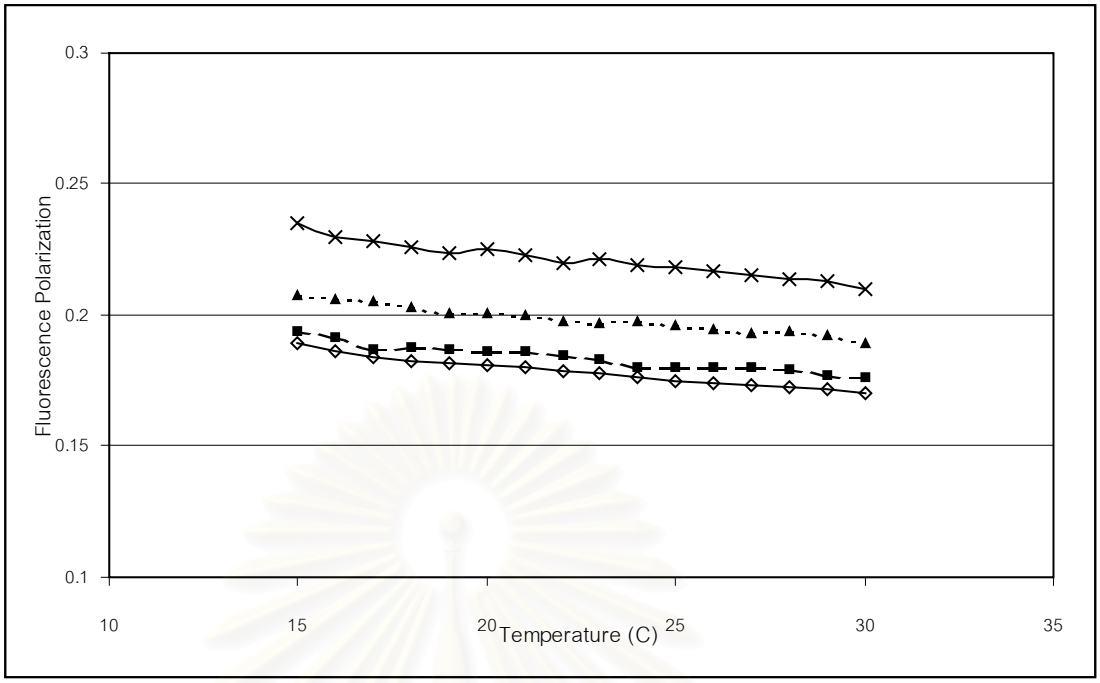
(A) fluorescence polarization (B) anisotropy

Concentration of EPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

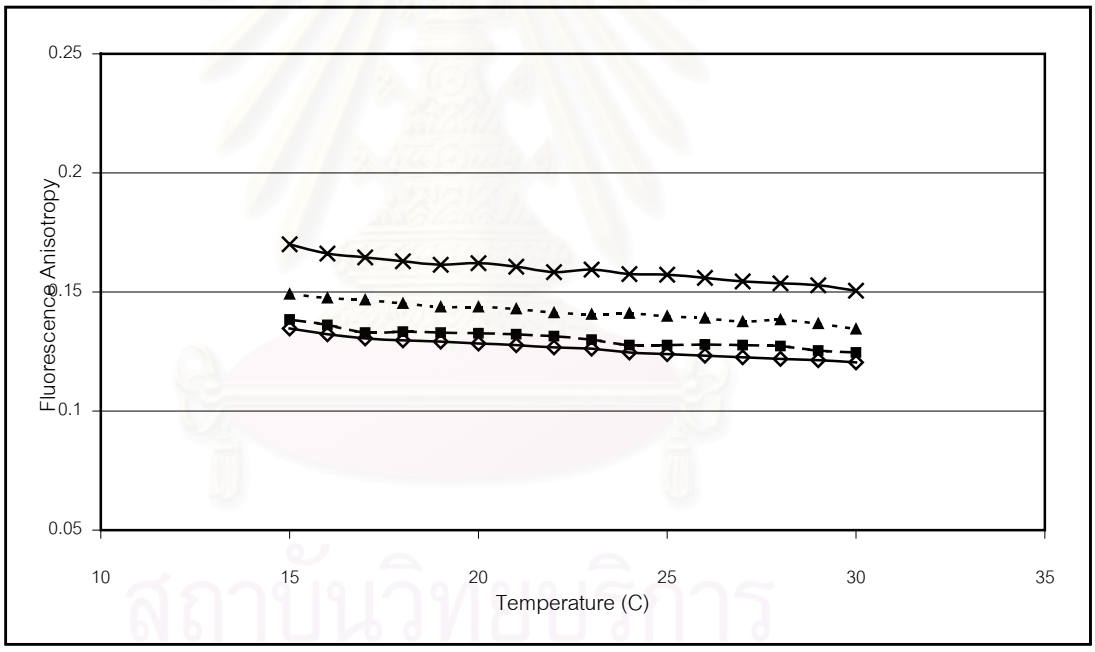



Figure 85 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol and DCP (100:10:10, by mole).

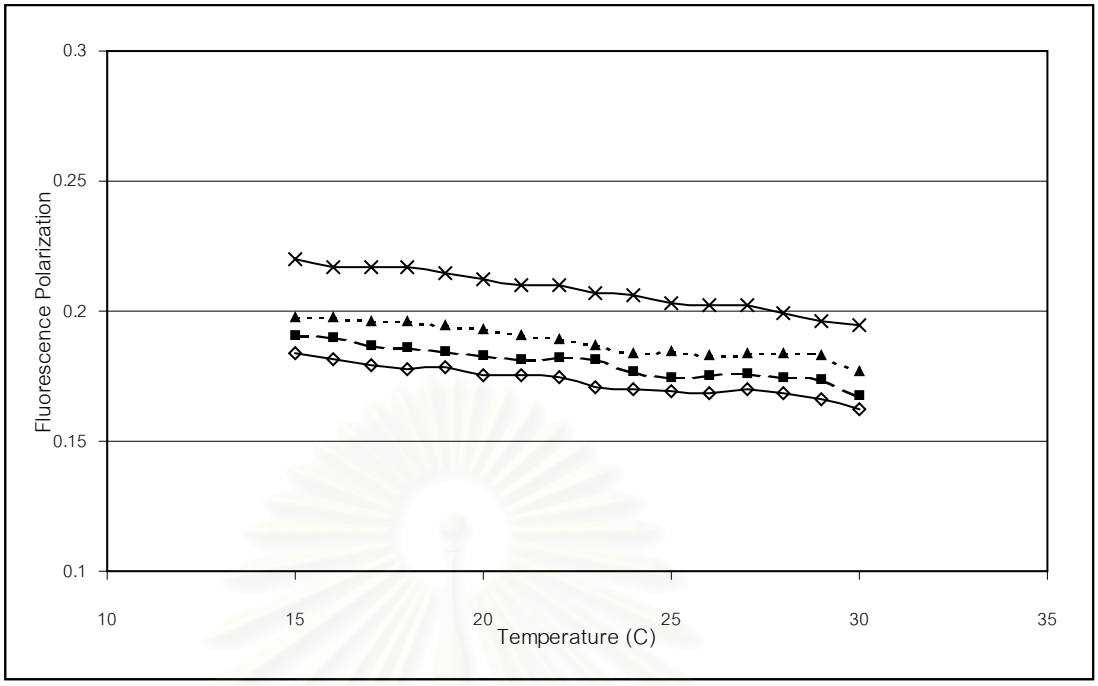
(A) fluorescence polarization (B) anisotropy

Concentration of EPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

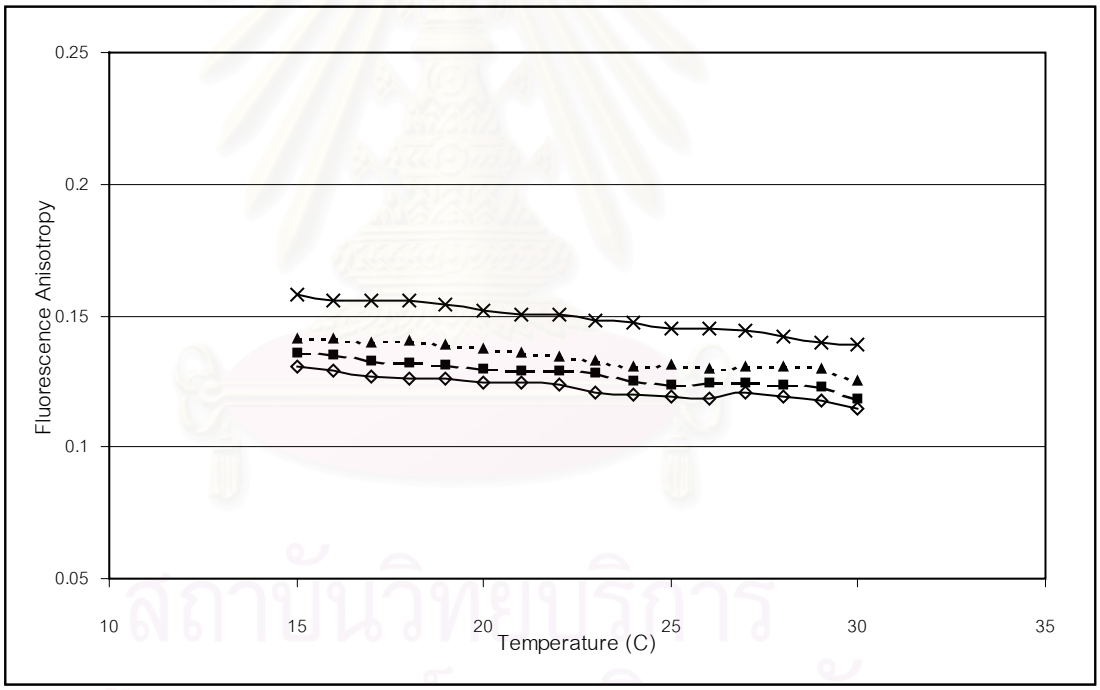



Figure 86 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol and SL (100:10:5, by mole).

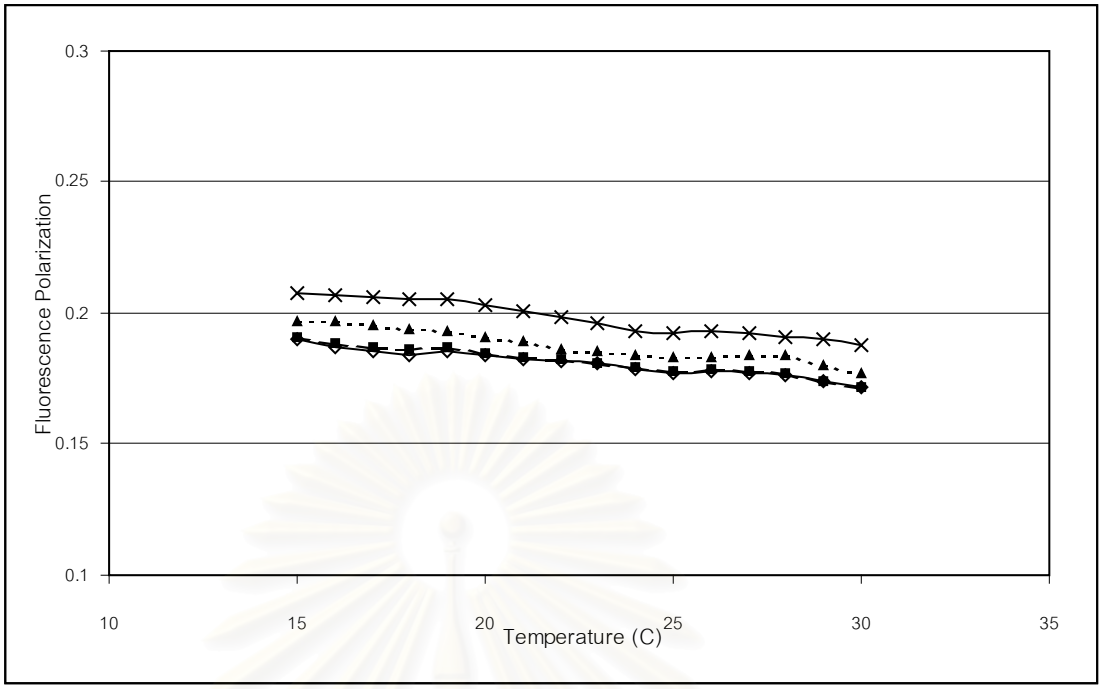
(A) fluorescence polarization (B) anisotropy

Concentration of EPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

A.




B.

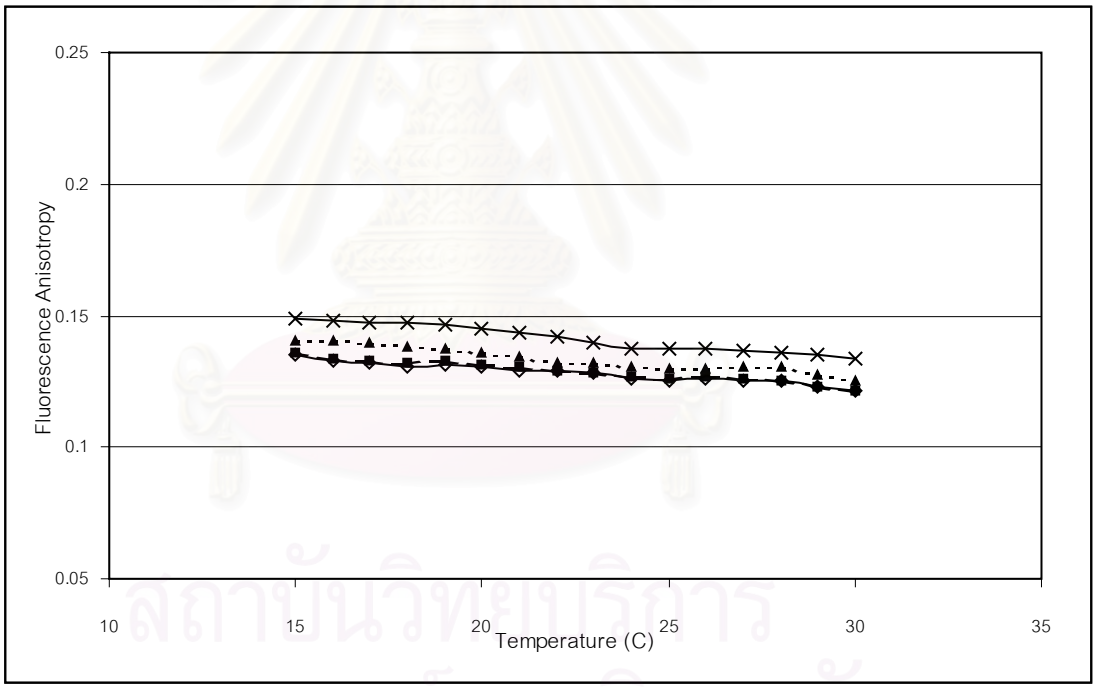



Figure 87 Effect of TRZ on thermotropic phase transition of EPC liposomes containing cholesterol and SL (100:10:10, by mole).

(A) fluorescence polarization (B) anisotropy

Concentration of EPC to TRZ, by mole:

◇ , without TRZ ; ■ , 75:1 ; ▲ , 25:1 and × , 7.5:1.

Each point represents the mean of at least three samples±S.D., bar was omitted for clarity.

From the experiments, it was shown that surface charge of lipid membrane had an important role on the interaction of phenothiazines with liposomes. Phenothiazines, both CPZ and TRZ had one positive charge at the experimental pH, then they should be interacted differently with negative or positive surface charged liposomes. Such an interaction might be exhibited by ionic interaction between cationic molecule and negative or positive surface charged liposomes. This membrane surface charge had a pronounce effect on the interaction of CPZ or TRZ with membrane when liposomes had negative surface charge because it could produce the interaction between cationic phenothiazine and negative surface charged liposome by electrostatic attraction therefore phenothiazine interacted with negatively charged liposome in a greater extent than did the neutral and positive ones. In contrast, charge-charge repulsion between cationic phenothiazine and positive surface charged liposome would be generated, therefore cationic drugs penetrated hardly into positive surface charged membrane, consequently by producing much smaller effect than the case of negative one. Moreover, it was found that the interaction of both CPZ and TRZ to negatively charged liposome, in general increased with increasing DCP content (Figure 64-65 and 68-69 for DMPC, Figure 72-73 and 76-77 for DSPC and Figure 80-81 and 84-85 for EPC) and vice versa in the case of positive surface charged liposomes (Figure 66-67 and 70-71 for DMPC, Figure 74-75 and 78-79 for DSPC and Figure 82-83 and 86-87 for EPC liposomes).

From investigations, it could be concluded that surface charge of liposomal membrane played an important role in the drug-membrane interaction.

# CHAPTER IV

# CONCLUSION

General structure of biological membrane is composed of a complex mixture of different lipids where intrinsic proteins are embedded in a liquid-crystalline lipid bilayer and peripheral proteins interact through polar interaction forces with the surface of the lipid bilayer. Lateral mobility of the phospholipids is in term related to the high flexibility of their hydrophobic fatty acyl chains may take place by various parameters in some regions of some biological membranes, concomitance with lateral motion of tightly bound intrinsic protein. The lateral motion of certain components are responsible for specific biological functions. Various factors such as drugs, temperature may cause lipid immiscibility in biological membrane since they modulate bilayer phase structure into gel or liquid-crystalline phase and whether tilted or non-tilted gel phase and their ability depend on kind of lipids, e. g., head-group structure and charge, as well as chain length and type of acyl chains of the lipid. This immiscibility can give a lateral phase separation into fluid domains in the plane of the membrane, and for transverse phase separation into an asymmetrical bilayer membranes, and/or possibly discontinuous bilayer membranes of different composition whose lipids are partially in the gel state and partially in the liquid-crystalline state should have a high lateral compressibility and extensibility. A lateral compressibility can facilitate the insertion of newly synthesized protein, lipid, or new membrane into old membrane and may also facilitate the kinetics of the translocation of protein carriers from one side of membrane to the other or motion of proteins within the membrane.

Phenothiazines are known to bind to biological membrane [Leterrier and Kersante, 1975] which may be a site of pharmacological action. It had been suggested that pharmacological action of phenothiazines could be due to physicochemical changes in the lipid part of membranes. Since small changes in membrane order can produce large changes in membrane functions and membrane lipid environment can directly influence the properties of the receptors which embedded in the phospholipid bilayer. To get insight in details of the event occurred in biological membrane, artificial membrane such as liposome was selected as a model membrane with known lipid compositions and alteration of physical state of liposomal membrane caused by phenothiazines was characterized by fluorescence polarization technique. A fluorescent probe molecule, 1-6-diphenyl-1,3,5-hexatriene (DPH) was used to monitor fluidity within the hydrophobic portion of the phospholipid bilayer and to detect changes in fluidity accompanying the gel to liquid crystalline phase transitions in these systems. For imitation of natural cell membrane, synthetic phosphatidylcholine with various acyl chain lengths, e.g., DSPC (C18:0) and DMPC (C16:0) , a natural phospholipid, egg yolk phosphatidylcholine , cholesterol, major constituents of sterol in biological membrane were selected as compositions of liposomal membrane and liposomal surface charged property was modified by using charged amphiphiles such as dicetyl phosphate (DCP) as negative charge and stearylamine (SL) as a positive one. Chlorpromazine (CPZ) and thioridazine (TRZ) were selected as phenothiazine drug of choice.

Data presented in this investigation suggested that both CPZ and TRZ fluidized lipid bilayers composed of pure synthetic phosphatidylcholine (DMPC and DSPC) below lipid thermotropic phase transition temperature ($T_m$) but above $T_m$ it exhibited condensing effect and $T_m$ was also shifted to lower temperature. However, phenothiazine provided only condensing effect on liposomes composed of natural

phospholipid (egg yolk phosphatidylcholines) since these liposomes were in fluid state overall temperature range studied. Incorporation of cholesterol into phosphatidylcholine liposomes, similar action of phenothiazines on liposomal membrane as previously described in pure synthetic phosphatidylcholine liposomes was still obtained but with the less extent. This might obtain by reduction of drug penetration caused by dense packing in liposomal membrane upon incorporation of cholesterol. At high molar ratios of phospholipid:cholesterol (100:50 and 100:75), thermotropic phase transition was abolished. Modification of liposomal surface properties by addition charged amphiphiles into lipid bilayer composed of phosphatidylcholine-cholesterol enhanced $T_m$ and exhibited ordered state in liposomal membrane, probably by coexistence of binary phase and/or mismatch at hydrocarbon termini in hydrophobic region of bilayer. Same phenomena as mentioned earlier in action of phenothiazines on pure phosphatidylcholine liposomes were also achieved, with pronounced effect in negatively charged liposomes, consequently by neutral and positively charged liposomes, respectively. Electrostatic attraction between positive charge on phenothiazine and negative charge on liposomal membrane might promote drug penetration into lipid bilayer and vice versa in positively charged liposomes. Action of phenothiazines on all lipid systems presented was concentration dependence, therefore the fluorescence polarization technique studied here is proved to be one of an highly efficient method in characterization membrane properties, especially phase transition.

In conclusion, chlorpromazine and thioridazine modulated physical properties of membrane by localizing themselves near the hydrophobic tail region of the lipid acyl chain and perturbed their structure depending on the state of membrane phase. They provided disordering effect to ordered structure (gel phase), hence making the membrane more fluid (fluidizing effect), on the contrary condensing effect to membrane which lipid components rearranged in disordered state (fluid

phase) was exhibited upon addition of phenothiazines. The substantial evidence demonstrated here supported site of action of phenothiazine and their effect on lipid constituents in artificial membrane (liposome) which may be explained natural phenomena occurring in biological membrane and may be used as a clue or prototype for studying interaction of another drug with artificial or native membrane.

Since fluorescence polarization are changed depending on state of lipid compositions in membrane and effects of phenothiazine on membrane were also concentration dependence therefore manipulation effective dose of phenothiazine on drug therapy may be possible if there are quantitative physiological changes in nerve tissues of schrizophrenic patient. Further comparative studies with other phenothiazines and also with other independent experiments are under investigation.

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