*Biochimica et Biophysica Acta*, 510 (1978) 230—241


BBA 78069

# ASYMMETRIC DISTRIBUTION OF PHOSPHATIDYLETHANOLAMINE FATTY ACYL CHAINS IN THE MEMBRANE OF VESICULAR STOMATITIS VIRUS

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(Received December 9th, 1977)

## Summary

The membrane of vesicular stomatitis virus (VSV) contains two distinct pools of phosphatidylethanolamine molecules which reside in the inner and outer phospholipid monolayers, respectively. 36% of the total membrane phosphatidylethanolamine is found in the outer monolayer while 64% is found in the inner. The two pools of VSV phosphatidylethanolamine can be distinguished operationally by the fact that only outer phosphatidylethanolamine is reactive in intact virions with the membrane-impermeable reagent trinitrobenzenesulfonate (TNBS). We have made use of this property to separate inner from outer VSV phosphatidylethanolamine and to determine the fatty acyl chain compositions of the two phosphatidylethanolamine pools separately. The results show that compared to outer phosphatidylethanolamine, inner phosphatidylethanolamine molecules contain a significantly higher proportion of unsaturated fatty acyl chains. Furthermore, whereas the proportion of unsaturated fatty acyl chains was found to be quite similar at the 1 and 2 glycerol carbon atoms in inner phosphatidylethanolamine, a marked dissimilarity was observed in outer phosphatidylethanolamine; outer phosphatidylethanolamine was enriched in saturated fatty acyl chains at the 1 position and in unsaturated fatty acyl chains at the 2 position. The differential fatty acyl chain composition of inner compared to outer phosphatidylethanolamine indicates that rapid, random transmembrane migration (flip-flop) of phosphatidylethanolamine does not occur in the VSV membrane. The nature of the fatty acyl chain asymmetry observed in VSV phosphatidylethanolamine does not support the view that the

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Abbreviations: VSV, vesicular stomatitis virus; TNBS, trinitrobenzenesulfonate; Tnp, trinitrophenyl.

identity of the fatty acyl chains can uniquely specify or determine which side of the membrane individual phosphatidylethanolamine molecules come to occupy. Although fatty acyl chain asymmetry and phosphatidylethanolamine asymmetry are correlated in VSV, no simple rules can be discerned which uniquely relate the two paramaters.

## Introduction

Recent experimental studies have clearly demonstrated the existence of phospholipid asymmetries in biological and synthetic membranes. The two lipid monolayers which together make up a membrane are often found to have quite different phospholipid compositions. For example, in the human erythrocyte membrane 76% of the phosphatidylcholine, but only 20% of the phosphatidylethanolamine is found in the outer phospholipid monolayer; 24% of the phosphatidylcholine and 80% of the phosphatidylethanolamine reside in the inner monolayer [1—3]. Similar phospholipid asymmetries have been observed in the membranes of influenza virus [4], vesicular stomatitis virus [5], *Bacillus megaterium* [6] and other sources (for review see ref. 7). In spite of the widespread occurrence of phospholipid asymmetries in biological membranes, the molecular mechanisms involved in generating and maintaining them are still unclear. The simplest systems where phospholipid asymmetry has been observed are the small single-bilayer liposomes composed of mixed phospholipids. In these cases the differential packing requirements of different phospholipid species and the small radius of curvature in the vesicles appear to be the factors responsible for generating overall phospholipid asymmetry [8—10]. In biological membranes, however, the small radii of curvate found in liposomes are not the rule and it is most likely that other factors are involved in generating and maintaining phospholipid asymmetry.

The experimental studies described here were undertaken to test the idea that phospholipid fatty acyl chains may be functionally involved in determining or specifying phospholipid asymmetry. Are individual phospholipid molecules incorporated specifically into one side or the other of a biological membrane depending on the identity of their fatty acyl chains? If they are, then pools of a particular phospholipid species on the two sides of a membrane ought to differ in fatty acyl chain composition. Fatty acyl chain asymmetry should be associated with phospholipid asymmetry. We have tested this expectation by comparing the fatty acyl chain compositions of phosphatidylethanolamine molecules found in the inner and outer monolayers of the vesicular stomatitis virus (VSV) membrane. Inner and outer VSV phosphatidylethanolamine were separated for our studies by making use of the fact that only outer phosphatidylethanolamine reacts with the membrane-impermeable reagent trinitrobenzenesulfonate (TNBS) intact virions [5]. Previous studies of the type described here have demonstrated that the fatty acyl chain compositions are quite similar in the inner and outer phosphatidylethanolamine components of mixed phosphatidylcholine/phosphatidylethanolamine liposomes [11] and in the inner and outer phosphatidylcholine found in the human erythrocyte membrane [12].

## Materials and Methods

*Reaction of VSV phosphatidylethanolamine with TNBS and separation of phospholipids by thin-layer chromatography.* The Indiana strain of vesicular stomatitis virus (VSV) was grown on monolayer cultures of BHK-21 cells and purified as previously described [5]. The membrane of virus particles prepared in this way was found to contain phospholipids in the following proportions: 37% phosphatidylethanolamine, 29% phosphatidylcholine, 21% sphingomyelin and 10% phosphatidylserine plus phosphatidylinositol. The remaining phospholipid material (3%) was not identified. The molar ratio of cholesterol/phospholipid in the virus membrane was 0.75. Purified virus particles were reacted with TNBS by incubating between 0.5 and 2 mg of virus in 5 ml phosphate-buffered isotonic saline pH 7.2, containing 1.5 mM TNBS for 90 min at room temperature. In our earlier study it was demonstrated that all the externally located phosphatidylethanolamine is labeled under these conditions [5].

After reaction with TNBS phospholipids were extracted from derivatized VSV with chloroform/methanol (2 : 1, v/v) and the two forms of phosphatidylethanolamine (derivatized and underivatized) were separated by thin-layer chromatography as previously described [5]. All solvents employed for phospholipid extraction and for thin-layer chromatography contained 0.02% $\alpha$-tocopherol to inhibit oxidation. $\alpha$-Tocopherol does not overlap with any of the fatty acid methyl esters in the subsequent gas-liquid chromatographic analysis employed here [13].

*Fatty acid methyl ester formation.* Methyl esters of the phosphatidylethanolamine-associated fatty acyl chains were prepared according to the procedure of Litman [11]. After thin-layer chromatography the phosphatidylethanolamine and trinitrophenyl-phosphatidylethanolamine (Tnp-phosphatidylethanolamine) spots were scraped from the chromatography plate into screw-cap vials. 8 ml of 3% methanolic HCl (w/w; formed by adding acetyl chloride to dry methanol) was added and the sample was heated at 60° C for 1 h. It was then cooled and mixed with 2 ml 5% NaCl. The fatty acid methyl esters formed in this way were extracted into pentane (pesticide grade), dried in a stream of $N_2$ and finally dissolved in 10—20 $\mu$l of lipopure hexane for gas-liquid chromatography.

*Gas-liquid chromatography.* Analysis of the fatty acid methyl esters was performed on a Beckman GC-45 gas chromatograph fitted with a flame ionization detector. Chromatography was carried out on a column containing 10% Silar 10C adsorbant and 90% 100/120 mesh Gas Chrom Q support packed in a stainless steel column 3 m long with an internal diameter of 1.8 mm (Supelco Co., Bellefonte, Pa). The column temperature was programmed to operate isothermally at 145° C for 16 min and then to initiate a temperature rise to 210° C in 32 min. 0.2-$\mu$l samples containing fatty acid methyl esters were injected and a flow rate of carrier $N_2$ gas was maintained at 80 ml/min. Under these conditions all the fatty acid methyl esters indicated in Tables I, III, IV and V were well separated from each other. In many cases longer chain methyl esters containing higher levels of unsaturation were also well resolved. For example, the methyl esters of arachidic (20 : 0), 20 : 1, 20 : 2, 20 : 3, 20 : 4 and 20 : 5 were all thoroughly resolved from one another.

The methyl esters present in each sample were determined quantitatively by

their respective peak areas in the chromatographic profiles. Areas of peaks were estimated by multiplying the amplitude of the peak by its half band width; these values were corrected for the methyl ester carbon chain length to arrive at the mol percent values given in Results. Individual fatty acid methyl esters were identified on the chromatographic profiles by comparison of their retention times with those of standard fatty acid methyl esters purchased from Applied Science Laboratory (State College, Pa.) or from Supelco.

*Phospholipase $A_2$ hydrolysis.* The reaction conditions described by De Haas et al. [14] were employed for phospholipase $A_2$ digestion of phosphatidylethanolamine and Tnp-phosphatidylethanolamine. 200—500 $\mu$g of purified phosphatidylethanolamine or Tnp-phosphatidylethanolamine, 7 mg sodium deoxycholate, 4.5 mg bovine serum albumin, 5 mM $CaCl_2$, 1 M borate buffer, pH 8.0 and 20 units of bee (*Apis mellifera*) venom phospholipase $A_2$ (Sigma Chemical Co.) were incubated together in a total reaction volume of 1 ml at 30°C for 3 h. The reaction was stopped by the addition of chloroform/methanol (2 : 1, v/v) and the lipids were extracted as previously described [5]. Up to 10 mg of standard phosphatidylethanolamine could be hydrolyzed under these conditions. The 1-lyso-phosphatidylethanolamine or 1-lyso-Tnp-phosphatidylethanolamine produced by this procedure was separated from the other reaction components by chromatography on activated silica gel G thin-layer plates developed in chloroform/methanol/water (62 : 34 : 4, v/v) containing 0.02% $\alpha$-tocopherol [13]. The lyso-phosphatidylethanolamine and lyso-Tnp-phosphatidylethanolamine derivatives migrate more slowly than the unmodified species in this solvent system while free fatty acids migrate near the solvent front.

The specificity of the bee venom phospholipase $A_2$ employed in these studies was verified by testing it with synthetic 1-palmitoyl-2-myristoyl phosphatidylcholine as substrate. Myristic acid was found to constitute greater than 95% of the fatty acid material removed from this substrate after incubation with bee venom phospholipase $A_2$. The small amount of free palmitic acid released was attributed to the presence of lipid contaminants having palmitic acid at the 2 position in the phosphatidylcholine standard. The presence of a phospholipase $A_1$ impurity in the phospholipase $A_2$ preparation was considered to be unlikely because the amount of palmitic acid released did not increase after prolonged incubation with the enzyme.

## Results

*Fatty acyl chain composition of inner and outer phosphatidylethanolamine*

The strategy we have adopted for determining the fatty acyl chain composition of inner and outer VSV phosphatidylethanolamine involves reaching VS virions with the membrane-impermeable reagent trinitrobenzenesulfonate (TNBS) under conditions where only outer phosphatidylethanolamine can react. Virus phospholipids are then extracted and derivatized phosphatidylethanolamine (trinitrophenyl-phosphatidylethanolamine or Tnp-phosphatidylethanolamine) is separated from underivatized phosphatidylethanolamine by thin-layer chromatography. The fatty acyl chain compositions of phosphatidylethanolamine and Tnp-phosphatidylethanolamine are then determined

separately by gas-liquid chromatography of the corresponding methyl esters. The results obtained for phosphatidylethanolamine and for TNP-phosphatidylethanolamine ought to yield the fatty acyl chain compositions of inner and outer phosphatidylethanolamine, respectively. This overall strategy depends critically on the fact that TNBS does not penetrate the lipid barrier of intact VS virions under the conditions employed. Experimental evidence demonstrating that this is the case can be found in our previous study of this topic [5].

The fatty acyl chain compositions of inner and outer phosphatidylethanolamine determined in one particular experiment are shown in Table I. The results indicate that the predominant species of fatty acyl chains are the same

TABLE I

PHOSPHATIDYLETHANOLAMINE-ASSOCIATED FATTY ACYL CHAINS IN THE VSV MEMBRANE

After reaction of VSV with TNBS outer (trinitrophenylated) and inner (underivatized) phosphatidylethanolamine were extracted with chloroform/methanol (2 : 1, v/v) and separated by thin-layer chromatography as described in Materials and Methods. Their fatty acid methyl esters were prepared by incubating the intact phospholipids with 3% methanolic HCl for 1 h at 60°C; methyl esters were then separated from each other and determined quantitatively by gas-liquid chromatography on a column of Silar 10C adsorbant. The Table lists the average mol percent and the range (in parentheses) of three determinations.

| Fatty acyl chain | Average mol % (range) | | |
|---|---|---|---|
| | Inner phosphatidylethanolamine *** | Outer phosphatidylethanolamine | Total phosphatidylethanolamine † |
| 12 : 0 | 0.2 (0.2—0.2) | 0.7 (0.6—0.7) | 0.4 |
| 12 : 1 | trace | trace | trace |
| 14 : 0 | 1.8 (1.7—2.0) | 6.3 (5.9—6.5) | 3.4 |
| 14 : 1 | 5.5 (5.2—5.7) | 4.5 (4.5—4.6) | 5.2 |
| 16 : 0 | 8.9 (8.8—9.1) | 12.6 (12.1—13.3) | 10.1 |
| 16 : 1 | 3.6 (3.1—3.9) | 4.6 (4.2—5.0) | 4.0 |
| 16 : 2 | 3.9 (3.9—4.0) | 2.5 (2.2—2.8) | 3.4 |
| 17 : 0 | 1.1 (0.4—1.5) | 6.5 (5.8—7.3) | 3.0 |
| 18 : 0 | 15.9 (14.5—16.9) | 12.9 (12.4—13.5) | 14.9 |
| 18 : 1 | 36.9 (35.6—38.9) | 33.0 (32.4—33.5) | 35.6 |
| 18 : 2 | 1.5 (1.4—1.8) | 1.6 (1.5—1.7) | 1.5 |
| 18 : 3 | 2.5 (2.4—2.7) | 1.5 (1.5—1.5) | 2.2 |
| 20 : 0 | 0.2 (0.1—0.2) | 0.1 (0.1—0.2) | 0.2 |
| 22 : 0 | 1.1 (0.9—1.3) | 0.9 (0.8—0.9) | 1.0 |
| >18 unsaturated * | 16.7 (16.2—17.0) | 12.3 (11.6—13.3) | 14.8 |
| Total unsaturated fatty acyl chain | 70.8 (69.7—72.7) | 59.7 (58.9—60.3) | 66.7 |
| Total saturated fatty acyl chain | 29.1 (27.2—30.1) | 40.3 (39.5—41.2) | 33.1 |
| Unsaturation ratio ** | 2.4 (2.3—2.7) | 1.5 (1.4—1.5) | 2.0 |

* The ">18 unsaturated" entry represents the proportion of monoenes and polyenes having 20—22 carbon atoms.

** The unsaturation ratio is the molar ratio of the total number of unsaturated fatty acyl chains to the number of saturated ones.

*** In this experiment inner phosphatidylethanolamine represented 65.5% and outer phosphatidylethanolamine 34.5% of the total VSV phosphatidylethanolamine as determined by inorganic phosphate determination of phosphatidylethanolamine and Tnp-phosphatidylethanolamine, respectively.

† The fatty acyl chain content of total VSV phosphatidylethanolamine was calculated as (65.5%) (inner phosphatidylethanolamine) + (34.5%) (outer phosphatidylethanolamine).

in inner and outer phosphatidylethanolamine. In both cases the most abundant species are palmitic (16 : 0), stearic (18 : 0) and oleic (18 : 1) acids. The monoenoic and polyenoic fatty acids of 20—22 carbon units long are represented as ">18 unsaturated" fatty acyl chains in Table I. These accounted for a considerable amount of the total fatty acyl chain content, 16.2% for inner phosphatidylethanolamine and 12.3% for outer phosphatidylethanolamine, respectively.

The overall degree of unsaturation was employed as a parameter for comparing the fatty acyl chain distribution in inner and outer phosphatidylethanolamine. This was computed as an "unsaturation ratio" which we define as the total number of unsaturated fatty acyl chains divided by the total number of saturated ones. For the experiment shown in Table I the unsaturation ratios for inner and outer phosphatidylethanolamine were 2.4 and 1.5, respectively. Table II shows similar unsaturation ratios obtained for inner and outer phosphatidylethanolamine in four different experiments. It was observed that the unsaturation ratios of both inner and outer phosphatidylethanolamine differed from one virus preparation to another. This is most probably due to variations in the fatty acyl chain composition of the virus when different lots of serum were employed to culture the BHK-21 cells for virus growth [18]. In all instances, however, the unsaturation ratio was found to be higher for inner than for outer phosphatidylethanolamine, the former being greater than the latter by 1.4—2.9-fold (see Table II). We conclude, therefore, that a higher proportion of unsaturated fatty acyl chains is associated with inner compared to outer phosphatidylethanolamine in the VSV bilayer.

The low unsaturation ratio observed for outer phosphatidylethanolamine was found to be qualitatively similar to the unsaturation ratios observed for total VSV phosphatidylcholine and sphingomyelin as shown in Table III. The values of 1.4 and 1.2 observed for phosphatidylcholine and sphingomyelin, respectively, more closely resemble the low range of values obtained for outer phosphatidylethanolamine than the higher values observed for inner phosphatidylethanolamine. Since most (greater than 70%) of the VSV phosphatidylcholine and sphingomyelin are found in the outer phospholipid monolayer

TABLE II

COMPARATIVE UNSATURATION RATIOS FOR INNER AND OUTER VSV PHOSPHATIDYLETHANOLAMINE

The fatty acyl chains of inner and outer phosphatidylethanolamine were prepared and determined quantitatively in four independently grown preparations of VSV. The number in parentheses is the percent of the total phosphatidylethanolamine found in the outer VSV monolayer as determined by inorganic phosphate analysis of phosphatidylethanolamine and Tnp-phosphatidylethanolamine.

| Experiment | Phosphatidylethanolamine | | Proportional unsaturation ratio inner/outer phosphatidylethanolamine |
|---|---|---|---|
| | Inner | Outer | |
| 1 (33%) | 2.3 | 0.8 | 2.9 |
| 2 (39%) | 4.1 | 2.6 | 1.6 |
| 3 (34%) | 4.6 | 3.3 | 1.4 |
| 4 (34.5%) | 2.4 | 1.5 | 1.6 |

TABLE III

FATTY ACYL CHAINS COMPOSITION OF VSV MEMBRANE PHOSPHOLIPIDS

The table lists the average mol percent and the range (in parentheses) of three determinations.

| Fatty acyl chain | Average mol % (range) | | |
|---|---|---|---|
| | Total phospholipid | Phosphatidylcholine *** | Sphingomyelin |
| 12 : 0 | trace | trace | trace |
| 12 : 1 | trace | trace | trace |
| 14 : 0 | 1.4 (1.2—1.6) | 1.5 (1.4—1.6) | 1.8 (1.7—1.8) |
| 14 : 1 | 0.3 (0.3—0.3) | 0.6 (0.5—0.6) | 0.6 (0.5—0.6) |
| 16 : 0 | 20.0 (19.9—20.1) | 27.9 (27.5—28.3) | 10.8 (10.4—11.2) |
| 16 : 1 | 4.5 (4.2—4.8) | 8.3 (8.2—8.4) | 3.5 (3.1—3.9) |
| 16 : 2 | 0.5 (0.4—0.5) | 0.6 (0.5—0.6) | trace |
| 17 : 0 | 0.6 (0.5—0.7) | 1.7 (1.6—1.7) | 2.1 (0.8—2.3) |
| 18 : 0 | 17.8 (17.5—18.0) | 11.2 (10.9—11.5) | 31.0 (29.7—32.3) |
| 18 : 1 | 39.7 (38.9—40.4) | 40.2 (38.8—41.6) | 37.7 (36.2—39.2) |
| 18 : 2 | 2.0 (1.8-2.1) | 1.1 (1.0—1.2) | 0.8 (0.7—0.8) |
| 18 : 3 | 1.9 (1.8—1.9) | 2.6 (2.4—2.8) | 1.1 (0.9—1.3) |
| 20 : 0 | 0.3 (0.2—0.4) | trace | trace |
| 22 : 0 | 1.6 (1.5—1.7) | 0.2 (0.1—0.2) | 0.7 (0.6—0.7) |
| >18 unsaturated * | 9.6 (9.1—10.0) | 4.2 (4.1—4.2) | 10.1 (9.3—10.8) |
| Total unsaturated fatty acyl chains | 58.3 (58.0—58.6) | 57.5 (56.5—58.4) | 53.7 (51.6—55.7) |
| Total saturated fatty acyl chains | 41.6 (41.2—42.0) | 42.5 (41.6—43.4) | 46.3 (44.2—48.4) |
| Unsaturation ratio ** | 1.4 (1.4—1.4) | 1.4 (1.3—1.4) | 1.2 (1.1—1.3) |

* The ">18 unsaturated" entry represents the proportion of monoenes and polyenes having 20—22 carbon atoms.

** The unsaturation ratio is the molar ratio of the total number of unsaturated fatty acyl chains to the number of saturated ones.

*** Phosphatidylcholine and sphingomyelin were separated from the other VSV membrane phospholipids by the two-dimensional thin-layer chromatography system described in Materials and Methods. Phospholipids were detected on the developed plate by ultraviolet light after the plate had been sprayed with 2',7'-dichlorofluorescein [17]. Fatty acid methyl esters were prepared and determined quantitatively as described in Table I.

(Patzer, E. and Wagner, R., personal communication), it is possible that the low unsaturation ratio observed for outer phosphatidylethanolamine may be characteristic of all phospholipids in the outer lipid leaflet.

*Fatty acyl chains at the 1 and 2 glycerol carbon atoms of phosphatidylethanolamine*

The positional specificity of fatty acyl chains at the 1 and 2 carbon atoms in the glycerol moiety of inner and outer phosphatidylethanolamine was examined by employing phospholipase $A_2$ from bee venom. This enzyme specifically removes the fatty acyl chains from the glycerol 2 carbon atom in either phosphatidylethanolamine or Tnp-phosphatidylethanolamine. The phosphatidylethanolamine and Tnp-phosphatidylethanolamine extracted after reacting VSV with TNBS as described above were treated separately with phospholipase $A_2$ under conditions where both Tnp-phosphatidylethanolamine and phosphatidylethanolamine are equally good substrates for the enzyme. The 1-lyso-phosphatidylethanolamine and 1-lyso-Tnp-phosphatidylethanolamine derivatives pro-

duced by complete phospholipase $A_2$ digestion were isolated by thin-layer chromatography as described in Materials and Methods. Methyl ester of the 1-lyso fatty acyl chains were then prepared and analysed by gas-liquid chromatography. The free fatty acids liberated by phospholipase $A_2$ hydrolysis could not be recovered quantitatively from the thin-layer plates and they were, therefore, not analyzed. Instead, the 2 position fatty acyl composition was calculated from the experimentally determined compositions of the 1-lyso and the untreated phospholipids.

The results for the 1 and 2 positions of inner phosphatidylethanolamine are shown in Table IV. Oleic acid (18 : 1) was found to be the predominant fatty acyl chain at both the 1 and 2 positions; it accounted for 45 and 29% of the fatty acyl chains at these positions, respectively. The other major fatty acyl chains found at the 1 position, in order of decreasing abundance, were palmitic (16 : 0), stearic (18 : 0) and the long chain unsaturated fatty acids. At the 2 position the long chain unsaturated fatty acyl chains were nearly as abundant

TABLE IV

FATTY ACYL CHAIN COMPOSITION OF PHOSPHATIDYLETHANOLAMINE IN THE INNER LEAFLET OF THE VSV ENVELOPE

The table lists the average mol percent and the range (in parentheses) of three determinations.

| Fatty acyl chain | Average mol % (range) | | |
|---|---|---|---|
| | 1 + 2 position | 1 position *** | 2 position † |
| 12 : 0 | 0.2 (0.2—0.2) | 0.8 (0.7—1.0) | 0 |
| 12 : 1 | trace | trace | 0 |
| 14 : 0 | 1.8 (1.7—2.0) | 2.6 (2.4—2.8) | 1.0 |
| 14 : 1 | 5.5 (5.2—5.7) | 5.6 (5.1—5.9) | 5.4 |
| 16 : 0 | 8.9 (8.8—9.1) | 14.6 (14.0—15.1) | 3.2 |
| 16 : 1 | 3.6 (3.1—3.9) | 2.4 (2.1—2.6) | 4.8 |
| 16 : 2 | 3.9 (3.9—4.0) | 3.5 (3.1—3.9) | 4.3 |
| 17 : 0 | 1.1 (0.4—1.5) | 0.3 (0.3—0.4) | 1.9 |
| 18 : 0 | 15.9 (14.5—16.9) | 12.5 (11.0—13.4) | 19.3 |
| 18 : 1 | 36.9 (35.6—38.9) | 44.6 (42.4—47.5) | 29.2 |
| 18 : 2 | 1.5 (1.4—1.8) | 0.8 (0.6—1.2) | 2.2 |
| 18 : 3 | 2.5 (2.5—2.7) | 1.4 (1.3—1.4) | 3.6 |
| 20 : 0 | 0.2 (0.1—0.2) | trace | 0.4 |
| 22 : 0 | 1.1 (0.9—1.3) | 0.6 (0.5—0.7) | 1.6 |
| >18 unsaturated * | 16.2 (16.7—17.0) | 10.1 (9.7—10.7) | 22.4 |
| Total unsaturated fatty acyl chains | 70.8 (69.7—72.7) | 68.5 (67.2—70.5) | 71.9 |
| Total saturated fatty acyl chains | 29.1 (27.2—30.1) | 31.5 (29.4—32.4) | 27.4 |
| Unsaturation ratio ** | 2.4 (2.3—2.7) | 2.2 (2.1—2.4) | 2.6 |

* The ">18 unsaturated" entry represents the proportion of monoenes and polyenes having 20—22 carbon atoms.

** The unsaturation ratio is the molar ratio of the total number of unsaturated fatty acyl chains to the number of saturated ones.

*** The 1 position fatty acyl chain composition was determined from the 1-lyso-phosphatidylethanolamine derivative produced upon complete hydrolysis of purified inner phosphatidylethanolamine by bee venom phospholipase $A_2$. The lipolytic reaction was carried out in the presence of sodium deoxycholate and bovine serum albumin.

† For each fatty acyl chain the percent content at the 2 position was calculated as: 2 × [(% 1 + 2 positions) —0.5 (% in l-lyso-phosphatidylethanolamine)].

as oleic (18 : 1 = 29.2%; >18 unsaturated = 22.4%) while stearic was the next most abundant fatty acyl chain (18 : 0 = 19.5%). Despite the quantitative differences observed for individual fatty acyl chains, however, the unsaturation ratios for the 1 and 2 positions of inner phosphatidylethanolamine were found to be quite similar, 2.2 and 2.6, respectively. As indicated by this ratio, the degree of fatty acyl chain unsaturation at the 2 position in inner phosphatidylethanolamine must be just slightly higher than that at the 1 position.

A quite different situation was observed in the case of outer phosphatidylethanolamine. Its fatty acyl chain compositions at the 1 and 2 glycerol carbon atoms are shown in Table V. As with inner phosphatidylethanolamine, unsaturated fatty acyl chains predominate at the 2 position (18 : 1 = 41.3%; >18 unsaturated = 18.8%). At the 1 position, however, the distribution differs significantly. Saturated fatty acyl chains are most abundant at the 1 position where they account for more than 60% of the total fatty acyl chains present.

TABLE V

FATTY ACYL CHAIN COMPOSITION OF PHOSPHATIDYLETHANOLAMINE IN THE OUTER LEAFLET OF THE VSV ENVELOPE

The table lists the average mol percent and the range (in parentheses) of three determinations.

| Fatty acyl chain | Average mol % (range) | | |
|---|---|---|---|
| | 1 + 2 position | 1 position *** | 2 position † |
| 12 : 0 | 0.7 (0.6—0.7) | 1.5 (1.4—1.6) | 0 |
| 12 : 1 | trace | 0 | trace |
| 14 : 0 | 6.3 (5.9—6.5) | 7.2 (6.9—7.4) | 5.4 |
| 14 : 1 | 4.5 (4.5—4.6) | 3.1 (3.0—3.3) | 5.9 |
| 16 : 0 | 12.6 (12.1—13.3) | 22.4 (22.0—23.0) | 2.8 |
| 16 : 1 | 4.6 (4.2—5.0) | 2.4 (2.2—2.6) | 6.8 |
| 16 : 2 | 2.5 (2.2—2.8) | 0.6 (0.5—0.6) | 4.4 |
| 17 : 0 | 6.5 (5.8—7.3) | 6.5 (6.3—6.6) | 6.5 |
| 18 : 0 | 12.9 (12.4—13.5) | 22.0 (20.1—24.0) | 3.8 |
| 18 : 1 | 33.0 (32.4—33.5) | 24.7 (23.6—26.2) | 41.3 |
| 18 : 2 | 1.6 (1.5—1.7) | 1.3 (1.0—1.5) | 1.9 |
| 18 : 3 | 1.5 (1.5—1.5) | 0.6 (0.4—0.8) | 2.4 |
| 20 : 0 | 0.1 (0.1—0.2) | 0.6 (0.6—0.7) | 0 |
| 22 : 0 | 0.9 (0.8—0.9) | 1.2 (1.0—1.3) | 0.6 |
| >18 unsaturated * | 12.3 (11.6—13.3) | 5.9 (5.7—6.1) | 18.8 |
| Total unsaturated fatty acyl chains | 59.7 (58.9—60.3) | 38.6 (37.5—39.9) | 81.5 |
| Total saturated fatty acyl chains | 40.3 (39.5—41.2) | 61.4 (60.0—62.8) | 19.1 |
| Unsaturation ratio ** | 1.5 (1.4—1.5) | 0.6 (0.6—0.7) | 4.3 |

* The ">18 unsaturated" entry represents the proportion of monoenes and polyenes having 20—22 carbon atoms.

** The unsaturation ratio is the molar ratio of the total number of unsaturated fatty acyl chains to the number of saturated ones.

*** The 1 position fatty acyl chain composition was determined from the 1-lyso-phosphatidylethanolamine derivative produced upon complete hydrolysis of outer phosphatidylethanolamine by bee veno venom phospholipase $A_2$. The lipolytic reaction was carried out in the presence of sodium deoxycholate and bovine serum albumin.

† For each fatty acyl chain the percent content at the 2 position was calculated as: 2 × [(% 1 + 2 positions) —0.5 (% in 1-lyso-phosphatidylethanolamine)].

This positional specificity is manifested in the quite different unsaturation ratios obtained for the 1 and 2 positions of outer phosphatidylethanolamine (1 position = 0.6; 2 position = 4.3).

## Discussion

The results in Tables IV and V show clearly that inner and outer VSV phosphatidylethanolamine differ significantly in overall fatty acyl chain content. Inner phosphatidylethanolamine has a higher proportion of unsaturated fatty acyl chains and there is a significant difference in the distribution of fatty acyl chains between the 1 and 2 glycerol carbon atoms in inner compared to outer phosphatidylethanolamine. These results are the first to show a difference in fatty acyl chain composition between pools of the same phospholipid on the two sides of a biological membrane; they differ qualitatively from the situation with the human erythrocyte membrane where inner and outer phosphatidylcholine were found to have nearly the same fatty acyl chain content [12]. In the future, therefore, it will be of interest to know whether fatty acyl chain asymmetries exist in membranes other than the VSV membrane and in phospholipids other than phosphatidylethanolamine.

The phosphatidylethanolamine fatty acyl chain asymmetry indicated by the results in Tables IV and V could not exist if migration of phosphatidylethanolamine molecules from one phospholipid monolayer to the other (trans-membrane migration or flip-flop) occurred at random in VSV and rapidly with respect to the time required for virus growth and purification (about a day). Random trans-membrane migration would extinguish any existing fatty acyl chain asymmetry. It is possible to conclude from our results, therefore, that rapid, random trans-membrane migration of phosphatidylethanolamine does not take place in the VSV membrane. This conclusion is consistent with the results of Shaw (Shaw, M., personal communication) who has observed that trans-membrane migration of phosphatidylcholine in the VSV membrane takes place at a very slow rate ($t_{1/2}$ >24 h at 37°C) and with the earlier studies of Rothman et al. [4] who showed that trans-membrane migration of phospholipids in the influenza virus membrane is also a very slow process.

The nature of the fatty acyl chain asymmetry observed for phosphatidylethanolamine in the VSV membrane cannot be interpreted to support the view that the identity of the fatty acyl chains somehow underlies or is functionally releated to the overall phospholipid asymmetry. No simple parameter of the fatty acyl chain distributions shown in Tables IV and V can reliably distinguish inner from outer phosphatidylethanolamine. For example, the most significant difference between the inner and outer phosphatidylethanolamine fatty acyl chains occurs at the 1 position of the phosphatidylethanolamine glycerol moiety. In outer phosphatidylethanolamine, greater than 60% of the fatty acyl chains at this position are fully saturated while only approx. 30% are saturated in inner phosphatidylethanolamine. It is clear, however, that some outer phosphatidylethanolamine molecules have an unsaturated fatty acyl chain at the 1 position and that some inner phosphatidylethanolamine molecules have a saturated one. Presence of unsaturation at the 1 position fatty acyl chain is therefore not a parameter than can distinguish reliably between inner and outer

phosphatidylethanolamine. Other simple parameters of the fatty acyl chain distributions shown in Tables IV and V also fail to correlate perfectly with either inner or outer phosphatidylethanolamine. One must conclude that although membrane asymmetries may exist in both the phospholipids and in their fatty acyl chains, these two asymmetries need not be functionally related. They could arise and be maintained by separate mechanisms.

Although the source of the VSV phosphatidylethanolamine fatty acyl chain asymmetry cannot be identified at the present time, it is nevertheless instructive to consider how this type of asymmetry could arise. VSV acquires its membrane from the surface membrane of its host cell through which it escapes by "budding" [15]. In this process the virus becomes coated with a small region of the host cell plasma membrane which contains virus-specific proteins but host cell lipids [16]. Since trans-membrane migration of phospholipids in mature VS virions is slow, the phsopahtidylethanolamine fatty acyl chain asymmetry observed in VSV must exist, at least temporarily, in the host cell membrane prior to virus maturation. The problem of explaining the origin of the viral fatty acyl chain asymmetry, therefore, resolves into explaining the origin of fatty acyl chain asymmetry in the host cell membrane. Two possibilities exist. On the one hand, phosphatidylethanolamine fatty acyl chain asymmetry could be unique to just those regions of the host cell plasma membrane through which virus particles are destined to bud. Asymmetry could be generated in these regions, for example, by interaction of host cell phospholipids with virus-specific proteins. On the other hand, phosphatidylethanolamine fatty acyl chain asymmetry may be a usual and normal feature of the uninfected host (BHK-21) cell plasma membrane. According to this model the virus would simply acquire a representative sample of the host cell plasma membrane. It is not known which of these two possibilities applies in the BHK-21 cell-VSV system we have studied. It is clear, however, that resolution of this issue would be a useful step in attempting to determine the origin of the phosphatidylethanolamine fatty acyl chain asymmetry observed in VSV.

## Acknowledgements

We thank Drs. A. Christopher, P. Holloway, R. Hunt, E. Patzer, M. Shaw and R. Wagner for their advice during the course of these studies. We also thank Mr. K. Klotz for his excellent technical assistance. This work was supported by grants from the National Institute of Child Health and Human Development and the National Cancer Institute.

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