Biochem. J. (1987) 248, 961–964 (Printed in Great Britain)

Occurrence of unusual molecular species of sphingomyelin containing 28–34-carbon polyenoic fatty acids in ram spermatozoa

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The high levels of very long chain fatty acids found in ram spermatozoa are located almost exclusively in one of two separable species of sphingomyelin. Mass spectral analysis, including fast atom bombardment of the purified sphingomyelin, has shown the fatty acids to have a carbon chain length of between 28 and 34, with between four and six double bonds, and to belong predominantly to the $n-3$ series.

INTRODUCTION

Polyenoic long chain fatty acids with carbon chain lengths greater than 22 (polyenoic VLCFA) have been detected in a number of mammalian tissues including brain (Poulos *et al.*, 1986*a*), retina (Aveldano, 1987; Aveldano & Sprecher, 1987) and endocytes (Rosenthal & Hill, 1984). In bovine retina these fatty acids, which include both $n-3$ and $n-6$ series derivatives, are located mostly in unusual di-polyunsaturated molecular species of phosphatidylcholine (Aveldano & Sprecher, 1987). However, in human brain, the polyenoic VLCFA are almost exclusively $n-6$ series acids and are found mostly in cholesterol esters although the ultra-long chain polyenoic fatty acids, i.e. those with carbon chain lengths greater than 32, are confined exclusively to a minor unidentified phospholipid (Sharp *et al.*, 1987).

We have recently reported that mammalian spermatozoa contain significant amounts of polyenoic VLCFA (Poulos *et al.*, 1986*b*). In particular, ram and bull spermatozoa contain high proportions of $n-3$ fatty acids with 32 and 34 carbon atoms.

In view of the reported differences in lipid distribution of polyenoic VLCFA we undertook an investigation into the distribution of the polyenoic VLCFA in ram spermatozoa. It was hoped that these studies could provide a clue as to their possible function.

We report that the $n-3$ polyenoic VLCFA with 30–34 carbon atoms are located exclusively in sphingomyelin.

MATERIALS AND METHODS

Ram semen was collected by electrical stimulation with a bipolar rectal electrode (Blackshaw, 1954). Spermatozoa were isolated from semen by centrifugation at 1000 *g* for 20 min at room temperature. The spermatozoa from four ejaculates were pooled and then extracted by the technique of Folch *et al.* (1957). The total lipids were separated into neutral lipid, glycolipid and phospholipid fractions by silicic acid column chromatography, and the phospholipids were separated into neutral and acidic components as described by Sharp *et al.* (1987). The total non-acidic lipids were applied as 15 cm bands to two (20 cm × 20 cm) silica-gel 60 (Merck) plates and chromatograms were developed in chloroform/methanol/water (70:30:4, by vol.). The various lipid zones were located by spraying the plates with 0.2% (w/v) dichlorofluorescein in 95% (v/v) ethanol and eluted with 10 ml of chloroform/methanol/water (5:5:1, by vol.). After partitioning to remove the dichlorofluorescein, portions of the eluates were *trans*-esterified (Poulos *et al.*, 1986*a*). The resulting fatty acid methyl esters were subjected to combined g.c.–m.s. as described earlier (Poulos *et al.*, 1986*a*). Determination of the *n*-series number of polyunsaturated fatty acids was carried out using the mass spectrometric technique described earlier (Fellenberg *et al.*, 1987). Phosphorus analyses were performed on the eluates as described by Owens (1966). Identification of the various lipids was based on a comparison of their t.l.c. mobility with authentic standards, using chloroform/methanol/water (70:30:4, by vol.) as developing solvent. In the case of sphingomyelin, identification was based on collision-activation mass-analysed ion kinetic energy spectroscopy (CAMIKES) of ions produced by fast atom bombardment as described by Easton *et al.* (1988). For these studies, mass spectra were measured on a Vacuum Generator ZAB 2 HF mass spectrometer operating in the positive ion fast atom bombardment mode. Additional proof of structure was obtained by g.l.c.–m.s. identification of sphingosine released after acid hydrolysis (Polito *et al.*, 1968).

RESULTS AND DISCUSSION

Polyenoic VLCFA ($n-3$ series) with 30–34 carbon atoms are the major VLCFA in ram spermatozoa (Poulos *et al.*, 1986*b*). Silicic acid column chromatography of ram sperm lipids confirmed that most of these fatty acids were phospholipid components. Further fractionation of the sperm phospholipids by preparative t.l.c. indicated that virtually all of the polyenoic VLCFA were present in one phosphate-containing lipid (zone 2). Acid hydrolysis released two lipid products identified by g.c.–m.s. as sphingosine and a mixture of long chain fatty

Abbreviations used: VLCFA, very long chain fatty acids; CAMIKES, collision-activation mass-analysed ion kinetic energy spectroscopy.

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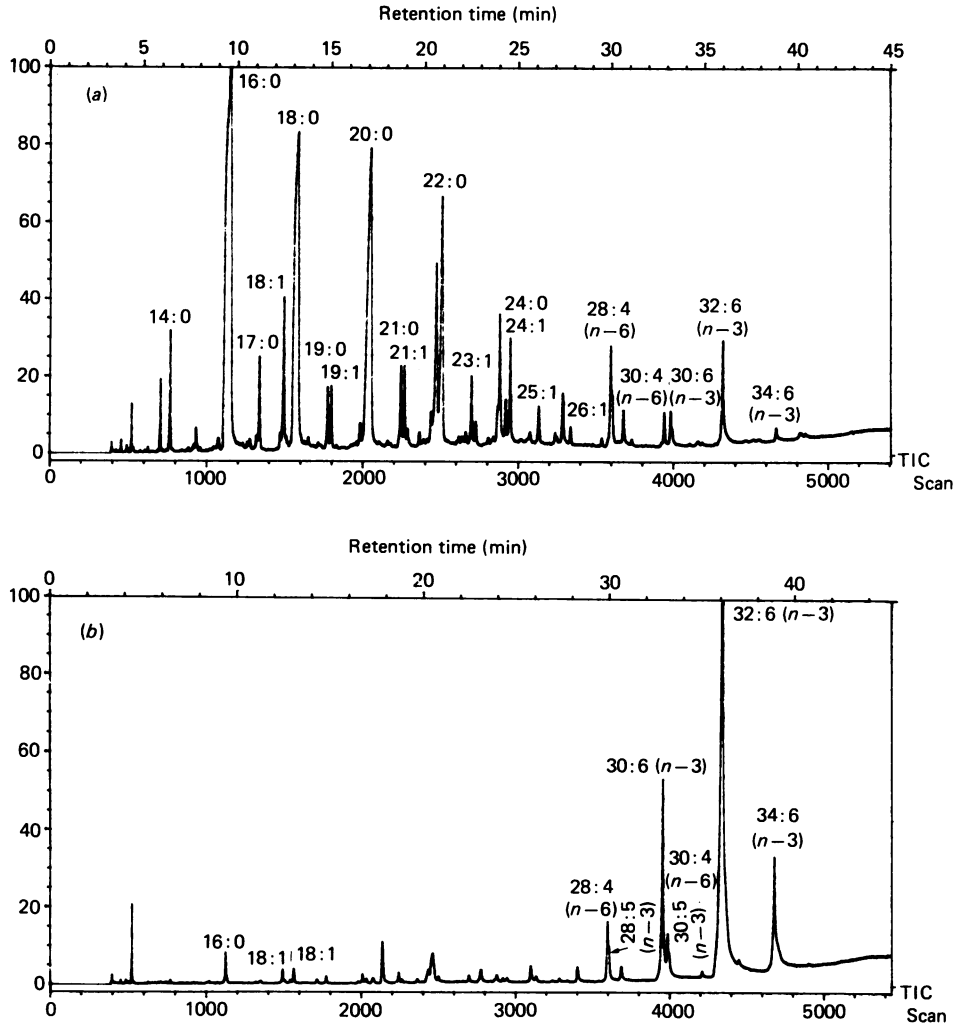

Fig. 1. G.c. of fatty acids derived from ram sperm sphingomyelin

Total ion chromatograms of the fatty acids of ram sperm sphingomyelin were obtained as described in the Materials and methods section. (*a*) Zone 1 sphingomyelin. (*b*) Zone 2 sphingomyelin.

acids, indicating that the lipid was sphingomyelin. The fatty acids released were almost exclusively (> 90%) 28–34 carbon polyenoic fatty acids with a predominance of 30:6($n-3$) and 32:6($n-3$) fatty acids (Fig. 1*a*). The latter comprised nearly 50% of the total fatty acids released from this particular lipid. In contrast, the slower-moving sphingomyelin (zone 1) contained predominantly shorter chain fatty acids, i.e. 16:0, 18:0, 18:1, 20:0 fatty acids with smaller amounts of polyenoic VLCFA (Fig. 1*b*). Accurate quantification of the fatty acids was not possible because polyenoic fatty acid standards are not available.

The spectrum obtained by fast atom bombardment mass spectrometric analysis (Fig. 2*a*) of the faster-moving sphingomyelin (zone 2) contains strong signals corresponding to ions at m/z 184 and 465, as well as bands of signals corresponding to ions between m/z 864 and m/z 944. The ion with m/z 184 afforded a CAMIKES mass spectrum containing ions at m/z 60, 86, 125 and 166 which is consistent with the structure of phosphorylcholine (Fig. 2*b*). The ion at m/z 465 can be attributed to sphingosylphosphorylcholine formed by deacylation of the parent lipid. The presence of an ion at m/z 184 in the CAMIKES spectrum generated from the m/z 465 fragment, presumably due to the phosphorylated base, provides strong supporting evidence for its identity (Fig. 2*c*). The ions at m/z 864, 888, 916 and 944 are ($M+1$) ions of individual molecular species of sphingomyelin containing 28:4, 30:6, 32:6 and 34:6 fatty acids (Fig. 2*a*).

To our knowledge, the presence of such unusual molecular species has not been previously reported although sphingomyelin from some tissues, notably brain (Stallberg-Stenhagen & Svennerholm, 1965), does contain high proportions of saturated and monounsaturated VLCFA while shorter chain polyenoic fatty acids, such as arachidonic acid, have been detected in human and rat tissue (Kokatnur *et al.*, 1985).

It should be emphasized that these lipids are not minor components of sperm. Thus, approx. 9% of the total lipid phosphorus in the lipids isolated from ram spermatozoa is found in the faster-moving sphingomyelin band (Darin-Bennett, 1975) and, therefore, the major molecular species which contains a 32:6 fatty acid

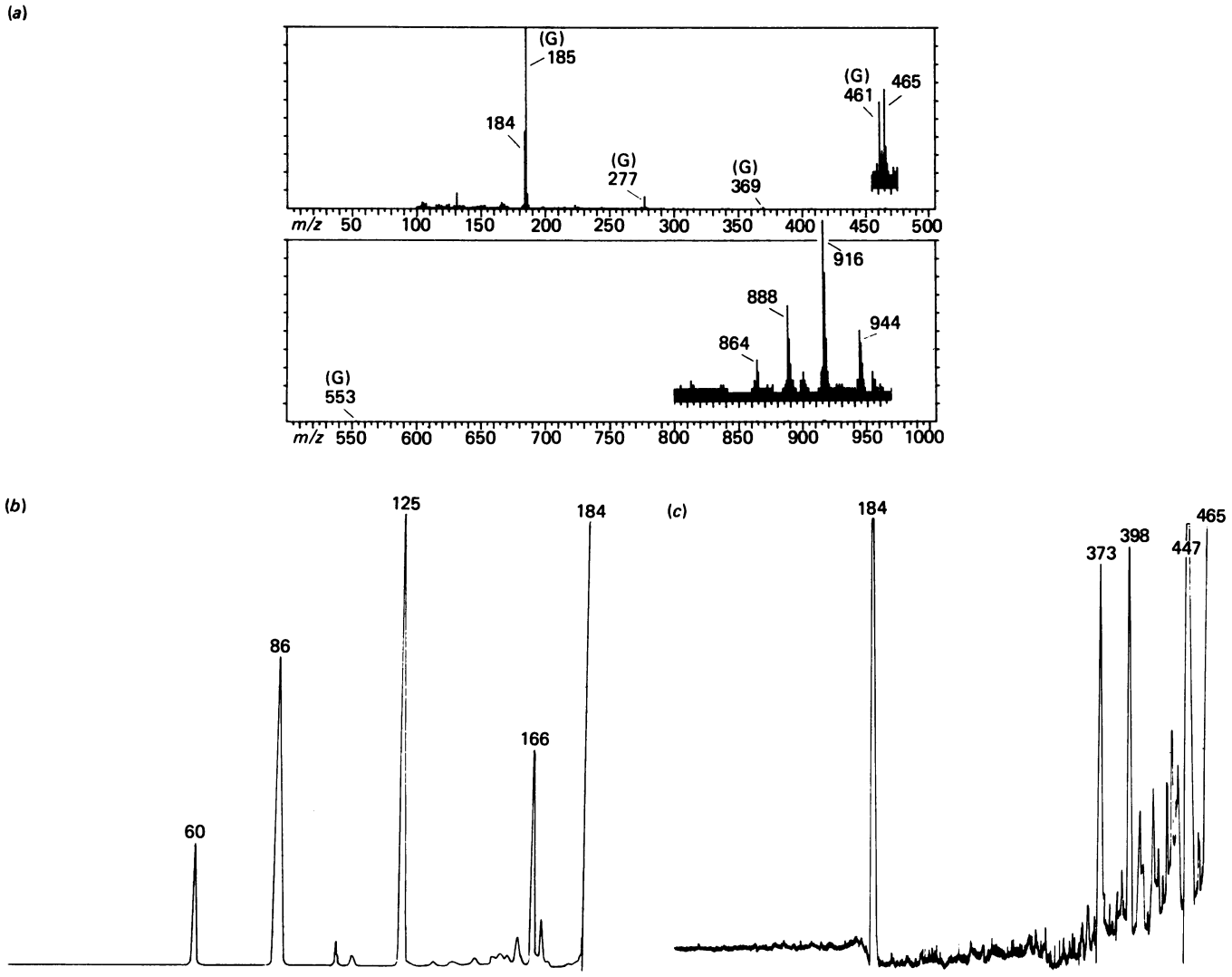

Fig. 2. M.s. of sphingomyelin isolated from ram spermatozoa

(*a*) Positive-ion fast atom bombardment mass spectrum of sphingomyelin. The peaks labelled (G) are protonated adducts of the solvent glycerol. (*b*) CAMIKES spectrum of the fragment of *m/z* 184 in the fast atom bombardment mass spectrum of sphingomyelin (*a*). (*c*) CAMIKES spectrum of the fragment of *m/z* 465 in the fast atom bombardment mass spectrum of sphingomyelin (*a*).

comprises nearly 4% of the total lipid phosphorus of ram spermatozoa. The occurrence of these unusual lipids raises a number of important questions concerning their mode of synthesis, degradation and their sub-cellular location.

The lipid distribution of the $n-3$ polyenoic VLCFA in ram sperm differs quite markedly from the distribution of the corresponding fatty acids in bovine retina where they occur largely in photoreceptor membranes as di-polyunsaturated phosphatidylcholines (Aveldano & Sprecher, 1987). Although we are unable to discount the possibility that the latter are constituents of ram spermatozoa, clearly there are fundamental differences in lipid composition between the two tissues because 30–34-carbon polyenoic fatty acids are virtually undetectable in sperm phosphatidylcholine. It has been speculated that retina $n-3$ polyenoic fatty acids may be required for the normal functioning of photoreceptor membrane proteins and therefore play a significant role in the visual process (Aveldano & Sprecher, 1987). Because of the major structural and physiological differences between retina and spermatozoa it is not unreasonable to speculate that the function of polyenoic VLCFA in these two tissues also differs. Some supporting evidence is provided by the differences in lipid distribution. Thus, while glycerolipid-bound fatty acids are metabolically active through the action of various phospholipases, the release of the corresponding amide-linked fatty acids is thought to involve the sequential action of sphingomyelinase and ceramidase (Mooibroek *et al.*, 1985) and is therefore probably slower. Whether this difference reflects a difference in function is possible, but remains to be determined.

This work was supported by grants from the National Health and Medical Research Council of Australia and the Adelaide Children's Hospital Research Trust.

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Received 28 July 1987/21 September 1987: accepted 5 October 1987