Distribution of Ceramide 2-Aminoethylphosphonate in Nature and Its Quantitative Correlation to Sphingomyelin

By

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Recently a large quantity of ceramide 2-aminoethylphosphonate (CAEP) was prepared in our laboratory from the fresh-water bivalve, *Corbicula sandai*, purified through a silicic acid column, and its properties and analytical data were obtained.[1] More recently, our distribution study of CAEP shows that CAEP is widely present in aquatic invertebrates.[2]

These finding led us to observe whether there was a quantitative correlation between contents of CAEP and sphingomyelin in nature

This paper describes the results so far obtained, although the present study is not yet completed.

EXPERIMENTEL

Materials——Whole tissues from locust, earthworm, ascarid, shrimp, crawfish and shellfish, whole viscera from red-snapper, eel, crucian, carp, squid and octopus, and the rat liver were used. Human and bovine erythrocytes gifted from Prof. Yamakawa of Tokyo University and the gonad of sea urchin and starfish from Dr. Nagai of Tokyo University were used together.

Extraction of Lipids——Each of about 500 g. of the materials was homogenized with acetone in a Waring blender and filtered. The residue was treated with 100 ml. of acetone, and filtered again. The residue obtained was extracted with 200 ml. of chloroform-methanol (2:1, v/v) at room temperature, and this operation was repeated three times. These three extracts were combined, evaporated to dryness *in vacuo*, and the solid obtained was fractionated with acetone. The acetone insoluble fraction was dissolved in 5 ml. of chloroform-methanol (1 : 1, v/v) and thiss olution was once washed with water according to the method of Kimura and Taketomi.[3] The lower lipophilic phase was filtered, and the filtrate was evaporated to dryness.

Preparation of Sphingolipid Fraction——Since CAEP and sphingomyelin are alkali-stable lipids, mild alkaline hydrolysis of the above lipid extract was performed to remove glycerophospholipids by the method of Schmidt.[4] The alkali-stable lipid fraction obtained was throughly dried over P_2O_5 in a vacuum desiccator, and then was weighed. This dried material was tentatively designated as sphingolipid fraction.

Thin-layer Chromatography——A basic type of Silica Gel G plate was used, and prepared by making a slurry of 30 g. of Silica Gel G with 65 ml. of 0.01 M aqueous solution of sodium carbonate. The best separation was achieved with the solvent mixture of chlorofom-methanol-acetic acid-water (100 : 20 : 12 : 5 , v/v). The spots

were visualized with three kinds of reagents ; 0.2% 2,'7'-dichlorofluorescein in 50%aqueous ethanol, 0.2% ninhydrin in butanol and the Dittmer's reagent, respectively.

Total Phosphorus——Phosphorus analyses were performed by the King's method.[5]

Phosphonate Phosphorus——The sphingolipid fraction was heated at 100°C with 6 N HCl for 40 hours. After this drastic hydrolysis, the resulting inorganic phosphorus was estimated by the same procedure as used for total phosphorus, except for digestion with perchloric acid. Then the amount of the phosphonate phosphorus was figured out from the difference between total phosphorus above described and present phosphorus, and percentage of CAEP was obtained as follows ; (amounts of phosphonate phosphorus/ total phosphorus) × 100.

Quantitative Analysis of Sphingomyelin —— Ten milligrams of sphingolipid fraction was used for analysis of choline for the purpose of measuring sphingomyelin. The material was heated under reflux with saturated aqueous solution of barium hydroxide for 3 hours. Then, the choline content of hydrolysate was estimated as Reinecke's salt by the Glick's method.[6] The percentage of sphingomyelin in the sphingolipid fractions was figured out as follows ; (amount of choline × 31/121 × 100)/(total amount of phosphorus).

RESULTS AND DISCUSSION

Observation of CAEP and Sphingomyelin by Thin-layer Chromatography——It was previously demonstrated[1] that both CAEP and sphingomyelin are "alkali-stable" lipids. Therefore, the original lipid extracts were subjected to the mild alkaline hydrolysis in order to remove glycerides. The yields of the alkali-stable fractions ranged from 70 to 150 mg. as examined by thin-layer chromatography. Fig. 1 shows thin-layer chromatogram of the phosphosphingolipids of five species of animals as typical examples. As known from the figure, the thin-layer chromatographic examination revealed that CAEP was present in the pond-snail, the corbicula and the locust, though not present in human erythrocytes and the ascarid. On the other hand, sphingomyelin which

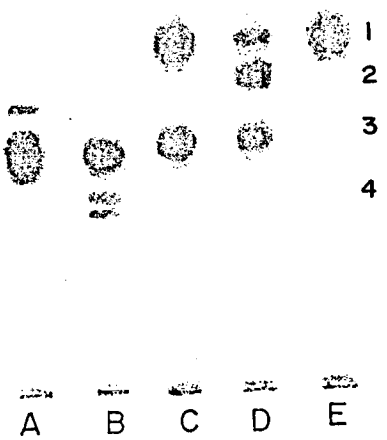


Fig. 1. Thin-layer chromatogram of the "alkali-stable" lipid fractions

The chromatogram was run on the basic plate using a solvent system of chloroform-methanol-acetic acid-water 100 : 20 : 12 : 5 (by volume), and then visualized by spraying with Dittmer's reagent.

A ; from human erythrocytes
B ; from ascarid, *Ascaris lumbriocodes*
C ; from locust, *Oxya vicina*
D ; from pond-snail, *Heterogen longispira*
E ; from corbicula, *Corbicula sandai*
1 ; CAEP
2 ; sphingoethanolamine
3 ; sphingomyelin
4 ; unidentified lipids

is so widely encountered in animal tissues was not seen in the corbicula, and the pond-snail and the locust seem to contain the above two sphingolipids. Thus, by

MAMMALIA

*Homo sapiens** (Human)

*Bos taurus** (Bovine)

*Rattus norvegicus** (*Rat*)

OSTICHYES

Anaguilla japonica (Eel)

INSECTA

Oxya vicina (Locust)

CRUSTACEA

*Cambarus clarkii** (Crawfish)

*Leander paucidens** (Shrimp)

CEPHALOPODA

Polypus vulgaris (Octopus)

BIVALVIA

Pinctada martensii (Pearl oyster)

Corbicula sandai (Corbicula)

Anodonta lauta

GASTROPODA

Cellana eucosmia

Haliotis gurneri (Abalone)

Monodonta labio

*Batillaria multiformis***

*Heterogen longispira*** (Pond-snail)

*Semisulcospira bensoni***

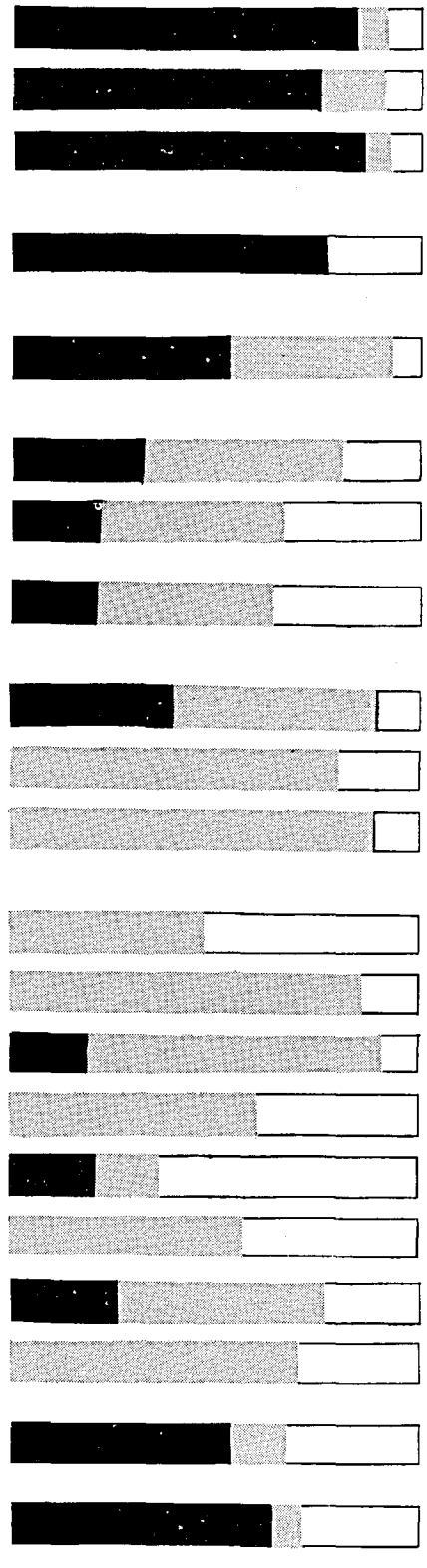
Purpura bronni

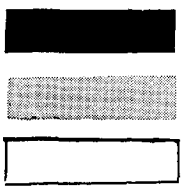
Pugilina ternatana

ANNELIDA

*Pheretinra communissima** (Earthworm)

*Ascaris lumbiocodes** (Ascarid)





; Sphingomyelin

; CAEP

; Other Phosphosphingolipids

* contains phosphonolipids other than CAEP.

** contains sphingoethanolamine in other phosphosphingolipids.

Parenthesized words show the popular name of animals.

Fig. 2. Relative Amounts of Phosphosphingolipids in Animals

using this technique which can separate CAEP and sphingomyelin, the distribution of these phosphosphingolipids in the other animals was investigated, and the results of thin-layer chromatography agreed favorably with those obtained from analytical data which will be described below.

Compensatory Correlation of Amounts between CAEP and Sphingomyelin——If all 2-aminoethylphosphonic acid (figured out by differential phosphorus amounts mentioned above) is considered to be present as CAEP, the appearance of considerable amounts CAEP in the corbicula, abalone and other five species of shellfish was amazing as seen in Fig. 2, and no sphingomyelin was present.

The amount of CAEP in the pond-snail, however, was small as compared with that in the corbicula, and sphingomyelin was present in a quite small amount. In the locust, the shrimp, the crawfish and the pearl oyster the roughly equal amounts of CAEP and sphingomyelin were present. Most of the phosphospingolipids of vertebrates (human, bovine, rat and eel) are sphingomyelin, and they seem to contain other phosphonolipids which have not been identified yet.

Thus, there seems to be a compensatory correlation of amounts between CAEP and sphingomyelin in some species of the invertebrates, although the present study has not yet given clear-cut results because of lack of investigated data on other phosphosphingolipis than sphingoethanolamine.

SUMMARY

1. A quantitative distribution of CAEP and sphingomyelin in some animals was inverstigated.

2. CAEP widely occurs in animals, and especially predominantly in shellfish.

3. There is no obvious relation between the animal classification and a quantitative distribution of CAEP and sphingomyelin, but there seems to be a compensatory correlation between amounts of CAEP and sphingomyelin in some species of invertebrates so far studied.

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