THE OCCURRENCE OF CYCLOPROPANE FATTY ACIDS IN THE PHOSPHOLIPIDS OF SHEEP RUMEN TISSUES

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1. Introduction

Cyclopropane fatty acids have been found in some seed oils [1] and in a variety of bacteria [2–6]. They also make an important contribution to the fatty acid composition of some species of protozoa [7] and certain insects [8]. A comprehensive review on these acids and their derivatives has been published by Christie [9]. As far as the author is aware, cyclopropane fatty acids have not been reported to occur naturally in mammalian tissues. However, in experiments carried out with rats fed on diets rich in cyclopropane fatty acids, metabolic derivatives of these constituents were found in their body fat [10].

In this laboratory, the phospholipid fractions isolated from sheep rumen tissues have been examined and this communication reports the natural occurrence of cyclopropane fatty acids in this material. They appeared associated only with the phosphatidylethanolamine fraction.

2. Experimental

Total lipid extracts (13.49 g, 61.0 mg P) from rumen tissues of Romney sheep, as obtained by established techniques [11], were fractionated on a silicic acid column (120 g, >325 mesh, Bio-Rad Laboratories, Richmond, Calif., USA) and eluted with chloroform–methanol mixtures as outlined earlier [12]. This yielded a fraction (396 mg, 13.95 mg P) which was identified as phosphatidylethanolamine by comparing its thin-layer chromatography (TLC) behavior with authentic phospholipid standards under identical conditions. Full details of the various TLC solvent systems and micro-phosphorus analytical methods applied here were described previously [11, 12].

The fatty acid methyl esters from the phosphatidylethanolamine fraction were prepared by methanolysis under milk alkaline (0.5 N methanolic NaOH) conditions [13]. The resultant esters were separated according to their degree of unsaturation by preparative TLC using 10% $AgNO_3$-impregnated silica gel G (E. Merck, A.G. Darmstadt, Germany) with a solvent system of benzene–hexane (1:1 v/v).

Gas-liquid chromatography (GLC) analyses were carried out on the separate methyl ester fractions using both polar (10% EGSS-X at 170°) and non-polar (3% ApL at 200°) liquid phases on Gas-Chrom Z (60–70 mesh, Applied Science Laboratories State College, Penn., USA). Oxygen-free nitrogen carrier gas was applied at 15 and 40 ml/min, respectively, and authentic fatty acid methyl esters were used as internal standards to calibrate both columns.

Methyl 9,10-methyleneoctadecanoate was synthesized by the Simmon–Smith reaction of diiodomethane with methyl octadec-9-enoate over a copper-zinc couple catalyst under reflux with diethylether [14].

Hydrogenolysis procedures [15] to convert cyclopropane fatty acids into their corresponding branched-chain derivatives were carried out under two sets of conditions. The milder occurred with PtO_2/methanol at 50° for 6 hr and the more severe case with PtO_2/glacial acetic acid at 50° for 16 hr.

The hydrogenolysis products were oxidized with CrO_3 [4,5] and extracted with diethylether (3X) and washed (4X) with distilled water. This material

Table 1

Gas-liquid chromatography ECL* values of A, B and 9,10-methyleneoctadecanoic acid methyl esters and their hydrogenolysis products.

Methyl esters	ECL 10% EGSS-X (170°)‡	ECL 3% ApL (200°)‡
A	17.66	16.60
B	19.67	18.70
9,10-Methyleneoctadecanoate	19.56	18.74
Hydrogenolysis products		
A: 2-Methylhexadecanoate	16.00	16.00
B: 2-Methyloctadecanoate	18.00	18.00
9,10-Methyleneoctadecanoate:		
9-Methyloctadecanoate / 10-Methyloctadecanoate	18.20	18.33
Nonadecanoate	19.00	19.00
Oxidation products	(180°)	
A: Tetradecanoate	14.00	
2-Hexadecanone	15.37#	
B: Hexadecanoate	16.00	
2-Octadecanone	17.35#	

The oxidation products of A and B are also included.

* Equivalent chain length.

‡ Details described in the text.

Expressed in relationship with *normal* straight-chain fatty acid methyl esters.

was fractionated on preparative TLC plates (silica gel G) with benzene. Three bands developed corresponding to (i) methyl esters, (ii) methyl ketones and (iii) free fatty acids. Band (iii) was methylated with diazomethane [16] and all bands were analysed independently by GLC (10% EGSS-X at 180°).

Selective release of β-fatty acids from the phosphatidylethanolamine fraction was obtained by the enzymatic hydrolysis with snake venom (*Crotalus adamanteus*, Ross Allen Reptile Institute, Silver Springs, Flor., USA) according to the methods of Long and Penny [17] modified by Land and Hart [18]. The free β-fatty acids and the remaining α-fatty acids, after being saponified from the lyso-component, were both esterified with diazomethane [16] and analysed by GLC.

3. Results

The phosphatidylethanolamine fraction of the lipids from sheep rumen tissue gave rise to two unusual fatty acids, A and B. When their methyl esters were examined by $AgNO_3$-impregnated TLC, A and B appeared in the saturated fatty acid ester zone. GLC analyses with both polar and non-polar columns showed that A and B differed from *normal* saturated fatty acids and B had a GLC retention time value similar to that of synthetic methyl 9,10-methyleneoctadecanoate. When this TLC saturated fatty acid ester fraction was treated with Br_2 [19], neither A or B could be detected by further GLC analysis.

On hydrogenolysis of A and B methyl esters under mild conditions, each yielded only a single product

with GLC properties identical to those of hexadeca- and octadecanoic acid methyl esters, respectively. More vigorous hydrogenolysis conditions were required to cleave methyl 9,10-methyleneoctadecanoate and three different products were produced (table 1).

When oxidized, the hydrogenolysis product from A gave two components, 2-hexadecanone and tetradecanoic acid. The identity of the methyl ketones was established by comparison with authentic samples by GLC. Similarly, the accompanying fatty acid oxidation products were also identified by GLC.

Enzymatic hydrolysates of the intact phosphatidylethanolamine fraction showed that the acids corresponding to A and B were located only in the α-position of the glycerol molecule.

4. Discussion

The results provided in the above section showed that A was 2,3-methylenehexadecanoic acid and B, 2,3-methyleneoctadecanoic acid. This was first indicated by GLC analysis of the saturated fatty acid methyl ester fraction from the argentation TLC fractionation, revealing that A and B, in contrast to *normal* saturated components, were no longer detectable by GLC after treatment with Br_2 [19]. Furthermore, the GLC properties of A and B in polar and non-polar columns resembled those of C_{17}- and C_{19}-cyclopropane fatty acid methyl esters, respectively [14].

The hydrogenolysis products from cyclopropane fatty acids are expected to contain branched-chain fatty acid isomers and *normal* fatty acid components [15]. However, those from A and B provided only single products with GLC properties coinciding with *normal* straight-chain fatty acid esters. A study of the reported GLC data on a series of monomethyl-octadecanoic acid methyl esters [20,21] suggests that the product from B, with GLC properties equivalent to methyl octadecanoate, is methyl 2-methyloctadecanoate. Consequently, it is assumed that the product from A with GLC properties similar to methyl hexadecanoate, would be 2-methylhexadecanoic acid methyl ester. The formation of only one product from either A or B by an asymmetric cleavage under mild conditions is consistent with the cyclopropane ring being influenced by the proximity of the electrophilic carboxyl-group. When this carboxyl-group is farther away from the reaction site, as in the synthetic methyl 9,10-methyleneoctadecanoate, the hydrogenolysis products formed under more intensive conditions were a mixture of (80%) 9-methyl- and 10-methyloctadecanoic acid and (20%) nonadecanoic acid methyl esters, as expected [15]. The site of the cyclopropane ring adjacent to the carboxyl-group in A and B was further established by oxidation after hydrogenolysis when A produced 2-hexadecanone and tetradecanoic acid whilst B yielded 2-octadecanone and hexadecanoic acid. From the above evidence it appeared that A and B represent part of a homologous series of cyclopropane fatty acids with the structures of 2,3-methylenehexadecanoic and 2,3-methyleneoctadecanoic acids.

Generally, cyclopropane fatty acids are associated in nature with the phosphatidylethanolamine fraction of bacterial lipids [22] where they are located in the β-position. There are exceptions to this rule [23,24], as in the present case, where the cyclopropane fatty acid is in the α-position. Steric hindrance considerations from molecular structures would expect the 2,3-methylene fatty acids to be preferably located in the α-position.

Cyclopropane fatty acids have not hitherto been reported as constituents of ruminant tissues. They could be derived from rumen micro-organisms because these acids have been suspected [25] and reported [26] to occur in some rumen bacteria. These cyclopropane fatty acids would be expected to have molecular structures similar to 11,12-methyleneoctadecanoic acid (lactobacillic acid), an isomer frequently with bacterial lipids. Therefore, the appearance of such 2,3-cyclopropane fatty acids was not anticipated but were unexpectedly found to comprise approx. 6% of the phosphatidylethanolamine fatty acids. They were not located in the other major phospholipids (phosphatidylcholine and sphingomyelin) and only represent 0.2% of the total rumen tissue fatty acid content. It is proposed to carry out further investigations in this field of work.

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