Biological membranes are highly dynamic structures. The fundamental concept underlying the structure of biological membranes was formulated in the form of the 'Fluid Mosaic Model'. This was latter modified to account for the existence of structurally and functionally distinct domains in the 'Fluid Mosaic' structures. Lipids are capable of executing two types of motional modes - intramolecular movements and intermolecular movements. The intramolecular motions refer to the rotational, segmental and polar head group movements exhibited by the molecule. The intermolecular movements on the other hand refer to the rotational and translational movements of the molecule as a whole with respect to its neighbors. Both rotational and translational motions are expressed in terms of the membrane microviscosity. In biological membranes, the transbilayer motions of lipids, that is, the translational movement of the lipids across the bilayer has been found to result in large concentration gradients of the various lipid components on either monolayer. The apparent stability of such gradients suggests that they must have some functional implications. Investigations revealed that the asymmetric disposition of membrane lipids play an important role in several biological phenomenon such as, membrane fusion, endocytosis, exocytosis, regulation of membrane protein activity, modulation of surface potential, procoagulant activity of the blood platelets and the clearance of aged and diseased cells from the circulation[170-173]. Due to the relative simplicity of the red blood cell, most of the initial investigations on lipid asymmetry have been conducted using this cell as the model and its has been concluded that, the plasma

membrane of erythrocytes possess an ATP-dependent, protein- mediated pumping mechanism that shuttles lipids across the bilayer[172].

The asymmetric transbilayer distribution of the phospholipids in erythrocytes is widely accepted as a paradigm for the plasma membrane of a variety of cell types [467]. However, inspite of two decades of dedicated experimentation, the translocase has eluded precise identification. Moreover, it has been difficult to ascertain whether the membrane skeleton has any crucial role to play in maintaining the asymmetric lipid distributions. Due to the non-specificity in the biochemical methods of investigation, it becomes essential to carry out further studies using mutants that have genetically defined modifications in the cytoskeletal proteins and the putative translocase. Due to the ease of propagation and the ability to introduce defined mutations, microorganisms such as the bacteria, virus and yeast have been used extensively in correlating protein structure and biological function. Of these, the yeasts stand out in having a phospholipid distribution pattern that is typical of the higher eukaryotes. In addition, the yeasts also possess a membrane skeleton that is lacking in both bacteria and viruses. Hence, they might serve as good models in lipid asymmetry studies.

Transbilayer phospholipid distribution in intact yeast cells and spheroplasts

The yeast cell wall constitutes a complex mesh work of carbohydrates and glycoproteins, and serve as a protective shield for the underlying plasma membrane. Since lipid asymmetry studies involve the use of chemical labeling reagents such as TNBS and fluorescamine, and enzymatic probes such as

phospholipases, it is very essential to determine as to whether these reagents are capable of penetrating the cell wall to reach the plasma membrane surface. Also, it is important to establish whether or not the presence of a cell wall affects the transbilayer distributions of the plasma membrane phospholipids.

Studies on the labeling characteristics of the spheroplasts revealed that due to their osmotic fragility, spheroplasts were unsuitable for studies on lipid asymmetry. The heterogeneity in cell wall composition among different species of yeast, makes it very difficult to effect a controlled degradation of the cell wall to generate spheroplasts from different cell types, that have similar osmotic stability. A comparison of the data would hence be impossible. The primary interest in using spheroplasts as a model for lipid asymmetry studies stemmed from the requirement for accessibility of the outer leaflet lipids of the plasma membrane to phospholipases. Unfortunately, treatment of spheroplasts with phospholipase A_2 results in lysis. In addition, control spheroplasts that is, spheroplasts suspended in buffer having no enzyme, exhibit appreciable lysophospholipid formation, obviously due the effect of calcium ions present in the buffer on endogenous phospholipases. As a result of such difficulties, the data on phospholipase action is expected to be very exaggerate and hence not useful for asymmetry studies.

Contrary to the observations on spheroplasts, intact yeast cells seemed to respond very well to the chemical labeling reagents and hence might serve as potential models for studies on the lipid organization in yeasts.

Transbilayer phospholipid distribution in the yeast sterol mutants

Sterols are known to affect the rate of flip-flop of the membrane phospholipids. Studies on lipid asymmetry in higher eukaryotes have shown

that, due to the presence of a protein mediated lipid segregation, the phase state (the degree of fluidity) of a membrane has only a little role to play in the steady state distribution of the phospholipids on the basis of the polar head group. However, since systematic studies of lipid asymmetry in yeast have not yet been undertaken, it would be rather unwise to assume a protein mediated mechanism for generation and maintenance of the lipid asymmetry in yeast cells as well. Moreover, due to the increasing amount of literature on the correlation between the lipid phase transition and the kinetics of membrane bound enzymes in many cell systems including the yeast[229,299,508-510], the indirect influence of the sterol structure on the translocase/flippase activity still remains an open possibility. It would therefore, be an interesting exercise to assess the nature of the compositional asymmetry in the plasma membrane of yeast cells having an altered phase-state, as is the case with the sterol mutants.

All the three *erg* mutants studied were insensitive to the action of nystatin, while the wild type was sensitive to this antibiotic. Since, sensitivity of yeasts to polyenes is largely determined by the structure of the sterols in the membrane, it is reasonable to assume that none of the sterol intermediates in these mutants meet the minimum requirements for maintaining the effective sterol-nystatin interactions. Also, since most of the structural and geometric requirements for effective sterol- polyene interactions are the same as that for sterol-phospholipid interactions [530-532], it is expected that the sterol intermediates would be incapable of interacting effectively with the membrane phospholipids.

Most of the sterol intermediates accumulated by the *erg* mutants have structures that can introduce defects in the acyl chain packing. Such defects would be transmitted transversely to the neighboring chains leading to disorganization of the membrane. The lower anisotropy values for the mutants as compared to the wild type cells in the fluorescence polarization experiments showed that the plasma membrane was indeed relatively more disorganized in the mutants than the wild type.Chemical labeling of intact yeast cells showed that the wild type and *erg* mutants have comparable levels of PE at the plasma membrane outer leaflet. Saturable labeling could not be achieved for *erg*-6, apparently due to penetration of the reagents into the cells. This observation is in agreement with the reported hypersensitivity of this mutant to ions and other solutes[526,527]. It was also realized that, though TNBS gave the same extent of labeling as fluorescamine, the reaction times required to achieve saturable labeling with this reagent(TNBS) vary largely with the yeast species. Although, prolonged incubations at 10°C in alkaline buffers do not appreciably deplete energy sources for normal cells, the utility of this method (TNBS labeling) in mechanistic studies, where modified cells are to be used, becomes questionable. Unlike TNBS, fluorescamine displays a relatively stable labeling pattern. The percentage of external PE evaluated by this probe is in agreement with the TNBS data. The limited use of fluorescamine in mammalian systems probably stems from its tendency to penetrate to the cell interior and bring about non-specific labeling of the plasma membrane as well as the organelle membranes. This could well be attributed to the pH of the mammalian cell cytoplasm (pH7.5). Unlike the mammalian cells, yeast cells maintain their cytoplasmic pH at around 6.3. Even when the external pH is as high as 8.2, the

cytoplasmic pH does not increase beyond pH 7.0 [535-537]. Therefore, even if fluorescamine does enter the yeast cells, it would not be able to bring about any chemical modification at such a low pH, given the short life span of the molecule. This probably explains the good response of yeast to fluorescamine labeling. The fast reacting nature of fluorescamine circumvents the long term incubations in alkaline buffers. Moreover, fluorescamine being an uncharged molecule would be able to approach the plasma membrane surface more easily than TNBS. Both these properties give fluorescamine a good advantage over TNBS in reporting the status of the aminophospholipids at the plasma membrane surface of the yeast cells.

Mechanism of maintenance of the transbilayer phospholipid asymmetry in yeast cells

Investigations on the nature of phospholipid asymmetry in higher eukaryotes have revealed a great deal about the preference of phospholipids for the inner and outer leaflets. Studies on the mechanism of maintenance of this asymmetric distribution have suggested the involvement of some membrane protein(s) as well as of ATP [253,368,393,396,397,401,402,538,539]. However, till date, no systematic studies on transbilayer phospholipid organization have been undertaken for the lower eukaryotes, such as yeast. Since the presence of a phospholipid translocase has not been established in the yeasts, the experiments conducted were aimed at attributing properties characteristic of translocases to the transbilayer distribution of the aminophospholipids in the yeast plasma membrane.

The use of sodium azide, a mitochondrial ATP synthesis inhibitor suggests towards the possible involvement of ATP in the maintenance of the transbilayer PE asymmetry. This has further been supported by the use of the ATPase inhibitors; such as sodium ortho vanadate, DES, and miconazole nitrate. Thus,the inhibitor studies suggest towards the possible existence of ATPase-dependent process(es) that shuttle PE transversely across the lipid bilayer. The inhibition of this putative activity by NEM further confirmed the involvement of protein-mediated process(es) in the transbilayer PE movements. Further proof implicating the role of ATP in the regulation of PE distribution was provided by the reversible inhibition of this putative activity by decreased cellular ATP levels. The use of NEM in ATP-depleted and ATP-repleted yeast cells also showed, that the observed effects were due to an alteration in the activity at a single locus.

Thus, taken together, the inhibitor, protein modification and energy depletion/repletion studies strongly suggest towards the possible existence of protein mediated, ATP-dependent process(es) in the yeast plasma membrane that regulates the transbilayer distribution of PE.

The steady state transbilayer distribution of the phospholipids is a combined effect of selective localization of certain phospholipids at the inner leaflet of the plasma membrane and others at the outer leaflet. From a comparison of the present observations with those of the higher eukaryotes, it may be speculated that the yeast plasma membrane probably has a bidirectional energy driven lipid pumping mechanism that maintains a high concentration of the aminophospholipids at the inner leaflet. The putative in-to-out translocating pump is probably an NEM-sensitive, low affinity

ATPase that has a poor specificity for the various phospholipid classes, as opposed to the out-to-in translocating pump which is an NEM-insensitive, aminophospholipid-specific high affinity ATPase. This would lead to a situation, in which lipids are continuously though slowly translocated to the outer leaflet in a lipid species independent manner, while the aminophospholipids are rapidly and specifically translocated to the inner leaflet, leading to a steady state condition in which they are at relatively higher concentrations in the inner monolayer. The above hypothesis can explain the observations on aminophospholipid distribution. In addition to this, the hypothesis can also accommodate the observations made by Kean *et al.* on the out-to-in PC movements [502]. The complexity of intact yeast cells make it impossible to analyze the direct or indirect influence of other factors such as the plasma membrane H^+-ATPase in modulating the working of these two pumps. For a more quantitative picture it becomes essential to use refined methods for obtaining yeast plasma membrane preparations of exceptionally high purity which may then be utilized to study the dynamics of lipid movements.