CHAPTER IV

RESULTS AND DISCUSSION

Nakamaru and his coworkers (1972,1974,1977) concluded from their studies that BCP, one of the sulfonphthalein dye, presented two parameter changes when used as an optical probe for biological membrane, i.e., a spectral shift of λ_{max} (wavelength of maximum absorption) and a change of absorption intensity at λ_{max}. Spectral shift of 10-15 nm. to longer wavelength was resulted from encountering to the hydrophobic environment of the BCP molecules compared to the maximum absorption wavelength in Tris - HCl buffer pH 8.8 at 589 nm. At this longer wavelength, BCP oriented in monomer conformation. Another absorption band appearing at 554 nm. was due to membrane-dimer formation. In addition to these two parameters, spectral shape as well as peak area were used in the present study for interpretation the binding of BCP to various model membranes. Tris - HCl pH 8.4 was used instead of pH 8.8 used in the previous experiment due to lesser binding of BCP to sarcoplasmic reticulum membrane at high pH, [Nakamaru, 1974; 1977]. Moreover, BCP also completely ionized to divalent anion form at pH 8.4 (pKa_2 of BCP was 6.3) and this form of dye only could be used as an optical probe because spectral changes were illustrated when its environment was altered.

A. Effect of acyl chain length of phospholipids.

The absorption spectra of BCP in the presence of DLPC, DMPC, DPPC and DSPC liposomes were shown in Figure 6. and their absorption intensities were summarized in Table 1. The absorption maxima around 598 nm. were belonged to monomeric form of the dye in membrane, [Nakamaru;1974, 1977]. In the cases of DLPC and DMPC which were fluid at experimental temperature, 25 °C, absorption intensity at this wavelength was higher than those of DPPC and DSPC. These might be caused by increasing amount of bound BCP to fluid phase compared to gel phase counterparts. Since enhancement of a fatty acyl chain length from 12 to 18 carbon atoms resulted in increasing rigidity of liposomal membranes as revealed by fluorescence polarization value concomitant with reduction in intensity at 598 nm. was observed. It was resulted from diminution of membrane - monomers, [Lentz, R.B.,et al., 1976] . Similarity in spectral shape around 582 nm. between fluid phase and gel phase lipids might be explained that it was the general pattern of BCP spectrum. However, the combinable peak at 557 and 575 nm. might be due to two conformations of membrane - dimer as suggested by Nakamaru (1977), eventhough this dimer peak was not clearly defined. The 2nd derivative of normal spectra could separate monomer peak from dimer one. Measurement of peak area by using cutting and weighing technique was performed and results were illustrated in Figure 7.

and Table 2. The largest membrane monomer and dimer were obtained in DMPC bilayer because membranes were unstable at temperature near T_C while the lowest monomer was demonstrated in DSPC due to the highest membrane rigidity. Consequently, it should be concluded that BCP binding to more fluid membrane oriented in monomer conformation, in contrast, dimer forms were obtained in more rigid membrane. Another evidence for dimer formation when membrane changed from liquid crystalline phase to gel phase was supported by study of merocyanine 540 as an optical probe, [Peter, I., et al., 1980].

Figure 8. depicted fluorescence polarization and anisotropy of phosphatidylcholine liposomes which acyl chain was varied from C 12 to C 18 atoms. High value of fluorescence polarization and anisotropy indicated high rigidity in hydrocarbon region which acyl chains were restricted motion. In contrast, low value referred to more fluidity. This was because above transition temperature, 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_C, it illustrated a high degree of orientation. In experiment, the longest acyl chain length phospholipid, DSPC, showed the highest fluorescence polarization compared to the shortest one, DLPC. These indicated that DSPC liposomes exhibited the highest rigidity, while DLPC illustrated the highest fluidity. Fluorescence polarization results corresponded with BCP in the previous experiment (Figure 6).

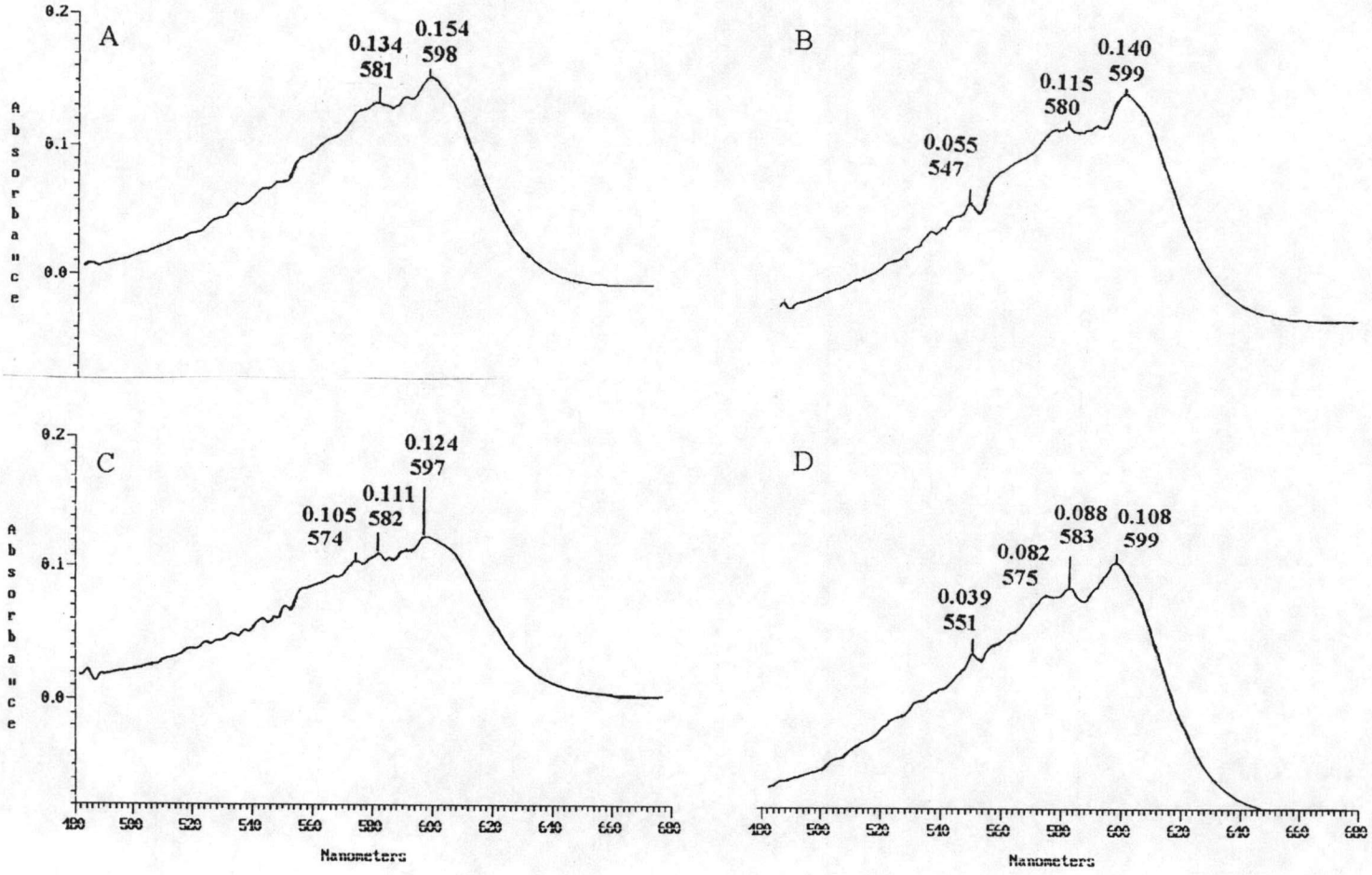


Figure 6 . Absorption spectra of BCP in liposomal membranes made from phosphatidylcholines at 25 °C
A. DLPC ; B. DMPC ; C. DPPC and D. DSPC

Table 1. Absorption intensity at each wavelength of BCP in membranes made from phosphatidylcholines.

Liposomal composition	Wavelength (nm.) and intensity (absorbance unit)			
DLPC	598 (0.154)	–	–	–
DMPC	599 (0.140)	–	–	547 (0.055)
DPPC	597 (0.124)	574 (0.105)	–	–
DSPC	599 (0.108)	575 (0.082)	551 (0.039)	–

Table 2. Peak area of monomer and dimer bands of BCP from membranes composed of various acyl chain length phospholipids [a].

Liposomal composition	Weight (mg.) [b]		Total weight (mg.)	Dimer / monomer
	Monomer	Dimer		
DLPC	4.095±0.035	7.885±0.064	11.98	1.926
DMPC	5.730±0.060	11.055±0.050	16.785	1.929
DPPC	2.380±0.030	9.865±0.071	12.245	4.145
DSPC	2.180±0.008	9.662±0.023	11.842	4.432

a Measurement was performed by weighing of peak area from second derivative spectrum.

b Each value represents the mean of three samples ± S.D.

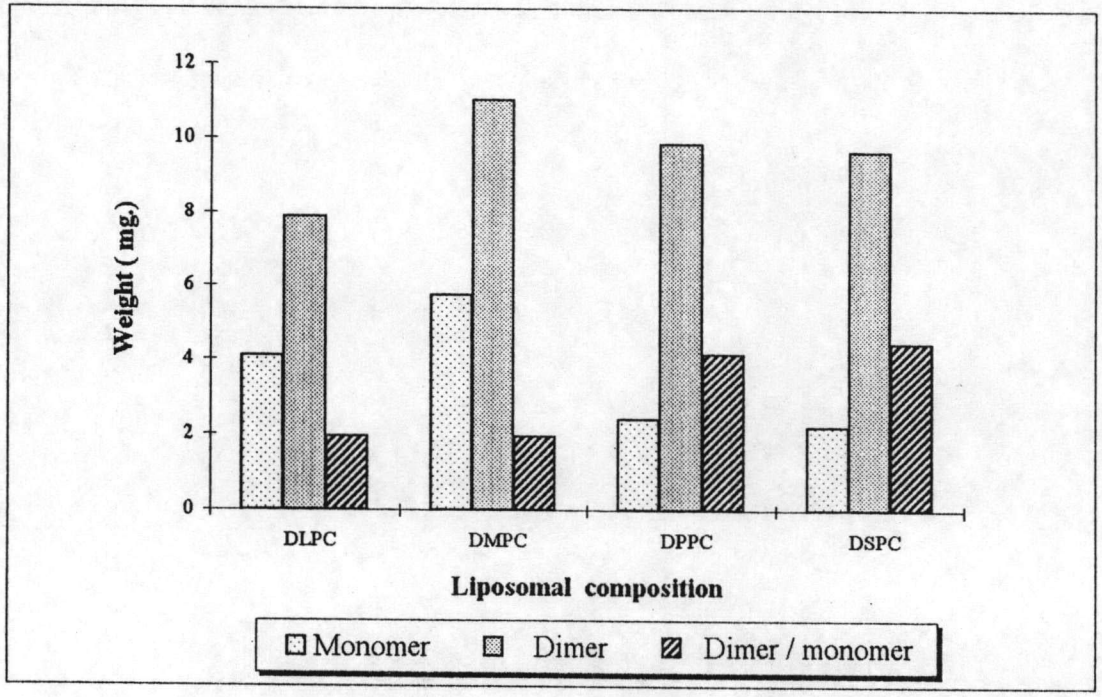
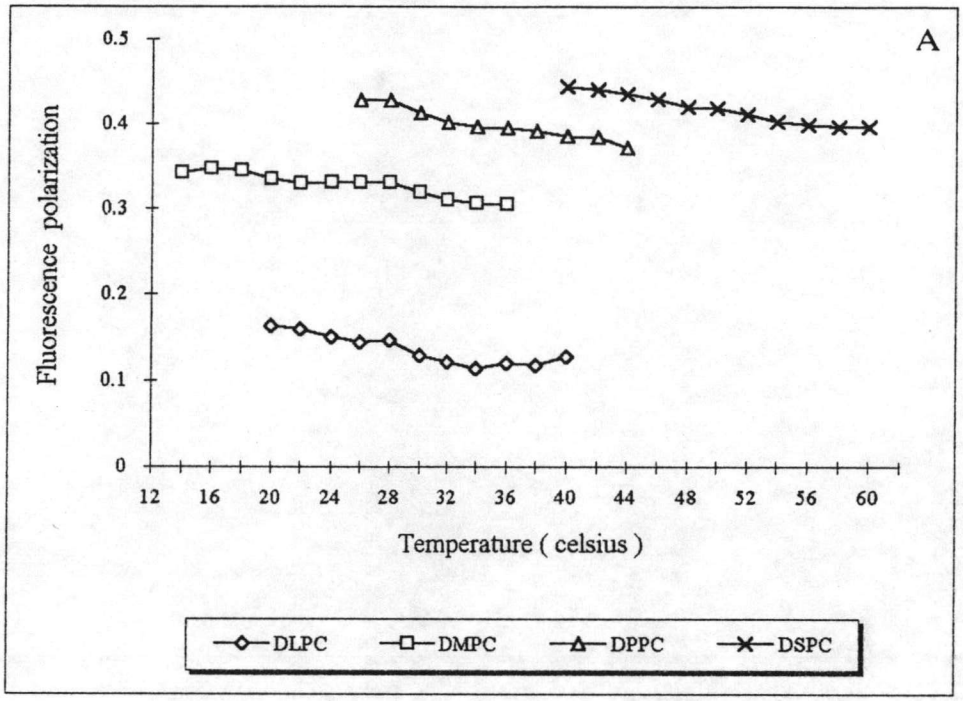


Figure 7. Peak area of monomer and dimer bands of BCP from membranes composed of various acyl chain length phospholipids.



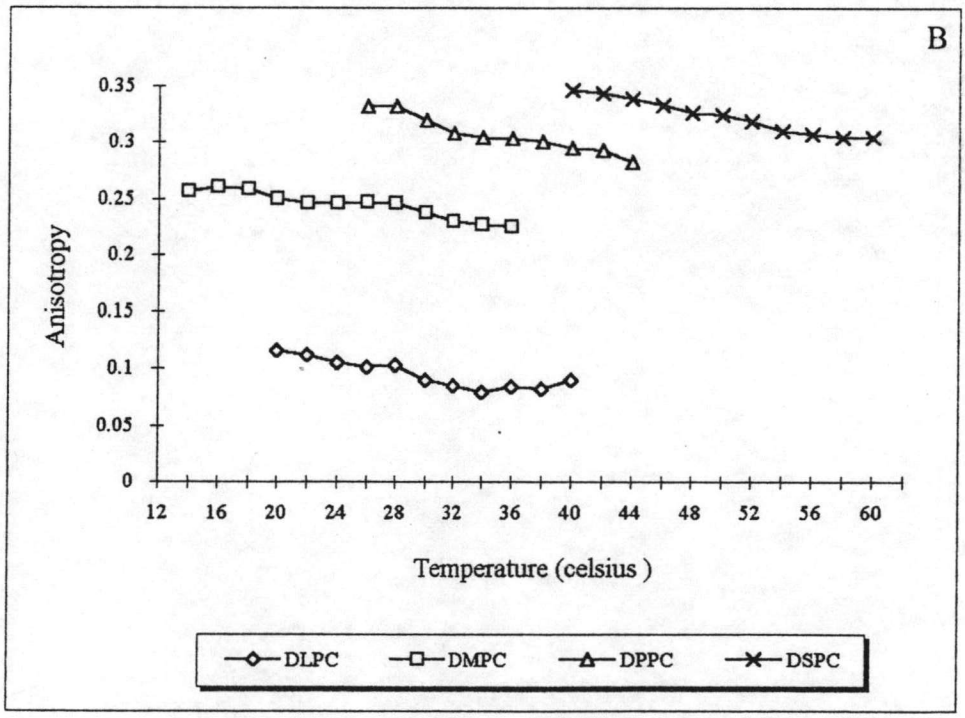


Figure 8. Fluorescence polarization (A) and anisotropy (B) of DPH in phosphatidylcholine liposomes having acyl chain length of C 12 - C 18.

B. Effect of temperature on binding of BCP on dimyristoylphosphatidylcholine.

To clarify binding properties of BCP to different state of membranes, dye was incubated with REV liposomes composed of DMPC at various temperatures. Significant shift to longer wavelength with increasing in intensity was observed when vesicles were incubated at temperature above phase transition temperature of DMPC (23 ^{o}C) as shown in Figure 9. and Table 3. This might be due to partitioning of bound monomer to more hydrophobic environment deep in membrane. The more the wavelength shift the deeper the located dye.

When temperature was risen to 32 ^{o}C, maximum absorption at 609 nm. in Figure 9E indicated that the location of dye was more hydrophobic than that of n-butanol as suggested by Nakamaru (1974). In Figure 9D and 9E, however, membrane dimer bands of both conformations showing λ_{max} at 551 and 575 nm. still appeared although slightly small amount compared to membrane monomer. These two dimer forms might be due to the overlaping of π-electrons on BCP molecules when they were close to each other in different conformations, [Nakamaru,1977]. Nevertheless, when temperature was decreased and bilayers transformed from fluid phase to gel phase, a fraction of membrane monomers still remained. Since BCP bound electrostatically to positive charge on lipid molecules and was holden in certain distance; thus hydrophobic part of

the dye molecule would easily partition to hydrophobic region near aqueous interface. Moreover, remained monomers would reorient to be the dimer conformations corresponding to high membrane density, as illustrated by diminution of monomer intensity with increasing dimer forms. Aqueous - dimer peak (544 nm.) appeared as shown in Figure 9A because the amount of free - dye in medium was too high to form dimer.

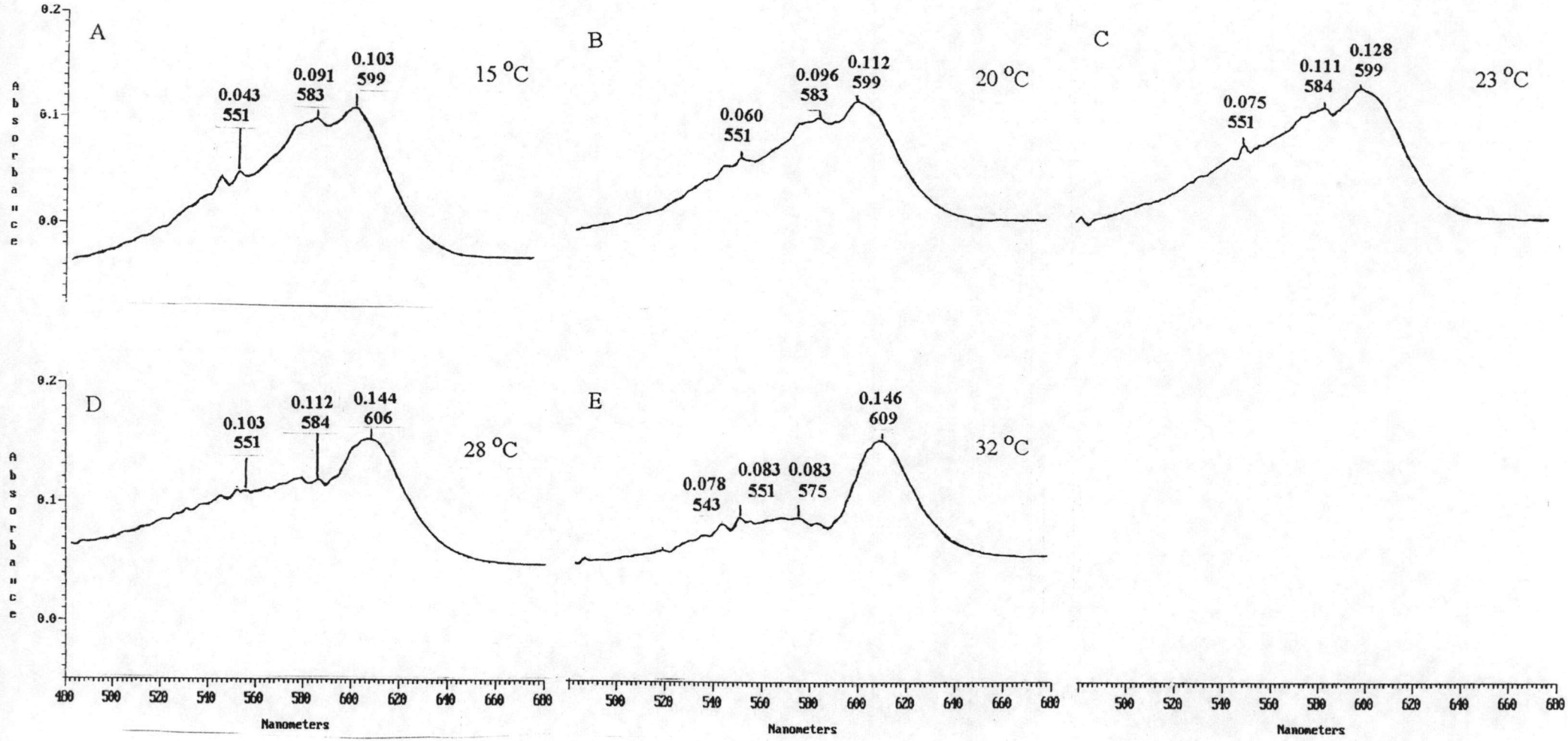


Figure 9 . Absorption spectra of BCP in liposomal membranes made from dimyristoylphosphatidylcholine at various temperatures as indicated

Table 3. Absorption intensity at each wavelength of BCP in membranes made from dimyristoylphosphatidylcholine at various temperatures.

Temperature (°C)	Wavelength (nm.) and intensity (absorbance unit)			
15	599 (0.103)	_	551 (0.043)	544 (0.037)
20	599 (0.112)	_	551 (0.060)	_
23	599 (0.128)	_	551 (0.075)	_
28	606 (0.144)	578 (0.112)	551 (0.103)	_
32	609 (0.146)	575 (0.083)	551 (0.083)	543 (0.078)

C. Effect of cholesterol on saturated phosphatidylcholine membranes.

1. On dilauroylphosphatidylcholine liposomes

The absorption spectrum of BCP in DLPC was compared to those obtained from bilayer membranes composed of various amounts of cholesterol as shown in Figure 10.There were three considerable features in this figure. (1) The longest wavelength (598 nm.) in DLPC slightly shifted to shorter wavelength (597 nm.) when cholesterol was added and remained constant at this wavelength up to 44.44 mole %, subsequently, red shift to 601 nm. was observed at 50.00 %. Peak intensity also decreased as cholesterol increased until 44.44 %, (Table 4) furthermore unchanged. Coincidently, remarkable peak around 591 nm. was observed owing to valley appearance. (2) A hump peak around 581 nm. was demonstrated in DLPC liposome (Figure 10A) and gradually enlarged concomitant with shifted the λ_{max} to 575 nm. upon increasing amount of cholesterol. Reduction of peak intensity was also obtained until 44.44 %, afterthat unchanged. (3) A group of peaks between 523-550 nm. was detectable when cholesterol was progressively added as a result of aqueous dimer formation, however, this peak was not observed in Figure 10A.

2. On dimyristoylphosphatidylcholine liposomes

Three noticeable changes in absorption spectra were also observed upon incorporation of cholesterol to DMPC REVs, (Figure 12).Absorption intensity (Table 5) and peak area around 599 nm. were diminished on increasing amount of cholesterol, moreover, a new peak appeared at about 575 nm. concomitant with enhanced peak area. When cholesterol content was higher than 16.67 %, another peak was also illustrated at 552 nm. The more the amount of cholesterol added the wider the peak obtained.

3. On dipalmitoylphosphatidylcholine liposomes

As illustrated in Figure 14 and Table 6, the remarkable changes were observed when DPPC was used instead of DMPC. The peak at 597 nm. did not show high intensity compared to those obtained from DLPC and DMPC. Investigation of the peak around 597 nm., it was found that intensity decreased and new peak at 576 nm. was observed when cholesterol was incorporated. The highest intensity showed at 16.67 mole %, afterthat declined, however, a broader peak was also illustrated. A bunch of peaks was observed at wavelength between 523 and 550 nm. and merged into one peak as progressively increased amount of cholesterol to 44.44 %. Nevertheless, further addition of cholesterol resulted in valley at 552 nm. and membrane - dimer at 557 nm. was dominant .

When Figure 10A was compared to Figure 12A and 14A, the main peak which altered in absorption intensity was the peak at 597 nm. This peak shifted to the longer wavelength compared to the aqueous peak of BCP at 589 nm. As a result of decreasing in intensity at about 597 nm. when DMPC or DPPC was used instead of DLPC, it could be explained in the term of changing of transition temperature of lipid used . At 25 °C, DLPC and DMPC were being in fluid phase, while DPPC was in gel phase. Eventhough DMPC was a liquid crystallline phase lipid, bilayers were stiffly higher than that of DLPC as supported by fluorescence polarization data [Lentz, R.B., 1976]. To understand more clearly about the state of membrane, cholesterol was incorporated as one of liposomal membrane components. The role of cholesterol can be interpreted as reducing or restricting the chain mobility of phospholipids above their transition temperatures (condensation effect), [Lentz, R.B.,et al, 1980; McIntosh, J.T.,1978; Marsh, D.,1973]. Therefore, its action was on changing of fluidity of membrane. It was remarkedly seen in decreasing intensity at about 597 nm.,while new peak at 575 nm. appeared. Intensity of this new peak decreased with increasing cholesterol content upto 44.44 %, and no change was observed upon further addition, however, peak area was enhanced in proportional to cholesterol content. At high concentration of cholesterol (50.00 %), the peak at 576 nm. might superimpose on the peak at 557 nm., therefore, a broad peak was obtained. This might be explained that, at a certain cholesterol content, dimers in membrane oriented in

two conformations showing λ_{max} at 575 and 557nm., respectively. A bunch of peaks between 523 and 550 nm. might be due to dimer form in aqueous medium, since, free dyes might be excluded from membrane as a result of high membrane density by incorporation of cholesterol.

For DMPC (T_C = 23 ^{o}C), due to very close value of DMPC's phase transition temperature to experimental temperature, the membrane bilayers were very unstable and clusters of gel phase could appear on fluid phase matrix. Thus the peak having λ_{max} around 552nm. might be illustrated, however, difficultly defined because of superimposition . When compared intensity at about 599 nm. to DLPC, however, DMPC bound to BCP in the amount smaller than DLPC because of higher membrane density. Similar to DLPC - cholesterol bilayers, addition of cholesterol to DMPC caused an appearance of the new peak at about 575 nm. coincided with occurrence of the peak at about 552 nm. It was noticeable that λ_{max} of membrane-dimer between 552 and 575 nm. was not reproducible depending on the oriented - conformation of each dimer in membrane, as previously reported by Nakamaru, (1977). Moreover, dimer conformation in DLPC - cholesterol bilayer should be different from DMPC- cholestertol system owing to distinction of spectral shape particularly at 50.00 %, however, their true structures should be under next investigation. Another group of peaks at wavelength between 523 and 550 nm. was similar to cholesterol-

DLPC vesicles. These small peaks were due to aqueous- dimer formation.

For observation with DPPC - cholesterol systems, DPPC (T_C = 41 ^{o}C) was being in gel phase at the experimental temperature (25 ^{o}C), consequently, membrane density of DPPC was higher than those of DLPC and DMPC bilayer. Thus, amount of bound dye was lesser extent than those fluid phase bilayers (DLPC and DMPC). Decreasing in dye binding was observed with declination of intensity at membrane-monomer band. Concomitantly, membrane - dimer formations at wavelength between 554 and 576 nm. were observed. However, when cholesterol content reached 50.00 %, monomer in membrane still remained eventhough very small amount, while dimer forms are the main oriented-conformation particularly dimer form having λ_{max} at 557 nm. Since, in highly fluid membrane dye could accumulate in restricted space and dimers easily occurred because they were close to each other. On examination involving cholesterol effects, cholesterol showed the different actions on lipids used depending on their statements, i.e., cholesterol caused DLPC membrane to be in high density; thus it had condensing effect or increased rigidity in DLPC membrane, however, fluidized DPPC bilayer.

As discussed previously, cholesterol was found to have a condensing effect on lecithins in their liquid crystalline phase and have a fluidizing effect on the gel phase one, [Marsh, D. and Smith, C.P.I.,1973]. From the fluorescence polarization experiment (Figure11,13), incorporation of cholesterol to fluid phase lipids, DLPC and DMPC, made membranes more rigid compared to pure lipid as indicated by high fluorescence polarization value. Membrane rigidity was proportional to amount of cholesterol added. In contrast, cholesterol produced fluidizing effect on gel phase lipid,DPPC, (Figure 15) by showing low value compared to pure lipid, (Figure 16). As demonstrated in BCP' s absorption spectra (Figure 10 ,12 and 14), BCP bound to cholesterol containing DLPC and DMPC liposome in amount lower than pure lipids but higher in cholesterol containing DPPC . These two studies supported to each other .

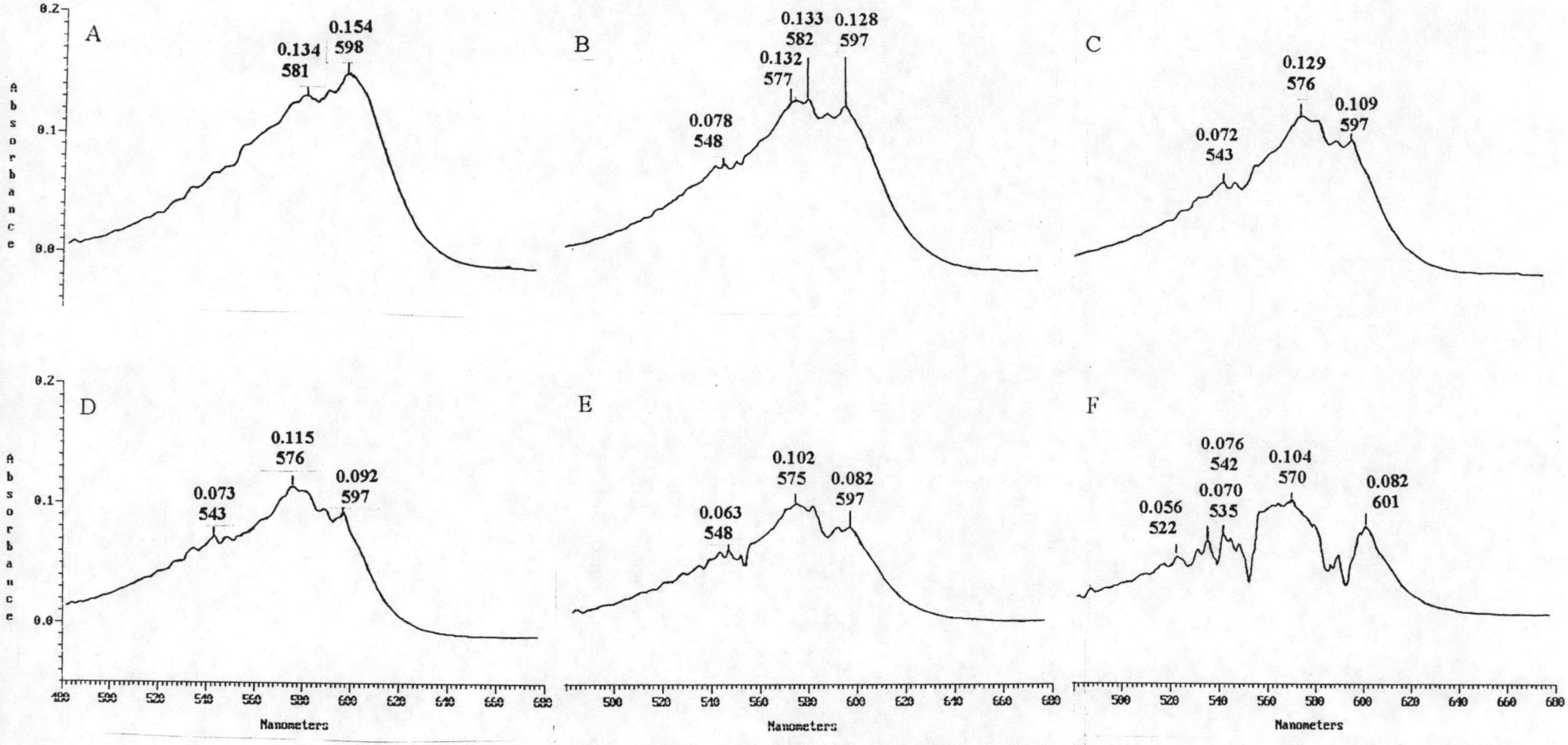


Figure **10**. Absorption spectra of BCP in membranes made from various mixtures of DLPC and cholesterol at 25 °C. The mole fraction of cholesterol is A. 0 % ; B. 16.67 % ; C. 28.57 % ; D. 37.50 % ; E. 44.44 % and F. 50.00 %

Table 4. Absorption intensity at each wavelength of BCP in membranes made from various mixtures of DLPC and cholesterol.

Liposomal composition (mole %)	Wavelength (nm.) and intensity (absorbance unit)				
DLPC	598 (0.154)	–	–	–	–
16.67	597 (0.128)	577 (0.132)	548 (0.078)	–	–
28.57	597 (0.109)	576 (0.129)	543 (0.072)	–	–
37.50	597 (0.092)	576 (0.115)	543 (0.073)	–	–
44.44	597 (0.082)	575 (0.102)	548 (0.063)	–	–
50.00	601 (0.082)	570 (0.104)	542 (0.076)	535 (0.070)	522 (0.056)

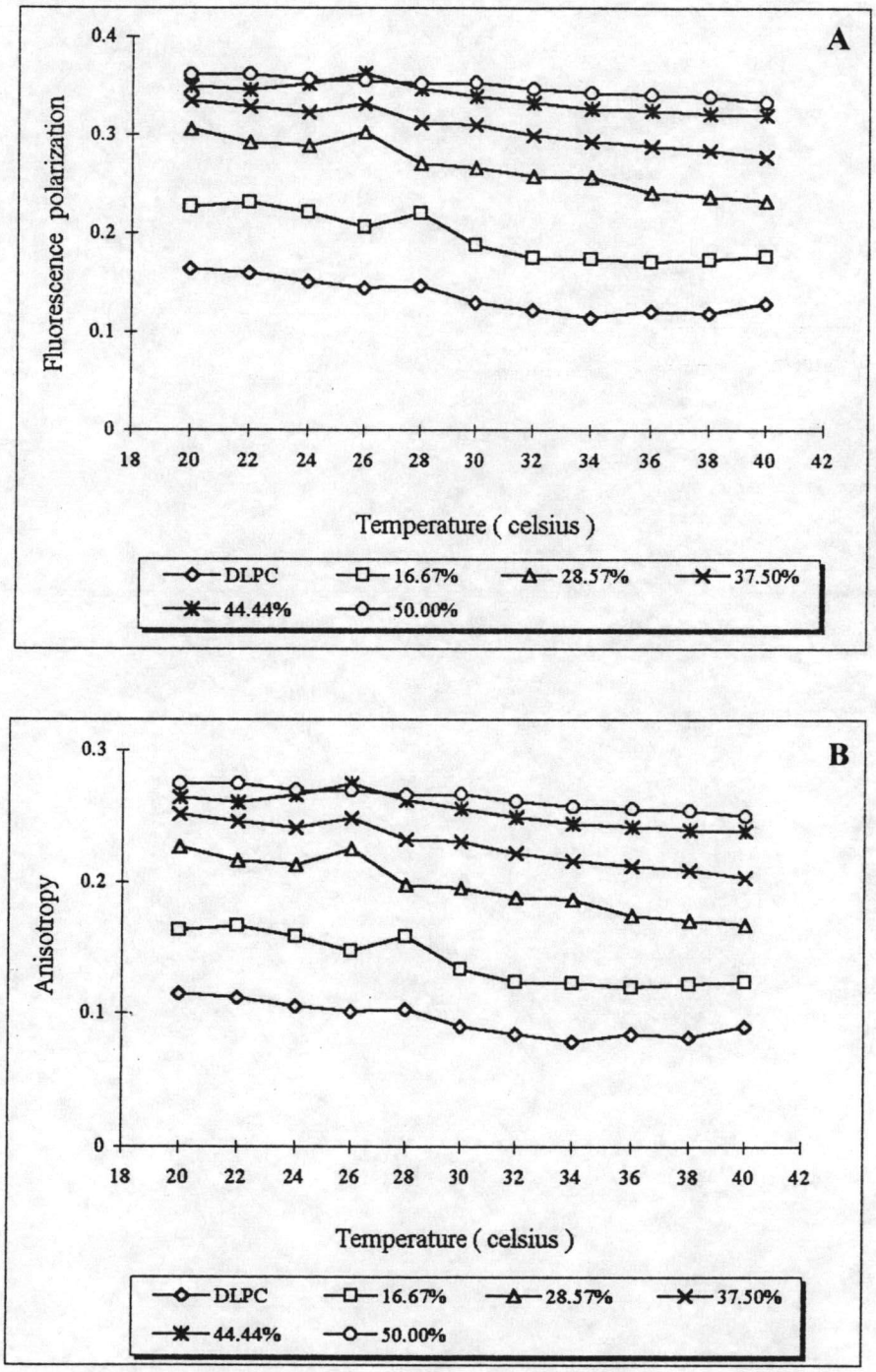


Figure 11. Fluorescence polarization (A) and anisotropy (B) of DPH in membranes made from various mixtures of DLPC and cholesterol.

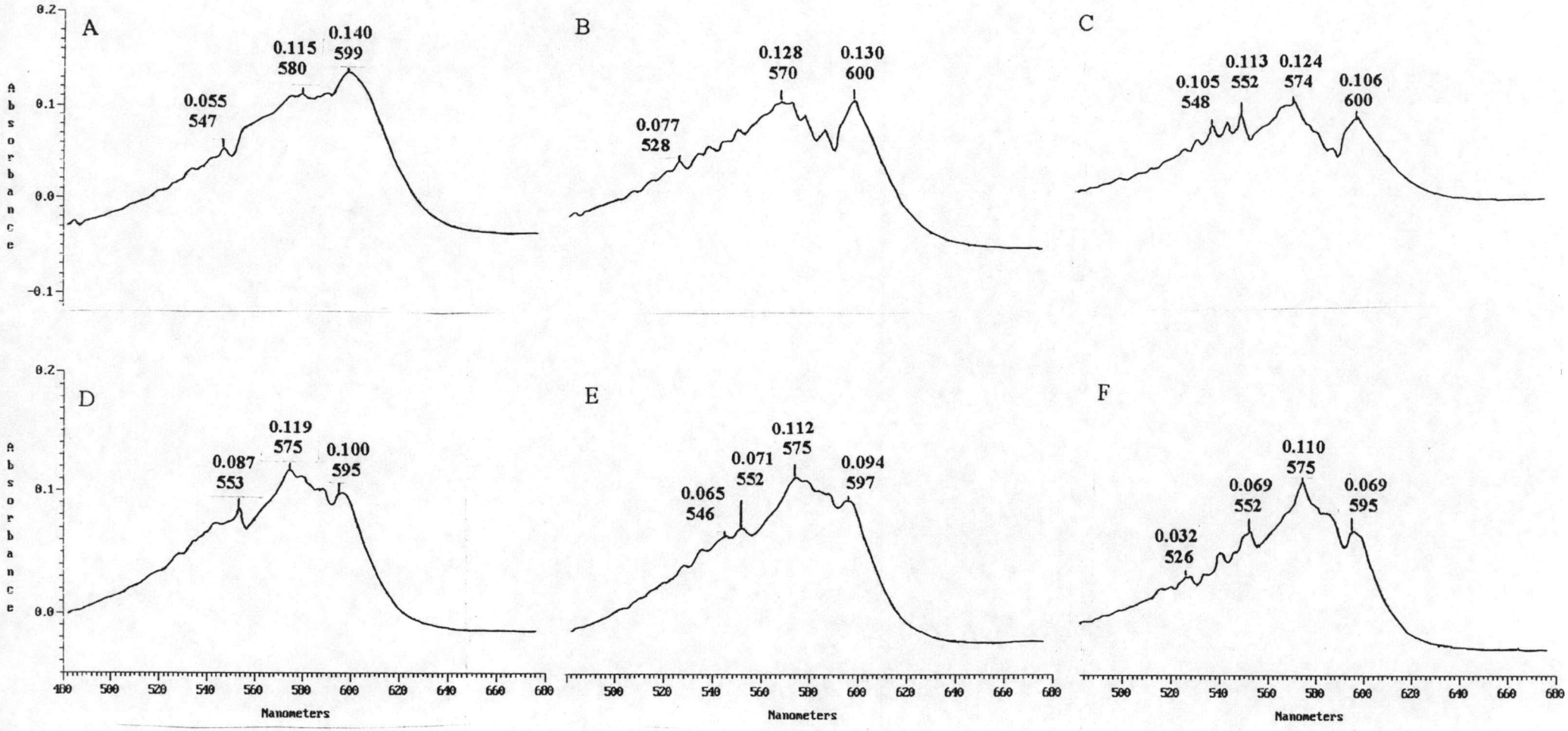


Figure 12. Absorption spectra of BCP in membranes made from various mixtures of DMPC and cholesterol at 25 °C. The mole fraction of cholesterol is A. 0 % ; B. 16.67 % ; C. 28.57 % ; D. 37.50 % ; E. 44.44 % and F. 50.00 %

Table 5. Absorption intensity at each wavelength of BCP in membranes made from various mixtures of DMPC and cholesterol.

Liposomal composition (mole %)	Wavelength (nm.) and intensity (absorbance unit)				
DMPC	599 (0.140)	–	–	547 (0.055)	–
16.67	600 (0.130)	570 (0.128)	–	–	528 (0.077)
28.57	600 (0.106)	574 (0.124)	552 (0.113)	548 (0.105)	–
37.50	595 (0.100)	575 (0.119)	553 (0.087)	–	–
44.44	597 (0.094)	575 (0.112)	552 (0.071)	546 (0.065)	–
50.00	595 (0.069)	575 (0.110)	552 (0.069)	542 (0.052)	526 (0.032)

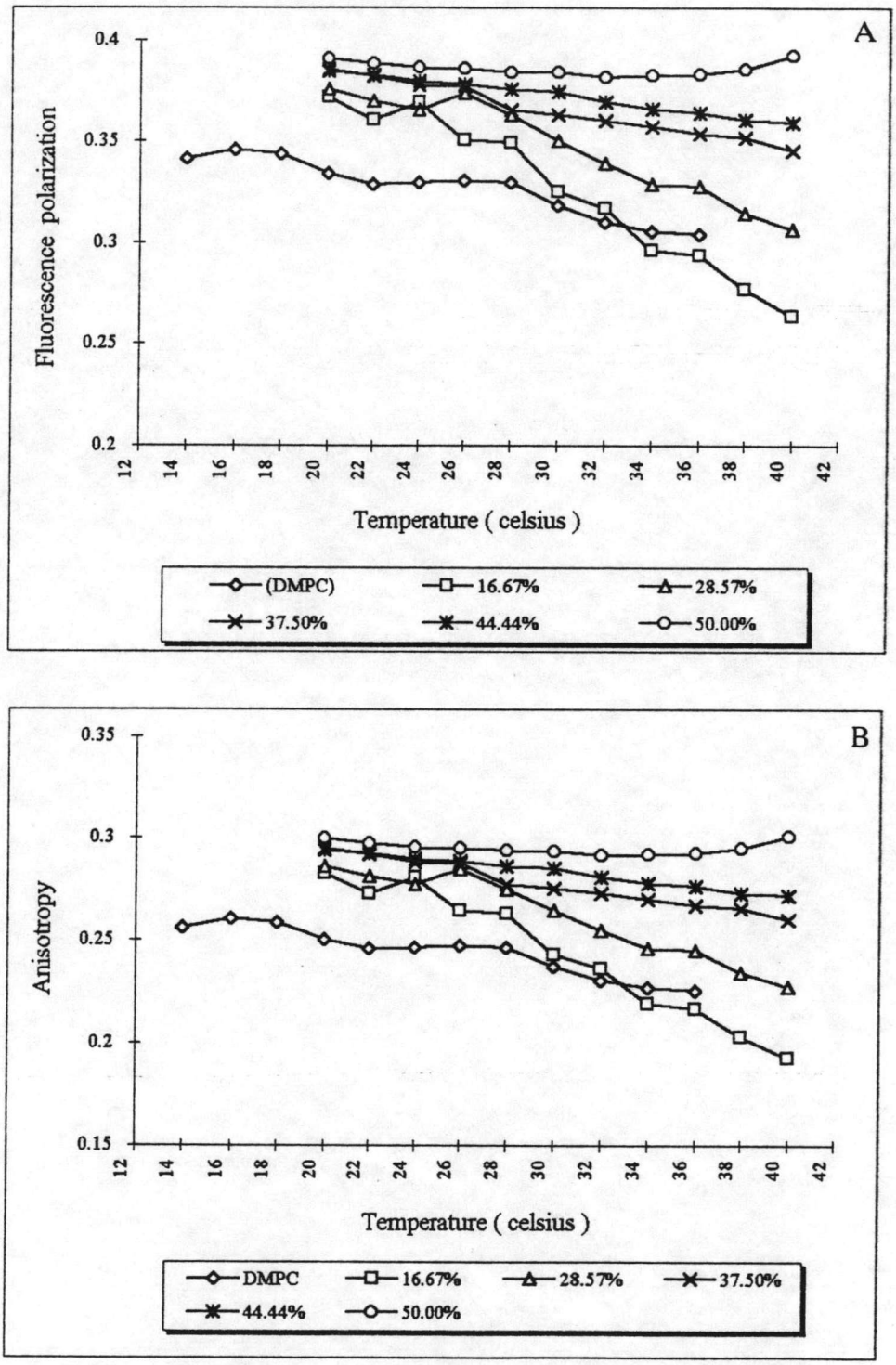


Figure 13. Fluorescence polarization (A) and anisotropy (B) of DPH in membranes made from various mixtures of DMPC and cholesterol.

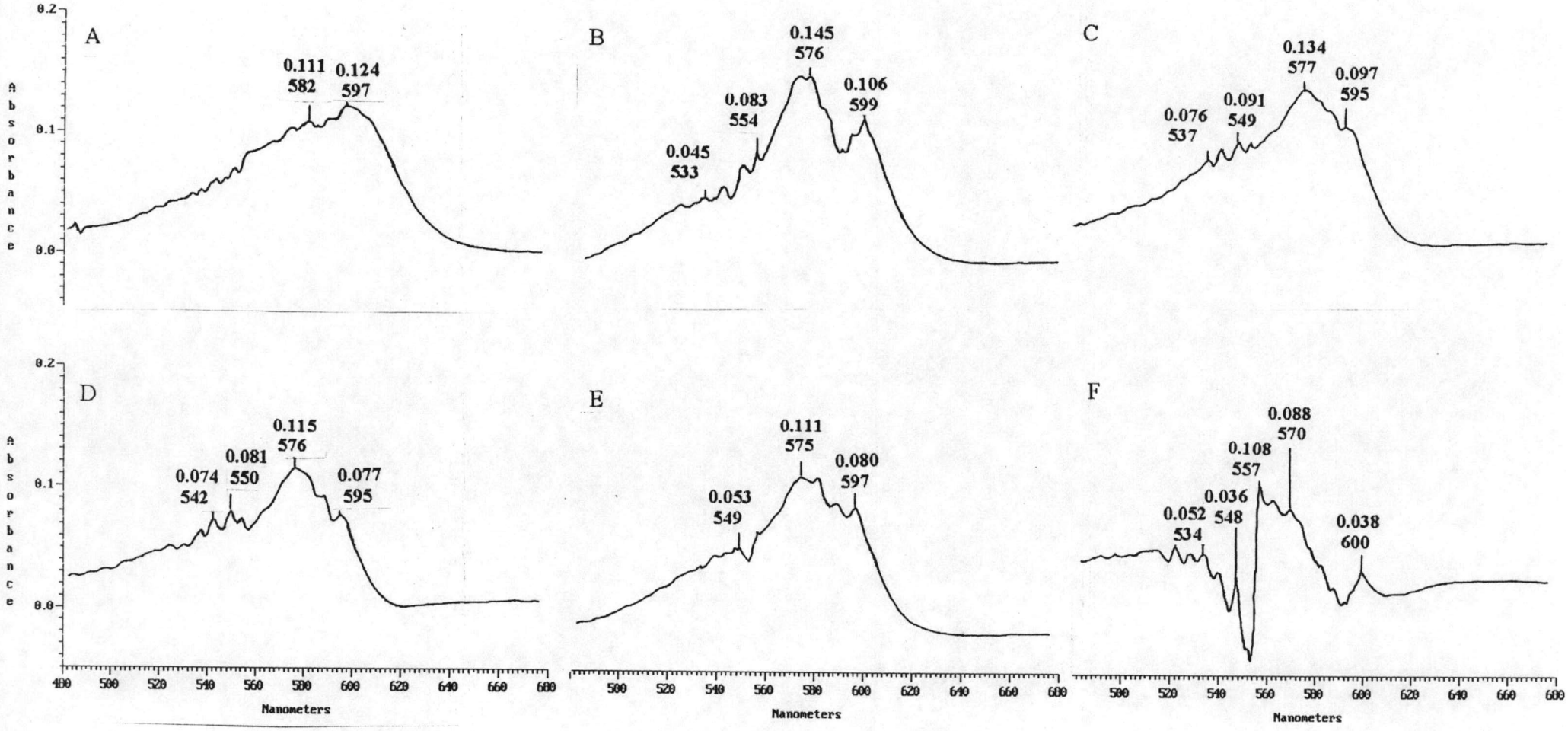


Figure 14. Absorption spectra of BCP in membranes made from various mixtures of DPPC and cholesterol at 25 °C. The mole fraction of cholesterol is A. 0 % ; B. 16.67 % ; C. 28.57 % ; D. 37.50 % ; E. 44.44 % and F. 50.00 %

Table 6. Absorption intensity at each wavelength of BCP in membranes made from various mixtures of DPPC and cholesterol.

Liposomal composition (mole %)	Wavelength (nm.) and intensity (absorbance unit)				
DPPC	597 (0.124)	–	–	–	–
16.67	599 (0.106)	576 (0.145)	554 (0.083)	–	533 (0.045)
28.57	595 (0.097)	577 (0.134)	549 (0.091)	542 (0.085)	537 (0.076)
37.50	595 (0.077)	576 (0.115)	550 (0.081)	542 (0.074)	524 (0.051)
44.44	597 (0.080)	575 (0.111)	549 (0.053)	–	–
50.00	600 (0.038)	570 (0.088)	557 (0.108)	–	534 (0.052), 523 (0.059)

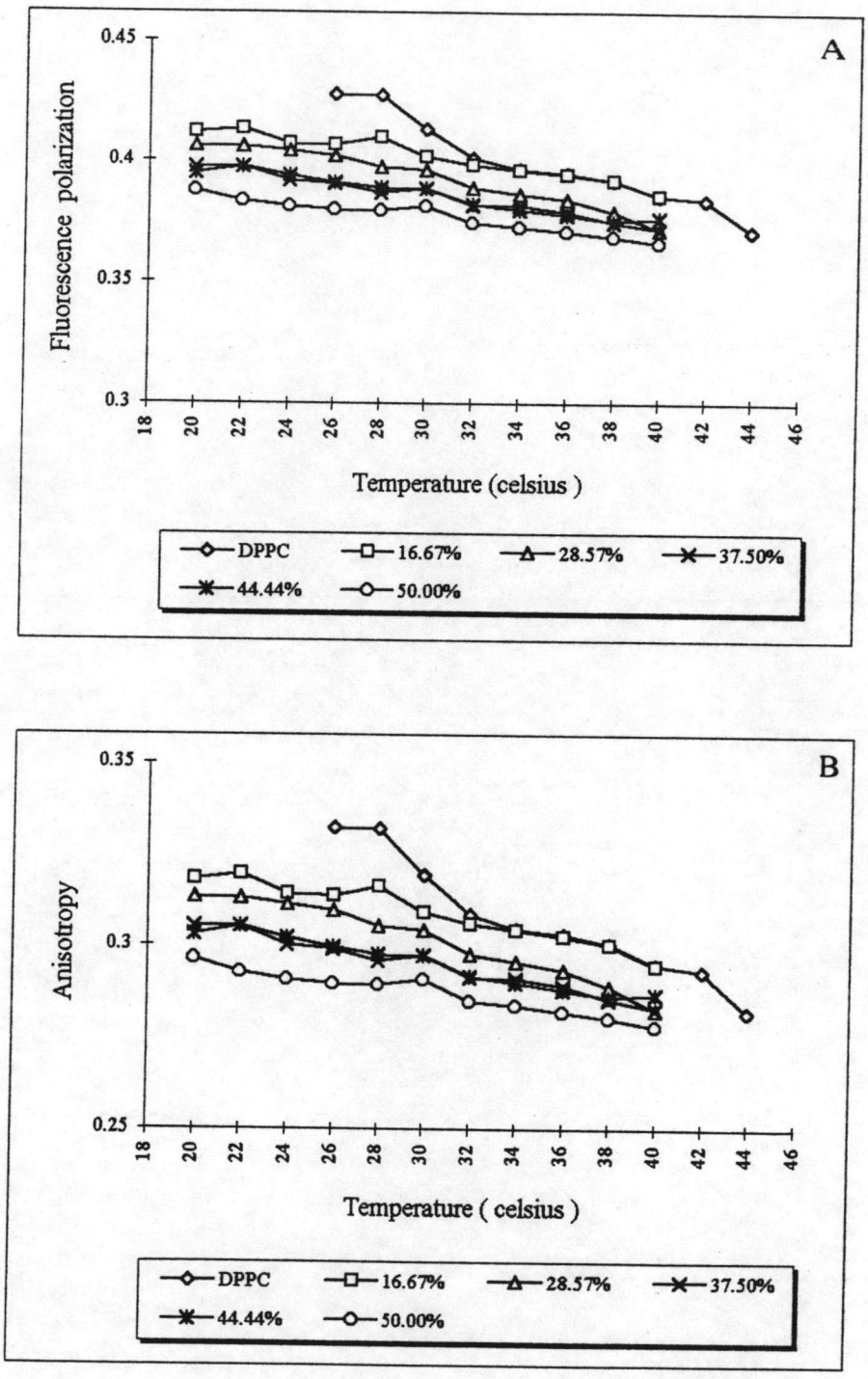


Figure 15. Fluorescence polarization (A) and anisotropy (B) of DPH in membranes made from various mixtures of DPPC and cholesterol.

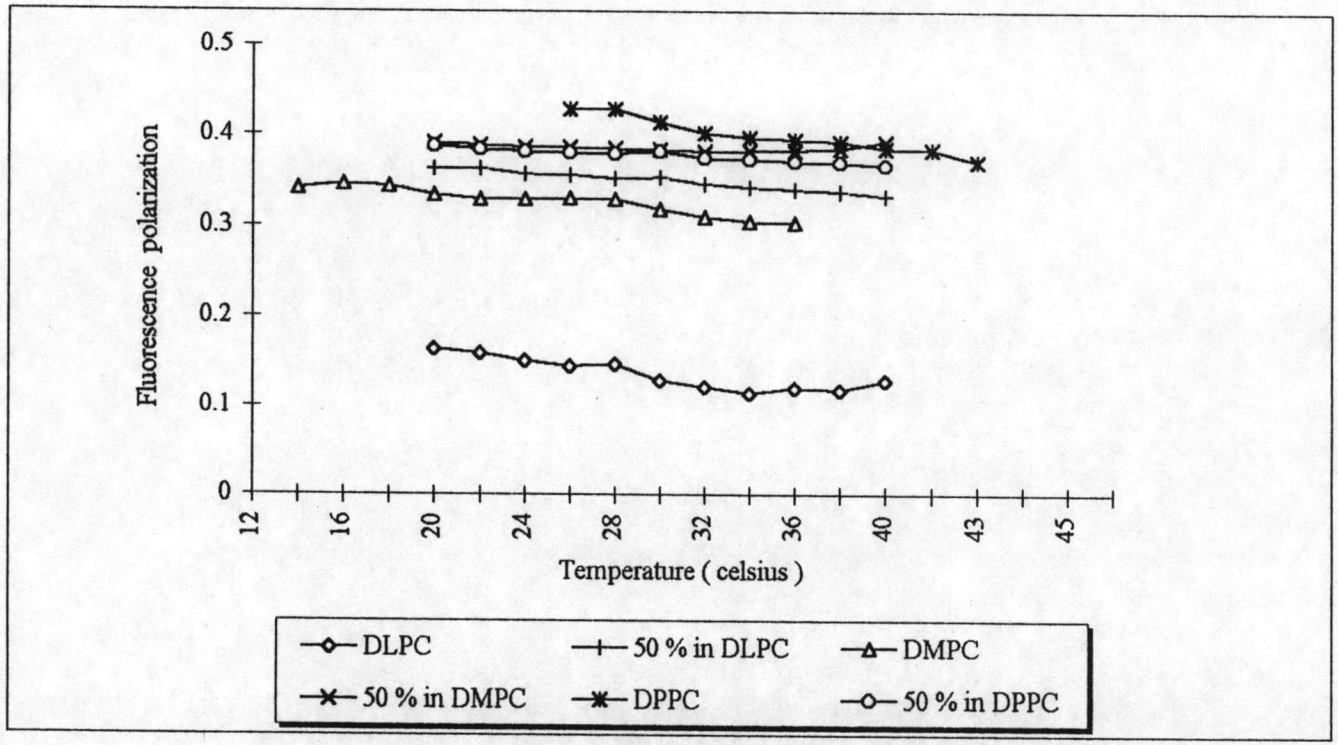


Figure 16. Effect of cholesterol on phospholipids differing in acyl chain length as illustrated by fluorescence polarization.

D. Effect of domain formations on membrane bilayers.

Figure 17. and Table 7. showed absorption spectra and intensity of BCP in membrane made from various mixtures of DLPC and DPPC. These two phospholipids differed by four carbon atoms of saturated acyl chain length; thus when they were mixed together, domain formation as a result of non-ideal mixing was obtained. As suggested by Lentz, R.B., et al., (1976f), DLPC preferentially colyophilized with DPPC in molar ratio of 1:1, i.e., when 20 % DLPC was combined with 80 % DPPC, domains were to be of 20 %. Free DPPC which did not form domains was 60 % and distributed through membrane bilayers. However, if 60 % DLPC was mixed with 40 % DPPC, domain formed was more than remained DLPC; thus it might distribute throughout liposomal membranes surrounding free DLPC. When it was so, binding of BCP in both cases should be different. In Figure17B, domains were surrounded by free DLPC, however, in Figure 17C and Figure 17D, mixture of DLPC: DPPC (1:1) distributed throughout membranes surrounding free DLPC and DPPC domains, respectively. Additionally, in Figure 17E, DPPC scattered and surrounded mixture domains.Distinction between Figure 17B and Figure 17E should be explained that binding of BCP to membrane was affected by their environment. Membrane monomer band at 599 nm. in Figure 17B was due to binding of BCP to free DLPC concomitant with dimer band at 582 nm. was from binding to domain portions. Moreover, dimer band at about 579 nm. was also observed in Figure 17E

owing to binding to free DPPC. Dimer band at 550 nm. increased with increasing domain region as shown in Figure 17C and Figure 17D. However, monomer band intensity in Figure 17C was higher than Figure 17D due to monomer in DLPC portion. It should be concluded that binding of BCP depended not only on amount of domains formed but on surrounding environment.

For an investigation of liposomes composed of DLPC and DPPC mixed together in various molar ratioes, non-ideal mixing of these two phospholipids obtained and domains on liposomal membranes frequently occurred. Due to the fact that DPH equally partition between liquid crystalline lipid and domain; thus domains occurred were not be detected by using DPH. Membrane fluidity illustrated by fluorescence polarization value was average of all membrane statements. When amount of DPPC incorporated to DLPC was increased, liposomal membranes exhibited more rigid than DLPC alone. Fluidity of membrane bilayers was inversted proportional to amount of DPPC incorporated as demonstrated in Figure 18. Therefore, the fluorescence polarization evidence agreed with BCP results.

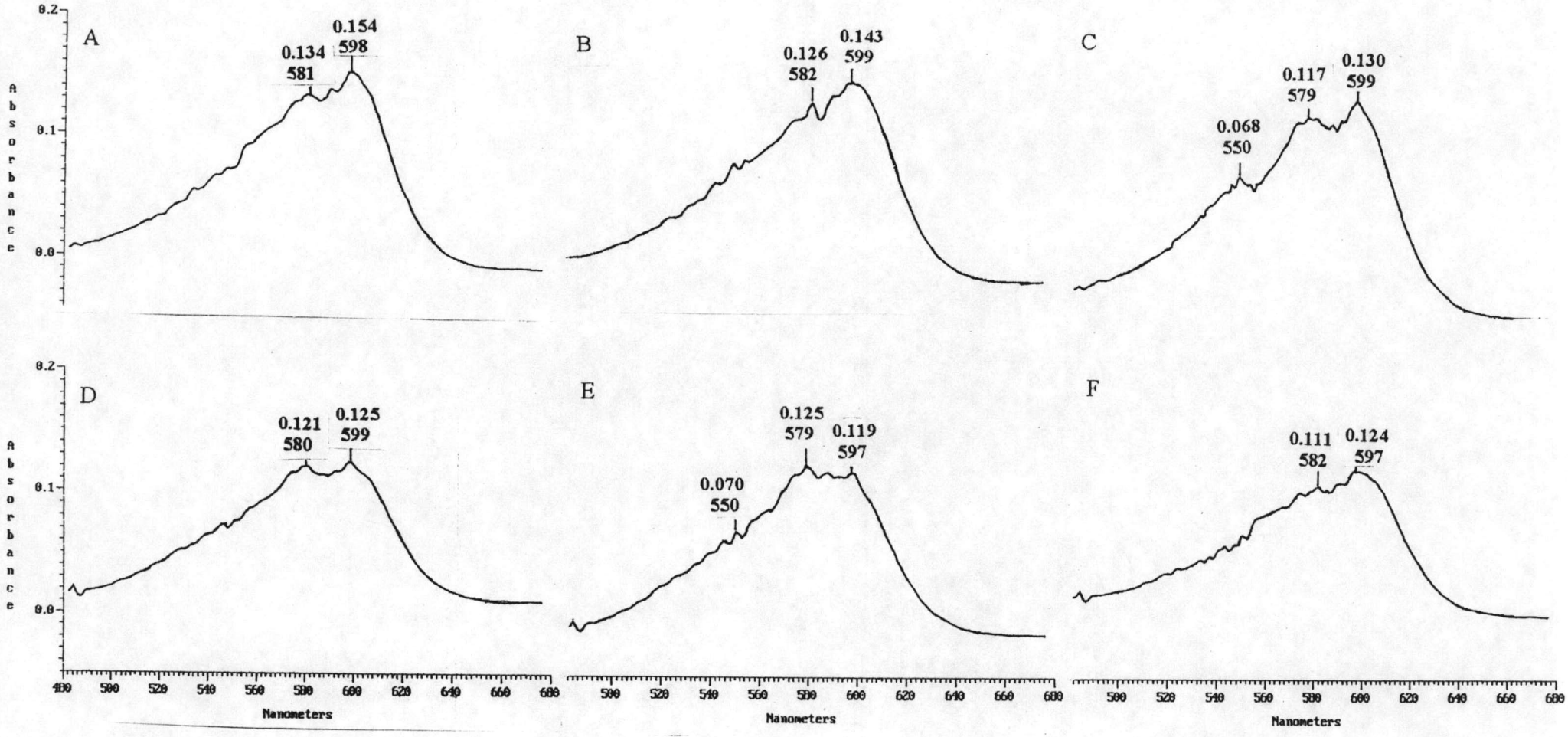


Figure 17 Absorption spectra of BCP in membranes made from various mixtures of DLPC and DPPC at 25 °C. The mole % of DPPC in total amount of phospholipid is A. 0 % ; B. 20 % ; C. 40 % ; D. 60 % ; E. 80 % and F. 100 %

Table 7. Absorption intensity at each wavelength of BCP in membranes made from various mixtures of DLPC and DPPC.

Liposomal composition (% DPPC)	Wavelength (nm.) and intensity (absorbance unit)		
DLPC	598 (0.154)	581 (0.134)	_
20	599 (0.143)	582 (0.126)	_
40	599 (0.130)	579 (0.117)	550 (0.068)
60	599 (0.125)	580 (0.121)	_
80	597 (0.119)	579 (0.125)	550 (0.070)
DPPC	597 (0.124)	582 (0.111)	_

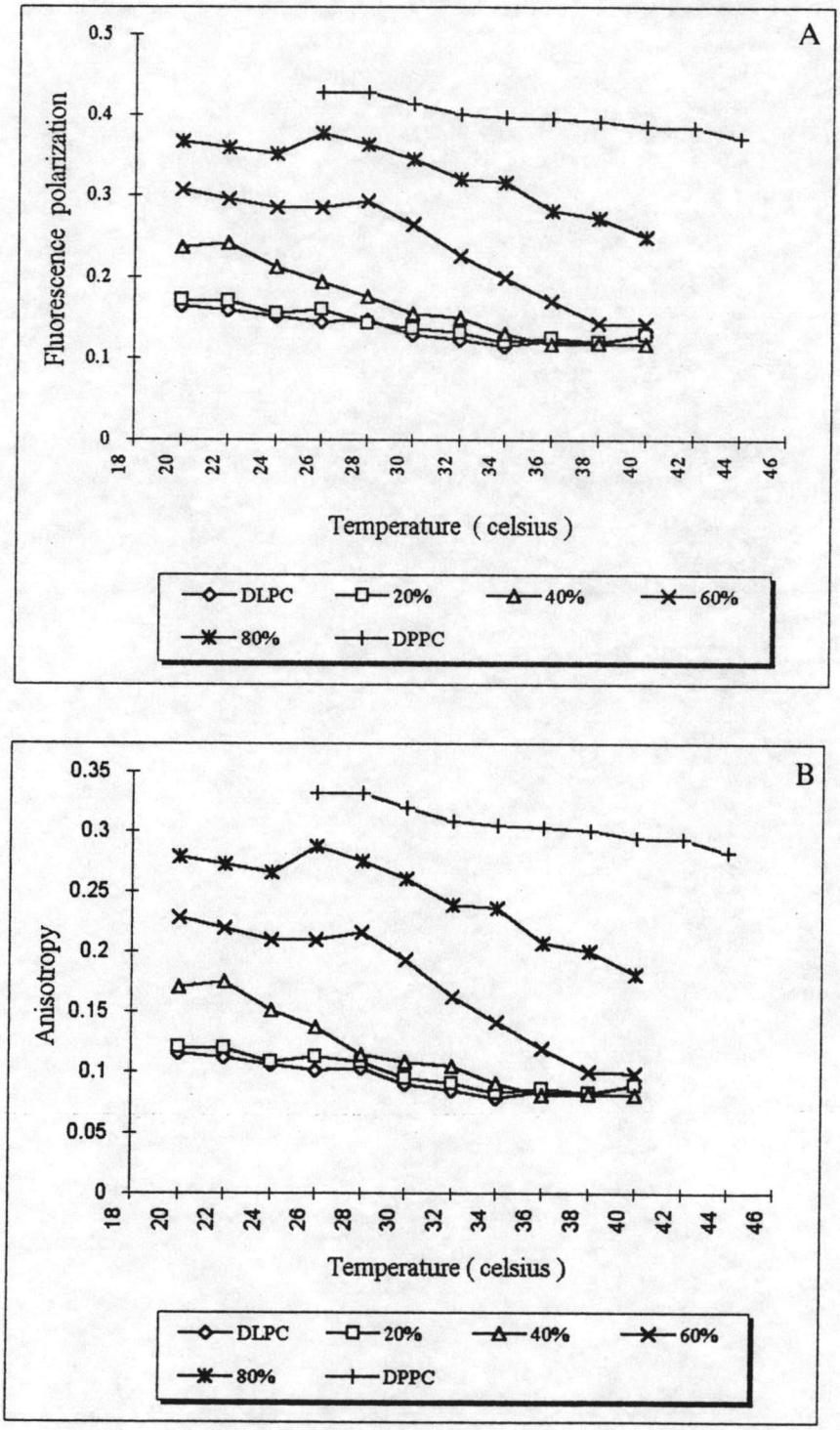


Figure 18. Fluorescence polarization (A) and anisotropy (B) of DPH in membranes made from various mixtures of DLPC and DPPC.

E. Effects of charged amphiphiles on binding of BCP to cholesterol containing dilauroylphosphatidylcholine liposomes.

In this section of the experiment, dicetylphosphate (DCP) and stearylamine (SL) were used as negatively and positively charged amphiphiles, respectively. Cholesterol in molar ratio of 0.4 and charged amphiphiles of 0.1 to DLPC were incorporated and the effects of charges as well as medium ionic strength were shown in Figure 19. and Table 8. At this molar ratio of cholesterol used, BCP absorption spectrum was sensitive to adding of another component and altering an environment, moreover, spectral changes could easily be observed. In each case of charge, effect of low ionic strength medium was compared to that of high ionic strength one. In Figure19C, BCP bound to membrane bilayers in the amount smaller than that obtained in Figure 19B .This was because of negatively charged repulsion between dye molecules and membranes.Eventhough, membranes were more loosely because repulsion between negative charges in membrane was higher and prevented BCP binding . When ionic strength of buffer was increased (Figure 19D), shielding effect that occurred on both dye and membrane was dominant. However, the region deep in membrane, negative charges were not shielded; thus loosely membranes were also obtained. Repulsion between negative charges of BCP and dicetylphosphate did not occur, consequently, BCP could penetrate by using hydrophobic property of dye molecules. Membrane - monomers and dimers

in Figure 19D showing λ_{max} at 600 and 579 nm., respectively enhanced when compared to Figure 19C. Since, membrane bilayers had wider space for dye to accumulate; thus dimers could easily form by closing of two monomers and occurred in proportional to amount of monomer in membrane. This dimer conformation resulted in broad peak at 579 nm.

For study the effect of positively charged amphiphile, identical procedure was performed but stearylamine was used instead of dicetylphosphate. It was found that BCP when encountered to hydrophobic environment or bound to positive charges shifted λ_{max} of bound monomer to longer wavelength as previously suggested by Nakamaru,(1974). Stearylamine was protonated at the pH used and positive charge was obtained. Repulsion between positive charges in membrane made it more loosely in similar way to negative charges. In low ionic strength solution (Figure 19E), negatively charged dyes bound electrostatically to those positive charges in membrane concomitant with partitioning of the hydrophobic part to more hydrophobic region deep in membrane. These might be the causes of shifting to the longest wavelength (604 nm). Moving of monomer band to this wavelength might be explained that ring structure formation was obtained by approaching of SO_3^- group of BCP molecules to trimethylammonium residues of phosphatidylcholine molecules (Figure 20); thus polarity of surrounding environment around dye molecules was reduced resulting in great shift of monomer band.

In comparison of Figure 19E and 19F, higher amount of BCP monomers with lesser dimers was found in low ionic strength solution compared to the high ionic strength one. Since negatively charged dyes could bind easily with stearylamine, a positively charged amphiphile, in liposomal membrane by electrostatic attraction, therefore evenly distribution of dye over the membrane might be obtained . Thus, the possibility of clustering of BCP molecules within membrane to form dimers was reduced, consequently, high monomer with low amount of dimers were achieved in low ionic strength solution (Figure 19E). In contrast, shielding effect of salt in high ionic strength solution (Figure 19F) resulted in increasing amount of dimers in compensation with monomers, similar to dicetyl-phosphate, (Figure 19D). However, it should be noted that monomer peak in the positively charged membrane (Figue 19F) was larger than the negatively charged one (Figure 19D). This might be attributed to a lesser chance of clustering of BCP molecules in positively charged membrane while electrostatic repulsion between negatively charged dye and dicetylphosphate in membrane allowed this.

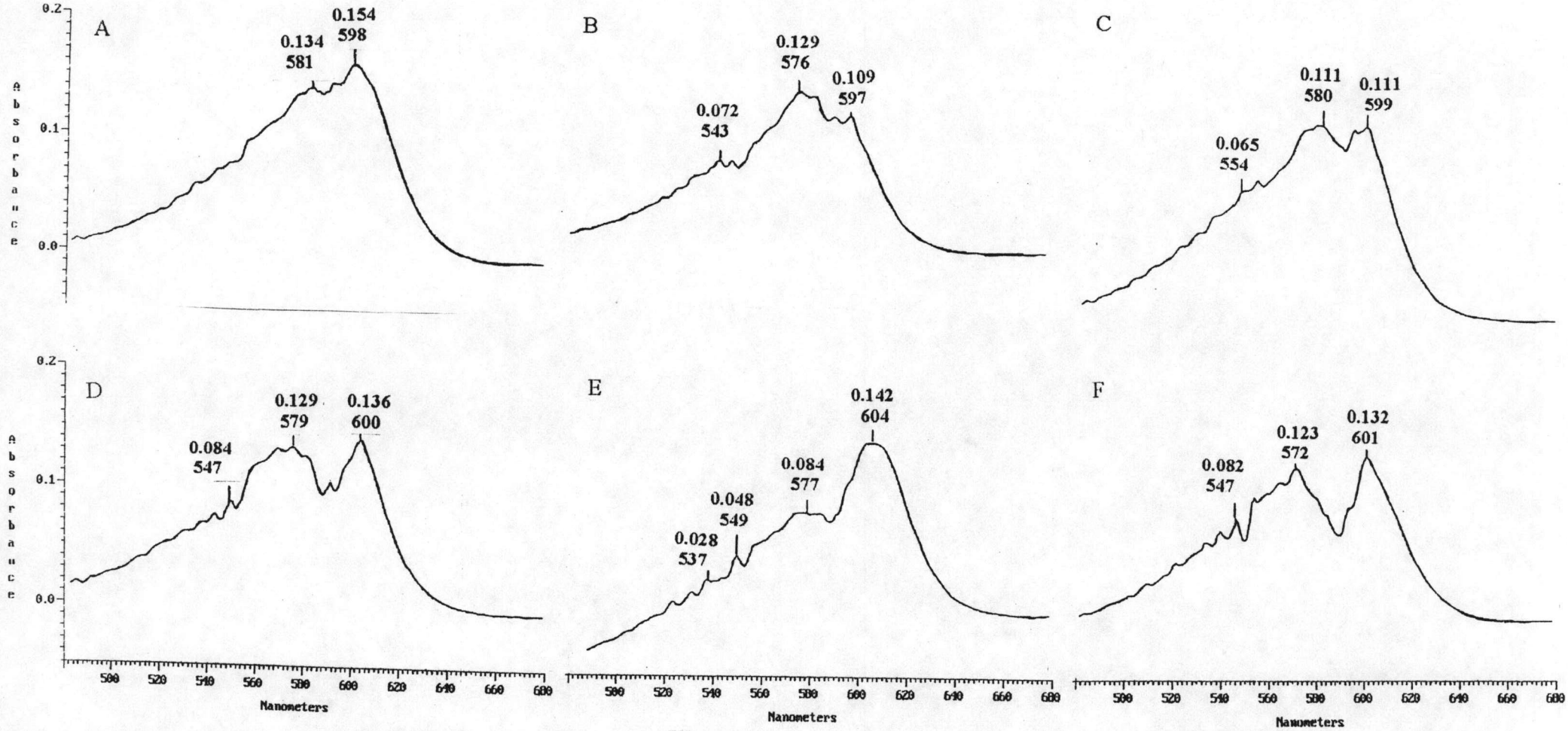


Figure19. Absorption spectra of BCP in membranes made from various mixtures of DLPC , cholesterol and charged amphiphiles (DCP / SL) in molar ratio as indicated 1. In Tris - HCl pH 8.4 or 2. In Tris - HCl pH 8.4 + 150 mM NaCl : A. 1:0:0 (2) ; B. 1:0.4:0 (2) ; C. 1:0.4:0.1DCP (1) ; D. 1:0.4:0.1DCP (2) ; E. 1:0.4:0.1SL (1) and F. 1:0.4:0.1SL (2).

Table 8. Absorption intensity at each wavelength of BCP in membranes made from various mixtures of DLPC, cholesterol and charged amphiphiles, DCP and SL, suspended in media with difference in ionic strength.

Liposomal composition (molar ratio)	Wavelength (nm.) and intensity (absorbance unit)				
DLPC (1)	598 (0.154)	581 (0.134)	–	–	–
DLPC : chol (1) (1 : 0.4)	597 (0.109)	576 (0.129)	–	543 (0.072)	–
DLPC : chol : DCP (2) (1 : 0.4 : 0.1)	599 (0.111)	580 (0.111)	–	554 (0.065)	–
DLPC : chol : DCP (1) (1 : 0.4 : 0.1)	600 (0.136)	579 (0.129)	–	547 (0.084)	–
DLPC : chol : SL (2) (1 : 0.4 : 0.1)	604 (0.142)	577 (0.084)	–	549 (0.048)	537 (0.028)
DLPC : chol : SL (1) (1 : 0.4 : 0.1)	601 (0.132)	–	572 (0.123)	547 (0.082)	–

Note : (1) in Tris - HCl pH 8.4 + 150 mM NaCl ; (2) in Tris - HCl pH 8.4

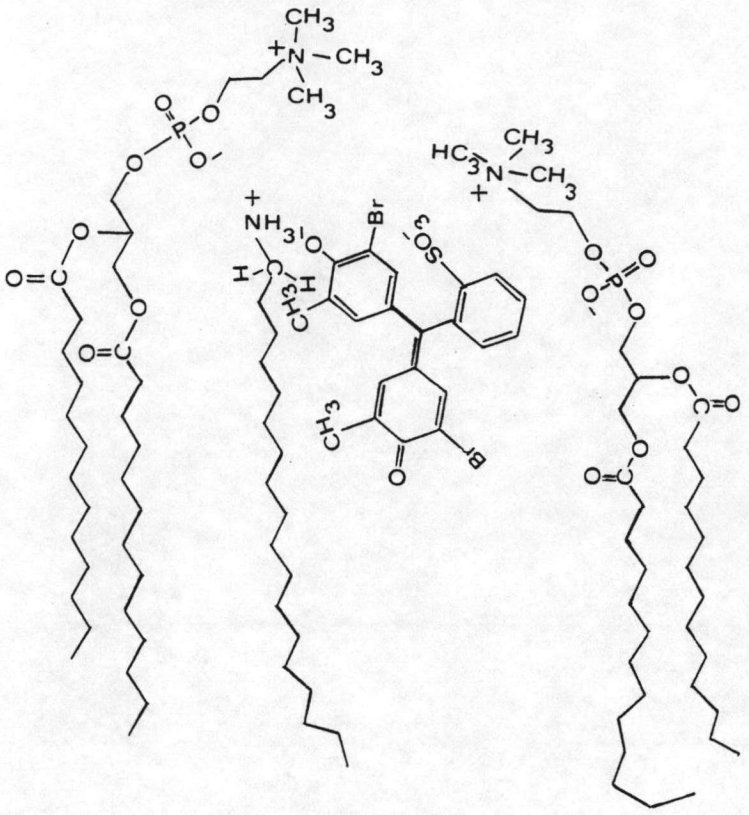


Figure 20. Proposed ring structure occurred between SO_3^- group of BCP molecules and trimethylammonium group of phosphatidylcholine.

F. Effects of cholesterol derivatives on binding of BCP to dimyristoylphosphatidylcholine and cholesterol containing dimyristoylphosphatidylcholine liposomes.

1. Dimyristoylphosphatidylcholine liposomes

From the previous experiment involving the effects of cholesterol on binding of BCP to DMPC liposomes, it was shown that cholesterol in molar ratio of 0.6 to DMPC produced the greatest changes in absorption spectra (Figure 12); thus cholesterol derivatives at this molar ratio were used instead of cholesterol for comparative purpose. As illustrated in Figure 21 and Table 9, the effects of all cholesterol derivatives on membrane fluidity were not similar to cholesterol. When derivative 0 was incorporated to DMPC, absorption intensity of membrane monomer band decreased and maximum absorption shifted to longer wavelength, coincidently, membrane dimer band increased showing λ_{max} at 557 nm.

As illustrated in Figure 3, length of side chain attached at 3-position of cholestene nucleus increased from derivative 0 (chol 0) to derivative II (chol II), respectively. The proposed orientation of cholesterol and its derivatives in membrane bilayer might be that oxygen atom at 3-position of cholestene nucleus bound to carbonyl group of phospholipids by dipole - dipole interaction, [Presti, T.F. et al, 1982].

In the case of cholesterol, it did not have side chain beyond that oxygen atom. Consequently, dipole - dipole interaction acted as crosslinkage between phospholipid and cholesterol; thus membrane density enhanced. Perturbation of membrane bilayer caused by steric interaction of cholesterol derivatives' side chain was obtained, resulting in enhancement of membrane fluidity which proportional to the length of side chain. As a consequence, BCP could easily penetrate and accumulate in a large amount within membrane as could be seen from Figure 21C, 21D and 21E, comparing with cholesterol containing membrane (Figure 21B). Increasing of peak area of dimer and monomer were demonstrated in Figure 23 and Table 11.These might be due to a large amount of dyes existing in restricted area between lipid molecules; thus monomer could come closely to another molecule and formed dimer as described previously in effect of charged amphiphiles. However, a significant amount of monomer still remained and some monomer might be in equilibrium with dimer .

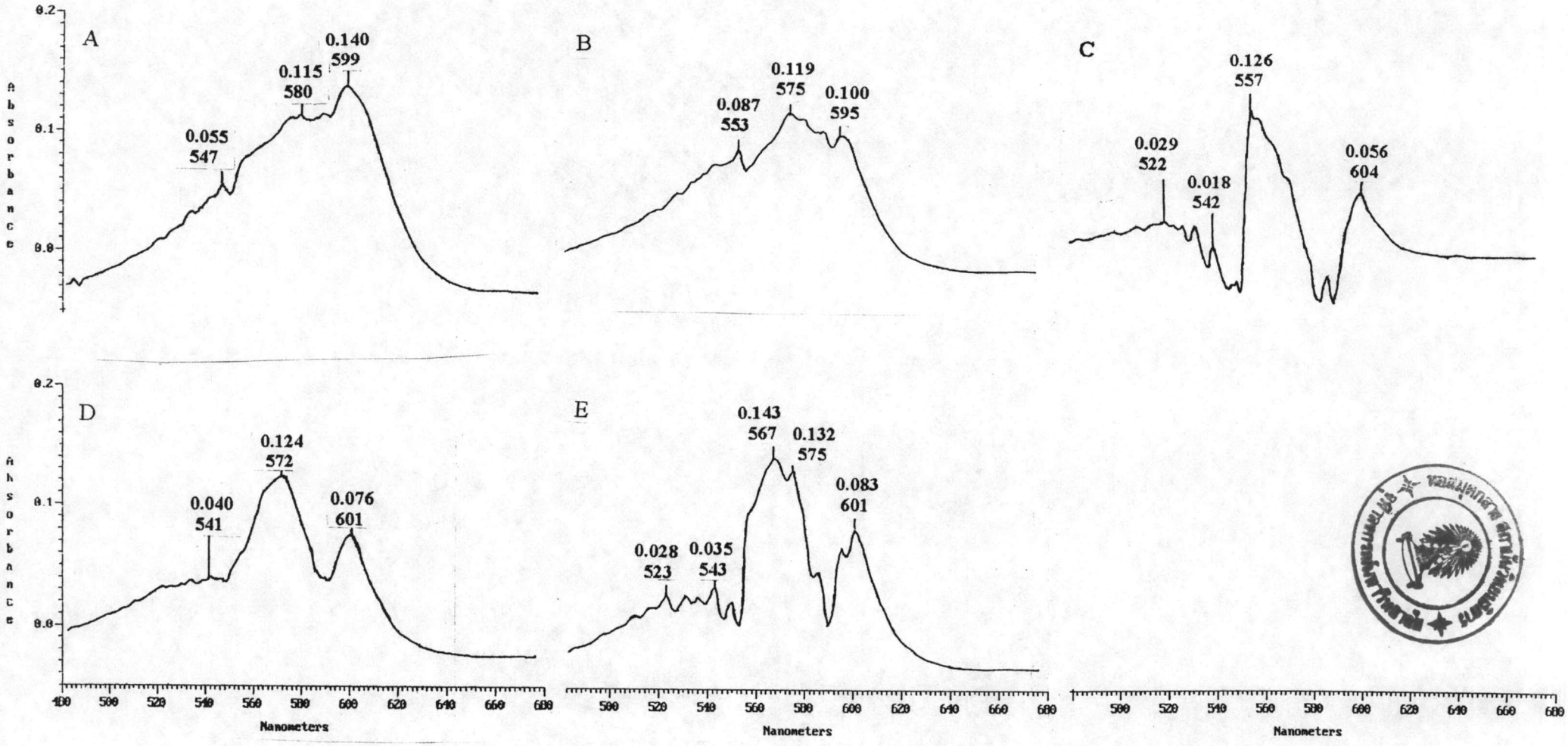


Figure 21 Absorption spectra of BCP in membranes made from various mixtures of DMPC and cholesterol / cholesterol derivatives in molar ratio as indicated. A. 1:0 ; B. 1:0.6 chol ; C. 1:0.6 chol O ; D. 1:0.6 chol I and E. 1:0.6 chol II.

Table 9. Absorption intensity at each wavelength of BCP in membranes made from mixtures of DMPC and cholesterol or DMPC and cholesterol derivatives ; i.e., chol O , chol I and chol II.

Liposomal composition (molar ratio)	Wavelength (nm.) and intensity (absorbance unit)				
DMPC	599 (0.140)	580 (0.115)	–	547 (0.055)	–
DMPC : chol (1 : 0.6)	595 (0.100)	575 (0.119)	–	553 (0.087)	–
DMPC : chol O (1 : 0.6)	604 (0.056)	–	–	557 (0.126)	542 (0.018), 522 (0.029)
DMPC : chol I (1 : 0.6)	601 (0.076)	572 (0.124)	–	–	541 (0.040)
DMPC : chol II (1 : 0.6)	601 (0.083)	575 (0.132)	567 (0.143)	550 (0.023)	543 (0.035), 523 (0.028)

2. Cholesterol containing dimyristoylphosphatidylcholine liposomes .

For an attempt to ratify fluidizing effect of cholesterol derivatives on liposomal membrane, they were incorporated to rigid membrane composed of DMPC and cholesterol in molar ratio of 1:0.6 by comparing with their parent, cholesterol, (Figure 22 and Table 10)

It was found that in the case of 1:1.2 molar ratio of DMPC to cholesterol (Figure 22C) a number of dyes binding to bilayers were small when compared to liposomes formed by ratio of 1 : 0.6 (Figure 22B).This might be due to the fact that cholesterol produced condensing effect in membrane above its phase transition temperature,[McIntosh,J.T., 1978; Marsh, D.,1973]; thus enhancement of membrane stiffness reduced penetration of dyes into bilayers. Furthermore, capable penetrated dyes reoriented mostly in dimer forms (Figure 22C) as a result of high rigidity in membrane. When cholesterol derivatives were additionally incorporated, remarkable variations in both monomer (600 nm.) and dimer bands (557, 575 nm.) were observed and enlarged in the order of increasing side chain length, therefore, derivative II produced the greatest effect as demonstrated in Figure 22F, 24. Since hydrophobic part of membrane now was also crowded by cholestene nucleus of both cholesterol and its derivatives; thus condensing effect in this area should be also high, however, much more dyes could

penetrate into these membranes containing cholesterol derivatives as seen from Figure 22D, 22E and 22F, in contrast to Figure 22C. This might be due to high stiffness of membrane caused by high concentration of cholestene nucleus also made membrane more fragile and might be more sensitive; thus disturbance by long chain head group of cholesterol derivatives provided greater effect (Figure 22F, 24 and Table 11) than in liposomal membrane composed of DMPC and cholesterol derivative alone (Figure21C, 21D and 21E). Therefore, side chain in 3-position of cholestene nucleus in cholesterol derivatives should act in a different way to that of cholesterol since epicholesterol (α - isomer) which differed from cholesterol (β-isomer) just only in its configuration also gave different effect on membrane,[Presti,T.F. et al., 1982]. This experiment provided an evidence of elevation of fluidity in liposomal membrane by cholesterol derivatives and also ascertained the ability of BCP for that detection.

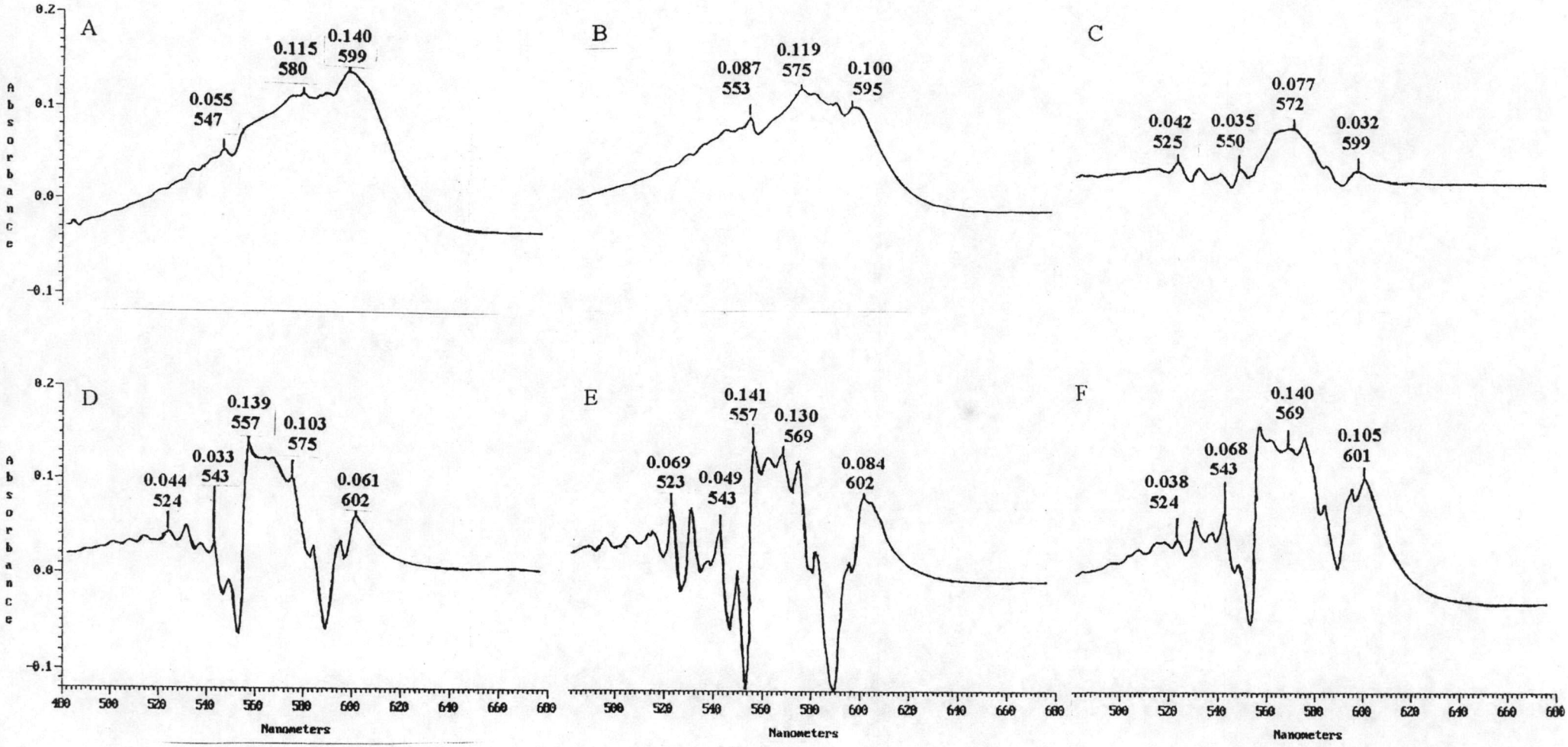


Figure 22 Absorption spectra of BCP in membranes made from various mixtures of DMPC , cholesterol and cholesterol derivatives in molar ratio as indicated. A. 1:0:0 ; B. 1:0.6:0 ; C. 1:1.2:0 ; D. 1:0.6:0.6 chol O E. 1:0.6:0.6 chol I and F. 1:0.6:0.6 chol II.

Table 10. Absorption intensity at each wavelength of BCP in membranes made from mixtures of DMPC, cholesterol and its derivatives.

Liposomal composition (molar ratio)	Wavelength (nm.) and intensity (absorbance unit)				
DMPC	599 (0.140)	580 (0.115)	_	_	547 (0.055)
DMPC : chol (1 : 0.6)	595 (0.100)	575 (0.119)	_	553 (0.087)	525 (0.042)
DMPC : chol (1 : 1.2)	599 (0.032)	572 (0.077)	_	550 (0.035)	542 (0.028), 533 (0.035)
DMPC : chol : chol O (1 : 0.6 : 0.6)	602 (0.061)	575 (0.103)	564 (0.124)	557 (0.139)	543 (0.033), 524 (0.044)
DMPC : chol : chol I (1 : 0.6 : 0.6)	602 (0.084)	576 (0.118)	569 (0.130)	557 (0.141)	543 (0.049), 523 (0.069)
DMPC : chol : chol II (1 : 0.6 : 0.6)	601 (0.105)	576 (0.147)	569 (0.140)	558 (0.161)	543 (0.068), 524 (0.038)

Table 11. Peak area of monomer and dimer bands of BCP from membranes composed of DMPC, cholesterol derivatives and / or cholesterol [a].

Liposomal composition (molar ratio)	Weight (mg.) [b]		Total weight (mg.)
	Monomer	Dimer	
DMPC : chol O (1 : 0.6)	10.315 ± 0.346	24.635 ± 0.502	34.950
DMPC : chol I (1 : 0.6)	13.970 ± 0.056	40.705 ± 0.615	54.675
DMPC : chol II (1 : 0.6)	15.025 ± 0.473	40.980 ± 0.603	56.005
DMPC : chol (1 : 1.2)	5.460 ± 0.240	24.025 ± 0.007	29.485
DMPC : chol : chol O (1 : 0.6 : 0.6)	10.270 ± 0.509	33.170 ± 0.451	43.440
DMPC : chol : chol I (1 : 0.6 : 0.6)	14.575 ± 0.064	31.680 ± 0.311	46.255
DMPC : chol : chol II (1 : 0.6 : 0.6)	20.710 ± 0.707	47.260 ± 0.442	67.970

a Measurement was performed by weighing of peak area from absorption spectrum.

b Each value represents the mean of three samples ±S.D.

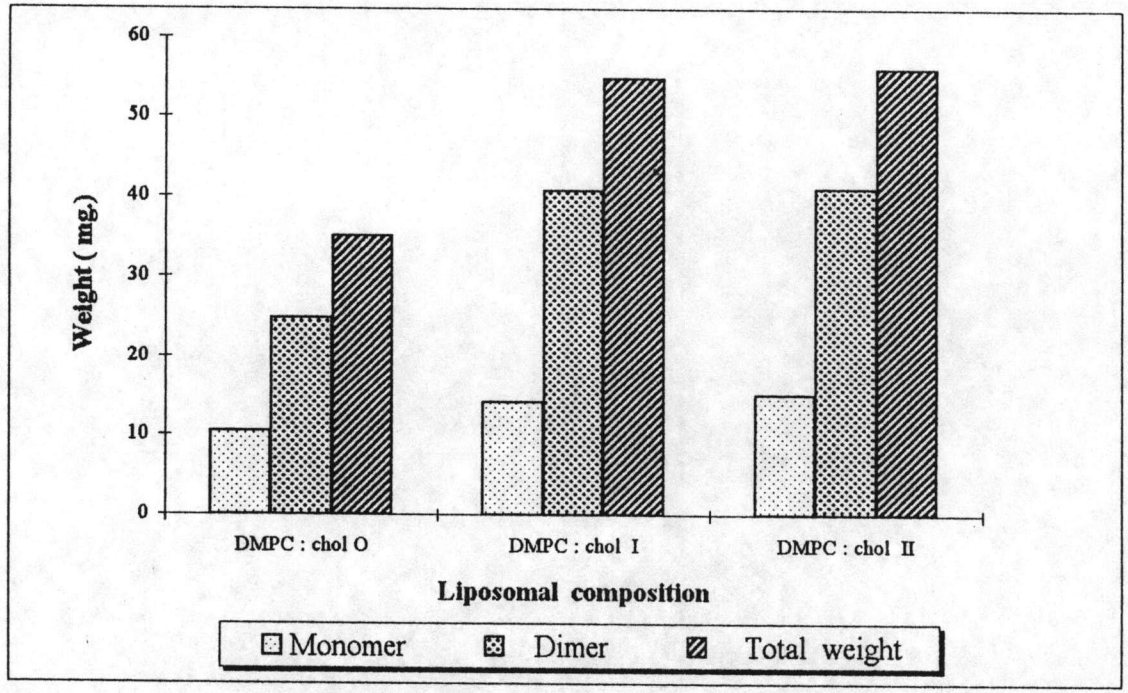


Figure 23. Peak area of monomer and dimer bands of BCP from membranes composed of DMPC and cholesterol derivatives (1 : 0.6, by mole).

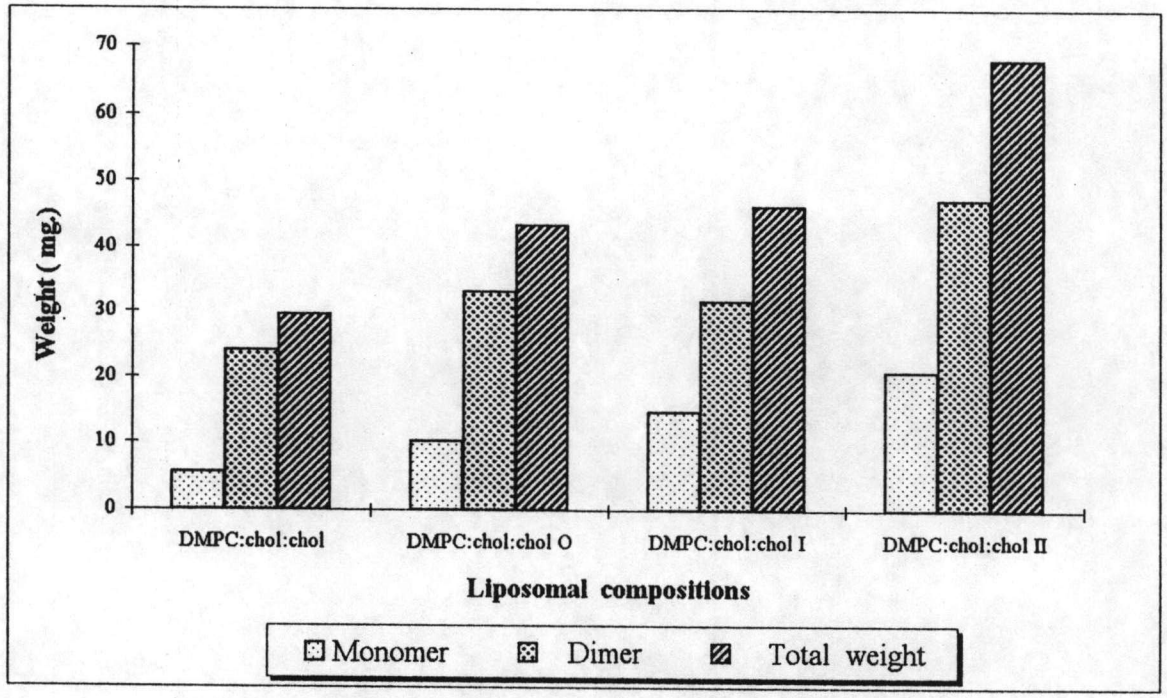


Figure 24. Peak area of monomer and dimer bands of BCP from membranes composed of DMPC, cholesterol and its derivatives (1:0.6:0.6, by mole).