Arora, Ashish (1996)
Some Synthetic and Biophysical studies on Glycerol Backbone Modified Phospholipid Analogs.

# 5. SUMMARY

All biological membranes, including the plasma membrane and the internal membranes of eukaryotic cells, have an overall common structure, which is comprised of macromolecular aggregates of amphipathic lipids and proteins held together by virtue of the hydorphobic force. The amphipathic lipid components, which are mainly the phospholipids and the glycolipids, are arranged as a continuous bilayer, 40-50Å in thickness. Glycerophospholipids, especially phosphatidylcholines, are the major component of eukaryotic cell plasma membranes.

Membrane lipids are composed of one or two fatty acyl chains and a polar (head) group that are linked either to glycerol or sphingosine. In bilayer configuration, the hydrocarbon chains are sequestered away from the aqueous phase, towards the interior of the bilayer, while the usually hydrated polar (head) groups are in contact with the aqueous phase.

Phosphatidylcholines bearing symmetric linear-saturated hydrocarbon chains, for ex. DPPC, can adopt four well-characterized lamellar phases, which in order of increasing temperature are the subgel phase, the gel-crystalline phase, the ripple phase and the liquid-crystalline phase. At subtransition, the subgel phase converts into the gel phase, at the pretransition, the gel phase converts into the ripple phase, and at the main transition, the ripple phase converts into the liquid-crystalline phase.

A number of studies have been performed to determine the structure and dynamics of membrane phospholipids to understand their functional and structural roles in biomembranes. These molecules in crystals, aggregated form or solution have a preferred conformation which seems to be determined primarily by the glycerol backbone. Further, the preferred conformation of the glycerol backbone has been shown to remain unaffected by the length of the acyl chains in phosphatidylcholines. Moreover, it has been

Acc. No. TH-018

suggested that the conformational preference around the glycerol bond between the carbon atoms that carry the two acyl chains could be governed by the parallel alignment of the acyl chains or by the acyl chain stacking in phospholipid aggregates. However, a similar conformation preference around this bond has also been observed for lyso phospholipids where only *sn-1* acyl chain is present. Consequently, it has been proposed that the above conformational preference is perhaps not induced by the chain stacking requirements. To further analyse the role of the acyl chain stacking in determining the preferred conformation of the glycerol backbone in phosphatidylcholines, phosphatidylcholine analogs having butanetriol residue, instead of glycerol moiety, as their backbone, and the two acyl chains in 1,2 or 1,3-arrangement (Fig.17) were synthesized, and the resulting phospholipids were analysed in organic solvents as well as in vesicles by high-resolution NMR spectroscopy. The aqueous dispersions of these phospholipids were further studied by electron microscopy, fluorescence polarization and differential scanning calorimetry.

The 500 MHz $^{1}H$ and $^{13}C$-NMR spectra were recorded for phospholipids dissolved in deuteriated chloroform. The $^{1}H$-NMR spectra of 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (Ic) differed markedly from that of the 1,3-dioctadecanoyl -*rac*-but-4-yl-[2-(trimethylammonium)ethyl]phosphate (IIc) and 1,2-dioctadecanoyl -*rac*-but-4-yl-[2-(trimethylammonium)ethyl]phosphate (IIIc), not only in chemical shift but also in the splitting patterns of some peaks. Coupling constants of vicinally coupled protons were determined by computer simulation of the experimental spectra, and from these coupling constants, the fractional populations of the three staggered-state rotamers about the various C-C bonds were calculated by using a generalized Karplus equation. This analysis revealed that there was a high degree of conformational preference around the C1-C2 bond of glycerol in Ic. The two preferred conformations are the rotamer A

(Fig.12a) characterized by the torsion angle $\theta_3/\theta_4$ = *ap/sc* and rotamer B with the torsion angle $\theta_3/\theta_4$ = *sc/-sc*. Both rotamers A and B are *synclinal* conformations, indicating that the two oxygen atoms on carbon atoms C1 and C2 to which the two acyl chains are attached have a *synclinal* arrangement, which would facilitate the parallel alignment of the two acyl chains. In contrast, these oxygen atoms in rotamer C (Fig.12a) are *antiperiplanar* and, therefore, the parallel alignment of the two acyl chains is difficult to achieve in this rotamer. The rotamer C has a very low population (5%) as compared to A (56%) and B (39%) (Table 22).

Similar analysis of IIIc about the C1-C2 bond gives values which indicate a preference of the rotamers A (55%) (Fig.31) and B (42%) over C (3%) (Table 22), suggesting that like Ic, the parallel alignment of the two hydrocarbon chains is preserved also in IIIc. Apparently, the intramolecular acyl chain stacking seems to be primary factor that determines the preferred conformation about the C1-C2 bond in the glycerol and butanetriol backbones. This arrangement of the acyl chain is energetically favorable, because it optimises both the van der Walls and hydrophobic interactions. However, no conformational preference is observed for the C2-C3 bond in Ic or IIIc. But, for the C3-C4 bond in IIIc, conformation G (Fig.31) with $\theta_1^{II}$ = +*sc* is the preferred conformer.

For IIc, no conformational preference was observed for the C1-C2 and the C3-C4 bond. However, for the C2-C3 bond, the conformational distribution is somewhat similar to that obtained for the C1-C2 bond of the glycerol backbone in Ic or the butanetriol backbone in IIIc. These results may be interpreted to suggest that a vicinal arrangent of the two acyl chains is essentially required for an effective intramolecular acyl chain stacking, and that structurally the C2 methylene residue in IIc probably represents the proximal beginning of the primary acyl chain.

The headgroup conformation appears to be similar in the phospholipids Ic-IIIc, since these compounds exhibit identical chemical shifts for the choline carbons in the $^{13}C$ spectra (Table 18). Further, all the three phospholipids had $^{31}P$-$^{1}H$ coupling constants in the range 6-7.2 Hz, which corresponds to a predominant *antiperiplanar* conformation about the C-C-O-P bond. Moreover, $^{1}H$-$^{1}H$ NOESY experiment confirm that the time-averaged orientation of the headgroup could be such wherein the choline methyl protons may lie within a 5Å distance from the C2 methine proton of Ic (or IIIc) or the C3 methine proton of IIc.

To examine whether the above NMR spectral characteristics of II or III are retained even in bilayers, the vesicles formed of Ib-IIIb (Fig.17) in $D_2O$ were analysed by both $^{1}H$ and $^{13}C$ NMR spectroscopy at 50°C. Both the $^{1}H$ and $^{13}C$ NMR spectra of Ib, IIb and IIIb in vesicles were similar to the corresponding spectra of Ic, IIc and IIIc in $CDCl_3$. However, the nonequivalence of the C2 methylene protons could still be clearly seen in the proton spectra of IIb (Fig.26) suggesting that the butanetriol backbone conformation observed for II (or III) in $CDCl_3$ is largely retained even in the vesicles. This is further supported by the similar $^{13}C$ chemical shifts observed for Ib in vesicles and Ic in $CDCl_3$, IIb in vesicles and IIc in $CDCl_3$, and IIIb in vesicles and IIIc in $CDCl_3$ (Table 18).

To further investigate the thermotropic phase behavior and packing in aggregates of these phospholipids in aqueous dispersions, the multilamellar and sonicated small unilamellar vesicles were characterized by electron microscopy, DPH fluorescence polarization and DSC.

Negative-staining transmission electron microscopy revealed that all the three phospholipid species formed vesicles of similar outer diameter which increased (20-35%) by including 33 mole% cholesterol in the vesicles bilayer. The acyl chain disorder in the

phospholipid bilayers was ascertained by measuring the fluorescence depolarization of DPH as a function of temperature. The polarization values for the vesicles comprising IIb or IIIb were smaller as compared to the vesicles formed of Ib. However, this difference in the polarization values continuouly diminished and ultimately vanished by including increasing amounts of cholesterol in the phospholipid bilayers. At 33 mole% cholesterol, all the three phospholipid bilayers showed similar fluorescence polarization values and exhibited no detectable phase transition atleast up to 50°C.

The DSC scans of Ib-IIIb multilamellar dispersions, which were incubated at 0-1°C for a minimum period of 3 days, exhibited multiple transitions. The aqueous dispersions of Ib exhibited three transitions with thermodynamic characterisitics similar to those reported for this phospholipid earlier. Almost similar thermal phase behavior was exhibited by IIb, except that the main transition was centered at lower temperature and was of lower enthalpy and entropy ($T_m$ , 35.1°C; $\Delta H$, 6.83 KCal/mole; $\Delta S$, 22.17 Cal/K.mole), as compared to Ib ($T_m$ , 41.4°C; $\Delta H$, 8.99 KCal/mole; $\Delta S$, 28.60 Cal/K.mole). Also, it was not well separated from the pretransition, as in case of Ib (Table 21). Unlike Ib and IIb, the phospholipid analogue IIIb exhibited only two endothermic transitions; a very broad, low enthalpy transition at about 25°C and the main transition at approximately 40°C ($\Delta H$, 8.66 KCal/mole; $\Delta S$, 27.67 Cal/K.mole), which was broader than that for Ib and IIb. The smaller values of the main thermal phase transition temperature ($T_m$) and the associated $\Delta H$ and $\Delta S$ values suggests that the phospholipid packing in the gel-crystalline state of the IIb (or IIIb) bilayers is less ordered, compared to Ib. Moreover, it is interesting to note that the racemic analog of Ib does not exhibit subtransition. Since both IIb and IIIb are racemic, IIIb is more closer in its behavior to rac-Ib than IIb.

As the permeability propeties of the phospholipid vesicles are usually correlated with the lipid packing in their bilayers, the time-dependent efflux of 6-CF from phospholipids vesicles was measured by entrapping a quenched concentration (250 mM) of this dye into small unilamellar vesicles of Ib-IIIb at 25°C. The 6-CF release from the vesicles comprising IIIb was slower as compared to the vesicles formed of Ib or IIb. However, this 6-CF release pattern changed after including 33 mole% cholesterol in the vesicles bilayer (Fig.30). At this cholesterol concentration, the permeability of 6-CF across the Ib/cholesterol bilayers was almost identical to that observed for the IIIb/cholesterol bilayers, whereas it was higher in case of the IIb/cholesterol bilayers.

Overall, this study shows that an insertion of one methylene residue between the C2 and C3 carbons in I does not significantly affect the thermotropic, DPH fluorescence polarization and permeability properties of its bilayers. But these properties are appreciably altered if a similar chemical change is introduced between the C1 and C2 carbons in I. These results clearly indicate that a vicinal arrangement of the two fatty acyl chains is essential for their effective packing in phosphatidylcholine bilayers. Finally, it may be interesting to mention here that like *rac*-Ib, more than 40% of IIb could be hydrolysed by phospholipase $A_2$, whereas IIIb, in agreement with the earlier report was completely resistant to the action of this enzyme.