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THE USE OF FLUORESCAMINE AS A PERMEANT PROBE TO LOCALIZE PHOSPHATIDYLETHANOLAMINE IN INTACT FRIEND ERYTHROLEUKAEMIC CELLS

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Intact Friend erythroleukaemic cells (Friend cells) were incubated at 0–4°C with increasing amounts of fluorescamine. Phospholipids were extracted and the amounts of phosphatidylethanolamine and of its fluorescamine derivative were determined. (1). The plasma membrane of intact Friend cells appeared to be permeable to fluorescamine in a concentration-dependent way. (2). Three pools of phosphatidylethanolamine could be detected as the fluorescamine concentration was raised. The two first pools were ascribed to the outer monolayer (16–17% of the total cellular phosphatidylethanolamine) and inner (17–18%) monolayer of the plasma membrane, respectively, indicating an essentially symmetrical distribution of this phospholipid. The third pool of phosphatidylethanolamine (66%) corresponds to the contribution of intracellular membranes. (3). These data were used in turn, to calculate the relative amount of each phospholipid class present in the plasma membrane. The results are in perfect agreement with those obtained by an independent method involving the use of sphingomyelinase C (Rawyler, A., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1983) Biochim. Biophys. Acta 730, 130–138). The present method is discussed in terms of its applicability for the localization of phosphatidylethanolamine in eukaryotic cells.

Introduction

Specific reagents for amino groups have been widely used to localize aminophospholipids in biological membranes (see Refs. 1 and 2 for recent reviews). Interpretation of experimental data is generally based on the behaviour of putative non-permeant probes and/or on the comparison between the labeling patterns obtained with permeant and non-permeant reagents. However, it is often difficult to ascertain whether a probe is really non-permeant since the impermeability of a given membrane to the probe is largely dependent on the conditions used (probe concentration, temperature, pH, incubation time). Moreover, to interpret the extent of labeling of a major cytoplasmic protein component as a measure for the extent of probe penetration can be misleading, as was shown for instance by the work of Haest et al. [3]. Chemical labeling usually requires long incubation times, particularly at low temperatures, in order to achieve complete labeling of aminophospholipids at the outer surface of the cell. Since such prolonged incubations may alter the permeability barrier of the membrane as well as the uptake properties of the cell in an uncontrolled way, it may be safer to consider any chemical reagent of low molecular weight as a potential permeable probe.

In this respect, the use of a freely permeable probe to localize aminophospholipids in biological membranes may eliminate most of these problems,

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

provided that the reaction with its target molecules proceeds relatively fast when compared to the time it takes to permeate through the plasma membrane. As a consequence, the labeling pattern should only depend on the probe concentration and one may thus expect the different cellular pools of aminophospholipids (outer and inner plasma membrane pools, intracellular pool) to be detected as a function of the probe concentration.

This paper shows that it is possible to localize phosphatidylethanolamine in Friend erythroleukaemic cells using fluorescamine as a permeable, fast-reacting probe. The results indicate that in intact Friend cells, phosphatidylethanolamine is available in three pools, which correspond, respectively, to the outer and inner layer of the plasma membrane and to the phosphatidylethanolamine present in the intracellular membranes. In addition, the experimental data can be used to calculate the proportion of the total cell phospholipid which is present in the plasma membrane, the results of which are in full agreement with those earlier obtained from experiments involving various phospholipases [4].

Materials and Methods

Friend erythroleukaemic cells (derived from clone 745A) were cultured as described [5]. After washing and counting [4], cells were resuspended in labeling buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM $NaHCO_3$, 5 mM glucose and 20 mM Hepes or Tricine). The pH of this medium was adjusted at 20°C with NaOH, taking into account a ΔpK_a/deg. C of −0.014 (Hepes) or −0.021 (Tricine) [6], in order to obtain pH 8.0 at the chosen temperature (generally 0–4°C) for the labeling reaction.

Cell labeling was carried out as follows. Cells were equilibrated for 5 min at the desired temperature and a fixed number of cells ($6 \cdot 10^7$ cells in 1.5 ml labeling buffer) was pipetted into a series of centrifuge tubes. Increasing amounts of fluorescamine (up to 10 μmol) were added in 30 μl of dimethylsulfoxide and the suspension was gently vortexed for exactly 15 s. The reaction was stopped by adding 0.5 ml 1 M ammonia and vortexing for 10 s, followed by direct lipid extraction according to Renkonen et al. [7]. Briefly, 8.6 ml chloroform/methanol (5 : 8, by vol.) were added to the labeled cells (2.0 ml). The mixture was stirred for 30 min in the dark, then centrifuged 10 min at 2000 xg. The supernatant was transferred to another centrifuge tube and partitioned in two phases by addition of 7.4 ml chloroform and 2.0 ml 0.9% aqueous NaCl. After removal of the upper phase, the lower phase was dried and lipids were dissolved in 5 ml chloroform/methanol (2 : 1, by vol.). Total lipid phosphorus determination, two-dimensional thin-layer chromatography and phosphorus analysis of the various phospholipid classes were carried out as described elsewhere [4].

Phosphatidylcholine was used as internal standard. In separate experiments, the cell intactness after labeling was assessed by the determination of lactate dehydrogenase activity in the cell supernatant. In this case, the cells were labeled as described above, except that ammonia was not added, then diluted to 8 ml with labeling buffer in the presence or absence of glycyl-glycine (final concentration 20 mM). Lactate dehydrogenase activity was measured [4] on aliquots of this diluted cell suspension and of the 1000 xg (5 min) cell supernatant. Cell lysis was always less than 5% for all fluorescamine concentrations. Glycyl-glycine (added to destroy any excess of extracellular non-reacted fluorescamine) had no effect on the enzyme assay. Lactate dehydrogenase activity in labeled cells was the same as in control cells, indicating that penetration of fluorescamine into the cells did not result in an inhibition of this enzyme.

Fluorescamine (4-phenylspiro[furan-2(3H),1′-phtalan]-3,3′-dione) was from Sigma Chemical Company. Solvents (from J.T. Baker Chemicals) were redistilled before use, except dimethylsulfoxide. Other chemicals were analytical grade products from Merck.

Results and Discussion

Fluorescamine was introduced as a versatile reagent for primary amino groups by Weigele et al. [8] and Udenfried et al. [9]. This compound offers several attractive features, which are summarized in Fig. 1A. Fluorescamine also reacts with ammonia, with the additional advantage that the resulting coupling product displays only a weak

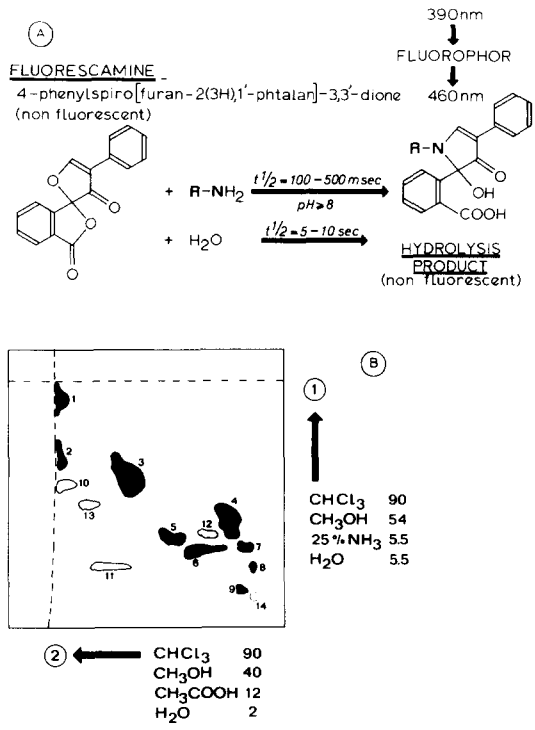

Fig. 1. (A) Chemical structure, reaction(s) and properties of fluorescamine (modified from Ref. 9). (B) Schematic representation of a two-dimensional thin-layer chromatographic separation of the lipids extracted from native and fluorescamine treated Friend cells. Lipids of intact, unlabelled cells are indicated as black spots. 1, cholesterol + neutral lipids; 2, diphosphatidylglycerol; 3, phosphatidylethanolamine; 4, phosphatidylcholine; 5, phosphatidylinositol; 6, phosphatidylserine; 7, sphingomyelin; 8, lysophosphatidylcholine; 9, gangliosides; 10 and 11, fluorescamine derivatives of 3 and 6; 12 and 13, lysophosphatidylethanolamine and its fluorescamine derivative; 14, origin.

fluorescence [9]. Finally, fluorescamine derivatives of phosphatidylethanolamine and phosphatidylserine can be clearly separated from all other cellular phospholipids, using a modification [10] of the two-dimensional thin-layer chromatography system of Broekhuyse [11], as shown in Fig. 1B.

Results from fluorescence microscopy indicated that above approx. 0.3 μmol/$6 \cdot 10^7$ cells, fluorescamine could indeed penetrate into Friend cells. This was evidenced by the fact that in most cells fluorescence was not restricted to a thin peripheric ring, but was also present intracellularly, although the lysis (as determined by the release of lactate dehydrogenase) was negligible (not shown). With respect to the short lifetime of fluorescamine in aqueous media (compare Fig. 1A), it can be concluded that this compound rapidly penetrates into the intracellular compartment, which suggests that in Friend cells, the plasma membrane is not an efficient barrier to this particular probe. This is not surprising since Schlegel et al. [12] provided evidence that another uncharged probe (a derivative of merocyanine 540) could label both plasma and intracellular membranes in Friend cells. In contrast to microorganisms, Friend cells (and other mammalian cells) are unable to maintain a pH gradient across the plasma membrane [13]. The intracellular pH easily equilibrates with the pH of the medium, thus providing conditions which equally favour intracellular labeling.

In preliminary experiments, Friend cells were labeled at 0–4°C with increasing amounts of fluorescamine. Cells were then diluted and spun down. Lipids were extracted from the cell pellet according to Reed et al. [14] and the respective amounts of phosphatidylethanolamine and of its fluorescamine derivative were determined. The results are depicted in Fig. 2A. The decrease in phosphatidylethanolamine was accompanied by a similar increase of its fluorescamine derivative as the concentration of the probe was raised. However, it was obvious that some uncontrolled labeling occurred since almost all of the cellular phosphatidylethanolamine could be labeled (> 95%) whereas cell lysis was still less than 5%. Indeed, if such a labeling pattern was entirely due to penetration of the probe, one should expect the efficiency of phosphatidylethanolamine labeling to decrease at increasing fluorescamine concentrations, since cytoplasmic proteins would act as a sink for the probe. Obviously, this was not the case (Fig. 2A). Therefore, we suspected that at high fluorescamine concentration, an artifactual labeling of the remaining phosphatidylethanolamine could have occurred, presumably during lipid extraction. Such an artifact may be explained if one assumes that part of the fluorescamine molecules which enter the cell can accumulate in the lipid phase of the cellular membranes, thus escaping the expected hydrolytic inactivation; addition of chloroform/methanol would then disperse the system, enabling the previously protected fluo-

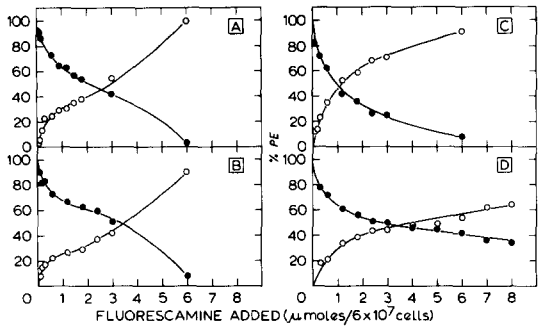

Fig. 2. Effect of the extraction conditions on the changes in the relative amounts of phosphatidylethanolamine (●) and of its fluorescamine derivative (○) in lipid extracts of Friend cells. Intact cells were treated at 0–4°C with increasing amounts of fluorescamine as described in Materials and Methods. Next, the following treatments were applied. (A) Cells were diluted to 8 ml with cold labeling buffer and centrifuged; 0.5 ml of 0.9% NaCl was added to the cell pellet, followed by extraction according to Reed et al. [14] (B) Cells were diluted to 8 ml with cold labeling buffer containing glycyl-glycine (final concentration 20 mM) and centrifuged; 0.5 ml 1 M acetic acid was added to the cell pellet (final pH approx. 3.5) followed by extraction [14]. (C) To the cell suspension (1.5 ml) was added 0.5 ml 60 mM glycyl-glycine in labeling buffer; after gently vortexing for 10 s, 0.67 ml 1 M HCl was added (final pH approx. 1) and the mixture was immediately extracted according to Renkonen et al. [7]. Due to the strongly acidic condition used, plasmalogen phosphatidylethanolamine and the corresponding fluorescamine derivative were recovered as their lyso forms. Total phosphatidylethanolamine was calculated from the sum (remaining phosphatidylethanolamine (diacyl form)+lysophosphatidylethanolamine (originating from the plasmalogen form)). Similar calculations were applied to the fluorescamine derivatives. (D) Cells were treated as described in Materials and Methods. Lipid extracts obtained in (A) and (B) were partitioned in two phases after addition of adequate volumes of chloroform and of 0.9% NaCl according to Folch et al. [15].

rescamine molecules to react with the remaining phosphatidylethanolamine. This is likely to occur since at the highest fluorescamine concentration used, the probe/(total cellular phosphatidylethanolamine) molar ratio is about 50. We therefore investigated our extraction conditions in greater detail.

The pH of the extraction medium was obviously the most important parameter to study, since it would control the degree of protonation of the remaining free amino groups in both proteins and aminophospholipids. Friend cells were labeled with fluorescamine as above, and the resulting cell suspensions were submitted to various treatments (presence or absence of glycyl-glycine, addition of weak or strong acid, or ammonia) before lipid extraction (see legend to Fig. 2 for additional details). Fig. 2 clearly shows that the neutral and the two acidified extraction methods all ended up with an almost total labeling of phosphatidylethanolamine, while in none of these cases any significant lysis of the cells had occurred prior to their extraction. Although the presence of 20 mM glycyl-glycine inactivates the residual extracellular fluorescamine more rapidly than water does, the increase in labeling efficiency at high probe concentrations (Fig. 2B) suggests that enough fluorescamine had accumulated inside the cell for an artifactual labeling of phosphatidylethanolamine still to occur. In addition, glycyl-glycine itself is only poorly permeable [6] so that it will not prevent unreacted fluorescamine molecules to accumulate in the lipid phase of the cellular membranes. Surprisingly, the conditions which favoured protonation of amino groups and which should thus prevent coupling to fluorescamine, were in fact promoting maximal labeling (compare Fig. 2A with Fig. 2B and Fig. 2C). Moreover, strongly acidic conditions significantly increased the apparent efficiency of labeling at low fluorescamine concentrations (Fig. 2C).

A plausible explanation for these apparently contradictory results may be that fluorescamine would not be subject to rapid hydrolysis in a neutral or an acidic medium.

As a consequence, fluorescamine would be stored in an acidified cell suspension as a 'latent' probe, since it will not be able to react with the amino groups which are completely protonated at this pH. Addition of chloroform/methanol, followed by phase separation, would extract most of the acid in the aqueous methanolic phase, which enables the labeling of the remaining phosphatidylethanolamine to take place in the chloroform phase.

Indeed, additional experiments showed that the hydrolysis of fluorescamine proceeds much slower in water or acidic solutions than in alkaline media. Moreover, the rate by which fluorescamine is inactivated in water or acidic media was further decreased after the addition of chloroform/methanol to form one phase. It is therefore obvious that partition of the extraction medium into

two phases would allow most of the unreacted fluorescamine to accumulate preferentially in the chloroform phase. When a small amount of an organic primary amine was added to such a chloroform layer, fluorescence appeared immediately. In summary, these results show that acidic extraction conditions should not be used, in order to avoid artifactual reactions. Similarly, neutral extraction conditions cannot prevent the fluorescamine, previously accumulated into the cell, to react with residual phosphatidylethanolamine during the extraction procedure. On the other hand, the use of an alkaline extraction medium turned out to eliminate any fluorescamine before it was able to react with phosphatidylethanolamine. By using ammonia, which freely permeates the plasma membrane, also intracellular residual fluorescamine could be destroyed. Therefore, the expected labeling pattern could only be observed under these conditions: a gradual decrease in labeling efficiency, taking place at increasing concentrations of fluorescamine (Fig. 2D)

When such data are plotted in a semi-logarithmic way, it becomes obvious that the labeling of intact Friend cells with fluorescamine reveals three pools of phosphatidylethanolamine (Fig. 3). The first pool, characterized by a high efficiency of labeling, obviously corresponds to that fraction of the phosphatidylethanolamine which is localized in the outer monolayer of the plasma membrane. The size of this outer pool can be determined by extrapolation of the second linear component (a), to a fluorescamine concentration equal to zero, and accounts for 16–18% of the total cell phosphatidylethanolamine. A second pool of intermediate labeling efficiency extends from 16–18% to 34–36% and may be identified as the phosphatidylethanolamine localized in the inner monolayer of the plasma membrane, which thus accounts for 16–20% of all of the PE present. A third pool is eventually evidenced; its very low efficiency of labeling strongly suggests that it represents the contribution of the intracellular membranes. The fact that both the decrease in phosphatidylethanolamine and the increase in its fluorescamine derivative yields similar values (Table I), emphasizes the reliability of the method. These data are in excellent agreement with those we recently obtained on Friend cells treated with pure phospholipases under non-lytic conditions (Rawyler, A et al., unpublished data). For instance, pure bee venom phospholipase A_2 degrades 17–18% of the total cell phosphatidylethanolamine under these conditions, which is essentially identical to the fraction of this phospholipid which can

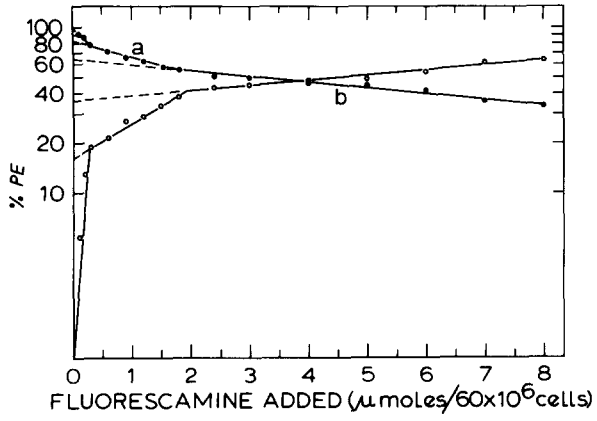

Fig. 3. Semi-logarithmic plot of the loss in phosphatidylethanolamine (●) and of the appearance of its fluorescamine derivative (○) in Friend cells labeled at 0–4°C with increasing amounts of fluorescamine, using the data of Fig. 2D. Phosphatidylethanolamine pools attributable to the outer and inner plasma membrane monolayer and to the intracellular membranes were derived by extrapolating lines a and b, respectively, of the curve representing the loss of phosphatidylethanolamine (●).

TABLE I

LOCALIZATION OF PHOSPHATIDYLETHANOLAMINE IN THE PLASMA MEMBRANE OF FRIEND CELLS, AS DETECTED BY TREATMENT OF INTACT CELLS WITH FLUORESCAMINE

Values are mean ± S.D.; number of experiments is given in parentheses.

Plasma membrane monolayer	Percentage of total cell PE present (based on)	
	Decrease in PE	Formation of fluorescamine derivative
Outer + inner [a]	34.2 ± 0.3 (3)	35.8 ± 1.3 (3)
Outer [b]	16.3 ± 1.6 (5)	16.9 ± 1.8 (5)
Inner [c]	17.9 ± 1.6	18.9 ± 2.2
Outer/inner	48/52	47/53

[a] and [b] Determined from semi-logarithmic plots of the fluorescamine-dose dependent labeling of PE in intact cells (see text).

[c] Calculated by substracting [b] from [a].

be labeled with fluorescamine in the outer monolayer of the plasma membrane of these cells (compare Table I). It is also of interest to note that the results obtained from the fluorescamine-dose dependent labeling of PE in the intact cells indicate an essentially symmetric distribution of this phospholipid over the two monolayers of the plasma membrane (Table I). Again, this result completely agrees with that derived from experiments involving bee venom phospholipase A_2, which indicated that 50–52% of the phosphatidylethanolamine in the plasma membrane is located in the outer leaflet.

We have previously shown that the plasma membrane of Friend cells contains 38.5% of the total cell phospholipids [4]; this value was obtained using a non-lytic sphingomyelinase C treatment of Friend cells, followed by isolation of plasma membranes from control and treated cells, and by comparison of the extent of phospholipid hydrolysis in the lipid extracts from both control and treated whole cells as well as in those from the corresponding isolated plasma membranes [4]. An analogous calculation can be made by using the value for the percentage of all of the cell phosphatidylethanolamine that is present in the plasma membrane, as determined by fluorescamine labeling (34.2%, Table I), and the relative amounts of the phosphatidylethanolamine present in the phospholipid fractions extracted from whole cells and purified plasma membranes, which are 18.5 and 16.5%, respectively (Table III in Ref. 4). Following the principle of the calculation outlined in Ref. 4, it follows that the amount of the total phospholipid fraction present in the plasma membrane is equal to: $[(18.5 \times 34.2/100)/16.5] \times 100 = 38.3\%$ of the total phospholipid content of the Friend cell. The fact that this value of 38.3% is in perfect agreement with the above mentioned figure of 38.5%, derived from experiments involving a treatment of the cells which is entirely different from the one reported here, once more illustrates the high degree of reliability of the results from the fluorescamine labeling experiments.

Knowing the composition of each of the phospholipid fractions derived from whole cells as well as from the plasma membrane [4], and taking into account that 38.3 to 38.5% of the total phospholipid complement of the cell is present in the plasma membrane, one can easily calculate the distribution of each of the individual phospholipid classes over plasma- and intracellular membranes of the Friend cell (Fig. 4).

We also attempted to scale down the method by taking advantage of the fluorescence properties of the fluorescamine derivatives (Fig. 1A). Aliquots containing one million of cells (Friend cells or erythroblasts isolated from mouse spleen according to Ref. 16) were labeled as described in Materials and Methods with increasing amounts of fluorescamine up to 150 nmol. The total lipid extracts, which contained about 13 nmol of total lipid phosphorus, including 2–3 nmol of aminophospholipids, were washed three times according to Folch et al. [15] and subsequently divided into equal portions. One of these portions was further treated with an excess of fluorescamine in order to derivatize all of the aminophospholipids to completion. Fluorescence intensity was determined on both this completely derivatized extract (set at 100%), as well as on the original extract. The ratio in fluorescence intensities of these two samples was considered to represent the percentage of aminophospholipid labeling. Since phosphatidylserine was found to react only very slowly in intact cells and the fact that phosphatidylethanolamine accounted for more than 82% of the total aminophospholipids, a simple correction allowed to calculate the relative amount of the

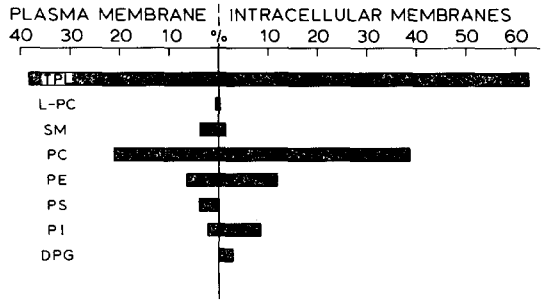

Fig. 4. Distribution of phospholipids between plasma membrane and intracellular membranes of Friend cells. The distribution of total phospholipid and individual lipid classes is expressed as percentage of the total amount of phospholipid present in the cell. Abbreviations are: TPL, total phospholipid; LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol and DPG, diphosphatidylglycerol.

phosphatidylethanolamine derivative formed, expressed as percentage of the total amount of this lipid present. The final results thus obtained were essentially identical to those presented in Fig. 3.

Finally, it is worth to summarize the specific advantages which are offered by the fluorescamine method described in this paper: Firstly, its simplicity and reliability are attractive features by themselves. Secondly, it must be emphasized that a complete picture of the phosphatidylethanolamine localization can be obtained on intact cells, thus obviating the need to work on opened structures or isolated membranes. The present method should thus be generally applicable for studies on the localization of phosphatidylethanolamine in most, if not all, eukaryotic cells,. Thirdly, the fluorescence properties of the fluorescamine enable an accurate determination of the reacted aminophospholipids in the nanomolar range, which implies a much higher sensitivity when compared to the usual phosphate analyses. This opens the interesting possibility of investigating the localization of aminophospholipids even when the cell supply is seriously limited, as for example in experiments involving differentiation of primary cells.

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References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
2 Etemadi, A.H. (1980) Biochim. Biophys. Acta 604, 423–475
3 Haest, C.W.M., Kamp, D. and Deuticke, B. (1981) Biochim. Biophys. Acta 640, 535–543
4 Rawyler, A., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1983) Biochim. Biophys. Acta 730, 130–138
5 Storm, G., Bosman, G.J.C.J.M., Boer, P., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1982) Biochem. Int. 5, 169–176
6 Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S. and Singh, R.M.M. (1966) Biochemistry 5, 467–477
7 Renkonen, O., Kosunen, T.V. and Renkonen, O.V. (1963) Ann. Med. Exp. Biol. Fenn. (Helsinki) 41, 375–381
8 Weigele, M., DeBernardo, S.L., Tengi, J.P. and Leimgruber, W. (1972) J. Am. Chem. Soc. 94, 5927–5928
9 Udenfried, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Science 178, 871–872
10 Roelofsen, B. and Zwaal, R.F.A. (1976) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 7, pp. 147–177, Plenum Press, New York
11 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457–461
12 Schlegel, R.A., Phelps, B.M., Waggoner, A., Terada, L. and Williamson, P. (1980) Cell 20, 231–328
13 Navon, G., Navon, R., Shulman, R.G. and Yamane, T. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 891–895
14 Reed, C.F., Swisher, S.N., Marinetti, G.V. and Ede, G.V. (1960) J. Lab. Clin. Med. 56, 281–289
15 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
16 Nijhof, W., Wierenga, P.K. and Goldwasser, E. (1982) Exp. Hematol. 10, 36–43