Lipid Composition and Structural Studies on Lipids from the Land Snail *Eobania vermiculata*

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The total lipids of the commercial land snail *Eobania vermiculata* (Gastropoda, Pulmonata, Stylommatophora) are found to constitute a small percentage (0.8%) of the wet tissue, which is comparable to that reported for other gastropods. Polar lipid components comprise 61.4% of the total lipids. The individual lipid classes obtained by column chromatographic fractionation were purified by preparative TLC or by column chromatography and their structure was confirmed by a combination of chromatographic and analytical determinations before and after mild alkaline hydrolysis and/or (dry) acid methanolysis and by IR analysis. Neutral lipids represent 36.4% of total lipids, containing cholesterol, cholesterol esters and triglycerides as their major components (26.2%, 29.1% and 25.5% respectively). They contain also a significant amount (14%) of free glyceryl ethers, which are found in a mollusc for the first time. The overall composition of the polar lipids (mol/100 mol lipid-P) was found as follows: Cardiolipin, 2.9; phosphatidylethanolamine, 24.9 (of which 19.8% plasmalogen analog); phosphatidylcholine, 49.2 (of which 45.6% glycerylether analog); ceramide aminoethylphosphonate, 7.5 plus 0.01 (another three minor species); diglyceride-aminoethylphosphonate, 6.3; Sphingoethanolamine 1.65 (for the first time found and structurally studied in a land gastropod); and phosphatidic acid 1.1. Unsaturated fatty acyl groups represent about 72.6 and 44.1 respectively in phosphatidylethanolamine and phosphatidylcholine. A significant amount (70.5%) of unsaturated fatty acids is concentrated in neutral lipids.

The $C_{16:0}$ alk-1-enyl chain was found to predominate (55.6%) in the side chains of ethanolamine plasmalogen. Batyl alcohol was found as the main glycerylether bound to choline phosphate (97.5%).

Saturated fatty acyl groups with 16 carbon atoms were main components (54%) of the major ceramide aminoethylphosphonate species.

Introduction

Mollusca phospholipids have been studied rather intensively with respect to some interesting components such as phosphonolipids and glycerylether phospholipids which are abundant in this phylum. Thus, the phosphonolipid ceramide aminoethyl phosphonate, originally discovered by Rouser *et al.* [1] in the sea anemona *Anthopleura elegantissima*, appears to be widely distributed within the phylum Mollusca [2–11], usually accompanied by a variety of other sphingophosphonolipids [12–17]. Moreover, mollusca are very rich in ether-containing phospholipids of both, the alkyl and alk-enyl types [18–22].

Although the lipid composition of marine mollusca is well documented, there is only a limited amount of information on the lipid composition of the terrestrial mollusca. *Cepaea nemoralis* is the only land snail which has been extensively studied concerning its lipid composition and metabolism [23–28]. Some other representatives of this order (Stylommatophora) such as *Euhadra herklotsi* [29], *Arianta arbustorum* [30], *Succinea putris* [31], and *Helix pomatia* [32–34] have been studied with regard to their fatty acid composition. Althaus *et al.* [35] have investigated the lipid composition of the single nerve cells

Abbreviations: STE, sterol esters; TG, triglycerides; FA, fatty acids; FAl, fatty alcohol; Chol, cholesterol; GE, glyceryl ethers; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPM, sphingomyeline; Car, cardiolipin; AEP_n, 2-aminoethylphosphonic acid; TMSi, trimethylsilyl-derivatives; Pnl, phosphonolipids; SPnL, sphingophosphonolipids; TLC, thin layer chromatography; IR, infrared; NL, neutral lipids; TL, total lipids; PL, phospholipids; P, phosphorus; GLC, gas liquid chromatography; MAH, mild alkaline hydrolysis; MAS, mild alkaline stable; GPE, glycerylphosphorylethanolamine; GPC, glycerylphosphorylcholine; DMA, dimethyl acetals; St, standards; Cerb, cerebrosides; GPnl, glycerylphosphonolipids; L-PE, lyso-PE; L-PC, lyso-PC.

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of *Helix pomatia* and Thompson and Hanahan [18] have studied the glyceryl ether phospholipids of two species of terrestrial slugs, *Arion ater* and *Ariolimax columbianus*.

In a long-term research program on the distribution of phosphonolipids in "Jelly fishes" [72] and in edible animals, we were tempted to investigate the lipids of the edible land snail, *E. vermiculatac* phylum: Mollusca, class: Gastropoda, order: Stylommatophora) which is widespread in Greece and other European countries.

In a previous paper [36] we reported the presence of complex glyco- and phosphonolipids among the water-methanol soluble lipids ("acidic lipids") of Eobania. In this paper we describe the qualitative and quantitative composition of the lipids of *E. vermiculata,* which were found to be rich in phosphonolipids and glyceryl ether phospholipids.

Experimental

Materials

Silica gel G, type 60 was purchased from Merck: silicic acid for column chromatography, 100 mesh, from Mallinckrodt; cardiolipin, sphingomyelin, phosphatidylethanolamine, lyso-phosphatidylethanolamine, lyso-phosphatidylcholine, phosphatidic acid, phosphatidylserine, phosphatidyl inositol were from SERVA; L-α-glycerophosphorylethanolamine, phosphatidylcholine, L-α-glycerophosphorylcholine, 2-aminoethyl-phosphonic acid, D, L-chimyl alcohol, D, L-batyl alcohol, ceramide, and sphingosine were from SIGMA.

Adult organisms of *E. vermiculata* were collected in coastal areas of East Crete (Greece), in August.

Extraction and fractionation of lipids

The animals were fed with flour for two days and kept in a perforated box at room temperature for approximately one month before lipid extraction. The total weight (with shells) of 390 specimen was 1276 g (wet tissue, 850 g).

Their lipids were extracted by a modification of the Bligh-Dyer method [37] as follows: To the intact snails (with shells) 1800 ml chloroform–methanol 1:2 (v/v) were added and immediately homogenized in an Omni-Mixer (0 °C, 5 min at medium speed). The liquid phase was separated by centrifugation (−4 °C, 10 min, 3000 rpm) and the residue was rehomogenized with, 1800 ml chloroform–methanol–water 1:2:0.8 (v/v/v), centrifuged again, and the two extracts were combined. To the pooled extracts (1530 + 1770 ml), 1100 ml chloroform and 1100 ml water were added. They were vigorously mixed and left for phase equilibration. The lower chloroform layer was evaporated to dryness in a rotary evaporator and redissolved in 100 ml of chloroform–methanol 9:1 (v/v).

A portion of the total lipid extract was evaporated to dryness, redissolved in a minimal amount of chloroform–methanol 98:2 (v/v) and applied on a column (4.5 × 13.3 cm) packed with 10 g of silicic acid activated overnight at 120 °C and mixed with 5 g of Hyflo Super Cel (filter aid). Elution was performed with solvent mixtures of increasing polarity as indicated in Table I. Monitoring was effected by TLC (see Fig. 1 and 2).

Preparation of derivatives

Fatty acid methyl esters and aldehyde dimethylacetals for GLC analysis were prepared by dry-acid methanolysis [38] of the lipid fractions followed by extraction with petr.ether (b.p. 40–60 °C), three times, and purification by preparative TLC with petr. ether–diethyl ether–acetic acid 70:30:1 (v/v/v) as developing solvent.

For the preparation of isopropylidene derivatives of glycerylethers, the phosphatidylcholine fraction was submitted to prolonged acid hydrolysis [39, 40], followed by extraction of the fatty acid methylesters for GLC analysis. The remaining methanol layer was made strongly alkaline and the glycerylethers were extracted with ethyl acetate (three times). They were purified by preparative TLC with petr. ether–diethyl ether–acetic acid 30:70:1 (v/v/v) as developing solvent mixture and they were converted to their isopropylidene derivatives according to Hanahan *et al.* [41].

For preparation of trimethylsilyl derivatives of long chain bases, a portion of purified sphingolipid was submitted to acid methanolysis [42] followed by extraction of fatty acid methylesters (three times) with petr.ether (b.p. 40–60 °C). The remaining aqueous-methanolic layer was evaporated to dryness in a stream of nitrogen. The residue was dissolved in 2 ml 2 N NaOH and the long chain bases were extracted with ethyl acetate (three times). After purification by preparative TLC, they were converted to

their TMS derivatives according to Sweeley *et al.* [43].

Mild alkaline hydrolysis (deacylation) [44] was used for the preparation of mild alkali stable components and of the respective water soluble products for further analysis or identification.

Analytical and chromatographic methods

Total-P and phosphonate-P were determined as previously described [45] and occasionally by the method of C. Long and D. A. Staples [46]. Esters were measured by the method of Snyder and Stephens [47], glycerylether according, to Hanahan and Watts [48] and long chain bases by the method of Lauter and Trams [49]. Sugars were determined according to Dubois *et al.* [50], plasmalogens by the method of Gottfried *et al.* [51] and cholesterol according to Courchaine *et al.* [52].

TLC analysis was carried out on chromatoplates of silica gel G 60, 0.25 mm thick, activated at 120 °C for 1 h.

Visualization of spots was effected by exposure to iodine vapors followed either by spraying with 50% sulfuric acid and charring or by spraying with ninhydrin reagent and/or phosphomolybdenum blue reagent for phospholipids [53] combined with the heating test of Stillway and Harmon [54] for phosphonolipids. Choline containing lipids were stained with Drangendorff's reagent [55] and glycolipids with α-naphtholsulphuric acid reagent [56].

GLC analysis of fatty acid methyl esters and long-chain aldehyde, dimethylacetals were performed on a Packard model 427 gas chromatograph equipped with flame ionization detector and connected with a Packard-611 recorder. A 6 ft stainless steel column (diam. 1/4 in) packed with 10% EGSS-X on Gas chrom Q (100–120 mesh) was used with a flow of nitrogen (carrier gas) 30 ml/min and column temperature 170 °C.

GLC analysis of isopropylidene derivatives of glycerylethers was performed on the same equipment. A 5 ft stainless steel column (diam. 1/8 in) packed with 5% SE-30 on chrom W AW (60–80 mesh) was used with column temperature 200 °C.

GLC analysis of sterols and of TMS derivatives of long-chain bases was performed using an 1-m glass column (diam. 2 mm) packed with 1% SE-30 on Chromosorb WHP (80–100 mesh) at 230 °C and 200 °C respectively, with a flow of nitrogen, 15 ml/min.

IR analysis was performed in a Perkin-Elmer IR spectrometer, model 157 with sodium chloride prisms.

Paper chromatographic analysis of water soluble products was carried out on paper whatman No. 1 with the ascending technique (8–10 h) in phenol–water–ethanol–acetic acid, 80:20:12:10 (by vol.) as developing solvent system.

Ninhydrin and Hanes-Isherwood [57] sprays were used for visualization of amino and phospho derivatives respectively. Choline was visualized with the Drangendorff's reagent [55].

Results

General observations

Initial experiments were designed to provide a deeper insight into the composition and nature of individual lipid classes present in *E. vermiculata.*

Such experiments indicated that *Eobania* lipids do not contain phosphatidyl serine and sphingomyelin.

In the final large scale experiment described in the present paper the total lipid content of *Eobania* was found 0.8% (w/w) of fresh tissue (average of 390 snails).

The total phospholipid content was 0.48% (w/w) of fresh tissue or 61.4% (w/w) of total lipids. On the basis of phosphonate-P determinations the sphingophosphonolipids were found to represent 7.5% (mol/mol) and the glycerophosphonolipids the 6.3% of total phospholipids.

By TLC separation in several developing solvent systems the mixture of *Eobania* phospholipids was resolved into 7 phosphorus positive spots, 4 of which cochromatographed with authentic standards of cardiolipin, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol. Two spots migrating between phosphatidylcholine and phosphatidylethanolamine were ninhydrin positive and gave positive Stillway-Harmon tests [54] for phosphonolipids.

A part of the total lipids (9 mg of lipid-P) was applied to a silicic acid column in chloroform–methanol, 98:2 (v/v) and stepwise sequential elution was performed with the solvents indicated in Table I. Thirteen bulk fractions were collected with the indicated respective bed volumes of solvents (Table I). According to the results of TLC analysis illustrated in Fig. 1 and 2, some of the above fractions were combined as indicated in Table I (last column) and Fig. 2 to obtain seven final fractions, I to VII, for

Table I. Distribution of lipid components of *Eobania vermiculata* obtained by chromatography on silicic acid column. Total sample, containing 9.0 mg total P (290 μmol) placed on column in chloroform–methanol, 98:2 (v/v) and 13 bulk fractions were eluted with indicated solvents. According to TLC analysis (Fig. 1 and 2) bulk fractions were combined as indicated into seven pooled fractions (I to VII) for further analysis (Table II). A, C, and M denote acetone, chloroform and methanol, respectively.

Column fraction No.	Eluting solvent (v/v)	Bed volumes	Total-P recovery [μmol]	[%]	Numbering of pooled fractions
1	CM(98:2)	4	2.24	0.78	I
2	AM(9:1)	2	0.75	0.26	II
3	AM(9:1)	2	1.26	0.44	
4	CM(9:1)	1	18.06	6.29	III
5	CM(9:1)	1	78.45	27.30	IV
6	CM(85:15)	4	3.23	1.12	V
7	CM(85:15)	3	110.32	38.40	
8	CM(4:1)	2	7.91	2.75	
9	CM(4:1)	2	43.10	15.00	
10	CM(3:2)	2	2.71	0.94	VI
11	CM(3:2)	1	11.87	4.13	
12	M	2	1.10	0.38	
13	M	3	6.30	2.19	VII
			287.30		

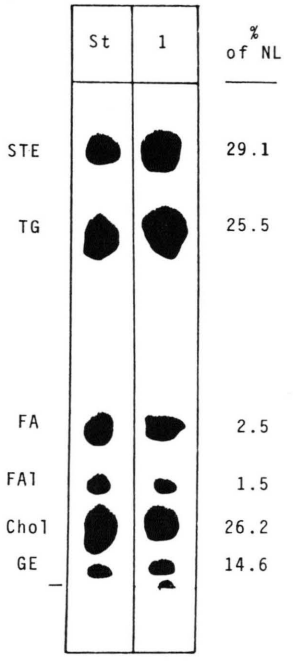

Fig. 1. TLC analysis of fraction 1 eluted from silicic acid column, as indicate in Table I. Developing solvent: petr. ether–ether–acetic acid, 70:30:1 (by vol.). Visualization: spraying with 50% H_2SO_4 and charring.

further analysis and structural study. The percent distribution of lipid-P in these final fractions and their analytical data are depicted in Table II. Results obtained by the differential hydrolysis method of Dawson [59] were found in good agreement (within ± 4%) [59] with the values of direct assays depicted in Table I.

Fraction I contained all the neutral lipids (about 36% w/w of total lipids) accompanied by a negligible amount (0.8%) of lipid-P. Based on their TLC chromatographic behavior before (Fig. 1) and after saponification, along with standards, the neutral lipids were found to consist mainly of sterols, sterol esters and triglycerides. Quantitation of neutral lipid components, was performed by a scanning TLC apparatus. The results are summarized in Fig. 1.

GLC analysis of sterols and sterol esters showed cholesterol as the main components. Glycerylethers and plasmalogens determination gave 5.24 μmol and 0.97 μmol respectively.

Fraction II contained the glycolipids components consisting of cerebrosides accompanied by at least five α-naphthol positive components with chromatographic behavior similar to that of acidic *Eobania* glycolipids reported earlier [36]. The small

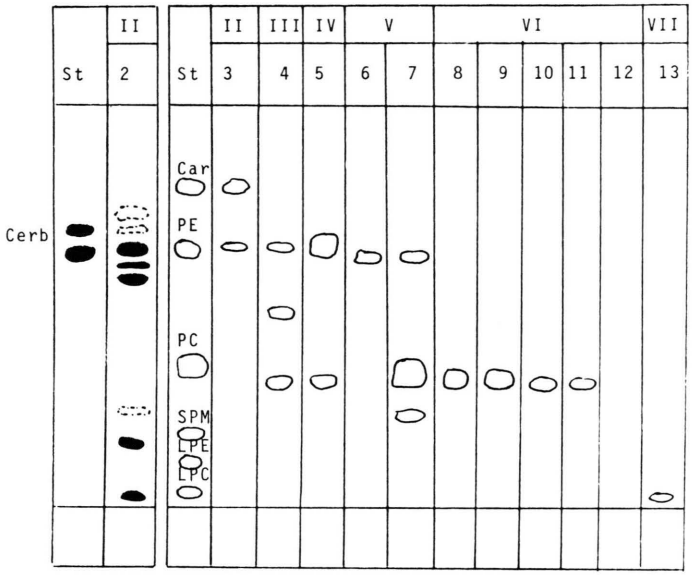

Fig. 2. TLC analysis of bulk fractions eluted from silicic acid column, as indicate in Table I. Developing solvent: chloroform–methanol–water, 65:25:4 (by vol.). Visualization: Dotted lines indicate spots positive to iodine vapors only; black spots were positive to naphthol reagent; white spots were positive to molybdenum blue reagent.

Table II. Analytical data of pooled column fractions. Values are given in total μmol found in each pooled fraction (I to VII) obtained as indicated in Table I (last column). Numbers in brackets indicate the respective values in mol per 100 mol of total-P recovered from column.

Pooled fraction No.	Total-P	Phospho-nate-P	Alkyl glycerol*	Alk-1-enyl glycerol**	Long-chain base	Alkyl ester
I	2.24 (0.78)	0	5.24 (1.82)	0.97 (0.34)	–	174 (60.6)
II	2.01 (0.70)	0	0	0.16 (0.055)	–	5 (1.7)
III	18.06 (6.29)	4.27 (1.49)	0	0.65 (0.23)	1.03 (0.36)	33 (11.5)
IV	78.45 (27.30)	8.02 (2.79)	6.16 (2.14)	14.20 (4.94)	3.70 (7.29)	140 (48.7)
V	113.55 (39.52)	27.50 (9.57)	27.9 (9.71)	4.90 (1.71)	21.60 (7.52)	151 (52.5)
VI	66.69 (23.21)	0	36.54 (12.72)	9.70 (3.38)	0	85 (29.6)
VII	6.30 (2.19)	0	–	–	–	–

* Corresponding to glyceryl ether analogs of the respective phospholipids.
** Corresponding to plasmalogen content of the fraction.

quantity of these components (total hexose 6 μmol) did not permit further analysis of this fraction.

Fractions III to VII were found to contain the phospho and phosphonolipids resolved just as illustrated in Fig. 2, *i.e.* each component (lipid class) was contaminated by small amounts of lipid components of adjacent polarities. Therefore, they were purified by column chromatography or preparative TLC for

structural studies which as described below, confirmed their identity indicated in Fig. 2.

By combining the analytical data of Table II with respective data of the purified fractions (see below and with the results of two dimensional TLC analysis according to Rouser *et al.* [7, 58] the following overall composition was inferred to the phospholipids of *E. vermiculata*. Cardiolipin 2.9%; phosphatidic acid, 1.1%; phosphatidylethanolamine 24.9% (of which 19.8% correspond to the plasmalogen analog and 8.6% to the glyceryl-ether analog); diacylglycero-aminoethylphosphonate, 6.3%; phosphatidylcholine, 49.2% (of which 10.3% correspond to the plasmalogen analog and 45.6% to the glycerylether analog); sphingoethanolamine 1.65%; ceramidyl-aminoethylphosphonate (major sphingophosphonolipid), 7.5% and three minor sphingophosphonolipids 0.01%; phosphatidylinositol, 4.0%; and uncharacterized lipid-P, 2.2% (fraction VII), remaining at the starting point on TLC with all developing systems used (see Fig. 2).

The fatty acid composition of the neutral lipids, phosphatidylethanolamine, phosphatidylcholine, ceramide aminoethylphosphonate and sphingoethanolamine was studied by GLC analysis of their methyl esters. As shown in Table III saturated fatty acids with 16–18 carbon atoms represent 65% of the ceramide animoethylphosphonate.

Structural studies

Major lipids: The main components of fractions IV and VI were phosphatidylethanolamine and phosphatidylcholine respectively. Their identification was based on the hydrolytic cleavage products such as glycerylphosphorylethanolamine, glycerylphos-

Table III. Fatty acid composition (%) of the main lipids of *E. vermiculata*.

Fatty acid	R.R.T.	NL	PE	PC	$CAEP_n$	Sphingoethanol-amine
C < 12		1.30		0.16	2.96	
$C_{12:0}$	0.33			1.04	1.59	2.41
$C_{12:1}$	0.44					2.99
$C_{13:0}$	0.45			0.95		
$C_{14:0}$	0.57	1.50	0.70	3.67		1.26
$C_{14:1}$	0.77	1.11				
$C_{14:2}$	0.86		0.39	2.57		
$C_{15:0}$	0.78	1.06		1.98		8.04
$C_{15:2}$	1.18					
$C_{16:0}$	1.00	9.22	2.21	18.54	54.02	9.30
$C_{16:1}$	1.21	3.94		5.46		
$C_{16:2}$	1.59					10.63
$C_{17:0}$	1.43	2.00	1.10	2.74	2.33	
$C_{17:1}$	1.56			5.23		
$C_{18:0}$	1.91	8.39	16.39	18.65	10.24	10.34
$C_{18:1}$	2.24	19.69	5.01	24.89	9.88	22.75
$C_{18:2}$	2.90	14.57	10.65	3.62	18.98	10.34
$C_{18:3}$	4.00	10.13	6.44			
$C_{19:0}$						
$C_{19:1}$	3.04					
$C_{19:2}$	3.77					
$C_{20:0}$	2.94	2.10	0.54	4.78		21.94
$C_{20:2}$	5.24		12.07			
$C_{21:0}$	3.68			3.41		
$C_{21:1}$	4.29	7.03	1.21	2.32		
$C_{21:2}$	7.14		26.07			
$C_{21:3}$	9.71		2.61			
$C_{22:0}$	6.29	5.20				
$C_{22:1}$	5.71	14.04				
$C_{22:3}$	13.09		8.10			
$C_{23:0}$	8.49		1.95			
$C_{24:0}$	10.86					

R.R.T.: relative retention time. $CAEP_n$: ceramidyl aminoethylphosphonic acid.

phorylcholine, ethanolamine/or choline and P, after mild alkaline hydrolysis or acid hydrolysis of the latter. The analytical results for glycerylethers and plasmalogens depicted in Table II were confirmed by the results differential hydrolysis according to Dawson [59].

The IR spectrum of lyso-phosphatidylcholine obtained by deacylation showed a doublet at 1080 cm^{-1} and 1050 cm^{-1} and a singlet at 964 cm^{-1} for quatternary ammonium attached to phosphate (Fig. 3).

As already mentioned, the phosphatidylethanolamine and phosphatidylcholine fractions contain glycerylether and plasmalogen analogs. By GLC analysis of the isopropylidene derivatives of the glyceryl ether mixture isolated from the phosphatidylcholine fraction it was found that the glycerylether analog of phosphatidylcholine contained 97.5% batyl alcohol and 2.5% of chimyl alcohol.

GLC analysis of the long-chain dimethylacetals derived from ethanolamine plasmalogen indicated that they consisted mainly (55.6%) of hexadecanal (Table IV).

Major sphingophosphonolipid: Fraction V was subjected to mild alkaline hydrolysis (to remove any traces of glycerophospholipids). As shown by TLC analysis, this treatment did not affect the chromatographic behavior of the main component.

Further two-dimensional TLC analysis (Fig. 5) showed that the main component was accompanied by traces another 4 components. A part of these mild alkaline stable lipids, containing 17.1 μmol of lipid-P, was applied to a silicic acid column. Stepwise sequential elution was performed with the solvents indicated in Fig. 4 together with the TLC patterns of the ten bulk fractions collected (Va to Vj). The main lipid components were recovered in fractions V_f and V_j (27.8% and 59.8% of total P, respectively). In fraction V_f over 95% of lipid-P was found to be phosphonate-P. Its infrared spectrum (Fig. 3) showed the typical absorption bands for amide at 1645 cm^{-1} and 1550 cm^{-1} and for phosphonate at 1200 cm^{-1}.

The lipid was subjected to dry acid methanolysis and the hydrolysis products were found to contain long-chain bases, fatty acid methyl esters and total P

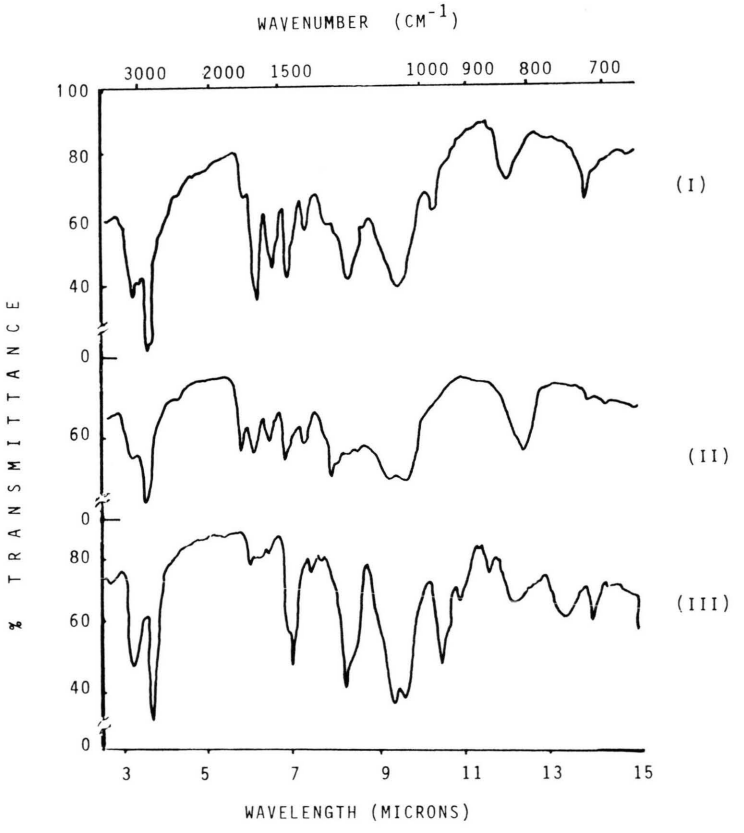

Fig. 3. Infra-red spectra of the major sphingophosphonolipid (I), sphingoethanolamine (II) and lysophosphatidylcholine (III) from *E. vermiculata*.

Table IV. Composition and retention times of long-chain aldehydes of ethanolamine plasmalogen on a 10% EGSS-X stainless steel column.

Component	Retention time [min]	Relative retention time $C_{16:0}$DMA	Aldehyde	[%]
1	1.00	0.57	$C_{14:0}$	1.25
2	1.30	0.74	anteiso-br. $C_{15:0}$	3.57
3	1.76	1.00	$C_{16:0}$	55.61
4	2.24	1.27	anteiso-br. $C_{17:0}$	21.45
5	3.30	1.87	$C_{18:0}$	18.12
Standard palmitic acid methylester		1.25		

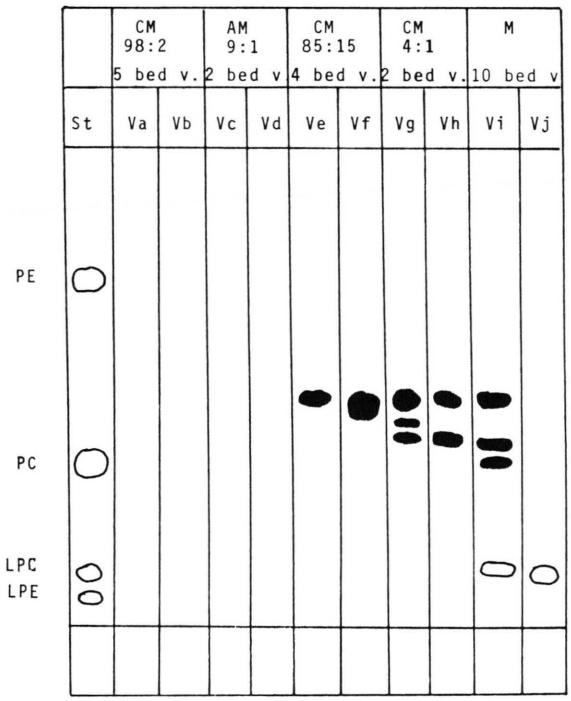

Fig. 4. Illustrative summary of data concerning column chromatographic fractionation of the sphingophosphonolipids of *E. vermiculata*. TLC developed with chloroform–methanol–acetic acid–water, 50:25:6:2 (by vol.). Visualization: All spots were positive to iodine vapors and molybdenum blue reagent. Black spots were positive to Stillway test [54]. Elution solvents and bed volumes are indicated on the top of the chromatoplate. The percent distribution of total-P in fraction Va to Vj was 0.0, 0.58, 0.19, 0.29, 0.06, 27.85, 3.01, 3.71, 4.46 and 59.75 respectively. A, C, and M denote acetone, chloroform and methanol.

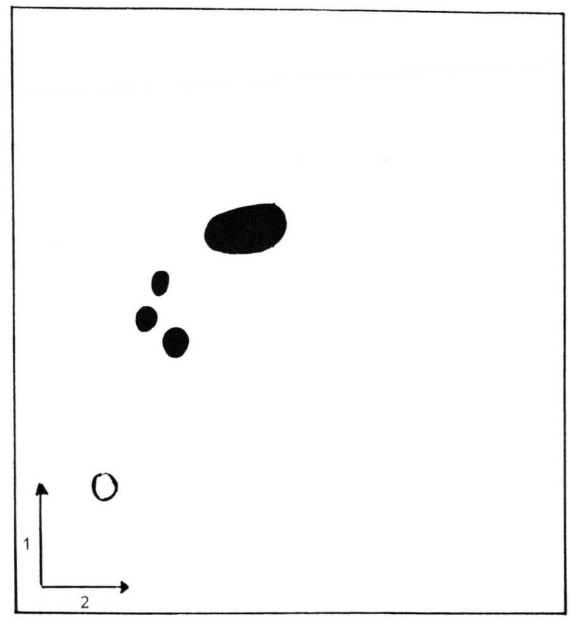

Fig. 5. Two-dimensional TLC of the MAS components of fraction V developed with 1. chloroform–methanol–water, 65:25:4 (by vol.); 2. chloroform–methanol–acetic acid–water, 50:25:6:2 (by vol.). Visualization: see Fig. 4.

in molar ratio. 0.86:1.04:1.00, respectively. Furthermore, the water soluble products of the above treatment were analyzed by ascending paper chromatography and found to contain only one component ninhydrin positive and comigrating with authentic 2-aminoethylphosphonate. Therefore, by combining all the data mentioned above, a structure of ceramide-aminoethylphosphonate was confirmed for this major sphingophosphonolipid component of *E. vermiculata.*

The trimethyl-silyl derivatives of long-chain bases were analyzed by GLC analysis. Six components were detected (Table V) the major of which was identified as sphingosine (41.6%).

Diacyl glycero aminoethylphosphonate: The water-soluble products of mild alkaline hydrolysis of fractions III, IV and V were found to contain 4.27, 8.02 and 5.25 μmol phosphonate-P, respectively. A ninhydrin-positive component, comigrating with authentic aminoethyl phosphonate on ascending paper chromatography was detected in the above water-soluble products after acid hydrolysis [60, 61a].

Minor phosphonolipids: The three components of fractions Vg, Vh and Vi which accompanied the major sphingophosphonolipid (see Fig. 4 and 5), as already mentioned, were stable to mild alkaline hydrolysis and positive to ninhydrin and Stillway test. Their Rf were not affected by mild alkaline treatment. These findings suggest that these components are another three molecular species of the ceramide aminoethyl phosphonate type, which are more polar than the major sphingophosphonolipid of fractions Vf, Vg, Vh, Vi, possibly due to additional hydroxyl groups in their long-chain base and/or fatty acyl moieties. The very small quantities of these components did not allow further examination.

Sphingoethanolamine (ceramide-phosphoryl ethanolamine): Fractions III and IV (Table I, Fig. 1) were subjected to mild alkaline hydrolysis (to remove cardiolipin, phosphatidylethanolamine and any traces of other components). TLC analysis, indicated that only one phosphorus containing and ninhydrin positive component was present. This component from fraction IV was purified by preparative TLC and further two dimensional TLC analysis confirmed that it was the only component. It was not a phosphonolipid since the phosphonate-P assay was negative. The molar ratios of LCB:FA:P was found 1:0.97:1.03. Its infrared spectrum (Fig. 3) showed typical absorption bands for amide at 1640 cm^{-1} and 1550 cm^{-1} but no band at 1200 cm^{-1} due to C-P bond. Also the spectrum in the region from 1100 cm^{-1} to 900 cm^{-1} showed a pattern expected for an ethanolamine containing lipid.

The lipid was subjected to acid methanolysis and the water soluble products of the above treatment were analyzed by ascending paper chromatography. They were found to contain a ninhydrin positive component co-migrating with authentic ethanolamine and another component at the starting point, which was inorganic phosphate. Therefore a structure of ceramide-phosphoryl ethanolamine was confirmed to it by combining all the data mentioned above.

The trimethyl-silyl derivatives of long-chain bases were analyzed by GLC analysis. Six components were detected (Table V) the major of which was identified as dihydrosphingosine (48.6%).

Table V. Composition (%) of sphingosides of ceramide-AEP_n and sphingoethanolamine. RRT denotes relative retention time with reference to sphingosine.

TMSi-sphingosides	RRT	Ceramide-AEP_n	Sphingo-ethanolamine
1	0.83*	3.2	1.2
2	1(30′ min)	41.6	3.6
3	1.14*	–	48.6
4	1.50	3.7	–
5	1.63	–	2.0
6	1.72	17.3	–
7	1.90	–	35.6
8	1.97	9.9	–
9	2.03	–	8.9
10	2.97	24.3	–

* RRT's of components 1 and 3 correspond to those of 3-O-methyl sphingosine and dihydrosphingosine respectively [42].

Discussion

The low percentage of the total lipid content (0.8% w/w) determined in the fresh tissue of the land snail *E. vermiculata* is comparable to that of other representatives of the order of Stylommatophora such as *Helix aspersa* and *Helix lucorum* [62]. The phospholipids comprise 61.6% of the total lipids, while the 36.4% of them are neutral lipids. Cholesterol, free or esterified and triglycerides, make up most of the neutral lipids (55.3% and 25.5% respectively) (Fig. 1), composition generaly similar to that previously found for other gastropods [10, 18].

A significant amount of free glycerylethers (14.6% of the neutral lipids) were found for the first time in a mollusc. Diacylglyceryl ether derivatives are previously found in the "triglyceride" fraction of the neutral lipids in other marine molluscs [19].

The major phospholipid fraction in *E. vermiculata* are choline and ethanolamine phosphoglycerides, which amount to 49.2% and 24.9% of the phospholipids respectively.

Ceramide aminoethylphosphonate is the third phospholipid in amount 7.5%. Diglyceryl aminoethylphosphonate 6.3% followed by phosphatidylinositol, cardiolipin, sphingoethanolamine and phosphatidic acid as minor component.

The above pattern of phospholipid composition of *E. vermiculata* is comparable to that given for other snails, such as the land snail *Cepaea nemoralis* [26], the fresh water snail *Lymnaea stagnalis* [8] and the marine snail *Aplysia kurodai* [10], as well as the slugs *Arion ater* and *Ariolimax columbianus* [18].

However, sphingoethanolamine (ceramide aminoethyl phosphate) is for the first time found, determined, and structurally studied in a land gastropod. Up today, sphingoethanolamine has been detected only in a limited number of aquatic snails of the order Mesogastropoda [63, 64] but it was not structurally studied. This substance was not present in the fresh water snail *Lymnaea stagnalis* [8]. The ceramide residue of sphingoethanolamine was found to be dihydrosphingosine bound with saturated or unsaturated C_{18} fatty acids and $C_{20:0}$ fatty acid.

The results of the present investigation reveal that a significant amount of phosphonolipids are present in *E. vermiculata*. The structure of the main component, which was identified as ceramide aminoethyl phosphonate was satisfactorily investigated. Its ceramide moiety was found to be mainly sphingosine bound to the extent of 71% with saturated fatty acids characterized by high percentages of palmitic acid (54%). This conforms to the ceramide aminoethyl phosphonate fatty acid composition from *Cepaea nemoralis* [26] as well as from other invertebrates [61b, 69, 70]. It is noteworthy that two of the major fatty acid components of ceramide aminoethylphosphonate of *E. vermiculata* are the unsaturated fatty acids $C_{18:2}$ (19%) and $C_{18:1}$ (9.9%).

It is of interest that a considerable amount of diglyceride-aminoethyl phosphonate (6.3 of PL) was detected mixed with ethanolamine phosphoglycerides. A lower amount of this substance (1% of the phospholipids) was found also in the water snail *Lymnaea stagnalis* [8]. It is worthy to note that the phosphonolipids in molluscs and marine invertebrates are reported to be of the ceramide-aminoethylphosphonate type [1–11] while in protozoa they are mostly of the phosphorylglycerol type [65–67].

The different phosphonolipid distribution in protozoa and molluscs would suggest certain differences in the pathway of biosynthesis of the aminoethylphosphonate portion of the phosphonolipids or in the distribution of the enzymes concerned with the formation of the diglyceryl-aminoethylphosphonate or ceramide-aminoethylphosphonate.

Another three unknown sphingophosphonolipids were detected which were not further identified because of their insufficient quantities (~0.01%). However they were considered to be of the ceramide-aminoethylphosphonate type with different structures of ceramide moiety. It has been established [61b] 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.

The presence of sphingophosphonolipids and sphingoethanolamine in *E. vermiculata* and the apparent absence of sphingomyelin may be viewed in the light of a compensatory correlation of their amount [10, 68–70].

Concerning the glyceryl ether type phospholipids in *E. vermiculata*, their content (26.15% of the total phospholipids), is higher than the respective percentage found in the slugs *Arion ater* and *Ariolimax columbianus* [18] and in the water snail *Lymnaea stagnalis* [8].

The quantitative distribution of the glyceryl ethers and plasmalogens among the different phospholipid classes showed that plasmalogens are concentrated in ethanolamine phospholipids (17.4% of it) while alkyl-glycerylethers are particularly high in the choline phosphoglycerides (54.8% of it). The glycerylether distribution in the phospholipids of *E. vermiculata* appears to be in conformity with that of the water snail *Lymnaea stagnalis* [8], with difference in that the amount of ethanolamine plasmalogens is lower (3.4% of ethanolamine phosphoglycerides) in the latter.

Among the vinylether side chains of ethanolamine plasmalogens, the $C_{16:0}$ chain (55.6%) predominates in contrast to that of the marine mollusca *Octopus*

dofleini and *Protothaca staminea* where the main side chains were with 18 carbon atoms [19].

The main glycerylether bound to choline phosphate in *E. vermiculata* was found to be batyl alcohol (97.5%), while the rest was chimyl alcohol. These data do not resemble the respective features of the slug *Ariolimax columbianus* where the main glycerylether (94%) was found to be chimyl alcohol [18]. The glycerylether composition of choline phosphoglycerides in the marine snail *T. lamellosca* is more complex [19].

The fatty acid composition of all the lipid fractions of *E. vermiculata* is characterized by high percentages of polyunsaturated fatty acids. The highest amount of unsaturated fatty acid was found in the ethanolamine phosphoglycerides and the lowest one in the ceramide aminoethylphosphonate. Similar results were obtained for *C. nemoralis* [26]. A significant amount (70.5%) of unsaturated fatty acids is concentrated in neutral lipids. These data would be comparable with the fact that the triglycerides of muscle and liver of the snail *Helix pomatia* were higher in unsaturated fatty acids (70.2%) than those of mammalian fat (55%) and were similar in this respect to fish oil triglycerides [71].

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