Phosphonolipids in the Mussel *Mytilus galloprovincialis*

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One of the possible roles of phosphonolipids is that they have a contribution to the protection of cellular integrity and survival of aquatic organisms (mollusca, cnidaria) as these lipids are included at high percentages. The total lipids of the edible mussel *Mytilus galloprovincialis* (Mollusca, Bivalvia, Mytilidae) were found to constitute 1.27% of fresh tissue. Polar lipid components constitute 61.5% of the total lipids. After separation by Solid Phase Extraction, the polar lipid fraction was separated by two dimensional thin-layer chromatography and the total phosphorus of each component, was determined. The main polar lipids found were: Phosphatidylcholine, 41.6 ± 0.8% (of which 11.3 ± 0.5% was glyceryl ether analog); ceramide aminoethylphosphonate 11.2 ± 0.2% plus 2.8 ± 0.1% another minor species; phosphatidylethanolamine, 26.6 ± 0.5% (of which 12.2 ± 0.3% was glyceryl ether analog). The individual ceramide aminoethylphosphonate species were isolated by preparative thin layer chromatography and the structure of the major one was confirmed by a combination of analytical and chromatographic methods. Saturated fatty acyl groups with 16 carbon atoms were the main components (48.4%) of the major ceramide aminoethylphosphonate species. Diglyceride aminoethyl phosphonates were not found in lipids of *M. galloprovincialis*.

Introduction

The phosphonolipids are a specific group of lipids containing a very resistant bond between carbon (C) and phosphorus (P). The accurate role and metabolic pathway of phosphonolipids are not yet well understood. Possible functional roles include the contribution to the protection of the cellular integrity, and consequently to the survival of aquatic organisms. Their presence plays a vital role to the cell adaptation when environmental conditions change (Hori and Sugita, 1984). The above properties depend not only on the chemical stability of the C-P bond, but also on the chemical inertness of phosphonolipids either due to existence a glyceryl ether bond in glycerophosphonolipids or due to the presence of hydroxy-fatty acids in sphingophosphonolipids. Moreover, phosphonolipids, apart from their ability to provide resistance to hydrolytic enzymes, have the property to provide cationic buffering capacity, to facilitate transport of essential small ions, to participate in specific metabolic pathways, to substitute the sphingomyelin and gangliosides, in some organisms and finally they enable inter- or intra- species communication or recognition processes (Hilderbrand and Henderson, 1983; Rosenberg, 1973). The presence of a phosphonolipid was first found by Rouser *et al.* (1963) in the sea anemone *Anthopleura elegantissima* as a sphingolipid ceramide aminoethylphosphonate. The same lipid, apart from its existence in cnidaria (Joseph, 1979; Nakhel, 1988), along with ceramide *N*-methyl-aminoethylphosphonate, is widely distributed in molluscs (Hori *et al.*, 1969; Matsubara, 1975; Joseph, 1982; Hori and sugita, 1984; Stavrakakis *et al.*, 1989; Matsubara *et al.*, 1990; Dembitsky *et al.*, 1992). Also, a new type of sphingophosphonolipids, aminoalkylphosphonyl cerebrosides, has been found in marine molluscs (Araki *et al.*, 1991; Yamaguchi *et al.*, 1992; Matsubara and Hayashi, 1993).

We have decided to study the mussel *Mytilus galloprovincialis* (Mollusca, Bivalvia, Mytilidae) on the basis that according to references, molluscs are rich in phosphonolipids (Matsubara, 1975; Joseph, 1982; Matsubara *et al.*, 1990). Another reason was that this organism comprises one of the main fishery food resources commercially available in Greece, having a high degree of consumption among the fishery products. Therefore, this organism is extensively cultivated in well organized fish production units. *M. galloprovincialis* is

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present in the Mediterranean, Adriatic and Black seas and northwards as far as northern France and South-West Britain (Bayne, 1976). Its food consists of organic detritus, protozoans and diatoms which are filtered from the sea-water.

In this paper we describe the qualitative and quantitative lipid composition of the whole organism of *M. galloprovincialis.* Together with other lipids, two species phosphonolipids and glyceryl ether phospholipids were found.

Materials and Methods

Extraction and isolation of polar and neutral lipids

Extraction of lipids from wet tissue was performed according to a modification of the Bligh and Dyer (1959) method as follows: To 233 gr of wet tissue (without shells) 698 ml chloroform/methanol (1:2, by vol) were added and the mixture was blended for 5 min at high speed in a Sorvall Omni-Mixer. The liquid phase was separated by centrifugation (−4 °C, 10 min, 7710 × *g*) and the residue was re-homogenized with 698 ml chloroform/methanol/water (1:2:0.8, by vol), centrifugated again, and the two extracts were combined. To the pooled extracts (635+653 ml), 1288 ml chloroform and 1288 ml water were added. After phase equilibration, the lower chloroform layer (total lipids) was evaporated to dryness, redissolved in chloroform/ methanol (2:1, by vol), and stored at 0 °C (Stavrakakis *et al.,* 1989). Total lipids were fractionated by solid phase chromatography as follows: A portion of total lipid extract (~ 5.3 mg solid) was dissolved in 0.5 ml chloroform/hexane, 1:1, and applied to a pre-washed 100 mg silica column. Neutral lipids were eluted three times with 6 ml aliquots of chloroform, and polar lipids were eluted with 7 ml methanol. The apparatus used was "Waters SEP-PACK Vacuum manifold" and the column used was "Waters SEP-PACK VAR RC Silica catridges". (Mastronicolis *et al.*, 1996).

Thin-layer and paper chromatography of lipids

Analysis of *Mytilus galloprovincialis* polar lipids by single-dimensional thin layer chromatography was carried out on silica gel 60 plates with the following solvent systems: **A**, chloroform/methanol/acetic acid/water (50:25:6:2, by vol); **B**, chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol); **C**, chloroform/methanol/water (65:20:3, by vol) and **D**, chloroform/ methanol/28% ammonium hydroxide (60:35:5, by vol). Polar lipids of *Mytilus* were further separated by two-dimensional HPTLC analysis (2D-HPTLC) carried out on precoated silica gel 60 plates (E. Merck, Darmstadt, Germany) with the solvent system **E** which consisted of the following: dimension 1, chloroform/methanol/acetic acid/water (50:25:6:2, by vol); dimension 2, chloroform/methanol/water (65:20:3, by vol). Visualization of spots was effected by exposure to iodine vapours followed by spraying with ninhydrin reagent and/or the phosphomolybdenum blue reagent for phospholipids, (Dittmer and Lester, 1964) combined with the heating test of Stillway and Harmon (1980) for phosphonolipids. Dragendorff reagent was used for detection of choline (Christi, 1982). Lipid spots also were visualized using cupric sulphate spray and heating at 180 °C for 2–5 min (Bhat and Ansari, 1989; Pucsok *et al.*, 1988). Glycolipids were stained with a-naphtholsulphuric acid reagent (Jacin and Mishkin, 1965). Paper chromatographic analysis of water-soluble products was carried out on Whatman paper N_o1 with the descending technique (16–20 h) in the following solvent system: phenol/water/ethanol/acetic acid, (80:20:12:10, W/V/V/V). Hanes and Isherwood (1949) and ninhydrin sprays, were used for visualization of phospho- and amino- derivatives, respectively and Dragendorff spray was used for staining of L-α-glycerylphosphorylcholine. Additionally, as lipid standards the following were used: Phosphatidylethanolamine and phosphatidylcholine were isolated from egg yolk. Phosphatidylinositol, L-α-glycerylphosphorylethanolamine, L-α-glycerylphosphorylcholine, L-α-glycerylphosphorylinositol, lyso-phosphatidylethanolamine, lyso-phosphatidylcholine and 2-aminoethylphosphonic acid were purchased from Sigma. The major sphingophosphonolipid of medusa *P. noctiluca* which isolated and identified as a ceramide aminoethylphosphonate component, CAEP (Nakhel *et al.*, 1988) was used as a standard of ceramide aminoethylphosphonic acid.

Analytical methods and quantitation of lipid components

Total phosphorus and phosphonate phosphorus were determined by the methods of Long and Sta-

ples (1961) and Kapoulas *et al.* (1984) respectively. Sugars were determined according to the method of Dubois *et al.* (1956). Esters were determined by the method of Snyder and Stephens (1959), long chain bases by the method of Lauter and Trams (1962), glyceryl ethers by the method of Hanahan and Watts (1961) and nitrogen according to the method of Hashmi *et al.* (1962). Mild alkaline hydrolysis (deacylation) was performed with 0.1 ml NaOH (1.2 N) in methanol (50%, by vol), 45 °C, 20 min. (Wells and Dittmer, 1966). Dry-acid methanolysis of sphingophosphonocompounds was performed by a modification of Vance and Sweely (1967) method as follows: 500 µl of 3 N methanolic-HCI (10%, W/V; Supelco Inc.) reagent were added to 1 mg of the lipid sample, methylation was allowed to proceed (100 °C, 5–6 h) and molecular ratios for structural data were determined in the hydrolysis products. The polar lipids in bulk were quantitated by weight after elution from solid phase extraction columns as described above.

Individual polar lipid components were quantitated after separation on two-dimensional TLC (20 × 20 cm 0.25 mm thick) as follows: Polar lipid sample (10 µg phosphorus) was applied on the plate and separated with the two-dimensional solvent system **E**. Spots were visualized by exposure to iodine vapours and each of phospholipid species was directly scraped into digestion tubes, and the total phosphorus was determined by modification of Long and Staples (1961) method as follows: **1**. Addition of 0.5 ml 72% $HClO_4$ and heating (160°-170 °C,1h). **2**. Addition 1 ml of water + 3 ml 0.4% ammonium molybdate + 0.5 ml ANSA **3**. Development of blue colour after 10 min in a boiling water bath **4**. 20 min at room temperature and extraction of phosphomolybdenum blue colour with 5 ml ethyl acetate. After phase equilibration (15 min), the optical densities of the clear blue upper layers were measured at 780 nm against the corresponding reagent blank.

Gas chromatographic analysis of fatty acid methyl esters

Fatty acid methyl esters of major sphingophosphonolipid component was prepared by scraping 2D-HPTLC spot, directly into digestion tube and converting the fatty acids to methyl esters with dry-acid methanolysis as follows: To each lipid sample, 400 µl of 3 N methanolic-HCI (10% W/V; Supelco Inc.) reagent were added, and methylation was allowed to proceed at 100 °C for 5–6 h. Fatty acid methyl esters were extracted four times with 0.8 ml aliquots of petroleum ether. The samples were then dried and redissolved in hexane for gas chromatographic analysis (Mastronicolis *et al.*, 1996). Fatty acid methyl esters were separated using a Hewlett – Packard 5800A (Hewlett-Packard, Palo, Alto, CA) on a DB™ -23 30 m × 0.25 mm capillary column (J & W Scientific, Folsom, CA) with 50% cyanopropyl phase, 0.25 µm film thickness. The carrier gas was H_2 and the make-up gas was N_2. The oven temperature was programmed, from 90° to 170 °C at a rate of 70 °C min^{-1} and 170° to 210 °C at 5 °C min^{-1}, with initial and final holding times of 1 and 5 min respectively. The splitless injector temperature was 210 °C and the detector temperature was 280 °C. The fatty acid methyl esters were identified by comparison with a standard mixture of fatty acid methyl esters.

Results and Discussion

General observations

The total lipids of *M. galloprovincialis* extracted according to the modified procedure of Bligh and Dyer (1959), amounted to 1.27% (w/w) of fresh tissue (average of 44 specimens of Mediterranean *M. galloprovincialis,* 5–7 cm long , in June). The lipid phosphorus content of the total lipids was 1,79% and the total phospholipid content was 0.78% (w/w) of fresh tissue. On the basis of phosphonate-phosphorus determinations the phosphonolipids were found to represent 14 ± 0.3% (mol/mol) of total phospholipids. The carbohydrate content of the total lipids was about 0.2%. The polar lipids in bulk after separation from neutral lipids by solid phase extraction were quantified by weight and amounted 61.5% of total lipids. The polar lipid fraction contained 7.9 ± 0.2% glyceryl ethers. Initial large-scale experiments with single TLC mobilities of total lipids of *M. galloprovincialis* were designed to provide a deeper insight into the composition and nature of individual lipid classes. Experiments using several developing solvent systems **A, B, C** and **D** indicated that the more successful solvent system for the separation of polar lipids were the solvent system **A** and the solvent system **B**. The difference of solvent system

A against of **B** was that the molecular species of minor phosphonolipid component migrated slower than the major phosphonolipid component (Fig. 1) but simultaneously the minor phosphonolipid partially overlapped phosphatidylcholine component. In system **B**, both the molecular species of phosphonolipids components had similar Rf range, but the phosphatidylcholine mobility was very slow and it was situated far from both phosphonolipid species, therefore the isolation and purification of phosphonolipid species mixture from the rest polar lipid components was very easy by preparative TLC (Fig. 2). In the solvent system **C**, the polar lipid components were separated into several spots with similar Rf values for the most part close to that of phosphatidylcholine. Finally, in the alkaline system **D**, both phosphonolipid components were concentrated in similar Rf range near at the origin of the plate. As determined by single TLC with the solvent system **A** the polar lipid fraction contained eight components (Fig. 1). Six of them were phospholipids (molybdenum blue positive components). The major phosphorus positive components were those that co-chromatographed with standards of phosphatidylethanolamine and phosphatidylcholine. Two more phospholipids positive to ninhydrin and to Stillway test, were designated as phosphonate lipids (ceramide aminoethylphosphonic acids) and the major one (**CAEP-I**) was co-chromatographed with the **CAEP** standard from *Pelagia noctiluca*. The last two phospholipid species designated as **a** and those at the **origin**. Furthermore, two ninhydrin positive components (free phosphorus) designated as **b** and **c** (Fig. 1).

As determined by 2D-HPTLC in system **E** (n= 5) the polar lipid fraction revealed 10 components which were designated as **PE_1, PE_2, CAEP-I, f,**

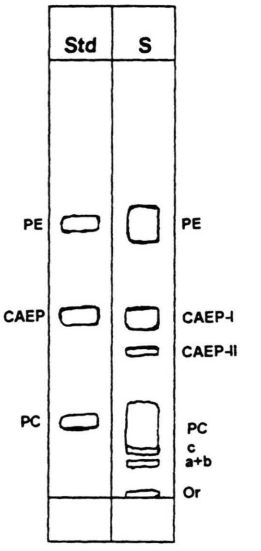

Fig. 2. Thin layer chromatographic separation of the polar lipid fraction of *Mytilus galloprovincialis* on a TLC silica gel 60 plate. The diagram on the left shows the next standards: PE, phosphatidylethanolamine; PC, phosphatidylcholine and CAEP, the sphingophosphonolipid of *P. noctiluca*. The right diagram shows the sample after spraying with cupric sulphate/ H_3PO_4 and charring. The solvent system was chloroform/methanol/acetic acid/ water (50:37.5:3.5:2, by vol). S, sample ; Std, standard; Or, origin; CAEP I and II, major and minor sphingophosphonolipids of *Mytilus* respectively. The letters a, b, c and origin denote also polar lipid components as described in the text.

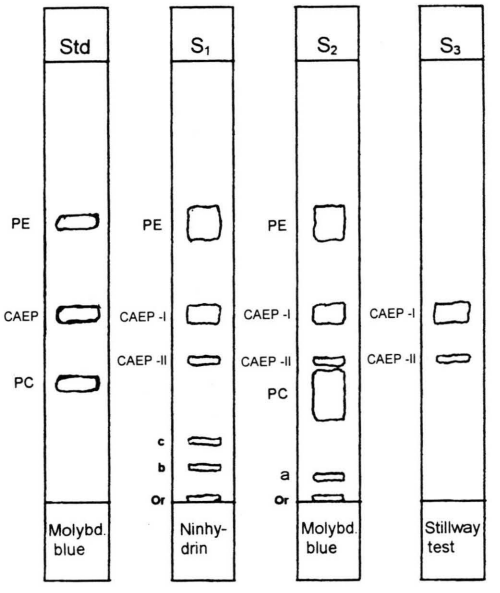

Fig. 1. Thin layer chromatographic separation of the polar lipid fraction of *Mytilus galloprovincialis* on a TLC silica gel 60 plate. The diagram on the left shows the next standards: PE, phosphatidylethanolamine; PC, phosphatidylcholine and CAEP, the sphingophosphonolipid of *P. noctiluca*. S_1 diagram shows the sample after spraying with ninhydrin reagent; S_2 diagram shows the sample after spraying with molybdenum blue reagent and S_3 diagram shows the sample after Stillway test. The solvent system was chloroform/methanol/acetic acid/water (50:25:6:2, by vol). S, sample; Std, standard; Or, Origin; CAEP I and II, major and minor sphingophosphonolipids of *Mytilus* respectively. The letters a, b, c and origin denote also polar lipid components as described in the text.

CAEP-II, PC, c, b, a and those at **origin** (Fig. 3). It means two more components than the eight components of the above single TLC separation were determined (Fig. 1, Fig. 2). The designated spots as $\mathbf{PE_1}$ and $\mathbf{PE_2}$ had the same Rf value on single TLC as **PE** standard (Fig. 1, Fig. 2) but could be resolved (by 2D-HPTLC) into two phosphorus and ninhydrin positive spots in chloroform/methanol/water (65:20:3, by vol) system (Fig. 3). The designated spots as **f** and **CAEP-I** had the same Rf value on single TLC as **CAEP** standard (Fig. 1, Fig. 2) but could be resolved by 2D-HPTLC into two phospholipid spots in a chloroform/methanol/water (65:20:3, by vol) system (Fig. 3). Species **f** was ninhydrin and Stillway test negative but the molecular species **CAEP-I** was positive to ninhydrin and Stillway test suggesting that it is a phosphonolipid component.The designated spot as **PC** had the same Rf value on single TLC as **PC** standard (Fig. 1, Fig. 2) Finally, the lipid component at the " **origin**" was positive to a-naphthol reagent test suggesting the presence of glyco- or sulpho-lipids in this spot.

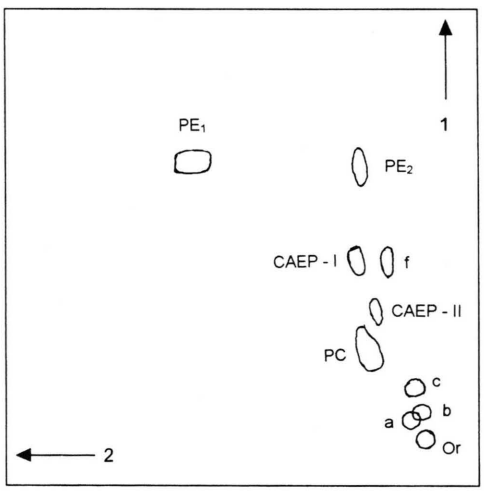

Fig. 3. Two-dimensional thin-layer chromatographic separation of *Mytilus galloprovincialis* polar lipids on high-performance thin layer chromatography silica gel 60. Plates were developed in direction 1 with chloroform/methanol/acetic acid/water (50:25:6:2, by vol) and in direction 2 with chloroform/methanol/water (65:20:3, by vol). The plate was stained by cupric sulphate/H_3PO_4 and charring. PE_1 and PE_2, two different molecular species of phosphatidylethanolamine; CAEP I and II, major and minor sphingophosphonolipids of *Mytilus* respectively; PC, phosphatidylcholine. The letters a, b, c, f and Origin (Or) denote the rest of polar lipid components of *Mytilus* as described in the text.

After the analytical data obtained by quantitation of total phosphorus using a polar lipid sample (up to 10 μg phosphorus) after separation on two-dimensional TLC by using the solvent system **E** as described in Materials and methods, the overall composition for the total phospholipids was inferred. The results are summarised: $\mathbf{PE_1}$, 13.5 ± 0.3%; $\mathbf{PE_2}$, 13.1 ± 0.2%; **CAEP-I**, 11.2 ±0.2%; **CAEP-II**, 2.8 ± 0.1%; **f**, 6.7 ± 0.4%; **PC**, 41.6 ± 0.8%; **a**, 1.6 ± 0.2% and **origin**, 4.5 ± 0.3%.

Structural studies

Major and minor sphingophosphonolipids: By using a polar lipid sample on preparative TLC with the solvent system **B** (Fig. 2) the band of the mixture of two phosphonolipid components (**CAEP-I** and **CAEP-II**) contaminated by the component **f** was isolated and the mixture of three lipid molecules was subjected to phosphonate-phosphorus determination. The results were revealed that the phosphonate-phosphorus represents about 75% of the total phosphorus content of this lipid mixture and so it was confirmed that the component **f** contained 25% of total phosphorus. Sample of the mixture of three lipid components (260.5 μg phosphorus) was subjected to mild alkaline hydrolysis in order to remove any amount of glycerophospholipids (component **f**) and the alkaline-stable products (197 μg phosphorus) were analysed by 2D-HPTLC using the solvent system **E**. The results confirmed that only two components (positive to ninhydrin, to molybdenum blue and to Stillway test) were present and their phosphonate-phosphorus analysis showed that 97% of lipid-phosphorus was found to be phosphonate-phosphorus. The mixture of two phosphonolipid species was further separated into two individual molecular species (major sphingophosphonolipid species and the minor one) by three successive preparative TLC using the solvent system **A** (Fig. 1).The minor sphingophosphonolipid, **CAEP-II**, was isolated and stored for further identification analysis. After testing the purity of major sphingophosphonolipid species, **CAEP-I**, by 2D-HPTLC analysis it was confirmed that only one component was present. Sample of this phosphonolipid was subjected to dry-acid methanolysis and the hydrolysis products, were found to contain total nitrogen, long-chain bases, (methyl) esters

and total phosphorus in molar ratios of 2.18 : 0.85 : 1.08 : 1.00 respectively. Furthermore, the water-soluble products of the above methanolysis were analysed by descending paper chromatography and found to contain only one component which co-migrated with 2-aminoethylphosphonic acid standard and was ninhydrin and phosphorus positive. Therefore, by all the results mentioned above a structure of ceramide aminoethylphosphonic acid, **CAEP-I**, is confirmed for this major sphingophosphonolipid component of *M. galloprovincialis*, and its main fatty acid composition was studied by gas liquid chromatographic analysis, as described in Materials and Methods. The results are listed in Table I.

Minor phosphonocomponent **CAEP-II** was isolated by preparative TLC with the solvent system **A** (see above). After testing the purity of minor sphingophosphonolipid species by 2D-HPTLC analysis only one component was present. Aliquot of this lipid was subjected to dry-acid methanolysis and the molar ratios for the total nitrogen, long-chain bases, (methyl) esters and total phosphorus were respectively 2.12 : 0.95 : 1.15 : 1.00. Therefore, by all the results mentioned above a structure of ceramide amino-phosphonic acid, **CAEP-II**, was confirmed for this minor sphingophosphonolipid component of *M. galloprovincialis*.

According to structural studies, the two sphingophosphonolipids of *M. galloprovincialis* belong to the ceramide aminophosphonic acid type, regardless of their chromatographic resolution into one major and one minor component. It has been established (Hori and Sugita, 1984) that additional hydroxyls in the long chain base and/or the fatty acyl group result in chromatographic resolution of the respective molecular species of the same type, owing to increased polarity.

Table I. Fatty acid composition of major sphingophosphonolipid isolated from *Mytilus galloprovincialis*.

Fatty acid	Weight %
2-OH $C_{10:0}$	9.4
$C_{14:0}$	6.7
$C_{15:0}$	9.1
$C_{16:0}$	48.4
$C_{18:0}$	12.7
$C_{18:1}$	7.8
$C_{18:2}$	2.6
$C_{20:0}$	3.3

Glycerol lipids

By using a polar lipid sample on preparative TLC with the solvent system **B** (Fig. 2) the band of the lipid component which co-chromatographed with phosphatidylcholine standard was isolated, and the extracted lipid was purified by two successive preparative TLC. After testing the purity of this phospholipid by 2D-HPTLC analysis, it was confirmed that only one component was present, which was positive to molybdenum blue and to Dragendorff reagents. This component was subjected to glyceryl ether determination and the result indicated that this lipid fraction contained 11.3 ± 0.5% glyceryl ethers (4.7 ± 0.2% of polar lipids). Furthermore, sample of this lipid (257 μg phosphorus) was subjected to mild alkaline hydrolysis and the alkaline stable products were found to contain 30 μg phosphorus. Sample of the latter alkaline-stable product was co-chromatographed with lyso-phosphatidylcholine standard on 2D-HPTLC with the system **E** and it was found to contain only one component, molybdenum blue and Dragendorff test positive. Also a water-soluble products sample of the above hydrolysis was analysed by descending paper chromatography and it was found to contain only one component which co-chromatographed with glycerylphosphorylcholine standard and was Hanes-Isherwood and Dragendorff positive. Therefore, by combining all the data mentioned above a structure of phosphatidylcholine was confirmed for this lipid.

By using the separation of polar lipids on a 2D-HPTLC (Fig. 3) and by comparing the mobilities of designated spots as $\mathbf{PE_1}$ and $\mathbf{PE_2}$ with that of **PE** standard we observed the following results. $\mathbf{PE_1}$ cochromatographed with **PE** standard but $\mathbf{PE_2}$ had a lower Rf in a chloroform/methanol/water (65:20:3, by vol) system in second dimension than that of **PE** standard. This was considered to be due to differences in the nature of their constituent fatty acids with the basic molecular structure the same as that of **PE** standard. There was a principal disadvantage to separate $\mathbf{PE_1}$ component from $\mathbf{PE_2}$ on a single TLC. This difficulty could be overcome by the technique of two-step single-dimensional TLC (Christie, 1982). According to

this technique, a polar lipid sample was applied to a silica gel G TLC plate, which was first developed in chloroform/methanol/acetic acid/water (50:25:6:2, by vol) to the top. It was then redeveloped in the same direction with chloroform/methanol/water (65:20:3, by vol) to the top. Lipids migrate in the order **PE_1**, **PE_2**, **CAEP-I**, **f**, **CAEP-II**, **PC**, **c**, **b**, **a** and **origin**. By using the advantage of the above technique that **PE_1** migrated faster than **PE_2**, a polar lipid sample was applied to a preparative two-step single-dimensional TLC and **PE_1**, **PE_2** lipid components were scraped and separated.

After testing the purity of **PE_1** component by 2D-HPTLC analysis it was confirmed that only one component was present which was positive to ninhydrin and to molybdenum blue reagents. The lipid-phosphorus recovery of **PE_1** was in agreement with the result of quantitation analysis for this lipid (13.5% of polar lipids). This component was subjected to glyceryl ether determination and the results indicated that **PE_1** contained glyceryl ether analogs in a proportion of 24.0 ± 0.7% (3.1 – 3,3% of polar lipids). Moreover, sample of **PE_1** lipid was subjected to mild alkaline hydrolysis and 24% of lipid-phosphorus was distributed in the alkaline-stable products. Sample of the latter alkaline-stable products was co-chromatographed with lyso-phosphatidylethanolamine standard on 2D-HPTLC with the solvent system **E** and it was found to contain only one ninhydrin and molybdenum blue positive component. Also, a water-soluble products sample of the above hydrolysis was analysed by descending paper chromatography and it was found to contain only one component which co-chromatographed with glycerylphosphosphorylethanolamine standard and it was ninhydrin and Hanes-Isherwood positive.Therefore, by combining all the data mentioned above a basic molecular structure of phosphatidylethanolamine was confirmed for this lipid.

We tested the purity of **PE_2** component by 2D-HPTLC analysis and also we tested its lipid phosphorus recovery as above described for **PE_1** species. Also, **PE_2** component was subjected to glyceryl ether determination and the results indicated that **PE_2** was free of glyceryl ethers. Moreover, sample of **PE_2** lipid was subjected to mild alkaline hydrolysis and almost the bulk of lipid-phosphorus was distributed in the water-soluble products. A water-soluble products sample of the above hydrolysis was analysed by descending paper chromatography and it was found to contain only one component which co-chromatographed with glycerylphosphosphorylethanolamine standard and it was ninhydrin and Hanes-Isherwood positive. Therefore, by combining all the data mentioned above a basic molecular structure of phosphatidylethanolamine was confirmed for **PE_2** species too.

Furthermore, **PC**, **PE_1** and **PE_2** lipid components were examined in relation to a possible existence of phosphonic acid analogs in them. For this purpose, phosphonate-phosphorus determination was carried out in the above lipids a well as in their respective water-soluble products obtained after their mild alkaline hydrolysis. The result of this determination was negative for all the above lipid fractions suggesting the absence of phosphonic acid analog in these glycerophospholipids fractions.

As previously mentioned (see *Major and minor sphingophosphonolipids* section above), a sample of the mixture consisted of the two phosphonolipids along with **f** component was subjected to mild alkaline hydrolysis and 25% of lipid-phosphorus was distributed in the water-soluble products. A water-soluble products sample was analysed by descending paper chromatography and found to contain only one component which co-chromatographed with glycerylphosphorylinositol standard and it was Hanes-Isherwood positive.Therefore, by the above findings a molecular structure of phosphatidylinositol (**PI**) could be possibly suggested for the **f** component.

The results of the present investigation revealed that the polar lipids comprise 61.5% of the total lipids while 38.3% of them were neutral lipids. The major phospholipid fraction in *M. galloprovincialis* were choline and ethanolamine phosphoglycerides, which amounted to 41.6 ± 0.8% and 26.6 ± 0.5% of the phospholipids respectively. Ceramide aminoethylphosphonates were the third phospholipid fraction in amount 14.0 ± 0.3%. Matsubara (1975) reported that the mussel *Mytilus edulis* has a ceramide aminoethylphosphonate species in amount of 25.6% of total lipids. Furthermore, long chain bases of this molecule were mainly the 16:1, 18:1, and 18:2 dihydroxy bases. Finally the main constituent fatty acid in CAEP of *M. edulis* was the palmitic acid (76.7%), and branched 17:0 was the second most abundant fatty acid (12.1%).

M. galloprovincialis was characterized by the absence of some lipid classes widespread in animals, such as sphingomyelin and phosphatidylserine. It was increasingly recognized that phosphonolipids may substitute sphingomyelin or other lipids and play their physiological roles. Also, diglyceride aminoethylphosphonates were not detected. This result is in agreement with the fact that the phosphonolipids in molluscs and marine invertebrates are reported to be of the ceramide aminoethylphosphonate type while in protozoa they are mostly of the phosphorylglycerol type (Hori and Sugita, 1984; Hilderbrand and Henderson, 1983). The different phosphonolipid distribution in protozoa and molluscs would suggest certain differences in the pathway of biosynthesis of the aminoethylphosphonate portion of phosphonolipids. It is interesting that the polar lipid fraction of the land snail *Eobania vermiculata* was characterized by the presence of a considerable amount of glyceride aminoethylphosphonate (6.3% of polar lipids) (Stavrakakis *et al.*, 1989). By comparing the sphingophosphonolipids contents in *Mytilus galloprovincialis* and medusa *Pelagia noctiluca* it is clear that medusa contains about double percentage of sphingophosphonolipids (Nakhel *et al.*, 1988).The percentage of glycerylether type phospholipids of *Mytilus galloprovincialis* was 7.9 ± 0.2%.The glyceryl ethers were concentrated in the molecular species of phosphatidylethanolamine component, (**PE_1**) and also in phosphatidylcholine component (**PC**) (3.20 ± 0.1% and 4.7 ± 0.2% of polar lipids, respectively).

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