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ASYMMETRIC LABELING OF AMINO LIPIDS IN LIPOSOMES

HON CHEUNG LEE and JOHN G. FORTE

Department of Physiology-Anatomy, University of California, Berkeley, CA 94720 (U.S.A.)

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Summary

Fluorescamine and trinitrobenzenesulfonate were used as chemical probes to differentially label amino phospholipids in liposomes. At low concentrations, fluorescamine reacts primarily with amino lipids on the external half of the bilayer. Further increase in fluorescamine concentration resulted in a linear increase of labeling indicating penetration and reaction with the internal half of the bilayer. Because of the pH requirements of the fluorescamine reaction, internal labeling was eliminated with a H^+ gradient: inside acidic/outside alkaline. Differential labeling was also achieved with trinitrobenzenesulfonate, which is normally not permeable but which can be transported by valinomycin-K^+ complex and react with internal amines. Thus, either half of the bilayer can be labeled with the same or different reagents.

When liposomes were double-labeled, the fluorescence of fluorescamine was quenched by the trinitrobenzenesulfonate label. This quenching was reversed by solubilizing the liposomes with acidic ethanol. No quenching occurred when fluorescamine-labeled liposomes were mixed with trinitrobenzenesulfonate-reacted liposomes (or trinitrophenylated methylamine) suggesting close proximity of two labels is required for quenching. Conditions which promoted vesicular fusion promptly produced quenching.

These differential labeling procedures can be usefully applied to quantitate aminolipids on internal and external vesicular surfaces, monitor vesicular fusion, and assess liposomal structure.

Introduction

Aminolipids (phosphatidylethanolamine and phosphatidylserine) are common constituents of biological membrane. Their distributions are some-

Abbreviations: TNBS, trinitrobenzene sulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid.

times highly asymmetric between the two halves of the lipid bilayer. For example, in red blood cells only about 5% of all phosphatidylethanolamine is on the external half of the membrane. Most of the phosphatidylethanolamine and virtually all of phosphatidylserine are located in the internal half (cytoplasmic) of the bilayer [1,2]. This kind of asymmetry may have important implications in the structure and function of biological membranes. Previous methods used to measure this asymmetry include the use of phospholipases [3,4] and the impermeable amino group reagent trinitrobenzenesulfonate [1,2,5,6]. A fluorigenic reagent, fluorescamine, developed by Weigele and coworkers [7,8], has been used to label amino groups on chick embryo fibroblasts [9], mitoplast [10], erythrocyte and sarcoplasmic reticulum [11]. It was concluded that this reagent was impermeable to these biological membranes. However, conflicting results have been reported on erythrocyte and coleoptile tissue [12].

In this report, we have used liposomes as a model system to test the feasibility of using fluorescamine to measure asymmetry in aminolipid distribution. We conclude that at low reagent concentration fluorescamine labels primarily external aminolipids on the bilayer. By combining the use of fluorescamine and trinitrobenzenesulfonate we were able to label asymmetrically either half of the bilayer with one reagent or the other. The use of these labeling techniques for monitoring membrane fusion phenomena and the structures of lipid in suspension were also demonstrated.

Materials and Methods

Materials. Highly purified phosphatidylethanolamine and phosphatidylcholine from egg yolk were generous gifts from Dr. Wayne Hubbell. Phosphatidylserine from bovine brain, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and valinomycin were from Sigma Chemical Company. Fluorescamine was from Pierce Company. Monesin was a gift from Eli Lilly Company. All lipids were stored in chloroform at −18°C under nitrogen. Ionophores were dissolved in ethanol.

Liposome preparation. Phosphatidylethanolamine-containing, phosphatidylcholine liposomes were prepared using the sonication method as follows: 0.45 ml of 20 mg/ml phosphatidylethanolamine was mixed with 0.5 ml of 50 mg/ml phosphatidylcholine. Chloroform was evaporated by a stream of N_2. 10 ml of 20 mM sodium citrate buffer (pH 4.0) was added to the dried lipid and the resulting suspension was sonicated at 4°C under N_2 atmosphere for 55 min using a Branson Sonifier (model W350). The pulsed mode of 50% duty cycle was used and the output power was at 75 W. After sonication, the liposomes were centrifuged at 48 000 × *g* for 60 min at 4°C to eliminate the titanium dust from the tip of the sonicating probe and the small amount of poorly sonicated lipid.

Phosphatidylserine liposomes were prepared by essentially the same method, except that a medium containing 100 mM NaCl, 0.1 mM ethylenediaminetetraacetate (EDTA) and 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.4, was used and the sonication was performed at room temperature under N_2 atmosphere for 15 min.

Negative staining electron microscopy. Liposomes were negatively stained by mixing with an equal volume of 2% sodium phosphotungstate (pH 7.4). A drop of the mixture was put on a 200 mesh formvar-carbon-coated copper grid. After 1 min, excess fluid was drawn off with a small piece of filter paper, and the grid was air-dried and examined with a JEM-100C electron microscope operated at 100 kV.

Labeling of the liposomes. The amount of free aminolipids on the liposomes was determined by a modified method of Litman [6]. Liposomes were reacted with 0.04% TNBS (final concentration) in 50 mM borate/NaOH, pH 8.2 (borate buffer), at room temperature for 2 h. The reaction was terminated by the addition of acidic propanol (0.5 N HCl in 96% *n*-propanol). The extent of the reaction was determined by measuring absorbance at 340 nm and compared with the control without liposomes.

Fluorescamine reacts with free amino groups to give a highly fluorescent product, while neither the hydrolyzed fluorescamine nor the reagent itself is fluorescent. Therefore the increase in fluorescence intensity is a direct measure of the extent of labeling. The fluorescent product also has a characteristic absorption maximum at 390 nm [13]; however, due to the superior sensitivity we will use the fluorescence method exclusively in this study. Liposomes were labeled with fluorescamine in a medium containing 50 mM borate buffer by rapid injection of a small aliquot of an acetone solution of fluorescamine through a micro syringe. The reaction mixture was vortexed during the injection to achieve rapid mixing which is essential to the reproducibility of the labeling. The final acetone concentration never exceeded 2%. The extent of the labeling was determined by dissolving small aliquots of the reacted liposome suspensions (50—100 μl) in 2 ml acidic alcohol (1 mM citrate, pH 4.0, in 99% ethanol) and the fluorescence measured at 480 nm with the excitation wavelength set at 380 nm.

Miscellaneous. All fluorescence measurements were made with a Perkin-Elmer MPF-44A spectrofluorimeter. Right-angle light scattering was measured in the same instrument with the excitation and emission wavelengths both set at 600 nm. H^+ gradient across the liposomes was measured by the accumulation of acridine orange as determined by the quenching of its fluorescence. It has been shown that acridine orange, like several other fluorescent amines, behaves as a weak base whose distribution across the membrane is a sensitive indicator of ΔpH [14,15]. Wave lengths were 493 → 530 (excitation → emission).

Results

Examination of negatively stained phosphatidylethanolamine-containing, phosphatidylcholine liposomes by electron microscopy revealed that the majority of liposomes were of diameters ranging from 25 to 35 nm. Occasionally vesicles with diameters up to 50 nm were encountered. From the general size range and substructre we conclude that the vesicles are unilamellar.

Fig. 1A shows the extent of labeling liposomes by fluorescamine in the absence and presence of Triton X-100. The fluorescence intensity was measured as a function of increasing amount of fluorescamine with a fixed amount

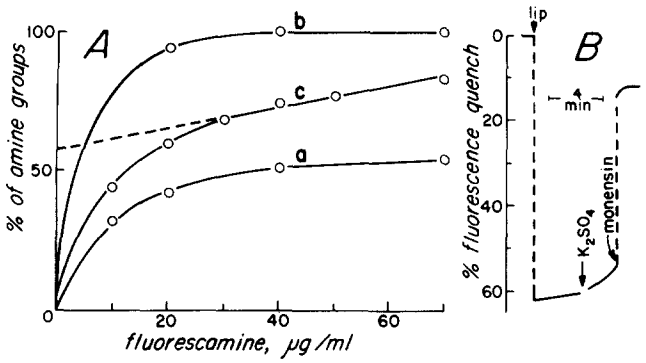

Fig. 1. Characterization of phosphatidylethanolamine-containing phosphatidylcholine liposomes prepared with a H^+ gradient. (A) Labeling of amine groups with fluorescamine. Liposomes were prepared in pH 4.0 buffer and transferred to 50 mM borate/NaOH buffer (pH 8.2) with a final concentration of 0.17 mg phospholipid/ml. The liposomes were then reacted with various amounts of fluorescamine, as indicated, and the fluorescence intensity of the product was measured as described in Materials and Methods. Total amino groups (100%) was taken as the saturating value in the presence of Triton (see curve b). Curve a: the reaction mixture contained only liposomes and fluorescamine. Curve b: Triton X-100 (1 mg/ml) was added to solubilize the liposomes before the labeling. Curve c: monensin (0.5 μg/ml) was added to dissipate the H^+ gradient across the liposome before the fluorescamine addition. (B) Measurement of H^+ gradient across the liposomes by the uptake of acridine oragne. An aliquot of suspended liposomes (0.68 mg in 200 μl), prepared in acidic citrate buffer (pH 4.0), was added to 1.8 ml 50 mM sodium borate buffer (pH 8.2) and 10 μM acridine orange at the indicated time (lip). This created a H^+ gradient across the liposomes and produced a quenching of the fluorescence of acridine orange as the weak base was accumulated by the liposomes. Addition of 2.5 mM K_2SO_4 partially dissipated the gradient. Further addition of 1 μg/ml monensin greatly enhanced the dissipation via Na^+-H^+ exchange.

of lipid. As can be seen, the extent of labeling is a saturable function of fluorescamine concentration with the amount of label in the absence of Triton (curve a) being about 54% of that in its presence (curve b). Thus, by solubilizing the liposomes more free amine groups are exposed for labeling. Since the liposomes are primarily unilamellar vesicles, the simplest interpretation of this effect is that only the external amino groups of the outer half of the bilayer are reactive in the native state and that solubilization exposes those additional groups on the inner half of the membrane. This would imply that either the membrane is impermeable to fluorescamine, or that the penetrated reagent is inactive in the intravesicular environment. The latter possibility gains support from the fact that the liposomes were prepared at an acidic pH. Even though the liposomes were subsequently transferred to a favorable alkaline reaction medium, a H^+ gradient (internal acidic) can still be maintained. That this is indeed the case is shown in Fig. 1B where the accumulation of the fluorescent amine (acridine orange) was used to monitor the H^+ gradient across the membrane. As the acidic liposomes were diluted ten-fold into an alkaline medium there was a marked accumulation of acridine orange, as revealed by the quenching of fluorescence, thus demonstrating a H^+ gradient across the liposomes. The gradient can be partially dissipated by adding K^+ as shown in the figure, indicating that the liposomes are somewhat permeable to both H^+ and K^+. The maintenance of the H^+ gradient before the addition of K^+ is thus due to the lack of counter ion flow rather than the impermeability to H^+. The rate of dissipation is, however, greatly accelerated by the addition of a Na^+-H^+

exchanger, monensin. Thus we established that under our labeling conditions, there was a H^+ gradient existing across the membrane such that even if fluorescamine were permeable it would be unreactive in the acidic intravesicular environment. Curve c in Fig. 1A shows the effect on the labeling pattern when the H^+ gradient was eliminated by the addition of monensin. Even in the absence of a H^+ gradient the extent of labeling is still less than that produced by Triton up to relatively high fluorescamine concentration, indicating that the reagent is not freely permeable to the membrane. The labeling curve shows a fast rise at low reagent concentration and a slow, nearly linear, increase as the reagent is further elevated. This is consistent with the interpretation that at low reagent concentration mainly the external groups are labeled giving the fast rise in the curve. As the external groups are saturated the further increase in the reagent concentration increases the flux of reagent into the vesicles which would be expected to be linearly dependent on the external reagent concentration. In fact, if the linear portion of curve c in Fig. 1A were extrapolated to zero reagent concentration (as shown by the dashed line), the intercept should give the total external label. Indeed, the extrapolated value of 57% is close to the saturating value of 54% in the presence of the H^+ gradient.

To further substantiate the interpretation that fluorescamine reacts with only the external amino groups when a H^+ gradient is present, we used TNBS as the external probe. As mentioned above, TNBS has been shown to be an impermeable reagent which reacts only with external amine groups in red blood cells and liposomal membranes [1,2,6]. The reaction of TNBS with amine groups results in a product that has an absorption maximum at 340 nm [17], which is far enough from the absorption maximum of 390 nm for the

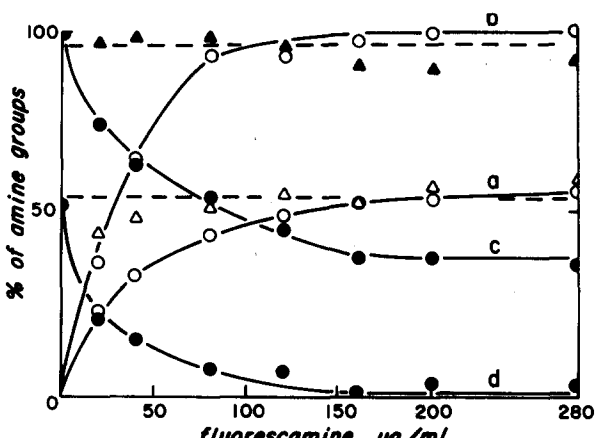

Fig. 2. Accessibility of amino groups on fluorescamine-labeled liposomes to TNBS. Phosphatidylethanolamine-containing phosphatidylcholine liposomes (0.68 mg/ml) were labeled with various amounts of fluorescamine as indicated and the reacted amino groups were measured without (curve a) or with (curve b) 1 mg/ml Triton X-100 as described in Fig. 1A. 0.3 ml aliquots of the reacted liposomes from experiments shown in curve a were further reacted with 0.1 ml of 0.8 mg/ml TNBS in 50 mM sodium borate (pH 8.2) for 2 h with (curve c) and without (curve d) 1 mg/ml Triton X-100. The TNBS reaction was terminated by addition of 0.3 ml acidic propanol and the resulting absorbance was measured at 340 nm as described in Materials and Methods. Control blanks consisted of parallel runs with TNBS added after the acid propanol. 100% of the TNBS reaction was taken as that with no fluorescamine label and in the presence of Triton X-100. The addition of curves a and d (△) should theoretically represent the total external amino groups available to both TNBS and fluorescamine. The sum of curves a and c (▲) represents the total amine groups on the liposomes.

fluorescamine product to allow for its determination without much interference from the latter. The liposomes were first labeled with fluorescamine, with or without Triton, as shown in curves a and b of Fig. 2. Again, the amount of labeling in the absence of Triton is saturated at about 56% of that in its presence. The amount of free amine groups left after the fluorescamine reaction in the absence of Triton was then assayed with TNBS reaction. This is shown in curves c and d of Fig. 2. When unlabeled liposomes were reacted with TNBS for 2 h at room temperature, the extent of the reaction in the absence of Triton was about 53% of that in its presence (i.e. zero fluorescamine concentration in Fig. 2). This corresponds well with the estimate of 56% external amine groups measured by the fluorescamine reaction. As the amount of fluorescamine labeling was increased, there was a corresponding decrease in the TNBS reaction. In fact, when curves a and d are added together, the result is a horizontal line (lower dashed line) of about 54% which is the total free amine groups on the external half of the bilayer available to both fluorescamine and TNBS. On the other hand, when the fluorescamine-labeled liposomes were solubilized before reacting with TNBS so that both the external and internal groups were available to the reagent, the resultant curve (curve c) was approximately parallel to curve d but shifted upward. When curves a and c were added together, the resultant was a horizontal line of about 96% (upper dashed curve) which corresponded to approximately the total amount of free amine groups on the liposomes (100%). From this we conclude that the same amine groups are accessible to both TNBS and fluorescamine.

Previous study has shown that TNBS can be transported through erythrocyte membranes as a K^+-valinomycin complex [18]. It was of interest to see if

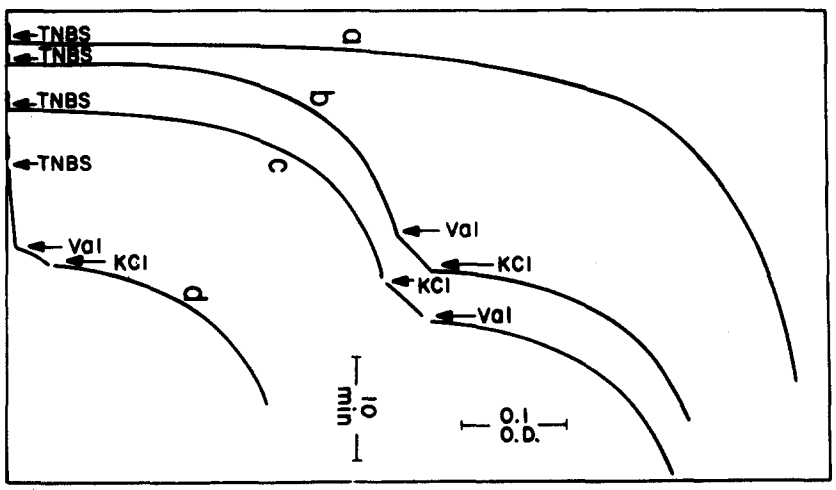

Fig. 3. Transport of TNBS into intravesicular space with valinomycin-K^+ complex. The TNBS reaction was monitored continuously by following the absorbance change at 350 nm after 0.22 mg/ml liposomes were reacted with 0.4 mg/ml TNBS (indicated by arrows labeled TNBS) in 50 mM sodium borate buffer, pH 8.2. Curve a: 0.5 mg/ml Triton was added to solubilize the liposomes before the TNBS reaction. Curves b and c: 2.5 μM valinomycin and 37 mM KCl were added in different order to transport TNBS into the intravesicular space. Curve d: the liposomes were first reacted with 50 μg/ml fluorescamine before the TNBS reaction. For all experiments the reference cuvette included identical solutions except for liposomes.

this is a general phenomena occurring also in liposomes. We therefore tested the effect of K^+ and valinomycin on the TNBS reaction with liposomes (Fig. 3). The time course of the TNBS reaction was followed by continuously monitoring the absorbance increase at 350 nm. As noted above, 50—55% of amine groups are available for reaction with TNBS. However, addition of valinomycin and KCl greatly increased the TNBS reactivity. Maximum stimulation required both valinomycin and KCl, and the extent of the reaction approached that in the presence of Triton. Thus we conclude that, similar to the erythrocyte membrane, valinomycin and K^+ complex can facilitate TNBS transport into the intravesicular space and react with the internal amine groups in liposomes. The TNBS reaction was not inhibited by an acidic internal environment because the H^+ gradient was dissipated by the addition of valinomycin and KCl.

Since the valinomycin-K^+ complex can mediate TNBS transport into the intravesicular space we attempted to asymmetrically label the external amine groups with fluorescamine and the internal groups with TNBS. As shown in curve d of Fig. 3, liposomes were first labeled with fluorescamine so that most of the external groups were reacted. Addition of TNBS gave no further change in absorbance indicating no free amine groups were available. However, when valinomycin and K^+ were added, to transport TNBS to the internal space, the absorbance was greatly increased as the TNBS reacted with internal amine groups.

We showed earlier that by eliminating the H^+ gradient and increasing the fluorescamine concentration, the reagent was able to penetrate the membrane and react with the internal groups. Thus, we can react the liposomes first with

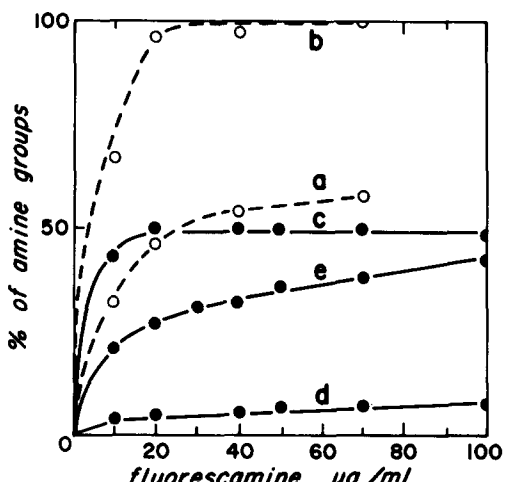

Fig. 4. Labeling of internal amino groups with fluorescamine. Phosphatidylethanolamine-containing phosphatidylcholine liposomes (0.17 mg/ml) were reacted first with 0.4 mg/ml TNBS for 2 h at room temperature to label all external amino groups. These TNBS-labeled liposomes (TNBS-liposomes) were further reacted with various amounts of fluorescamine as indicated. Curve c: TNBS-liposomes were solubilized with 1 mg/ml Triton before the fluorescamine addition. Curve d: TNBS-liposomes maintained intact and with H^+ gradient for the fluorescamine reaction. Curve e: 0.5 μg/ml monensin was added to dissipate the H^+ gradient before the fluorescamine reaction. Control experiments are also shown consisting of reacting identical amounts of liposomes, and with no prior TNBS labeling, with various concentration of fluorescamine with (curve b) and without (curve a) Triton. Total amine groups (100%) was taken as the saturating value in the control experiments in the presence of Triton.

TNBS so that all the external amine groups are reacted, then eliminate the H^+ gradient with monensin and label the internal groups with increasing amount of fluorescamine. When liposomes were first reacted with TNBS, subsequent reaction with fluorescamine under three sets of conditions produced the following results as shown in Fig. 4. After solubilization with Triton, 49% of the total amine groups were subsequently reactive with fluorescamine (curve c). Without solubilization, and in the presence of H^+ gradient, only about 7% of the amine groups were reactive with fluorescamine (curve d). When the H^+ gradient was eliminated by monensin the extent of fluorescamine reaction approached that of Triton-solubilized liposomes (curve e).

In all the double-labeling experiments presented above the fluorescence measurements were carried out in acidic ethanol as described in Materials and Methods. However, when the same experiments were performed in an aqueous buffer, it was observed that the TNBS reaction quenched the fluorescence of the fluorescamine label (Fig. 5). The liposomes were first labeled with fluorescamine so that most of the external groups were reacted. An aliquot of the reacted liposomes were put into borate-buffered aqueous medium and the fluorescence intensity was measured as a function of time. Addition of TNBS produced a slight quenching effect, possibly due to reaction with small amounts of unreacted external groups. Facilitation of TNBS entry into the liposomes by addition of valinomycin and K^+ produced a progressively more severe quenching. After about 30 min of reaction at room temperature, the fluorescence was quenched at about 25% of the fluorescence intensity before

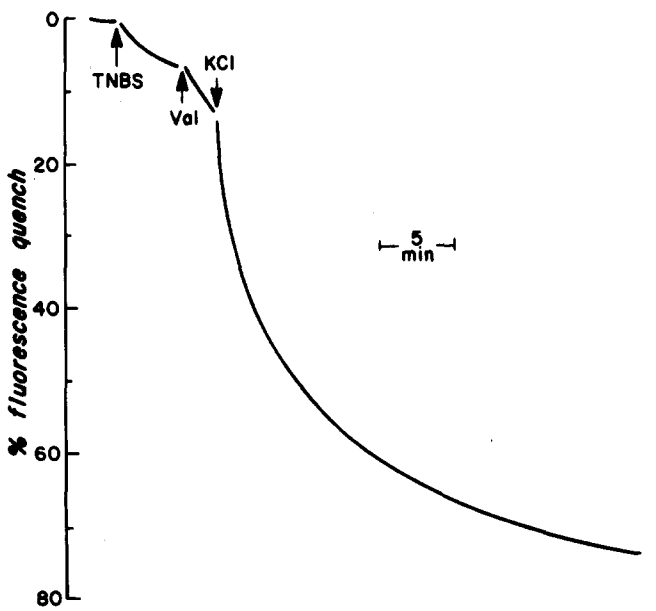

Fig. 5. Quenching of fluorescence of the fluorescamine label with the TNBS reaction. 0.17 mg/ml liposomes were first reacted with 25 μg/ml fluorescamine so that most of the external groups were labeled. 0.3 ml of reacted liposomes was diluted to 2 ml with 50 mM sodium borate buffer, pH 8.2, and the fluorescence intensity at 480 nm (excited at 380 nm) was monitored continuously. At the time indicated 0.1 mg/ml TNBS was added. Further addition of 2.5 μM valinomycin and 37 mM KCl was used to transport the TNBS to internal sites. This caused a large quenching of the fluorescamine signal.

TABLE I

THE EFFECT OF TNBS ON QUENCHING OF FLUORESCAMINE FLUORESCENCE IN AQUEOUS AND ACIDIC ALCHOHOL SOLUTIONS

Phosphatidylethanolamine-containing phosphatidylcholine liposomes (0.17 mg lipid/ml) were reacted with fluorescamine (15 μg/ml) as described in Materials and Methods, KCl (15 mM) and valinomycin (2.5 μM) were then added to the liposomes. Some aliquots were used directly for measurements (fluorescamine label) while others were treated with TNBS (0.1 mg/ml) to produce doubly labeled liposomes (fluorescamine and TNBS). After reaction of amine groups, 50 μl of the liposomal suspension was diluted into 2 ml of either acidic alcohol (99% ethanol, 1 mM citrate, pH 4.0) or aqueous borate buffer (50 mM, pH 8.2), as indicated. Fluorescence intensity at 480 nm (excitation 380 nm) was then measured and the mean ± 1 S.D. was calculated for a number of samples (n). The fluorescence of the fluorescamine-labeled liposomes dissolved in acidic alcohol was set arbitrarily at 100%.

Liposome label (suspending solvent)	Fluorescence intensity
Fluorescamine (acidic alcohol)	100 ± 2.1 (n = 5)
Fluorescamine (aqueous medium)	51.5 ± 1.0 (n = 4)
Fluorescamine and TNBS (aqueous medium)	20.8 ± 1.0 (n = 4)
Fluorescamine and TNBS (acidic alcohol)	97.0 ± 2.5 (n = 5)

the addition of TNBS. As indicated above, when the fluorescence was measured in acidic alcohol, no such quenching was observed.

Since it appeared that the quenching effect was eliminated by solubilizing the lipids into monomers we further investigated the effect of solvents on the fluorescence of fluorescamine label. We found that the fluorescence is a function of the hydrophobicity of the solvent, confirming previous findings by others on fluorescamine-labeled cloacin from *Escherichia coli* [19]: thus, the more hydrophobic the solvent, the higher the fluorescence intensity. However, when the intensity was plotted against the empirical hydrophobicity index of Kosower [20], the linear relationship was not obeyed unless the solvents were acidified (data not shown). Therefore it appears that acidification neutralizes the charges on the phospholipids and allows them to go into truly homogeneous solution. In Table I, the effect of hydrophobicity and the reversal of TNBS quenching by acid alcohol is shown. Liposomes were first reacted with fluorescamine. Aliquots were taken either for direct measurement, or doubly labeled by TNBS in the presence of K^+ and valinomycin. When liposomes were labeled only with fluorescamine the fluorescence intensity in the aqueous medium was about half of that measured after solubilization in acidic alcohol. Doubly labeled liposomes in aqueous medium gave only about 20% of the fluorescence intensity. However, when the doubly labeled liposomes were solubilized in acid alcohol, all the fluorescence was recovered.

The above experiments suggest that the proximity between the fluorescamine and TNBS labels may be responsible for the fluorescence quenching. In support of this interpretation when liposomes are separately labeled with fluorescamine and TNBS, mixing them together in aqueous medium does not result in quenching. Thus it appears that the two labels must be close enough to interact through some energy transfer mechanism and/or screening, and produce the quenching of fluorescence. Solubilization by acidic alcohol into homogeneous solution increases the distance between labels, eliminating the quenching. This immediately suggested that this quenching phenomena might be used for monitoring fusion between liposomes.

It has been shown that phosphatidylserine liposomes can be induced to fuse by the addition of Ca^{2+} [21]. Thus, small, spherical vesicles were shown to change into large, scroll-like cylinders. Addition of EDTA to these cylinders chelates Ca^{2+} and transforms them to large spherical vesicles. This fusion phenomenon has been well-characterized by electron microscopy [21], dynamic light scattering and turbidity [22]. We will use this system to test the possibility of measuring the fusion process with our labels. If one population of phosphatidylserine liposomes is labeled with fluorescamine and the other with TNBS, mixing them together should produce no change in fluorescence. Induction of fusion of these two populations of liposomes by Ca^{2+} should bring the label into close proximity and should result in quenching. This is indeed the case as shown in Fig. 6. After mixing TNBS-labeled liposomes with fluorescamine-labeled liposomes there was a small decrease in fluorescence intensity, which was presumably due to the filtering effect of the highly colored TNBS-labeled liposomes. Subsequent addition of Ca^{2+} produced a large quenching (more than 60% of the intensity before Ca^{2+}). Chelation of Ca^{2+} resulted in only minimal restoration. As a control experiment only fluorescamine-labeled liposomes were used. Addition of Ca^{2+} resulted in a small increase in fluorescence, presumably due to the interaction of Ca^{2+} with the

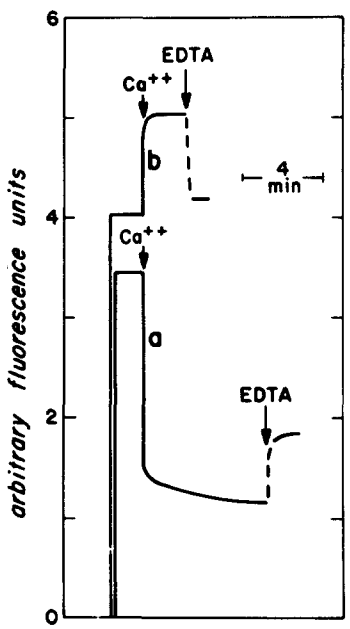

Fig. 6. Effect of Ca^{2+}-induced fusion on phosphatidylserine liposomes labeled with TNBS and fluorescamine. Phosphatidylserine liposomes (0.5 mg/ml) were separately labeled with either 100 μg/ml fluorescamine or 0.4 mg/ml TNBS for 2 h at room temperature. In curve a, aliquots of fluorescamine-labeled and TNBS-labeled liposomes (20 μl and 200 μl, respectively) were mixed and diluted to 2.0 ml with 50 mM sodium borate buffer. Fluorescence was measured at 480 nm (excitation 380 nm). At the indicated time, 5 mM $CaCl_2$ was added to induce massive fusion of the liposomes which resulted in dramatic quenching of the fluorescence. Addition of 6 mM EDTA resulted in only minimal reversal of the quenching. A control experiment was run in parallel which included only the fluorescamine-labeled phosphatidylserine liposomes (curve b). Addition of Ca^{2+} caused a slight enhancement of fluorescence, instead of quenching, and was fully reversible by EDTA.

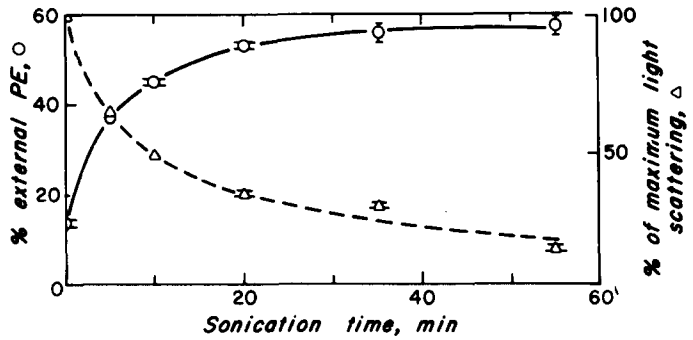

Fig. 7. Effect of sonication on the percentage of external amino groups. Phosphatidylcholine (25 mg) and phosphatidylethanolamine (9 mg) were vortexed vigorously in 10 ml of 20 mM sodium citrate buffer, pH 4.0, (zero sonication time), and subsequently sonicated for various periods of time. Aliquots (50 μl) were withdrawn, diluted to 1 ml with 50 mM sodium borate, pH 8.2, and reacted with 70 μg fluorescamine in the presence and absence of Triton. The ratio of fluorescence intensity between the two was taken as the percentage of external groups being labeled. Right-angle light scattering at 600 nm was measured in parallel experiment where 50 μl of liposomes were diluted to 2 ml with borate buffer. The maximum amount of light scatter (zero sonication time) was taken as 100%.

labels. For example Ca^{2+} might bind to the phospholipid head groups, reducing the electrostatic repulsion, and allow the fluorescamine label to move into a slightly hydrophobic environment. Chelation of Ca^{2+} restored most of the fluorescence as expected from a pure binding process.

Another possible application of the asymmetry labeling technique is to assess the extent of multilamellar structure in liposomes preparation. This is because the percentage of external groups to the total number of groups should be very low in a multilayered structure (much less than 50%), while, the number of external groups in unilamellar liposomes is proportional to the surface area of the external and internal surfaces. The ratio of these two surface areas is, in turn, proportional to [(external radius)^2/(internal radius)^2]. Therefore we can measure external groups and compare to the total (e.g. obtained by solubilization with Triton) at various sonication times to obtain some information on the preparation (Fig. 7). At zero sonication time, the liposomes should be multilamellar and thus should have low percentage of external groups (approx. 14% observed). As the sonication time increased, the percentage of external groups progressively increased toward a saturating value of about 58%. Also, for comparison, the corresponding decrease in 90° light scattering of the liposomal suspension is shown in Fig. 7.

Discussion

In this report we demonstrated that, at low concentration, fluorescamine can be used as a highly sensitive probe to primarily label external aminolipids on unilammelar liposomes. As the reagent concentration was increased more of the internal amine groups were also labeled. It has been pointed out that the highly hydrophobic nature of fluorescamine should allow rapid penetration through the membrane [9—11]. However, since the reagent is rapidly deactivated through hydrolysis and shows even faster reaction toward free amine groups [12], the permeability barrier may still be sufficient to favor external site

labeling and the asymmetry would be especially apparent at low concentrations of reagent.

Conditions can be arranged to insure an even more selective labeling of external groups by fluorescamine. By manipulating the internal environment of the liposomes (imposition of a H^+ gradient) the reaction with internal amine groups was selectively eliminated. We have also observed that liposomes made from the dipalmitoylphosphatides of ethanolamine and choline have very low permeability to fluorescamine, even wihout a H^+ gradient (unpusblished observations). In this case the lipids are fully saturated and, at room temperature, are in a crystalline form, thus the additional barrier to permeation favors the more selective external labeling.

For the phosphatidylethanolamine-containing, phosphatidylcholine liposomes used in this study we found that about 56% of the total amine groups appear on the external surface. For randomly distributed lipids the percentage of external groups should be equal to the percentage of the external surface area. If we take 30 nm as the representative diameter of these liposomes and a bilayer of thickness of 4 nm [23], then the area of the external surface should be 64% of the total. The reasons for this discrepancy with our estimated value of about 56% can be two-fold. Firstly, a population of slightly larger vesicles may contribute to significantly lower the relative proportion of external groups. Secondly, the distribution of phosphatidylethanolamine between the inner and outer halves of the bilayer may be asymmetric. The packing geometry of the phosphatidylethanolamine head groups and the small negative radius of curvature of the inner surface may serve as a driving force for the concentration of phosphatidylethanolamine on the inner surface [6,24].

By the sequence of exposure to both fluorescamine and TNBS we were able to selectively and asymmetrically label one-half of bilayer aminolipids with either reagent. This should serve to extend the versatility of these chemical methods in measuring membrane asymmetry. The fluorescamine reaction has the added advantages of being highly sensitive with fast reaction time and rendering unnecessary the separation of unreacted reagent due to its deactivation through hydrolysis.

The introduction of a highly fluorescent probe selective to only one-half of a membrane bilayer has a number of intriguing possibilities. Two applications were demonstrated in this study: namely, monitoring the fusion process and assessing the structure of a lipid preparation. Other possibilities are currently being investigated. The limitation, requiring a H^+ gradient for totally selective fluorescamine labeling, can be satisfied in the application of the method to biological membranes with the use of the acid-base transition developed by Jagendorf and Uribe [25] for chloroplast membranes. Our preliminary results in applying this approach to a preparation of gastric microsomal vesicles have been promising.

For biological membranes, amine groups on both proteins and lipids are obviously available for reaction. Separation can easily be achieved by solvent extraction. The proportion of reacted aminolipids can subsequently be followed by thin-layer chromatography. Fluorescamine-reacted ethanolamine and serine phosphatides are easily visualized under ultraviolet light and their rapid migration, relative to the free aminolipids, permits quantitative assessment.

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