Quantification of lysophosphatidylethanolamine in the nanomole range

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Summary A microanalytical procedure for the determination of trace amounts of lysophosphatidylethanolamine and other amine-containing lipids is described. The technique involves reaction of lipid extracts with fluorescamine to give fluorescent derivatives of lipids containing a free amino group, separation of the products by thin-layer chromatography, and quantification by fluorescence spectroscopy after elution from the adsorbent. Amounts of 100 pmol are easily determined, even in the presence of large amounts of other phospholipids, and 30–40 samples can be analyzed per day. Use of the procedure is illustrated with rat liver mitochondria undergoing a phospholipase A_2-dependent increase in inner membrane permeability due to treatment with Ca^{2+} plus N-ethylmaleimide.—**Schmid, P. C., D. R. Pfeiffer, and H. H. O. Schmid.** Quantification of lysophosphatidylethanolamine in the nanomole range. *J. Lipid Res.* 1981. **22:** 882–886.

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Trace amounts of lysophosphatidylethanolamine may be associated with permeability changes of biological membranes such as that recently demonstrated for the inner mitochondrial membrane (1–4). In order to relate minute changes in the levels of lysophosphatidylethanolamine to permeability changes of biological membranes, a rapid and sensitive assay method is required. Available methods proved inadequate for the measurement of the small amounts (0.5% of total phospholipids) produced during the permeability change. Although sensitive methods exist for phosphate determination (cf 5), the separation of lysophosphatidylethanolamine from other phospholipids can only be accomplished by time-consuming two-dimensional TLC. Similarly, high-performance liquid chromatography, which can be used for the analysis of derivatized ethanolamine and serine-containing phospholipids (6), may be somewhat cumbersome for the quantitative assay of large series of samples.

We have therefore developed a new method that is rapid, specific, and sensitive. It is based on the reaction of the free amino group with fluorescamine, isolation of the reaction product by one-dimensional TLC, and quantification by fluorescence determinations. By this method as little as 100 picomoles of lysophosphatidylethanolamine are measured accurately in extracts containing approximately 300 nmol of phospholipid. The method is demonstrated by assaying lysophosphatidylethanolamine levels in rat liver mitochondria under swelling conditions (1–3).

MATERIALS AND METHODS

Fluorescamine (4-phenylspiro[furan-2(3H),1-phthalan]-3,3′-dione) was purchased from Sigma Chemical

Abbreviations: TLC, thin-layer chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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Co., St. Louis, MO, and used without further purification. Egg yolk phosphatidylethanolamine was purified by silicic acid column chromatography (7) followed by preparative TLC. Hydrolysis with *Ophiophagus hannah* venom (Miami Serpentarium Laboratories, Miami, FL) yielded lysophosphatidylethanolamine, which was purified by TLC on silica gel H plates developed in chloroform–methanol–water 65:25:4 (by volume) and was found to be pure by two-dimensional TLC (8). The concentration of the standard solution was determined by phosphorus estimation (9). Thin-layer plates containing magnesium acetate were made from a slurry of 40 g silica gel H (Merck) and 3 g magnesium acetate (cryst.) in 100 ml of water (8).

Sample preparation

Rat liver mitochondria were prepared (3) and incubated in 0.21 M mannitol, 0.07 M sucrose, 10 mM succinate (Na^+), rotenone at 0.5 nmol/mg protein, and 3 mM HEPES (Na^+), pH = 7.4. Protein concentration was 1.0 mg/ml and the temperature was 25°C. Increasing inner membrane permeability was followed by monitoring swelling (decrease in apparent absorbance of mitochondrial suspensions at 540 nm) (cf 1–3). At specified times, samples of 1.5 ml were pipetted into 2 ml methanol, and 4 ml of chloroform was added. They were vortexed and stored on ice. After centrifugation, the lower phase was removed with a disposable pipet and transferred to a 15-ml conical tube with screw cap; the upper phase was reextracted with 4 ml of chloroform–methanol–water 86:14:1 (by volume) (10). After centrifugation, the second lower phase was added to the first.

Procedure

The lipid extracts were taken to complete dryness in a nitrogen stream and redissolved in 100 μl of chloroform–triethylamine 50:1 (by volume). The tertiary amine provided the free base necessary for the reaction to proceed without itself participating or interfering (11, 12). Fifty μl of a solution of fluorescamine in dry toluene (4 mg/ml) was added to each sample and the samples were immediately vortexed 8–10 sec. After ½ hr at room temperature, they were again taken to dryness and were redissolved in 150 μl of chloroform. The tubes were capped and stored on ice to prevent evaporation.

One hundred μl of each sample were applied with a Hamilton microsyringe to a lane 2.5 cm wide on a 10 × 20 cm silica gel H-magnesium acetate plate. Three samples can be applied to each plate. The plates were developed in chloroform–methanol–conc. NH_4OH–water 65:35:5:1 (by volume) in tanks lined with filter paper. After the plates were dried briefly, the lysophosphatidylethanolamine-fluorescamine spots were visible under UV light at an approximate R_f = 0.3. As little as 100 pmol can be detected visually if the sample is applied in a band 2 cm wide.

The marked lysophosphatidylethanolamine-fluorescamine bands were scraped into 3.5 ml of methanol–4% aqueous NH_4OH 95:5 (by volume) in conical centrifuge tubes and vortexed for 20 sec to elute the labeled compound. The fluorescent products were stable in this solvent mixture for several hours (12). After centrifuging, the supernatant was transferred to a cuvette and the fluorescence was determined in an Aminco-Bowman spectrophotofluorometer (excitation wavelength, 395 nm; emission wavelength, 468 nm).

A known amount (20–50 nmol) of egg yolk lysophosphatidylethanolamine was reacted in the same manner as the samples. After ½ hr, the solvents were evaporated and the reaction product was dissolved in 1.0 ml of chloroform. Appropriate aliquots (10–150 μl) were spotted on the plates, developed, and eluted to give a standard line encompassing the concentration of lysophosphatidylethanolamine in the experimental samples.

RESULTS AND DISCUSSION

Fluorescamine (13) has been used for the quantification of proteins and amino acids (11, 12), sphingosine bases (14, 15), and for labeling proteins (16, 17) and lipids (18, 19) on the outer surface of bilayer membranes. The present method is the first use of fluorescamine for the quantitative assay of phospholipids in trace amounts. It constitutes a more sensitive assay than phosphorus determination, has the advantage that lysophosphatidylethanolamine needs to be separated only from other lipids containing a free amino group (i.e., phosphatidylethanolamine and phosphatidylserine) after the reaction, and does not require the rigorous steps needed to obtain low phosphorus blank values. **Fig. 1** shows a typical TLC separation. The lysophosphatidylethanolamine-fluorescamine adduct is clearly separated from other fluorescent substances. Other lipids that co-migrate with the adduct do not interfere since they fail to react with the fluorescent reagent. The standard lysophosphatidylethanolamine-fluorescamine must be purified by TLC to remove unidentified fluorescent compounds that arise from the reagent.

The fluorescence intensity is linear with respect to concentration (**Fig. 2**). **Table 1** shows that recovery of exogenous lysophosphatidylethanolamine, added to

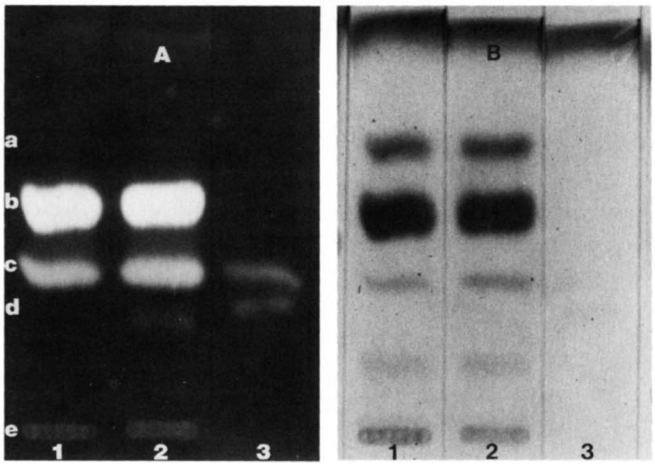

Fig. 1. Separation of the lysophosphatidylethanolamine-fluorescamine product by thin-layer chromatography. (A) Appearance of the chromatogram under UV light. (B) The appearance after spraying with 50% H_2SO_4 and charring. Lane 1: Mitochondrial extract containing 0.4 nmol LPE. Although the LPE-fluorescamine adduct (d) is not clearly visible in this photographic reproduction, as little as 0.1 nmol of this fraction can be detected visually. Lane 2: Mitochondrial extract plus 4 nmol egg yolk LPE. Lane 3: 4 nmol egg yolk lysophosphatidylethanolamine (LPE) standard. a: Diphosphatidylglycerol, b: phosphatidylcholine + phosphatidylethanolamine-fluorescamine, c: unidentified contaminant, d: LPE-fluorescamine, e: phosphatidylserine-fluorescamine plus other impurities.

the mitochondria during extraction and carried through the entire procedure, is quantitative and independent of the absolute amount over the concentration range indicated.

The utility of this method for investigating the control of lysophosphatidylethanolamine levels in mitochondrial membranes is illustrated in **Fig. 3.** When these organelles accumulate Ca^{2+} and are subsequently treated with the sulfhydryl reagent N-ethylmaleimide, they release Ca^{2+} and undergo a nonspecific but limited increase in the permeability of the inner membrane. In this medium the resultant Donnan potential leads to swelling, as reflected by the decrease in apparent absorbance at 540 nm (1–3). Utilizing the new assay procedure, changes in the level of lysophosphatidylethanolamine that accompany these changes in mitochondrial functional properties can be determined. After an initial period during which swelling becomes maximal, the mitochondria accumulate lysophosphatidylethanolamine at the rate of 50 pmol per min per mg protein. This linear rate extrapolates to a level of zero at the time when swelling was initiated by addition of the sulfhydryl agent. In contrast, when Ca^{2+} and N-ethylmaleimide are omitted or when N-ethylmaleimide is added alone, the lysophosphatidylethanolamine content slowly decreases and the inner membrane permeability barrier remains intact (i.e., mitochondria do not swell). These findings support our hypothesis that lysophosphatidylethanolamine, accumulating due to the action of the Ca^{2+}-activated phospholipase A_2 associated with the inner mitochondrial membrane, is responsible for the increase in permeability (1–4). During the initial portion of the incubation, it appears that other phospholipid-metabolizing activities are superimposed on the action of the phospholipase A_2, resulting in the

TABLE 1. Recovery of added lysophosphatidylethanolamine

Added	Measured	Recovery
nmol/mg protein		%
0	0.53	
0.73	1.31	96.2
1.46	1.99	100.0
2.20	2.67	97.8
2.93	3.35	96.8
3.67	4.03	96.0
4.40	4.69	95.1
5.13	5.70	99.3
5.87	6.30	98.4
Average (± S.D.) 97.5 ± 1.7		

Egg yolk lysophosphatidylethanolamine was added to the extraction solvent prior to the addition of mitochondria (1.5 mg protein) and the mixture was immediately extracted and analyzed as described in Materials and Methods.

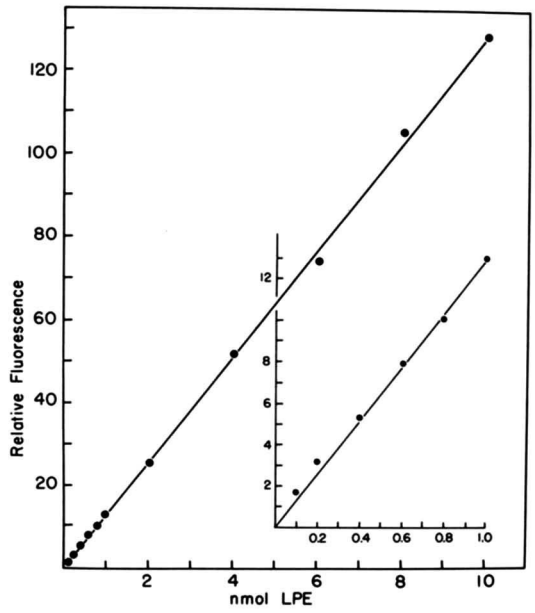

Fig. 2. The linear relationship between product concentration and relative fluorescence intensity. Egg yolk lysophosphatidylethanolamine (LPE) was reacted with fluorescamine and aliquots were purified by TLC and eluted as described in Materials and Methods. The insert shows the region from 0.1 to 1 nmol on an expanded scale.

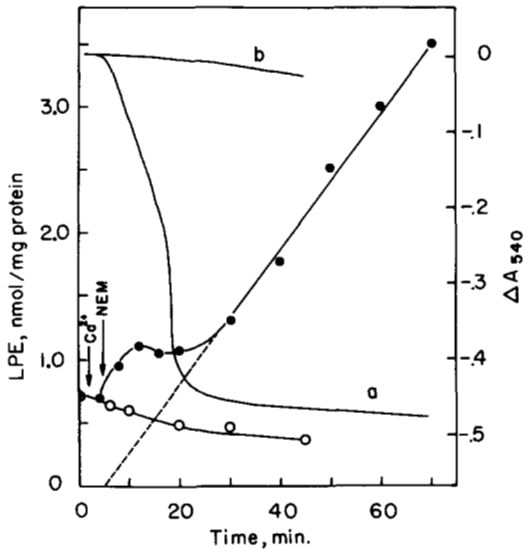

Fig. 3. The effect of Ca^{2+} plus *N*-ethylmaleimide on the permeability and lysophosphatidylethanolamine (LPE) content of rat liver mitochondria. Trace a and ●, the apparent absorbance at 540 nm (downward deflection indicates swelling) and the LPE content, respectively, when $CaCl_2$ (67 nmol per mg protein) and *N*-ethylmaleimide (NEM, 100 nmol per mg protein) were added where indicated. Trace b and ○, the apparent absorbance and LPE content when addition of Ca^{2+} and *N*-ethylmaleimide were omitted.

complex behavior of total lysophosphatidylethanolamine content.[2]

These data illustrate the usefulness of the present method in the study of the correlation between changes in permeability and lysophosphatidylethanolamine content of the inner mitochondrial membrane. It is equally suitable for the determination of lysophosphatidylethanolamine levels in other membrane systems. The extraction procedure described here, i.e., the transfer of the incubation mixture to methanol prior to the addition of chloroform, is necessary to avoid aggregation and afford complete extraction of lipids. While the present method is particularly useful for the assay of the products of phospholipase A_2 activity, i.e., 1-acyl lysophosphatidylethanolamine, it is also applicable to the quantification of 2-acyl lysophosphatidylethanolamine produced by phospholipase A_1 activity or by chemical or enzymic cleavage of ethanolamine plasmalogen. The presence of phosphatidylcholine even in μmol amounts does not affect the fluorescence properties of the adduct. Other amine-containing phospholipids can be assayed by the present technique if these fluorescamine adducts are completely separated from the artifact (fraction c in Fig. 1). The latter compound is always produced from the reagent even in the absence of biological material. As demonstrated in Fig. 1, the phosphatidylethanolamine-fluorescamine adduct migrates ahead of the contaminant in TLC and can thus be easily isolated. The phosphatidylserine-fluorescamine adduct appears to be separable by TLC into two fractions whose structures are currently under investigation.

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[2] Schmid, P. C., D. R. Pfeiffer, and H. H. O. Schmid. Unpublished results.

REFERENCES

1. Pfeiffer, D. R., R. F. Kauffman, and H. A. Lardy. 1978. Effects of *N*-ethylmaleimide on the limited uptake of Ca^{2+}, Mn^{2+} and Sr^{2+} by rat liver mitochondria. *J. Biol. Chem.* **253:** 4165–4171.
2. Pfeiffer, D. R., P. C. Schmid, M. C. Beatrice, and H. H. O. Schmid. 1979. Intramitochondrial phospholipase activity and the effects of Ca^{2+} plus *N*-ethylmaleimide on mitochondrial function. *J. Biol. Chem.* **254:** 11485–11494.
3. Beatrice, M. C., J. W. Palmer, and D. R. Pfeiffer. 1980. The relationship between mitochondrial membrane permeability, membrane potential and the retention of Ca^{2+} by mitochondria. *J. Biol. Chem.* **255:** 8663–8671.
4. Schmid, P. C., D. R. Pfeiffer, and H. H. O. Schmid. 1980. Permeability changes of mitochondrial membranes are related to the level of their constituent lysophosphatidylethanolamines. *Federation Proc.* **39:** 2188. (Abstract).
5. Hess, H. H., and J. E. Derr. 1975. Assay of inorganic and organic phosphorous in the 0.1–5 nanomole range. *Anal. Biochem.* **63:** 607–613.
6. Jungalwala, F. B., R. J. Turel, J. E. Evans, and R. H. McCluer. 1975. Sensitive analysis of ethanolamine- and serine-containing phosphoglycerides by high-performance liquid chromatography. *Biochem. J.* **145:** 517–526.
7. Kates, M. 1972. Techniques of Lipidology. North Holland Publishing Company, Amsterdam.
8. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorous analysis of spots. *Lipids.* **5:** 494–496.
9. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
10. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
11. Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigele. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science.* **178:** 871–872.

12. Castell, J. V., M. Cervera, and R. Marco. 1979. A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A reexamination of the conditions of the reaction. *Anal. Biochem.* **99:** 379–391.
13. Weigele, M., S. L. DeBernardo, J. P. Tengi, and W. Leimgruber. 1972. A novel reagent for the fluorometric assay of primary amines. *J. Am. Chem. Soc.* **94:** 5927–5928.
14. Kisic, A., and M. M. Rapport. 1974. Determination of long-chain base in glycosphingolipids with fluorescamine. *J. Lipid Res.* **15:** 571–577.
15. Naoi, M., Y. C. Lee, and S. Roseman. 1974. Rapid and sensitive determination of sphingosine bases and sphingolipids with fluorescamine. *Anal. Biochem.* **58:** 571–577.
16. Nakaya, K., M. Yabuta, F. Iinuma, T. Kinoshita, and Y. Nakamura. 1975. Fluorescent labeling of the surface proteins of erythrocyte membranes using cycloheptaamylose-fluorescamine complex. *Biochem. Biophys. Res. Commun.* **67:** 760–766.
17. Hawkes, S. P., T. D. Meehan, and M. J. Bissell. 1976. The use of fluorescamine as a probe for labeling the outer surface of the plasma membrane. *Biochem. Biophys. Res. Commun.* **68:** 1226–1233.
18. Krebs, J. J. R., H. Hauser, and E. Carafoli. 1979. Asymmetric distribution of phospholipids in the inner membrane of beef heart mitochondria. *J. Biol. Chem.* **254:** 5308–5316.
19. Lee, H. C., and J. G. Forte. 1979. Asymmetric labeling of amino lipids in liposomes. *Biochim. Biophys. Acta.* **554:** 375–387.