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Studies on Sphingolipids of Barnea (Umitakea) dilatata japonica. I

Cerebroside and Ceramide Aminoethylphosphonate

Yoshihiro MISHIMA* and Akira HAYASHI**

1. The complex lipids were extracted from the muscle of a marine bivalve, *Barnea (Umitakea) dilatata japonica* with chloroform-methanol (2:1, vol/vol).

2. The lipids were treated mildly with diluted alkali and the alkaline stable lipids were fractionated on silicic acid column chromatography.

3. Sphingolipids were isolated and characterized as ceramide monohexoside (CMH) and ceramide 2-aminoethylphosphonate (CAEP). Sphingomyelin and ceramide 2-N-methylaminoethylphosphonate were not found in the alkaline stable lipids fraction.

4. CMH was composed of galactosyl ceramide which contained octadecasphingadienine as the main long chain bases and hexadecanoic acid as the main fatty acids.

5. Ceramide moiety of CAEP was mainly composed of hexadecanoic acid and octadecasphingadienine, and only 2-aminoethylphosphonic acid was detected in the water-soluble components of CAEP.

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1. INTRODUCTION

Aquatic molluscs contain many varieties of sphingolipids, among which globoside mucolipids of complex structure constitute the majority of the glycolipids[1]. On the other hand, ceramide monohexoside (CMH) which has a simpler structure is known to be present in Corbicula sandai REINHARDT[2], Hyriopsis (Limnoscapha) schlegelii (v. MARTENS)[3], Chlorostoma argyrostoma turbinatum (A. ADAMS)[4], etc..

There have been a relatively large number of studies on sphingolipids, and it is particularly interesting to note that phosphonolipids with C-P bonds were found in large concentrations. For example, it is known that ceramide aminoethylphosphonate (CAEP) is present in bivalves such as Corbicula sandai[5], Hyriopsis (Limnoscapha) schlegelii[3,6], Crassostrea gigas (THUNBERG)[7], Tapes (Amygdala) philippinarum (A. ADAMS et REEVE)[8], and in several species of spiral shell. It has also been reported[9] that ceramide 2-N-methylaminoethyl phosphonate(CMAEP), an N-methyl derivative of CAEP, is present in Turbo cornutus, etc.. It appears that the distribution of sphingophosphonolipids varies according to the variety of aquatic mollusc . However, the correlation between the lipid distribution and the biological classification of molluscs has not yet been established.

The authors have been studying marine molluscs in order to clarify the above matter and to investigate the relationship between the habitat of the molluscs and the structure of sphingolipids, which are considered to be a component of the biomembrane. Among marine molluscs there have not been many studies on the sphingolipids of bivalves and no reports have been published on the lipids of Barnea (Umitakea) dilatata japonica. Barnea dilatata japonica belongs to the class Bivalvia, family Adapedonta and lives in muddy and rocky seashores on southern Honshu and further south. The shell of this bivalve is about 7 cm long and is 93
thin. It has a suction tube three times as long as the shell, and it typically lives by feeding itself via this tube. The Barnea dilatata japonica in the Ariake Sea are particularly famous for their succulent meat which includes the suction tube. This meat is packed in cans or dried for food.

This report concerns a study of the sphingolipids in this rich shellfish meat, including the suction tube. It was found that Barnea dilatata japonica meat contained very small amounts (about 0.2%) of CMH, and CAEP which was the main sphingolipid component (about 20%) was present in high concentrations. The structures of CMH and CAEP were also clarified, and are reported below.

2. METHOD OF EXPERIMENT

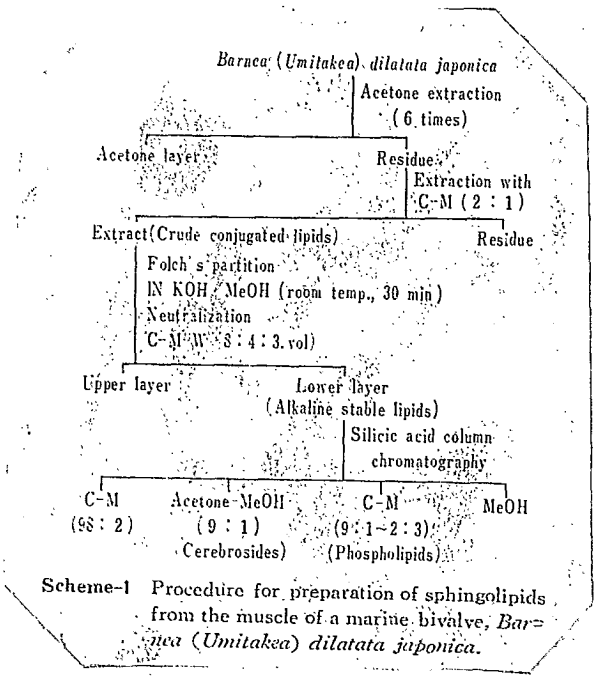
2.1 Extraction of Test Material and Complex Lipids

Purchased fresh Barnea dilatata japonica from the Ariake Sea were used. The shells and viscera were removed, and the meat was dried with a cloth to remove excess moisture. 4.6 kg of meat prepared in this way was cut thinly, and three times its volume of acetone was added. The acetone-soluble lipids and water were then removed, and this acetone extraction process was repeated until no further coloration of the acetone was observed. After filtration, chloroform methanol (C-M) (2:1) extraction was carried out six times on the remaining shellfish meat (601 grams, dry weight). The extraction was conducted by agitation with a stream of nitrogen. After removing the solvent from the extract, 23.6 g (0.5% of the fresh Barnea dilatata japonica meat) of crude complex lipids were obtained.

Folch's partition[10] was performed on the lipids as shown in Scheme 1, and after weak alkali hydrolysis[11] the Folch's partition was repeated. Regarding the chloroform layer, it was washed with acetone and 8.5 g (the P content was 2.6%) of crude sphingolipids was obtained.

2.2 Thin Layer Chromatography (TLC)

A plate with silicic acid (Silica gel G. Merck) of thickness 0.25 mm was prepared for use, and C-M-W (32:8:1),



Scheme-1 Procedure for preparation of sphingolipids from the muscle of a marine bivalve, *Barnea (Umitakea) dilatata japonica*.

C-M-W (65:25:4) and C-M-2N ammonium hydroxide (40:10:1) were used as developers for the glycolipids, phospholipids and long chain bases (LCB) respectively. Ninhydrin reagent, Dittmer reagent[12] and α-naphthol reagent[13] were used as detecting reagents.

2.3 Silicic Acid Column Chromatography

Silicic acid (Wakogel C-300) was activated at 120°C for 12 hours before being used in a quantity of one gram of silicic acid per ten milligrams of lipid. As elution solvents, C-M (98:2), acetone-methanol (9:1), C-M (9:1), (4:1), (3:2), (2:3) and methanol were used in sequence.

For the second silicic acid column chromatography, C-M (95:5) was used in the glycolipid fractionation instead of acetone-methanol, but this solvent was omitted in the phospholipid fractionation. TLC (refer to Section 2.2) was conducted on each eluate and the presence of CMH and CAEP was investigated.

2.4 Quantitative Analysis

Glycose was determined by the phenol sulfuric acid method[14] and phosphorus by the King method[15]. As for the fatty acids, methyl esterification was carried out according to the method in 2.5. Then from a colorimetric determination of the ester bonds using the method of Snyder and Stephens[16] and from gas chromatography (GC) results, the mean carbon numbers were obtained, and calculations were performed for fatty acids of these mean carbon numbers. The long chain bonds were determined by the method of Lauter et al.[17].

2.5 Hydrolysis by Hydrochloric Acid - Methanol

The methanolysis of the sphingolipids was performed using the improved method of Gaver and Sweeley[18]. Several milligrams of the lipids were methanolyzed at 78°C for 15 hours in 1N hydrochloric acid - methanol hydrate (water 10M). The fatty acid methyl esters were then extracted with petroleum ether (bp 30 to 60°C), and their composition was examined by GC.

The methanol layer was condensed and adjusted to pH 12 with 2N sodium hydroxide solution, then the LCB were extracted with diethyl ether. The extract was washed with water and part of it was dried for GC analysis and GC - mass spectrometry (GC-MS) as a trimethylsilyl (TMS) derivative according to the method of Yamakawa et al.[19]. The glycose was converted to a trimethylsilyl derivative for GC analysis.[19]

2.6 Hydrolysis by Hydrochloric Acid

Several milligrams of the sphingolipids were hydrolyzed at 110°C for 30 hours in 6N hydrochloric acid. The fatty acids were removed with petroleum ether. After Folch's partition, the water layer was fully dried in a steam bath. The acids remaining were removed to a reduced pressure desiccator which contained sodium hydroxide. To the remaining material were added 60 μl of acetonitrile, 80 μl of bistrimethyl silylacetoamido and 16 μl of trimethyl chlorosilane, and these were kept at 60°C for three hours to make a TMS derivative which was then analyzed by GC and GC-MS.

2.7 Gas Chromatography (GC)

Gas chromatography was carried out under the following conditions using a Shimadzu GC-5A (equipped with hydrogen flame ionization detector):

TMS-LCB: 3% SE-30 (Gas Chrom Q), Ø 3mm x 2m, column temperature 230°C, nitrogen gas 40 ml/min.

TMS-aminoethylphosphonic acid: 2% OV-1 (Chromosorb W), Ø 3mm x 2m, column temperature 160°C, nitrogen gas 40 ml/min.

TMS-glycose: 5% Neopentylglycol succinate polyester (Chromosorb W), Ø 3mm x 2m, column temperature 130°C, nitrogen gas 40 ml/min.

Fatty acid methyl esters: (a)20% Diethyleneglycol succinate polyester (Chromosorb W), Ø 3mm x 2m, column temperature 170°C, nitrogen gas 40 ml/min, (b)4% SE-30 (Chromosorb W), Ø 3mm x 2m, column temperature 195°C, nitrogen gas 40 ml/min.

2.8 Gas Chromatography - Mass Spectrometry (GC-MS)

A Shimadzu-LKB 9000 was used for GC-MS. For TMS-LCB a 3% SE-30 (Gas Chrom Q, 80 - 100 mesh) glass column (Ø 3mm x 2m) was used, and measurements were carried out with a column temperature of 220°C, separator temperature 250°C, ion source 270°C, electron energy 70eV, accelerating voltage 3.5kV, ionization current 60 μA. TMS-AEP analysis was carried out with a 2% OV-1 (Chromosorb W, 80 - 100 mesh, Ø 3mm x 1m) column, the column temperature was raised from 90° to 140°C at 3°C/min, separator temperature 210°C, ion source 230°C, and with the other conditions the same as

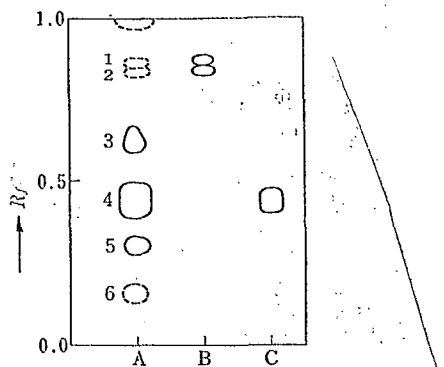
for TMS-LCB.

3. RESULTS

3.1 TLC of the Complex Lipids

The TLC (Fig. 1) of the crude complex lipids (see Scheme-1) extracted from the muscle of Barnea dilatata japonica using the method in Section 2.1 showed them to contain 2 glycolipids (1 and 2) and 4 positive ninhydrin phospholipids (3 to 6). Since the R_f values of the 2 glycolipids and phospholipids 4 to 6 did not change even under a weak hydrolysis treatment by alkalis and the R_f values of the glycolipids were well matched with those of CMH obtained from ox brains, they were named CMH-I and CMH-II respectively. Phospholipid 4 was well matched with the R_f value of CAEP obtained from Turbo cornutus. Since phospholipid 3 was hydrolyzed by alkaline treatment and changed to a positive ninhydrin phospholipid with a low R_f value, it proved to be a phospholipid with ester bonds.

The crude complex lipids were fractionated by the method shown in Scheme 1. Using silicic acid column chromatography, CMH-I and CMH-II were eluted with acetone-methanol (9:1), and CAEP was eluted in the first half of C-M (4:1) and phospholipids in the latter half. Silicic acid column



Solvent : $CHCl_3$-MeOH-H_2O (65:25:4, vol)
A : Complex lipids of the muscle of *Barnea dilatata japonica*
B : CMH of ox brain
C : CAEP of *Turbo cornutus*

Fig.-1 Thin layer chromatogram of the complex lipids obtained from a marine bivalve, *Barnea (Umitakea) dilatata japonica*. Spots were detected with α-naphthol, ninhydrin, and Dittmer's reagent.

chromatography was conducted repeatedly on the CMH and CAEP fractions and the CMH-I and CAEP were each isolated.

3.2 Analysis of the CMH-I Fraction

3.2.1 IR Spectrum

The CMH-I fraction IR spectrum (Fig. 2) exhibited absorption spectrum features unique to sphingoglycolipids, such as strong hydroxyl group absorption at 3400 cm^{-1} and 1080 cm^{-1}, amido absorption at 1640 cm^{-1} and 1545 cm^{-1}, and trans double bond absorption at 970 cm^{-1}. The spectrum was also well matched with the absorption pattern of CMH.

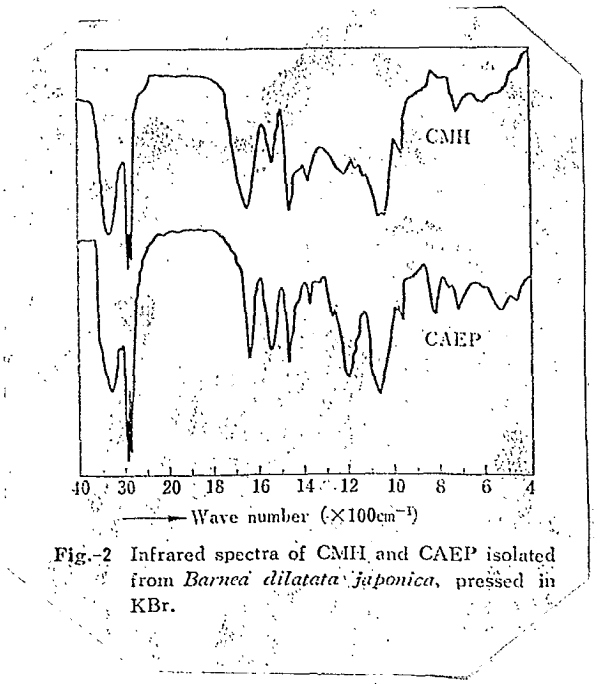
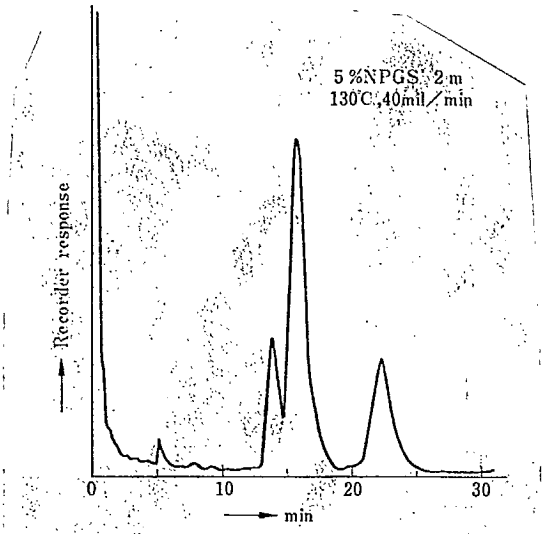


Fig.-2 Infrared spectra of CMH and CAEP isolated from *Barnea dilatata japonica*, pressed in KBr.

3.2.2 Composition of the Constituents of CMH 95

Through the results of the glycose and LCB determinations on the CMH-I fraction, the mole ratio was 1.0 : 0.9. From this and the IR spectrum results it was ascertained that this glycolipid was CMH. It was not possible to carry out the fatty acid determination due to lack of test material.

After the methanolysis of about one milligram of CMH-I according to the method of Section 2.5, fatty acid esters, LCB and glycose were extracted. The gas chromatography of TMS-glycose is shown in Fig. 3. Since the retention time and the peak pattern were well matched with those of standard material and no other peaks were observed, it was learnt that the constituent glycose is solely galactose.



Conditions of gas chromatography: a 2 m glass column of 5% neopentylglycol succinate on Chromosorb W (80~100 mesh), at 130℃.

Fig. 3 Gas chromatogram of trimethylsilylyl ether derivative of sugar obtained from CMH.

The composition of the LCB was determined by GC and GC-MS with the results shown in Table 1. In the mass spectrum of the main components (Fig. 4), m/e 132 was observed as the base ion, and M-15 was observed at m/e 426, M-103 at m/e 338, and M-132 at m/e 309. Therefore, this LCB was identified as octadecasphingadienine ($C_{18:2}$).

The results of GC analysis of the fatty acid methyl are shown in Table 2. The constituent fatty acids were mostly saturated acids, the main component being palmitic acid (92.7%). Through the results of the GC-MS analysis, it was found that 2 hydroxyhexadecanoic acid was contained in the $C_{17:0}$ peak (1.7%), and this was thought to be due to the

mixing of the constituent fatty acids of CMH-II (see Fig. 1) with low R_f values.

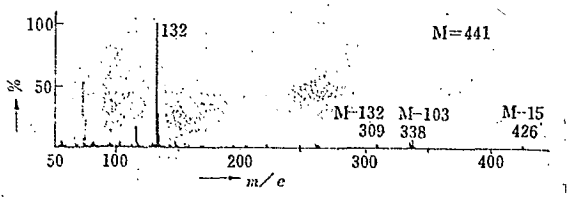
Table-1 Long chain base composition of CMH obtained from *Barnea dilatata japonica*. Trimethylsilyl long chain bases were analyzed by gas chromatography at the following conditions: a 2 m glass column of 3% SE-30 on Gas chrom Q (80~100 mesh), at 230℃. Abbreviations: m : n, m and n shows number of carbons and number of double bonds, respectively.

LCB	%
16 : 1+X_1	20.7
18 : 2	31.4
18 : 1	11.5
19 : 2 ?	17.1
19 : 1	12.4
X_2	6.9

X_1, X_2 : unidentified

Table-2 Fatty acid composition of CMH obtained from *Barnea dilatata japonica*. Condition for gas chromatography: (a) a 2 m glass column of 20% diethylene glycol succinate polyester, at 170℃, (b) a 2 m glass column of 4% SE-30, at 195℃. Abbreviations: Tr., trace; br, branched; h, hydroxy.

Fatty acid	%
14 : 0	Tr.
br 15 : 0	Tr.
15 : 0	Tr.
16 : 0	92.7
br 17 : 0	0.8
17 : 0	1.7
h 16 : 0	
18 : 1	0.6
18 : 0	1.2
19 : 0	1.1
br 20 : 0	Tr.
20 : 0	0.6
br 22 : 0	Tr.
22 : 1	0.6
br 24 : 0	0.7
24 : 0	Tr.



The spectrum was recorded on an LKB-9000 instrument under the following conditions: an electron energy of 70 eV, an accelarating voltage of 3.5 kV, a trap current of 60 μA, an ion source at 270℃, a separator at 250℃, and a 3% SE-30 column (2 m) at 220℃.

Fig.-4 Mass spectrum of trimethylsilyl ether derivative of long chain base obtained from CMH.

3.3 Analysis of the CAEP Fraction

3.3.1 IR Spectrum

The phosphorus, LCB and fatty acid molar ratio of 96
the CAEP fraction was 0.8 : 1.0 : 1.0. The IR spectrum of this fraction is shown in Fig. 2. The absorption around 1200 cm^{-1} indicated a C-P bond and this spectrum was well matched with that of CAEP. Although CMAEP exhibits a similar IR spectrum, the CAEP fraction showed a strong positive ninhydrin reaction in TLC and the water-soluble component was 2-aminoethylphosphonic acid (see Section 3.3.3). Therefore, this IR spectrum was concluded to be that of CAEP.

3.3.2 LCB and Fatty Acid Composition

The hydrolyzate obtained through methanolysis of the CAEP fraction using the method described in Section 2.5 was studied. In the TLC of the LCB, the R_f values were well

matched with those of sphingosine obtained from ox brain. From the results of the analysis of the TMS-LCB by GC and GC-MS (Table 3), the main component of the LCB was found to be $C_{18:2}$ (62.0%), and a large amount of what was assumed to be $C_{19:2}$ (14.3%) was also present.

With regard to the fatty acid composition of the CAEP, it was found through the results (Table 4) of GC analysis under the conditions of Section 2.7 that only non-hydroxyacids were present, their main component being palmitic acid (89%).

Table-3 Long chain base composition of CAEP obtained from *Barnea dilatata japonica*. Conditions for analysis were the same as for **Table-1**.

LCB	%
16 : 1	5.1
18 : 2	62.0
18 : 1	13.5
19 : 2 ?	14.3
19 : 1	5.0

Table-4 Fatty acid composition of CAEP obtained from *Barnea dilatata japonica*. Conditions of gas chromatography were the same as for **Table-2**. For abbreviations, see **Table-1** and **Table-2**.

Fatty acid	%
14 : 0	Tr.
16 : 0	89.5
br 17 : 0	6.3
17 : 0	2.2
18 : 0	1.9

3.3.3 Identification of 2-aminoethyl phosphonic acid

The fatty acids were removed with petroleum ether from the CAEP hydrolyzate produced by 6N hydrochloric acid. The water soluble component obtained using the method of Section 2.6 was analyzed by GC and GC-MS as a TMS derivative, and only peaks well matched with the retention time of 2-aminoethyl phosphonic acid were observed. As a result of GC-MS analysis of these peaks (Fig. 5), M-15 was observed as base ions at m/e 398, and fragment ions unique to phosphonic acid were observed at m/e 225 and m/e 174. Since the pattern of this spectrum was well matched with that of a standard product, it was ascertained that the water-soluble component of this phospholipid is 2-aminoethyl phosphonic acid and that this lipid is ceramide 2-aminoethyl phosphonate.

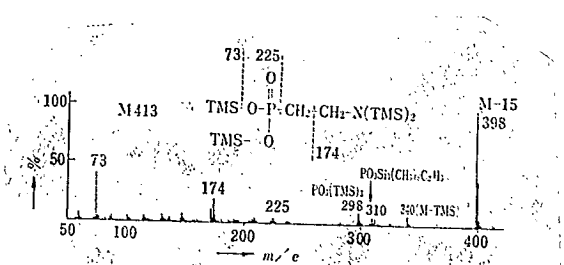


Fig.-5 Mass spectrum of tetra-trimethylsilyl derivative of the watersoluble component obtained from CAEP. Conditions of mass spectrometry were the same as for Fig.-4 except for the following: an ion source at 230°C, a separator at 210°C, a 1 m column of 2% OV-1 on Chromosorb W (80~100 mesh) at programmed temperature from 90 to 140°C (3°C/min).

4. DISCUSSION

It was observed that in the sphingolipids of the muscle of Barnea dilatata japonica, CMH was present as the main glycolipid component and CAEP was present as a phospholipid. Two kinds of CMH (CMH-I and CMH-II) were present and from the results of analysis in this study it was clarified that CMH-I was galactosyl ceramide composed of $C_{18:2}$ and $C_{16:1}$ LCB and palmitic acid. The fatty acid composition and LCB of CMH-II were different from those of CMH-I, and CMH-II was thought to contain hydroxyacids. However, due to the shortage of sample material further analysis could not be carried out in this study.

Regarding the CAEP, the ceramide part was composed mainly of the LCB $C_{18:2}$ and palmitic acid. It was notable that a high concentration (20%) of CAEP was present in the sphingolipids of Barnea dilatata japonica muscle. With regard to the LCB composition of the sphingophosphonolipids of Barnea dilatata japonica, a large content (62.0%) of $C_{18:2}$ was present as compared with other marine bivalves, for instance a 20 to 50% $C_{18:2}$ content in Crassostrea gigas (THUNBERG),[20] and among roll shells Turbo cornutus[9] has a 12 to 18% content, and if diene type LCB are included the Crassostrea gigas content is 20 to 60% and the Turbo cornutus content 20 to 43%. A large content of diene type LCB appears

to be a characteristic of sphingolipids in marine molluscs. Moreover, in the GC of the CMH-I and CAEP of Barnea dilatata japonica, a peak corresponding to the retention time of $C_{19:2}$ LCB was observed, and since the MS base ion was m/e 132, it was definitely a LCB. However, it was not possible to identify $C_{19:2}$ from the MS. There was a large quantity of this component and so its structure and the location of the main component $C_{18:2}$ double bond are presently being studied. 97

Regarding the fatty acid composition, palmitic acid was the main component in both the CMH-I and CAEP. The fatty acid compositions in both of the above were similar to those of the sphingolipids in other bivalves. CMAEP, which is a N-methyl derivative of CAEP, was not detected in Barnea dilatata japonica. The phospholipid 3 (see Fig. 1) of Barnea dilatata japonica can be hydrolyzed by alkalis even under moderate conditions, and in TLC it becomes a phospholipid with a ninhydrin positive R_f value close to that of CAEP. This lipid is being studied at present.

While there are a considerable number of reports on the sphingolipids of marine animals, there were no studies on the class Bivalvia, family Adapedonta prior to this paper. That the presence of CMH and CAEP has been established and their components determined provides suggestions not only in the field of comparative biochemical studies but also

for the biosynthesis route of the sphingolipids of molluscs.

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