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The occurrence of polyenoic fatty acids with greater than 22 carbon atoms in mammalian spermatozoa

Alfred POULOS, Peter SHARP, David JOHNSON, Ian WHITE* and Alan FELLENBERG

Department of Chemical Pathology, The Adelaide Children's Hospital, King William Road, North Adelaide, South Australia 5006, Australia, and *Department of Veterinary Physiology, Sydney University, Sydney, New South Wales, Australia

Fatty acids with carbon chain lengths greater than 22 (VLCFA) have been detected in boar, ram, bull and human spermatozoa. Saturated and mono-unsaturated fatty acids were present in all spermatozoa but, except for human spermatozoa, polyenoic fatty acids were quantitatively the most important components. Marked differences in polyenoic fatty acid composition were observed. Whereas human spermatozoa contain predominantly di-, tri- and tetraenoic fatty acids with up to 32 carbon atoms, boar, ram and bull spermatozoa also contain pentaenoic and/or hexaenoic acids with up to 34 carbon atoms. Human and boar spermatozoa differ markedly from those of the ram and bull in that only $n-6$ series acids are present.

INTRODUCTION

Polyenoic fatty acids with up to 22 carbon atoms are present in significant amounts in most mammalian tissues (Naughten, 1981). Apart from their role as structural membrane components some of these fatty acids, in particular arachidonic (20:4, $n-6$), eicosatrienoic (20:3, $n-6$) eicosapentaenoic (20:5, $n-3$), docosatetraenoic (22:4, $n-6$) and possibly docosahexaenoic (22:6, $n-3$), act as precursors for a variety of physiologically active oxygenated metabolites including prostaglandins, thromboxanes and leukotrienes (Lewis & Austen, 1984; Needleman *et al.*, 1979; Lee *et al.*, 1984; Aveldano & Sprecher, 1983; Van Rollins *et al.*, 1984). One of the richest sources of polyenoic fatty acids is the male mammalian reproductive tract, particularly testis and spermatozoa (Carpenter, 1971; Poulos *et al.*, 1973, 1975). Depending on the species the phospholipid-bound fatty acids present in the spermatozoa may contain up to 65% polyenoic derivatives, mostly 22:6, 22:5 and 20:4 (Poulos *et al.*, 1973). Although most studies thus far on the fatty acids of mammalian spermatozoa have focused on those fatty acids with carbon chain lengths of 22 or less, more recent reports indicate that polyenoic fatty acids with more than 22 carbon atoms (VLCFA) may also be present. Grogan & Lain (1982) and Grogan & Huth (1983) have reported that 24:5 and 26:5 are synthesized from arachidonic acid by mouse spermatocytes and spermatids in culture, while the probable occurrence of even longer chain fatty acids was suggested by the recent studies of Grogan (1984) who found 28:5 and 30:5 fatty acids in rat testis.

In the present paper we report on our investigations into the polyenoic VLCFA composition of the spermatozoa of a number of mammalian species. Our data confirm that these fatty acids are present in all spermatozoa examined thus far, and further, that the composition varies markedly according to the species.

MATERIALS AND METHODS

Ram semen was collected by electrical stimulation with the bipolar rectal electrode (Blackshaw, 1953). Bull semen was obtained by using an artificial vagina. Human semen was obtained by massage from normal subjects. Boar semen was obtained by using a dummy sow. Frozen Danish herring was purchased from a local supermarket (Glyngore brand).

Spermatozoa were isolated from semen by centrifugation at 1000 *g* for 20 min at room temperature. The spermatozoa were extracted according to the technique of Folch *et al.* (1957) and the extracts were stored at −20 °C until ready for analyses. Lipids were also extracted from herring flesh in the same manner. The total lipid extracted from single ejaculates of all species (or from 2 g of herring) was treated with 1.5% (v/v) (18 M) H_2SO_4 in methanol and the liberated fatty acid methyl esters were purified by preparative t.l.c. as described by Berkovic *et al.* (1983) Antioxidants were initially employed in the isolation procedures but were found to be unnecessary. Experience in the handling of polyunsaturated VLCFA (Poulos *et al.*, 1986) has shown them to be remarkably stable in comparison with short-chain polyunsaturated fatty acids.

G.l.c. of the fatty acid methyl esters was carried out as described previously (Poulos *et al.*, 1986). The peak heights of the various fatty acid methyl esters were determined with a Hewlett–Packard 3390 integrator. Data were expressed as a percentage of the individual fatty acid relative to the combined peak heights of all fatty acids with carbon chain lengths > 14. Gas chromatography–electron impact mass spectrometry (g.l.c.–m.s.) was performed on a JEOL JMS DX-303 single-beam double-focusing mass spectrometer equipped with a Hewlett–Packard 5890 gas chromatograph and a JEOL JMA-DA5000 data system. Chromatographic separation was achieved with a splitless injector coupled to a Scientific Glass Engineering (SGE) BP-1 bonded phase silica column (12 m × 0.22 mm internal diameter, 0.25 μm phase thickness) directly inserted into the mass spectrometer source. Chemical ionization mass spectra were obtained using methane as a reagent gas.

RESULTS AND DISCUSSION

The results of g.l.c. analyses of the fatty acid methyl esters derived from the total lipids extracted from ram,

Abbreviation used: VLCFA, very long chain fatty acid(s).

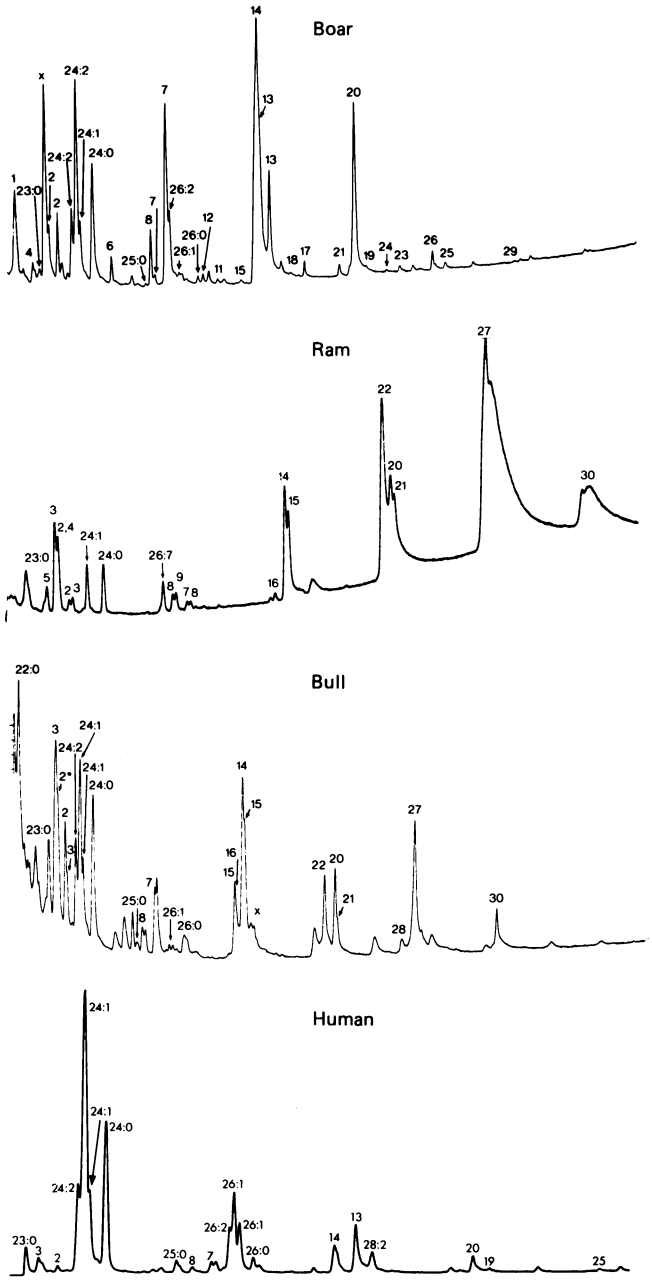

Fig. 1. G.l.c. of VLCFA in mammalian spermatozoa

Lipids were extracted and fatty acid methyl esters were chromatographed as described in the text. The data shown represent the total ion chromatograms and depict only the VLCFA. The tentative identity of individual fatty acids is shown in Table 1.

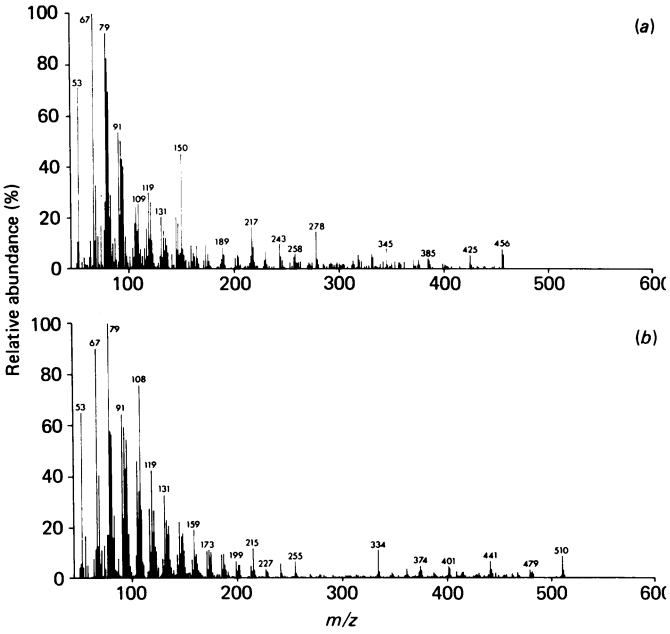

Fig. 2. Mass spectrometry of polyenoic VLCFA

Electron impact mass spectra of boar spermatozoa 30:5 ($n-6$) (*a*) and 34:6 ($n-3$) (*b*) fatty acid methyl esters was carried out as described in the text.

bull, boar and human spermatozoa are shown in Fig. 1. All extracts contained a number of fatty acids with carbon chain lengths in excess of 22. These collectively comprised (based on peak height measurements) up to approx. 5% of total recovered fatty acids. The identification of the VLCFA methyl esters was performed in several stages. The carbon chain length and degree of unsaturation were obtained from the mass of the molecular ion formed under electron impact (M^+) and chemical ionization $(M+H)^+$ conditions, and from a comparison of gas chromatographic retention times with those of available standards. Saturated and monounsaturated VLCFA (24:0, 24:1, 25:0, 26:0 and 26:1) were easily identified because of the presence of an intense ion of m/z 74 [$CH_2=C(OH)OCH_3^+$]. The methylene-interrupted polyunsaturated fatty acids of the $n-3$ and $n-6$ series were distinguished by the presence of high abundance fragments of m/z 79 ($C_6H_7^+$) and m/z 91 ($C_7H_7^+$) as illustrated in Fig. 2. These ions were also detected in the spectra of some highly unsaturated fatty acid methyl ester standards, belonging to both the $n-3$ and $n-6$ series, including arachidonic (20:4, $n-6$), eicosatrienoic (20:3, $n-6$), eicosapentaenoic (20:5, $n-3$), docasatetraenoic (22:4, $n-6$) and docosahexaenoic (22:6, $n-3$) acids and have been reported to be characteristic for this class of fatty acid (Araki *et al.*, 1976). Intense ions at m/z 53 ($C_4H_5^+$) and m/z ($C_5H_7^+$) in Fig. 2 are common to many unsaturated (including acetylenic) fatty acids.

Assignment of the double bond positional series was based on our observation that a characteristic fragment ion split from the alkyl end is produced by members of each series i.e. m/z 108 ($C_8H_{11}^+$) for the $n-3$ series, m/z 150 ($C_{11}H_{17}^+$) for the $n-6$ series and m/z 192 ($C_{14}H_{23}^+$) for the $n-9$ series (Fellenberg *et al.*, 1986). Comparison of the relative intensities of these three ions afforded an unambiguous assignment of the positional series for all of the polyenoic fatty acids identified (Table 1) which was consistent with their g.l.c. retention times and the observation from standards that an isomer of the $n-9$ series precedes that of the $n-6$ series which in turn precedes that of the $n-3$ series.

Corresponding ions at m/z 109, 151 and 193 were observed when the analysis was conducted under chemical ionization conditions. No greater sensitivity was afforded, however, in this mode because the relative intensities of the protonated molecular ions formed from

the polyenoic VLCFA esters were comparable with those of the molecular ions formed under electron impact conditions.

The VLCFA extracted from herring flesh have been shown by classical degradative methods to belong almost exclusively to the $n-3$ series (Linko & Karinkanta, 1970). As further confirmation of our assignment of structure, a herring flesh extract was subjected to argentation chromatography and the polyenoic VLCFA methyl esters concentrated. The concentrate was added to a human spermatazoa extract which by our method of assignment contained only $n-6$ series polyenoic VLCFA (Fig. 3). Acceptable chromatographic separations of all of the polyenoic VLCFA was observed, indicating that the human spermatozoa sample did not contain 30:4, 30:5, 30:6, 28:4, 28:5, 28:6, 28:6, 28:7, 26:3, 26:4, 26:5, 26:6 and other fatty acids of the $n-3$ series. The mass spectra of these compounds were, however, almost identical with those of the same assigned structure in the boar sperm extract and each contained dominant ions at m/z 79, 91 and 108.

Table 1 lists the VLCFA detected in ram, bull, boar and human spermatozoa. Fatty acids with even numbers of carbon atoms predominated in all four species, while significant amounts of odd-numbered fatty acids were detected only in boar spermatozoa. Saturated and mono-unsaturated VLCFA were present in all spermatozoa examined but, except for human spermatozoa, polyenoic fatty acids collectively comprised the greater proportion. These consisted mostly of di-, tri- and tetraenoic acids in human spermatozoa, while boar, ram and bull spermatozoa contained, in addition, pentaenoic acids. Hexaenoic acids were only detected in significant amounts in ram and bull spermatozoa. The maximum carbon chain length observed was 34 and these fatty acids were detected in ram, bull and boar spermatozoa.

An inspection of the data obtained for the ram and boar shown in Table 1 demonstrates that penta- and hexaenoic derivatives are found in the shorter as well as in the longer chain VLCFA. This could be interpreted to mean that elongation of all of these derivatives may take place without concomitant desaturation. It therefore appears unlikely that the longer chain hexaenoic acids are formed by a sequential elongation and desaturation mechanism as appears to operate for the synthesis of polyenoic fatty acids with up to 22 carbon atoms (Naughten, 1981). Whether this is due to the inability of polyenoic VLCFA to act as substrates for desaturases or whether some of the desaturases, notably Δ^4 desaturase, which is absent from some tissues (Robert *et al.*, 1977; Hyman & Spector, 1981; Naughten, 1981; Dunbar & Bailey, 1975), are also absent from testis and spermatozoa remains unclear.

It is clear that there are very significant structural differences between the different species. Of particular interest is the apparent absence of $n-3$ VLCFA in boar and human spermatozoa in contrast with ram and bull spermatozoa which contain $n-3$ as well as $n-6$ series fatty acids. This observation is particularly intriguing as boar and human spermatozoa are thought to contain large amounts of shorter chain $n-3$ precursors (Poulos *et al.*, 1973) which, at least theoretically, could be chain elongated. We have reported a similar preponderance of $n-6$ series VLCFA in the brain of patients with Zellweger's syndrome, an inherited disorder characterized by a deficiency of tissue peroxisomes (Poulos *et al.*, 1986). Studies by Bourre *et al.* (1978) have indicated that there may be three separate elongases for saturated fatty acid synthesis. Apart from a system which synthesizes palmitic acid *de novo* from acetyl- and malonyl-CoA, there is a C_{16} elongase which converts palmitate to stearate and a C_{18} elongase which is capable of converting stearic acid to longer chain fatty acids, including the saturated VLCFA, lignoceric and hexacosanoic acid (24:0 and 26:0 respectively). Goldberg *et al.* (1973) have reported that there is, in addition, a C_{20} elongase which produces lignoceric acid, but this has been disputed (Bourre *et al.*, 1978). At present it is not known whether any of these elongases are involved in the biosynthesis of polyunsaturated VLCFA or whether there is a separate elongase or individual elongases specific for $n-3$ and $n-6$ series acids. However, in view of the apparent specificity of the elongating mechanism in spermatozoa, human brain (Poulos *et al.*, 1986) rat testis (Bridges & Coniglio, 1970) and marine lipids (Linko & Karinkanta, 1970) it is tempting to speculate that there may indeed be separate elongases for $n-3$ and $n-6$ fatty acids, although our findings may also be explained by the existence of a single polyenoic VLCFA elongase with different species specificity.

Little is known about the possible function of these unusual fatty acids in biological systems. However their rapid turnover (Yoshida & Takeshita, 1984; Grogan &

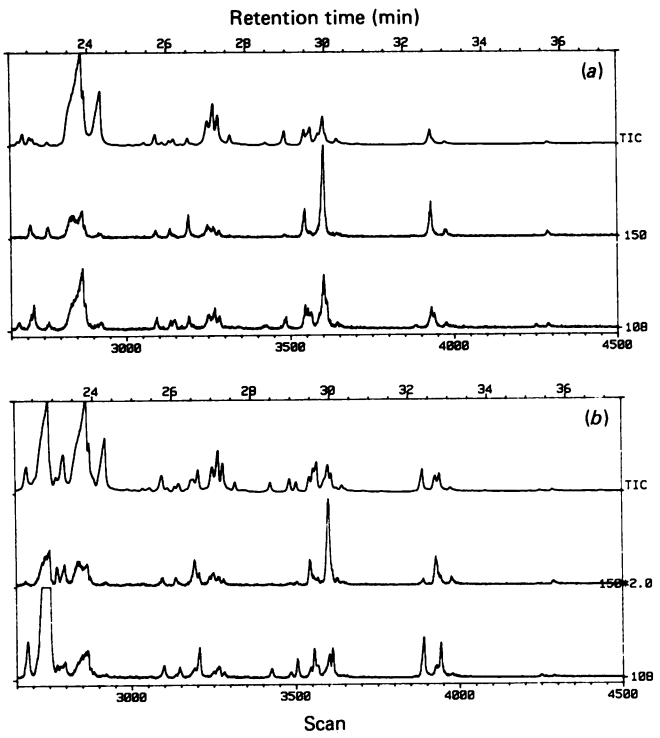

Fig. 3. Mass chromatograms of human semen extract before (*a*) and after (*b*) the addition of VLCFA concentrate from herring flesh

The total ion current (TIC) and contributions from ions with m/z of 108 and 150 for the VLCFA of carbon chain length 24–32 carbon atoms is displayed. The ion intensities have been normalized to full scale. Note that some of the large peaks in the m/z 108 trace of the human semen chromatogram belong to mono-unsaturated fatty acids.

Table 1. Polyenoic fatty acids in mammalian spermatozoa

The identification of the various fatty acids shown in Fig. 1 was based on g.l.c. retention times and mass spectrometry as described in the text.

Identification no.	Carbon number: double bonds	Boar		Ram		Bull		Human	
		n−3	*n*−6	*n*−3	*n*−6	*n*−3	*n*−6	*n*−3	*n*−6
1	23:4		+						
2	24:3		+		+		+		+
3	24:4			+	+	+	+		+
4	24:5		+	+					
5	24:6			+					
6	25:3		+						
7	26:3		+		+		+		+
8	26:4		+	+	+		+		
9	26:5			+					
10	26:6			+					
11	27:3		+						
12	27:4		+						
13	28:3		+						+
14	28:4		+		+		+		+
15	28:5		+	+		+			
16	28:6			+		+			
17	29:4		+						
18	29:5		+						
19	30:3		+						+
20	30:4		+		+		+		+
21	30:5			+		+			
22	30:6			+		+			
23	31:4		+						
24	31:5		+						
25	32:4		+						+
26	32:5		+						
27	32:6		+			+			
28	32:7			+		+			
29	34:5		+						
30	34:6			+		+			

Lain, 1982; Grogan & Huth, 1983; Rosenthal & Hill, 1984) and excretion (Rosenthal & Hill, 1984) coupled with their synthesis in human and pig brain, but not liver (Yoshida & Takeshita, 1984), suggests that they may have some, as yet unknown, function. In view of their structural similarities it is conceivable that they interfere with the metabolism of the shorter chain polyenoic acids, such as arachidonic acid (Lee *et al.*, 1985) or alternatively they may, themselves, act as precursors for the synthesis of a group of physiologically active compounds analogous to prostaglandins and leukotrienes.

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REFERENCES

Araki, E., Ariga, T. & Murata, T. (1976) Biomed. Mass Spectrom. **3**, 261–264
Aveldano, M. I. & Sprecher, H. (1983) J. Biol. Chem. **258**, 9339–9343
Berkovic, S., Zajak, J. D., Warburton, D. J., Merory, J. D., Fellenberg, A. J., Poulos, A. & Pollard, A. C. (1983) Aust. NZ J. Med. **13**, 594–600
Blackshaw, A. W. (1954) Aust. Vet. J. **30**, 249–250
Bourre, J. M., Pollet, S., Paturnea-Jonas, M. & Baumann, N. (1978) in Enzymes of Lipid Metabolism (Gatt, S., Freysz, L. & Mandel, P., eds.), Plenum, New York
Bridges, R. & Coniglio, J. G. (1970) J. Biol. Chem. **245**, 46–49
Carpenter, M. P. (1971) Biochim. Biophys. Acta **231**, 52–79
Dunbar, L. M. & Bailey, J. M. (1975) J. Biol. Chem. **250**, 1152–1153
Fellenberg, A. J., Johnson, D. W., Poulos, A. & Sharp, P. (1986) Biomed. Environ. Mass Spectrom., in the press
Folch, J., Lees, M. & Sloane-Stanley, J. (1957) J. Biol. Chem. **226**, 497–509
Goldberg, I., Schechter, I. & Bloch, K. (1973) Science **182**, 497–499
Grogan, W. M. (1984) Lipids **19**, 341–346
Grogan, W. M. & Huth, E. G. (1983) Lipids **18**, 275–284
Grogan, W. M. & Lain, J. W. (1982) Lipids **17**, 604–611
Hyman, B. T. & Spector, A. A. (1981) J. Neurochem. **37**, 60–69
Lee, T. H., Mencia-Huerta, J. M., Shih, C., Corey, E. J., Lewis, R. A. & Austen, K. F. (1984) J. Biol. Chem. **259**, 2382–2389
Lee, T. H., Hoover, R. L., Williams, J. D., Sperling, R. I., Ravalese, J., Spur, B. W., Robinson, D. R., Corey, E. J., Lewis, R. A. & Austen, K. F. (1985) New Engl. J. Med. **312**, 1217–1224
Lewis, R. A. Austen, K. F. (1984) J. Clin. Invest. **73**, 889–899
Linko, R. R. & Karinkanta, H. (1970) J. Am. Oil Chem. Soc. **47**, 42–46
Naughten, J. (1981) Int. J. Biochem. **13**, 21–32
Needleman, P., Raz, A., Minkes, M. S., Ferrendeli, J. A. & Sprecher, H. (1979) Proc. Natl. Acad Sci. U.S.A. **76**, 944–948

Poulos, A., Darin-Bennett, A. & White, I. G. (1973) Comp. Biochem. Physiol. **46B**, 541–549
Poulos, A., Brown-Woodman, P. D. C., White, I. G. & Cox, R. I. (1975) Biochim. Biophys. Acta **388**, 12–18
Poulos, A., Sharp, P., Singh, H., Johnson, D., Fellenberg, A. J. & Pollard, A. C. (1986) Biochem. J. **235**, 607–610
Robert, J., Rebel, G. & Mandel, P. (1977) Biochimie (Paris) **59**, 417–423
Rosenthal, M. D. & Hill, J. R. (1984) Biochim. Biophys. Acta **795**, 171–178
Sprecher, H., Van Rollins, M., Sun, F., Wyche, A. & Needleman, P. (1981) J. Biol. Chem. **257**, 3912–3918
Van Rollins, M., Baker, R. C., Sprecher, H. W. & Murphy, R. C. (1984) J. Biol. Chem. **259**, 5776–5783
Yoshida, S. & Takeshita, M. (1984) Biochim. Biophys. Acta **795**, 137–146

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