Determination of Molecular Asymmetry in the Phosphatidylethanolamine Surface Distribution in Mixed Phospholipid Vesicles†

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ABSTRACT: A method has been developed for selectively labeling the amino groups of phosphatidylethanolamine molecules located in the inner surface of mixed phosphatidylethanolamine–phosphatidylcholine vesicles. The results of these experiments demonstrate that as the mole fraction of phosphatidylethanolamine in the vesicle increases, phosphatidylethanolamine molecules preferentially distribute toward the inner vesicle surface. In addition, all the primary amino groups of phosphatidylethanolamine in the mixed phospholipid vesicles are reactive with trinitrobenzenesulfonic acid and can be localized in either the inner or outer vesicle surface. The mixed phospholipid vesicles are found to be impermeable to trinitrobenzenesulfonic acid at 20 and 37°, with essentially no change in the observed labeling pattern at these temperatures. Bovine retinal rod outer segment disc membranes label to about 70% of their primary amino groups at 20°, while labeling to 100% at 37° (W. J. DeGrip *et al.* (1973), *Biochim. Biophys. Acta 323*, 125). Rhodopsin, in disc membranes labeled at room temperature with trinitrobenzenesulfonic acid, is stable, but undergoes complete thermal bleaching at 37°. Unlabeled discs exhibit no thermal bleaching in the temperature range from 20 to 37°. The temperature dependence of the degree of labeling of the disc membrane is thought to arise from the inherent thermal instability of labeled rhodopsin, *i.e.*, the change in the permeability of the membrane to the labeling reagent being a direct consequence of the denaturation of rhodopsin.

During the past several years, a number of reagents have been employed for the purpose of labeling the reactive groups of proteins and phospholipids in the surfaces of biological membranes (Wallach, 1972); these have been grouped as penetrating and nonpenetrating reagents, respectively (Bretscher, 1972; Gordesky and Marinetti, 1973; Rifkin *et al.*, 1972; Arrotti and Garvin, 1972). We wish to report further evidence for the formation of an asymmetric phospholipid bilayer in the form of phospholipid vesicles, which is suitable for evaluating membrane labeling reagents. A large number of recent physical measurements (Singer, 1971) have supported the contention that the majority of phospholipid in biological membranes exists as a phospholipid bilayer in which proteins are inserted. Therefore, a phospholipid vesicle enclosed by a single bilayer wall represents an ideal model for phospholipid labeling experiments in biological membranes. Some controversy has arisen over the extent of labeling attainable with trinitrobenzenesulfonic acid in experiments carried out at room temperature and 37°. In order to clarify this problem and to demonstrate the utility of the system, we have evaluated this membrane labeling reagent with respect to completeness of labeling and extent of bilayer penetration. In addition, we have studied both the temperature dependence of the labeling of phosphatidylethanolamine in mixed phospholipid vesicles and the thermal stability of rhodopsin in labeled and unlabeled retinal rod outer segment disc membranes.

Experimental Section

TNBS[1] *Determination of the Phosphatidylethanolamine Concentration.* The procedure for the determination of phosphatidylethanolamine concentration by reaction with TNBS has been described previously (Litman, 1973). The TNBS assay yields a linear relationship between absorbance and phosphatidylethanolamine concentration over the range of 0–0.9 μM phosphatidylethanolamine. This plot yields a line with a slope of 1.445 A/μM of phosphatidylethanolamine and a correlation coefficient obtained by a linear least-squares fit of the data of 0.9997.

Determination of the Ratio of Phosphatidylethanolamine in the Inner Vesicle Surface to the Total Phosphatidylethanolamine Content by Reaction with TNBS. Vesicles were prepared for labeling of the inner surface phosphatidylethanolamine molecules by colyophilization of phosphatidylethanolamine and phosphatidylcholine in the desired mole fraction ratio; this was followed by hydration in pH 5 NaOAc buffer (0.1 M) containing either 0.2 or 0.3 M TNBS and ultrasonic irradiation for 1.5 hr at 2° under a constant flow of argon. After centrifugation at 100,000*g* for 30 min, the sample was concentrated by ultrafiltration to 2 ml and passed down a 1.5 × 20 cm G-50 column which was preequilibrated with NaOAc buffer. The void volume fraction of the G-50 column was concentrated to 2 ml by ultrafiltration and passed down a 1 × 30 cm Sepharose 4B column which was preequilibrated with NaOAc buffer; 2-ml samples were collected. Labeling of the inner vesicle surface was accomplished by diluting 100–200-μl aliquots of the Sepharose 4B column fractions to 2 ml with 1.8% $NaHCO_3$ buffer (pH 8.5). The reaction was allowed to proceed for 2 hr in the dark, at room temperature. After this development period, the reaction was stopped by addition of 2 ml of 0.5 N HCl in 96% propanol. Prior to reading at 410 nm, the samples were spun for a minute to a desk top centrifuge to remove any air bubbles. Labeling at 37° was carried out by equilibration of a mixed phospholipid vesicle and blank solution at the desired temperature, after which TNBS reagent was added. At specified times aliquots of both vesicle solution and blank were removed in triplicate and mixed with the appropriate acidic propanol solution. The solutions were allowed to attain room temperature and read as described above.

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[1] Abbreviation used is: TNBS, 2,4,6-trinitrobenzenesulfonic acid.

TABLE I: Inner Surface Labeling Experiments in Mixed Phosphatidylethanolamine–Phosphatidylcholine Vesicles.

Initial Mole Fraction of Phosphatidylethanolamine	Incorporated Mole Fraction of Phosphatidylethanolamine	Phosphatidylethanolamine in Inner Surface / Total Phosphatidylethanolamine
0.075[a]	0.084 (5)[c]	0.287
0.100[a]	0.115 (4)[c]	0.304
0.125[b]	0.141 (9)[c]	0.388

[a] Vesicles were formed in NaOAc buffer containing 0.2 M TNBS. [b] Vesicles were formed in NaOAc buffer containing 0.3 M TNBS. [c] Number of fractions analyzed.

Outer surface labeling, phospholipid purification, and phosphate assay have all been described in detail elsewhere (Litman, 1973).

Preparation of Bovine Retinal Rod Outer Segment Discs. Dark adapted bovine retinas were obtained frozen from the Hormel Co., Austin, Minn. All manipulations were carried out at 4° using dim red light (Wratten Safelight filter no. 1, Eastman Kodak, Rochester, N. Y.); 100 retinas were thawed and homogenized, using a Potter-Elvehjem tissue grinder, into 8 volumes of 0.1 M phosphate buffer. The homogenate was centrifuged at 27,000*g* for 20 min. The pellet obtained was purified by homogenization in 44% (w/v) sucrose, followed by flotation by centrifugation at 27,000*g* for 20 min. The float material was diluted with 3 volumes of 0.1 M phosphate buffer and centrifuged at 27,000*g* for 20 min. The pellet, representing 25 retinas, was homogenized into 30 ml of 40% (w/v) sucrose, placed in a centrifuge tube, and 5 ml of phosphate buffer was layered over the sucrose solution. A second flotation was carried out in the SW-27 swinging bucket rotor at 52,000*g* for 1 hr. The outer segments were collected at the buffer sucrose interface. The interfacial material was homogenized into 3 volumes of 0.1 M phosphate buffer and centrifuged for 20 min at 27,000*g*. The pellet obtained after an additional flotation was homogenized into 30 ml of cold water and spun for 30 min at 27,000*g*. The water wash was repeated three times.

TNBS Labeling of Retinal Rod Outer Segment Discs. A disc sample suspended in distilled water was diluted with 3.6 volumes of 1.8% bicarbonate buffer (pH 8.5). TNBS was added in a threefold excess of what was necessary for complete labeling of all the primary amino groups in the sample as 0.375 of the original sample volume. The sample was incubated for 2.5 hr at room temperature in the dark. After the incubation period, the sample was mixed with 4 volumes of 0.2 M sodium acetate buffer (pH 5); this shifts the solution pH to 5 and terminates the labeling reaction. The discs were then pelleted at 3000*g* for 5 min. The pellet was resuspended in 40 ml of 1.8% bicarbonate buffer (pH 8.5) and pelleted. The bicarbonate wash is repeated twice more, the disc being finally resuspended in 2 ml of bicarbonate buffer.

Thermal Stability of Rhodopsin in Unlabeled and TNBS Labeled Discs. All spectral measurements were made on a Cary 15 recording spectrophotometer employing a thermostated, 1-cm sample cell and standard rectangular, 1-cm reference cuvet. Temperature regulation was to within ±0.1° and was measured by a thermistor inserted in the flow line. The sample thermal equilibration time was determined, with the thermistor probe inserted in the cell, to be 3 min. Initial readings were made at 5 min after initiating the flow of thermostating solution; this value was taken as the time zero reading. Sample concentrations of both labeled and unlabeled disks were adjusted so as to yield 0.1 absorbance unit on bleaching. Measurements were made as follows: aliquot of the disc solution was placed in the thermostated sample cell at room temperature; another aliquot of the disc solution was placed in the reference cell. The instrument base line was set with both cells in place at room temperature. The thermostating solution was allowed to enter the cell and readings of the differential absorbance at 498 nm were made at the specified times on the 0.1 absorbance scale. After each reading the monochromator slits were closed and the cell was positioned away from the beam. The rate of bleaching was indicated by the growth of a negative difference trough at 498 nm.

Results

TNBS Labeling of Inner Surface Phosphatidylethanolamine Molecules. In experiments designed to obtain selective labeling of the inner vesicle surface, vesicle formation was carried out in a solution containing a high enough concentration of TNBS so that the internal volume of the vesicle would trap sufficient TNBS to label all the inner surface phosphatidylethanolamine. The solution was buffered at pH 5, which is acid enough to prevent the labeling reaction from proceeding, due to protonation of the primary amino groups. After removal of external label by molecular sieve chromatography, the pH of the sample was adjusted to 8.5, under which conditions the reaction with TNBS would proceed. In order to verify that no labeling had occurred prior to the adjustment of the pH to 8.5, aliquots of fractions from the Sepharose 4B column were diluted with 0.5 N HCl in 50% propanol and treated as a total phosphatidylethanolamine labeling test; absorbances for these controls were identical with those obtained for standard blanks, verifying that no labeling had occurred at pH 5. To ensure that sufficient TNBS to yield complete labeling of inner surface phosphatidylethanolamine molecules was being trapped during the sonication process, aliquots of Sepharose 4B column fractions were mixed with 1.8% $NaHCO_3$–50% propanol buffer (pH 8.5), without the further addition of TNBS. The absorbances observed in these tests were intermediate between those obtained from inner surface labeling tests and those obtained from total labeling experiments, indicating that TNBS was trapped in excess of what was required to completely label the inner surface phosphatidylethanolamine molecules. The results of selectively labeling phosphatidylethanolamine molecules in the inner vesicle surface of mixed phosphatidylethanolamine–phosphatidylcholine vesicles are shown in Table I. The ratio of the inner surface phosphatidylethanolamine to total phosphatidylethanolamine content is seen to increase with increasing mole fraction of phosphatidylethanolamine.

Temperature Dependence of the Labeling of Outer Surface Phosphatidylethanolamine Molecules in Mixed Phospholipid Vesicles. The color development in the reaction between TNBS and phosphatidylethanolamine, in 1.8% $NaHCO_3$–50% propanol (pH 8.5), reaches completion in 90 min and remains constant for at least an additional 85 min at room temperature. A similar time curve obtained in outer surface phosphatidylethanolamine labeling experiments on mixed phospholipid vesicles, in 1.8% $NaHCO_3$ buffer (pH 8.5) at room temperature, yielded a constant absorbance after 40 min which remained steady for at least another 200 min. The time dependence of the TNBS reaction with phosphatidylethanolamine in mixed phospholipid vesicles at 37° demonstrates the same plateau as was observed in the room temperature labeling experiments, but

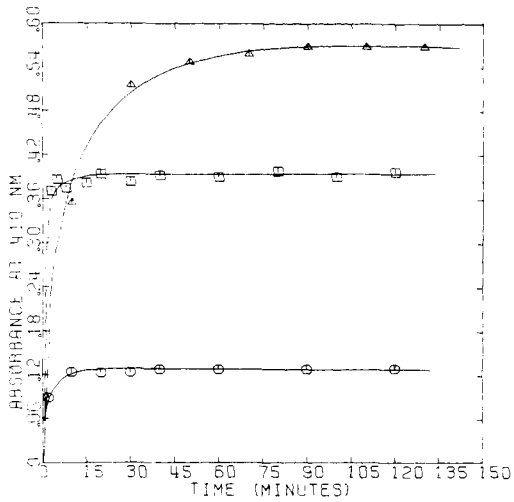

FIGURE 1: Time dependence of color development in TNBS assay for phosphatidylethanolamine: rate of color development in assay for total phosphatidylethanolamine content (△), and for outer surface labeling reaction at room temperature (○) and at 37° (□).

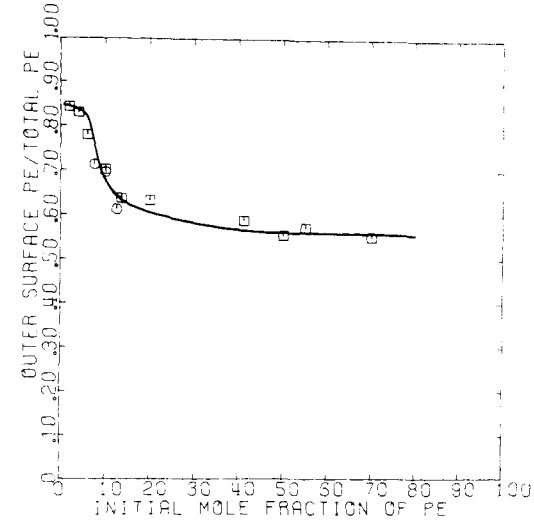

FIGURE 3: The dependence of the ratio of the outer surface phosphatidylethanolamine content to the total phosphatidylethanolamine content on the initial mole fraction of phosphatidylethanolamine as determined by selective labeling of the outer vesicle surface (□) and the inner vesicle surface (○).

shows an enhanced rate of reaction. The results of these experiments are summarized in Figure 1.

Thermal Stability of Rhodopsin in Labeled and Unlabeled Discs. The apparent absorbance at 498 nm of disc suspensions is made up of at least two components, that of the intrinsic absorption of the visual pigment rhodopsin and a scattering component due to the disc particles. The latter contribution is compensated for by using an aliquot of the disc suspension in the reference beam; this allows one to observe the decay of the 498-nm band of rhodopsin as a function of temperature. Measurements on unlabeled discs show that no thermal bleaching occurs in these membranes at 20 and 37°. At 20°, labeled discs exhibit no thermal bleaching, while at 37°, rhodopsin in labeled discs is completely bleached in about 2 hr (Figure 2).

Discussion

We have previously reported extensive chemical and physical studies on the characterization of mixed phospholipid vesicles formed by ultrasonic irradiation of lyophilyzed samples in aqueous buffer systems (Litman, 1973); these vesicles are formed of a single bilayer wall enclosing a trapped internal volume. During these studies it was found that the ratio of outer surface phosphatidylethanolamine to total phosphatidylethanolamine content was in qualitative agreement with the value of 0.73 which is the surface ratio for a vesicle with a diameter of 105 Å and a wall thickness of 40 Å. This ratio was found to drop as the mole fraction of phosphatidylethanolamine increased, reaching a plateau value of 0.56 at 0.5 mole fraction of phosphatidylethanolamine (Figure 3). This behavior was interpreted as indicating the formation of an asymmetric bilayer with a disproportionation of the phosphatidylethanolamine toward the inner surface of the bilayer. Reduced reactivity of phosphatidylethanolamine toward TNBS has been interpreted as being due to a masking of the primary amino groups of the phosphatidylethanolamine by hydrogen bonding to the phosphate head groups (Papahadjopoulos and Miller, 1967); evidence for this type of interaction has recently been presented from nuclear magnetic resonance studies (Phillips *et al.* 1972) and low angle X-ray diffraction studies (Rand *et al.*, 1971). If masking of otherwise reactive primary amino groups occurs due to interactions of this type, then the usefulness of labeling experiments to determine phospholipid asymmetry in biological membranes is cast in doubt; *i.e.*, a unique explanation of the basis of distribution is not possible. In order to clarify the interpretation of labeling experiments in general and to provide a unique explanation for the reduction of the outer surface phosphatidylethanolamine to total phosphatidylethanolamine ratio with increasing mole fraction of phosphatidylethanolamine, we developed a method for selectively labeling the phosphatidylethanolamine in the inner vesicle surface; this experiment was accomplished by trapping, during sonication, sufficient TNBS to label all of the phosphatidylethanolamine molecules on the interior vesicle surface. The trapping was carried out at pH 5, where the primary amino group of phosphatidylethanolamine is fully protonated and hence unreactive to TNBS. After removal of excess TNBS by molecular seive chromatography, the labeling reaction is activated by adjustment of the pH to 8.5; this activation step depends on the equilibration of the pH of the internal volume of the vesicle with the external pH. Previous studies have shown that phosphatidylcholine vesicles are impermeable to H^+ (Deamer *et al.*, 1972; Kornberg *et al.*, 1972); these studies demonstrate that gradients of several pH units can be developed across phospholipid bilayers and that equilibration of these gradients requires the presence of anions, A^-, so that the H^+ can traverse the membrane in the

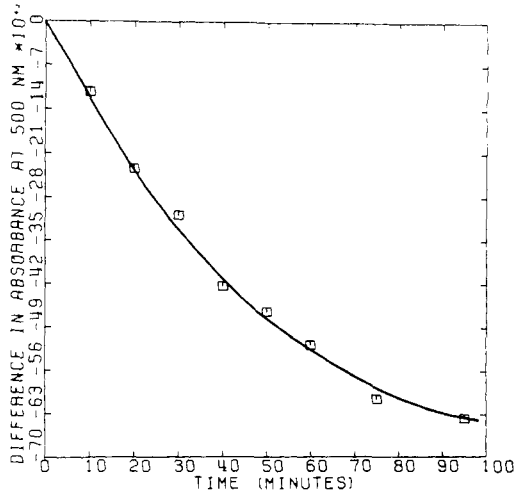

FIGURE 2: The time dependence of the difference in absorbance at 500 nm between TNBS labeled disc membranes at 37° and at room temperature (20 ± 1°).

form of HA. Thus it is likely that pH equilibration in these experiments occurs *via* the coupled transfer of H^+ and acetate ion in the form of acetic acid. In this way the internal pH of 5 is equilibrated with the external pH of 8.5, activating the labeling reaction.

Internal labeling experiments, coupled with the outer surface labeling experiments, will allow us to obtain complete labeling of all the phosphatidylethanolamine molecules if no masking due to head group interactions occurs. The values of the outer surface phosphatidylethanolamine to total phosphatidylethanolamine ratio obtained in labeling of the outer vesicle surface along with those calculated from inner vesicle surface labeling experiments are summarized in Figure 3. It is seen that both types of experiments yield the same ratio values. In addition, in separate labeling experiments on vesicles of identical mole fraction of phosphatidylethanolamine, the sum of the outer surface phosphatidylethanolamine and the inner surface phosphatidylethanolamine was identical with the total phosphatidylethanolamine content of the system. Thus all primary groups of phosphatidylethanolamine are accessible to reaction with TNBS. These results do not prohibit the kind of head group interactions suggested previously, but indicate that if they exist, they do not preclude labeling with TNBS.

The results obtained here support the contention that there is an asymmetric surface distribution of phosphatidylethanolamine in mixed phosphatidylethanolamine–phosphatidylcholine vesicles. The plateau region in the time course of the reaction of TNBS with phosphatidylethanolamine containing vesicles demonstrates the nonpenetrability of TNBS with respect to the phospholipid bilayer structure. Gordesky and Marinetti (1973) have studied the labeling of erythrocyte membranes with TNBS and also conclude that this reagent is of the nonpenetrating type. In contrast, Arrotti and Garvin (1972) have reported that TNBS penetrates the erythrocyte membrane with subsequent labeling of internal hemoglobin. The lack of agreement over the membrane penetrating nature of TNBS very likely lies in the conditions under which the labeling experiments were carried out. Gordesky and Marinetti (1973) and the work reported here employ a room temperature incubation for the labeling experiments, while Arrotti and Garvin (1972) employ an incubation at 37°.

Labeling of phosphatidylethanolamine containing vesicles at 37° and thermal stability studies of rhodopsin in labeled and unlabeled disc membranes were undertaken to evaluate the effect of labeling on the structural integrity of both membrane phospholipid and protein. The results of these studies indicate that the mixed phospholipid vesicles retain their impermeability to TNBS at 37°, demonstrating that any leakiness which develops in the membranes at this temperature is not due to the membrane phospholipid which exists in bilayers. The question then arises as to the effect of labeling on the membrane protein. Our results concerning the thermal stability of unlabeled discs are in good agreement with those of Hubbard (1958), *i.e.*, that these discs are completely stable to thermal bleaching at 20 and 37°. Our experiments indicate that disc membranes label to about 60% of their total amino groups in 2.5 hr at 20° (H. G. Smith, Jr., and B. J. Litman, unpublished results). Disc membranes labeled to this degree are stable for at least 3 hr at room temperature. These same disc membranes, in the absence of any excess TNBS, undergo complete thermal bleaching at 37°. After 2 hr at 37°, there is essentially no change in absorption upon exposing the sample cell to light for 20 min. These results demonstrate that while the integrity of the native membrane may be demonstrated at "physiological" temperature, *i.e.*, 37°, the labeled membrane is a new species with its own physical characteristics. The properties of the "native" membrane cannot be assumed for the labeled membrane. De Grip *et al.* (1973) have recently published experiments demonstrating that the labeling of disc membranes reaches a plateau at 70% of complete labeling at room temperature with no change in the absorbance at 500 nm. At 37° labeling goes to 100%, with a decay in the 500-nm absorbance above 70% labeling. Our result provides a basis for interpreting the findings of both De Grip *et al.* (1973) and Arrotti and Garvin (1972). The phospholipid portion of the membrane is assumed to remain intact during labeling as is indicated by our vesicle labeling experiments. The loss of the membrane permeability barrier at 37° can be explained in terms of the thermal stability of the membrane protein. At 37°, a labeled species is formed which is not stable at this temperature; this species undergoes denaturation, which exposes previously buried amino groups. This process can create holes in the membrane making it permeable to TNBS, which results in labeling of material internal to the membrane vesicle. In a membrane such as the disc, which is 80–90% rhodopsin (Heitzmann, 1972), one would expect complete collapse of the membrane structure upon denaturation of this protein; this would lead to 100% labeling. In the erythrocyte only limited regions may be affected, creating holes in the membrane which would allow labeling of amino groups internal to the erythrocyte membrane without complete loss of the erythrocyte membrane integrity.

Our results indicate that within the limits of the stability range of the modified membrane formed in a labeling experiment, valid information may be obtained concerning the surface distribution of molecular species in biological and model membrane systems. However, TNBS is a strongly perturbing reagent. It is known to cause a large shift in the pK of the amino groups with which it reacts; this would have the effect of changing the net charge on the species so labeled. This perturbation is not strong enough to disrupt a phospholipid bilayer; that this may be the case is indicated by the fact that bilayer vesicles can be formed from fully charged phospholipids such as phosphatidylinositol (Litman, 1973). Protein conformation is known to be strongly effected by the net charge of the protein, as is evidenced by the pH denaturation of many proteins (Tanford, 1968). Although altering the net charge of primary amine containing phospholipids does not disrupt a bilayer configuration, it may be expected to weaken the rhodopsin–phospholipid interactions which are thought to play an important role in stabilizing the protein structure. The importance of these interactions is evidenced by the change in helical content which occurs upon bleaching of rhodopsin in detergent solution, but which does not occur upon bleaching of rhodopsin in its native disc membrane (Shichi *et al.*, 1969). In addition, thermal bleaching at room temperature is brought about by either complete removal of lipid or detergent or solubilization by detergents which do not possess hydrocarbon side chains long enough to stabilize the protein structure. The latter tends to indicate that hydrophobic interactions may be dominant in stabilizing the native rhodopsin configuration. Although the exact nature of the forces leading to destabilization of the TNBS labeled rhodopsin structure cannot be exactly delineated at this time, it is apparent that reaction of rhodopsin with TNBS has greatly reduced its thermal stability. One would expect to observe similar effects for other membrane proteins. Hence it would appear that the loss of the permeability barrier in the outer segment disc membrane, and probably in the erythrocyte, is due to denaturation of one or more classes of membrane protein.

There is some evidence that structural variations occur in

membrane surfaces as a function of temperature (Taylor *et al.*, 1971). Hence the ideal temperature at which to carry out labeling experiments meant to elucidate the surface distribution of membrane components is the "physiological temperature" for that organism. Depending on the nature of the perturbation introduced by the labeling group, this "physiological temperature" may be outside the range of stability of the modified membrane. In this case a compromise must be reached, *i.e.*, those conditions which most closely approximate "physiological conditions" and which are within the stability range of the modified membrane.

In summary we have employed the labeling reagent TNBS to obtain selective labeling of the primary amino groups of phosphatidylethanolamine in the inner and outer surfaces of a mixed phosphatidylethanolamine–phosphatidylcholine vesicle. The results of these experiments demonstrate that as the mole fraction of phosphatidylethanolamine increases there is a disproportionation of phosphatidylethanolamine toward the inner vesicle surface resulting in an asymmetric surface distribution of this lipid. Such asymmetric phospholipid distributions have been observed in the erythrocyte membrane (Bretscher, 1972; Gordesky and Marinetti, 1973), the bovine retinal rod outer segment disc membrane (H. G. Smith, Jr., and B. J. Litman, unpublished results), and in a model system composed of phosphatidylglycerol and phosphatidylcholine (Michaelson *et al.*, 1973). The driving force for this type of asymmetry in phospholipid vesicles is thought to arise from the packing requirements imposed upon the system by the small radius of curvature of the phospholipid vesicles (Litman, 1973). Certain biological structures, such as the microvilli of the intestinal epithelium, also exhibit morphological regions of radius of curvature similar to that of the phospholipid vesicles; these structures may be expected to contain similar asymmetries in the surface distribution of their membrane components. The confirmation of the presence of asymmetries of this type in a wider range of systems, the elucidation of the driving force for their formation, and their effect on the functional properties of the membranes in which they exist will provide new insight into structure–function relationships in biological membranes.

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