Chem. Phys. Lipids 1 (1967) 157–164 © North-Holland Publ. Co., Amsterdam

MOLECULAR SPECIES OF EXTRACELLULAR PHOSPHATIDYLETHANOLAMINE FROM *ESCHERICHIA COLI*

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Received 10 October 1966

(1) Phosphatidylethanolamine was hydrolysed by phospholipase C from *Bacillus cereus* (E.C. 3.1.4.3) and the resultant diglycerides were separated into five subfractions on thin-layer plates of silica impregnated with silver nitrate.

(2) The positional distribution of the fatty acids in these diglycerides was determined by means of hydrolysis with pancreatic lipase (E.C. 3.1.1.3). The results were in good agreement with those obtained by hydrolysis of the phosphatidylethanolamine with snake-venom phospholipase A (E.C. 3.1.1.4).

(3) The following molecular species accounting for 97.3% of the phosphatidylethanolamine could be calculated: (1-octadecenoyl-2-hexadecenoyl)-, (di-octadecenoyl)-, (1-palmitoyl-2-hexadecenoyl)-, (1-hexadecenoyl-2-palmitoyl), (1-palmitoyl-2-octadecenoyl)-, (1-octadecenoyl-2-palmitoyl)-, (1-palmitoyl-2-cis-3, 10-methylene-hexadecanoyl)-, and (di-palmitoyl)-phosphatidylethanolamine.

Introduction

The analysis of phospholipids in terms of molecular species has recently been accomplished for a number of phospholipid classes from animal tissues[1–4]) and green leaves[5]). Although a separation of intact phospholipids can be achieved[6]) most investigators converted the phospholipids into less polar compounds before separation on silica plates impregnated with silver nitrate. This fractionation appears to depend on the total number of double bonds, the distribution of the double bonds among the fatty acid constituents present at both ester positions and the location of the double bond in the paraffinic chain. In the present paper the results are presented of a species analysis of a bacterial phospholipid containing cyclopropane fatty acids.

Materials and methods

Phosphatidylethanolamine

The phospholipid was donated by Dr. E. Work (Twyford Laboratories Limited, London). The compound had been isolated from an extracellular

lipopolysaccharide-phospholipid-protein complex produced by a lysine-requiring strain of *Escherichia coli*[7]). Details of the isolation procedure are given by Knox, Cullen and Work[8]). The phosphatidylethanolamine (P, 4.4%) moved as a single spot on thin-layer chromatograms.

Hydrolysis by phospholipase A

About 8 mg of the purified phosphatidylethanolamine were dissolved in 2 ml of freshly distilled ether. 2 mg of *Crotalus adamanteus* venom (Light & Co., England) in 0.25 ml of 0.1 M Borate buffer (*p*H 7.0) and 0.25 ml of 0.005 M $CaCl_2$ were added to this ethereal solution. This mixture was shaken during 1 hr with the aid of a Griffin shaker (Griffin & George, England). Thin-layer chromatography on micro-slides coated with silica was used to control if the degradation was complete. The resultant lysocephalins and fatty acids were separated by thin-layer chromatography on silica plates (20 × 10 cm), using chloroform-methanol-water (65:35:4, by vol.) as a developer.

Hydrolysis by phospholipase C

About 50 mg of pure phosphatidylethanolamine were taken up in 3 ml of freshly distilled ether. This ethereal solution was incubated with a crude enzyme preparation from *B. cereus*[9]). After 30 min the hydrolysis was complete as was demonstrated by thin-layer chromatography on silicic acid. The diglycerides formed were extracted from the incubation mixture with several portions of ether. The absence of 1,3-diglycerides was confirmed by thin-layer chromatography using synthetic 1,2- and 1,3-diglycerides as reference substances.

Subfractionation of the 1,2-diglycerides

After phospholipase C degradation of the phosphatidylethanolamine the 1,2-diglycerides could be subfractionated by thin-layer chromatography on silicic acid impregnated with silver nitrate. The plates (20 × 20 cm) were prepared as follows: 20 g of silica gel G-Stahl (Merck-Darmstadt, Germany) were slurred in 45 ml of a 6% (w/v) aqueous solution of silver nitrate. Coating was made with a Desaga applicator. The plates were activated during a period of 4 hr at 120 °C immediately before use. After application of the 1,2-diglycerides, separation was accomplished with chloroform-absolute ethanol (98:2, v/v). The lipids were visualized under UV. light after being sprayed with a 0.01% solution of Rhodamine 6G in water. The subfractions obtained were scraped off from the chromatoplate and extracted immediately by the procedure of Bligh and Dyer[10]). The ratio of the individual subfractions was determined by adding to each fraction as an internal

standard, a fatty acid not occurring in the phosphatidylethanolamine under investigation.

Hydrolysis with pancreatic lipase

The 1,2-diglyceride subfractions could be degraded enzymically by adding to about 5 mg of the diglycerides successively 9 mg of pancreatic lipase (Calbiochem., Los Angeles, U.S.A.), 1 ml of tris buffer (*p*H 8.0), 0.1 ml of 22% (w/v) aqueous solution of calcium chloride and 0.25 ml of 0.1% (w/v) aqueous solution of sodium deoxycholate. This mixture was first warmed during 1 min in a waterbath at 40 °C and then shaken vigorously during 5 min at 40 °C for complete degradation into 2-monoglycerides and free fatty acids. The reaction was then stopped by addition of 0.5 ml of 6 N HCl. The 2-monoglycerides and fatty acids could be separated by thin-layer chromatography using 20 × 10 cm glass plates coated with a $\frac{1}{2}$ mm of silica gel G. The plates were developed in light petroleum (b.p. 38.2–53 °C)-ether-formic acid (60:40:1.6, by vol.).

Gas-liquid chromatography

The methylesters of the fatty acids were prepared by treatment for a period of 2 hr at 70 °C with methanol which contained 26 g of HCl per liter. The methylesters were analysed by means of a F & M instrument equipped with a flame ionisation detection system and a 10% polyethylene glycol adipate column. The temperature of the column was maintained at 180 °C. The fatty acids were identified by comparison with retention data of standards or with published values.

Results and discussion

Composition and distribution of fatty acids of phosphatidylethanolamine

The overall fatty acid composition of the phosphatidylethanolamine is recorded in table 1. The results were in good agreement with those obtained by Knox, Cullen and Work[8]). The main saturated fatty acid was found to be palmitic acid (16:0), while the monoenoic fatty acids appeared to be hexadecenoic and octadecenoic acid only. In addition a 17:0 cyclopropane fatty acid was present. The identity of this cyclopropane fatty acid was characterized by several authors[11,12]) as cis-3, 10-methylene-hexadecanoic acid. Hydrolysis with snake venom phospholipase A (table 1) an enzyme which releases specifically the fatty acids from the 2-ester position[13,14]) indicated that the 17:0∇ fatty acid occupied exclusively the 2-position, this being in agreement with the findings of other investigators[15]). Also, hexadecenoic acid (16:1) from which the 17:0∇ fatty acid is formed[16–18])

TABLE 1

Fatty acid composition of extracellular phosphatidylethanolamine from *E. coli*. The fatty acid composition is given in moles %. The positional distribution was determined by hydrolysis with phospholipase A from snake-venom.

Fatty acids	Overall composition	1-position	2-position
14:0	2.5	2.4	+
16:0	43.5	75.2	6.9
16:1	22.0	2.9	44.1
17:0∇*)	5.5	—	10.6
18:0	+	3.2	—
18:1	26.6	16.1	38.4

*) The ∇ sign is used to designate a cyclopropane ring in the fatty acid chain.

was found to be nearly exclusively in the 2-position. The octadecenoic acid appeared to occur in both ester positions, though it was more abundant in the 2-ester position. In agreement with previous reports the 1-ester position contained for the greatest part the saturated fatty acid, palmitic acid (16:0).

Subfractionation of the diglycerides derived from phosphatidylethanolamine

The phosphatidylethanolamine was broken down completely by enzymatic hydrolysis with phospholipase C from *B. cereus*. The resultant 1,2-diglycerides were subjected to chromatography on silica plates impregnated with silver nitrate. This subfractionation yielded 5 spots (fig. 1). The fatty acid composition of these 5 subfractions is given in table 2. Subfraction 1 representing a component with two double bonds apparently consisted nearly exclusively of diglyceride containing hexadecenoic and octadecenoic acid. Spot 2 which was clearly resolved from spot 1 as can be seen in fig. 1, also represents species containing two double bonds. Fatty acid analysis of this fraction revealed the presence of octadecenoic acid only. The separation of these two neighbouring spots may be brought about by the minimal difference in chain length of the paraffinic chains* or by differences in the position of the double bond. The fatty acid constituents of subfraction 3 were found to be palmitic acid and hexadecenoic acid, this giving rise to

* A subfractionation of the methylesters of the fatty acids from this phosphatidylethanolamine on silver nitrate impregnated silica using oleic acid and vaccenic acid as reference substances, yielded three spots corresponding to hexadecenoic acid, oleic acid and saturated fatty acids (myristic acid, palmitic acid, stearic acid and the cyclopropane fatty acid). No appreciable quantities of vaccenic acid could be detected. Hence it seems likely that the separation of the diglycerides of subfractions 1 and 2 is brought about by differences in chain length of the fatty acid constituents.

species containing one double bond. A similar difference as noted between spot 1 and spot 2 was observed between spot 3 and spot 4; the mobility of the diglycerides of 4, in which octadecenoic acid is combined with palmitic acid, is greater than that of the corresponding hexadecenoic compound. Subfraction 5 reveals the presence of diglycerides without unsaturated fatty acids, the major components being palmitic acid and 17:0∇ acid. During the subfractionation of the 1,2-diglycerides no migration to 1,3-diglycerides

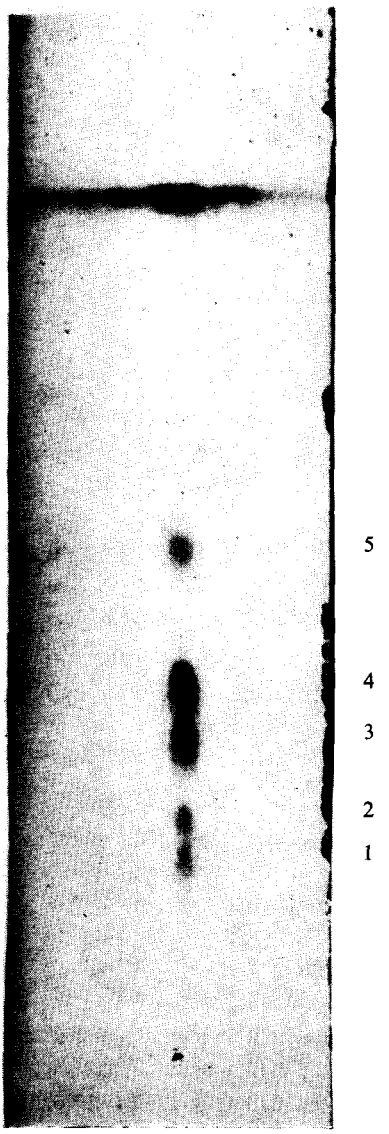

Fig. 1. Subfractionation of diglycerides obtained by phospholipase C hydrolysis of phosphatidylethanolamine, on silica impregnated with silver nitrate. The plates were prepared as described in the experimental part. The developing system was chloroform-absolute ethanol (98:2, v/v). The plates were stained by spraying with an aqueous 20% (w/v) solution of $(NH_4)_2SO_4$ and subsequent heating in an oven at 150 °C. The fatty acid composition and distribution among the 1- and 2-position of these 5 subfractions is given in table 2

TABLE 2

Fatty acid composition of the subfractionated diglycerides derived from phosphatidylethanolamine and the positional distribution of the fatty acids in each subfraction of diglycerides. The 1,2-diglycerides were derived from phosphatidylethanolamine by phospholipase C hydrolysis. The subfractionation of the 1,2-diglycerides was performed on silver nitrate impregnated silica. The fatty acid composition of the 2-position of the fractionated diglycerides was ascertained by pancreatic lipase hydrolysis. The fatty acid composition is given in moles %. The numbers of the subfractions correspond to the numbers of the spots on fig. 1.

Subfraction	1	2	3	4	5
Weight percentages	9.8	10.6	24.8	31.7	23.3
Fatty acid composition					
14:0	+	+	+	+	3.0
16:0	+	+	48.5	48.7	69.1
16:1	49.2	+	51.4	–	–
17:0∇*)	–	–	+	+	28.0
18:0	–	–	–	–	+
18:1	50.8	100.0	–	51.3	–
Fatty acid composition of the 2-position					
14:0	–	–	–	–	5.2
16:0	–	+	2.2	2.3	40.1
16:1	100.0	1.9	97.1	–	–
17:0∇	–	–	0.7	1.0	54.9
18:0	–	–	–	–	+
18:1	+	98.1	–	96.7	–

*) The ∇ sign is used to designate the presence of a cyclopropane ring.

took place, as was demonstrated by chromatography of the diglycerides after combining the subfractions with synthetic 1,2- and 1,3-diglycerides as reference substances (fig. 2).

Hydrolysis of diglycerides with pancreatic lipase and calculation of the molecular species.

Each of the 5 subfractions was hydrolysed with pancreatic lipase. This enzyme splits off specifically the fatty acids from the 1-position of 1,2-diglycerides[19,20]). The results were in good agreement with the information obtained by means of phospholipase A hydrolysis of the phospholipid. In subfraction 1, the 2-position was found to be occupied by hexadecenoic acid exclusively. This enables us to conclude that this subfraction represents the molecular species (1-octadecenoyl-2-hexadecenoyl)-phosphatidylethanolamine abbreviated as 18:1/16:1-PE. Because subfraction 1 was determined to account for 9.8% of the total phosphatidylethanolamine, species

18:1/16:1-PE is occurring to the same extent in the total phosphatidylethanolamine (table 3). Similarly 18:1/18:1-PE is present in this phosphatidylethanolamine to an extent of 10.6%. By hydrolysis of subfractions 3 and 4 with pancreatic lipase it was demonstrated that the 2-position of the 1,2-diglycerides of both subfractions was nearly completely occupied by hexadecenoic and octadecenoic acid respectively (species 16:0/16:1-PE and 16:0/18:1-PE). In both subfractions palmitic acid is also present in the 2-position, though in a very small amount. Taking into account the number of double bonds in these subfractions it seems legitimate to state the existence of 16:1/16:0-PE and 18:1/16:0-PE in subfractions 3 and 4 respectively. In full accordance with the results obtained by the phospholipase A hydrolysis of the phosphatidylethanolamine, hydrolysis of subfraction 5 with pancreatic lipase demonstrated that the cyclopropane fatty acid was located nearly exclusively in the 2-ester position. The amounts of the individual molecular species present in this subfraction can be derived in the following way. The 1-position was occupied almost completely by palmitic acid. Because the percentage of palmitic acid in the 2-position is 40.1%, this indicates that 40.1% of this subfraction $\frac{40.1}{100} \times 23.3\%$ or 9.3% of the total phosphatidylethanolamine was represented by the species 16:0/16:0-PE. In a similar way 16:0/17:0∇-PE was deduced to be present in the phosphatidylethanolamine to an extent $\frac{54.9}{100} \times 23.3\%$ or 12.8%. Using the analytical data described in this paper we could evaluate quantitatively 97.3% of the molecular species.

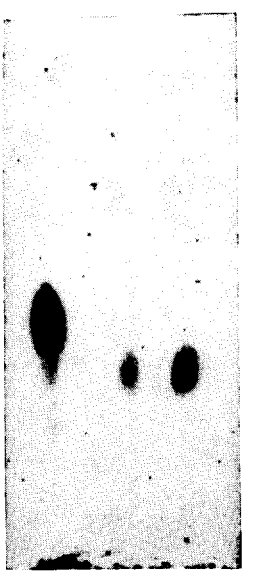

Fig. 2. Silica thin-layer chromatogram of diglycerides derived from phosphatidylethanolamine after subfractionation on silica impregnated with silver nitrate and recombining of the obtained subfractions (spot 3). Spot 1: Synthetic standard 1, 3-diglycerides. Spot 2: Synthetic standard 1,2-diglycerides. The developing mixture was ether-hexane (50:50, v/v). The plate was stained by charring with sulphuric acid.

TABLE 3

Molecular species of extracellular phosphatidylethanolamine from *E. coli*. The calculation of these species has been carried out as described in the text, using the data given in table 2.

1-position	2-position	
18:1	16:1	9.8%
18:1	18:1	10.6
16:0	16:1	24.1
16:1	16:0	+
16:0	18:1	30.7
18:1	16:0	+
16:0	17:0∇	12.8
16:0	16:0	9.3
		Total 97.3%

Acknowledgements

We are indebted to Dr. E. Work (Twyford Laboratories Ltd., London) for donating phosphatidylethanolamine and her keen interest in this study.

The present investigations have been carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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