Polyenoic very-long-chain fatty acids (VLCFA) have been shown to be localized in unusual molecular species of sphingomyelin in the testes and spermatozoa of the ram, bull, rat, and boar and in the spermatozoa of man. The composition of polyenoic VLCFA-sphingomyelin was comparable in the testes and spermatozoa of each mammalian species; however, the sphingolipid was more concentrated in spermatozoa. The composition of testicular and spermatozoan polyenoic VLCFA-sphingomyelin differed considerably between animal types. Human spermatozoa mainly contained n-6 polyenoic VLCFA with two to four double bonds and even-carbon chain lengths up to 32. In ram and bull testes and spermatozoa, n-3 and n-6, tetra-, penta-, and hexaenoic VLCFA with even-carbon chain lengths up to 34 predominated. In rat and boar testes and spermatozoa, the polyenoic VLCFA were mainly n-6 derivatives with three to five double bonds and even- and odd-carbon chain lengths up to 34. The testes and spermatozoa of the latter two animal species contained 2-hydroxyylated, in addition to non-hydroxyylated, polyenoic VLCFA in sphingomyelin. This is the first time that 2-hydroxyylated polyenoic VLCFA have been recognized in biological systems. Non-hydroxyylated polyenoic VLCFA were initially observed in the sphingomyelin of rat testes 25 days after birth, followed by 2-hydroxyylated derivatives at 30 days. The total amount of polyenoic VLCFA associated with rat testicular sphingomyelin increased dramatically from 25 to 40 days of postnatal life and then remained constant to 60 days (sexual maturity). The ratio of 2-hydroxyylated to non-hydroxyylated polyenoic VLCFA increased during this period. Polyenoic VLCFA-sphingomyelin seems to occur exclusively in the testes and spermatozoa of mammals, and it is postulated that this lipid plays a role in reproduction.

One of the richest sources of polyenoic long-chain fatty acids (LCFA) with up to 22 carbon atoms is the male mammalian reproductive tract, particularly testes and spermatozoa. Depending on the animal species, the phospholipid-bound fatty acids present in testes and spermatozoa may contain up to 65% polyenoic derivatives, mostly docosahexaenoic acid (C_{22:6,6}), docosapentaenoic acid (C_{22:5,6}), and arachidonic acid (C_{20:4,6}) (2, 3). The importance of polyenoic VLCFA in the testes is emphasized by reports that factors which adversely affect the testicular composition of these fatty acids (e.g., essential fatty acid deficiency) result in impaired spermatogenesis (5–8). There is evidence that prostaglandins and leukotrienes derived from polyenoic LCFA play a significant role in testicular (9–12) and spermatozoan (13–16) physiology. The differentiating germinal cells (spermatogonia → spermatocytes → round spermatids → condensing spermatids → spermatozoa) and non-germinal cells (Sertoli and Leydig) of the testes have polyenoic LCFA compositions which are different from each other. The later stages of germinal cell development are especially characterized by having high concentrations of 22 carbon penta- and hexaenoic fatty acids (17–19). Polyenoic LCFA have been shown to be synthesized by elongation and desaturation of shorter chain precursors in whole testes and isolated germinal and non-germinal cells (20–24).

Although most studies on the polyenoic fatty acids of mammalian testes and spermatozoa have focused on those with carbon chain lengths of 22 or less, polyenoic very-long-chain fatty acids (VLCFA) with more than 22 carbon atoms are also present. Bridges and Coniglio (20) observed that tetracosatetraenoic acid (C_{24:4,6}) and tetracosa-pentaenoic acid (C_{24:5,6}) are formed from linoleic acid (C_{18:2,6}) and arachidonic acid (C_{20:4,6}) in rat testes. Grogan and Lam (22) and Grogan and Huth (23) showed that cultured mouse spermatocytes and spermatids can synthesize tetracosatetraenoic acid (C_{24:4,6}) and tetracosa-pentaenoic acid (C_{24:5,6}) from arachidonic acid (C_{20:4,6}). It was subsequently demonstrated that arachidonic acid (C_{20:4,6}) is the precursor of even longer chain polyenoic fatty acids such as hexacosatetraenoic acid (C_{26:4,6}), hexacosapentaenoic acid (C_{26:5,6}), octacosapentaenoic acid (C_{28:5,6}), and triacosapentaenoic acid (C_{30:5,6}) in rat testes (25). Poulos et al. (26) reported that polyenoic VLCFA represent a significant component of the total fatty acids (approximately 5%) in the spermatozoa of a number of mammalian species, and that the composition varies markedly according to the species. Human spermatozoa contain predominantly di-, tri-, and tetraenoic fatty acids with up to 32 carbon atoms, while boar, ram, and bull spermatozoa also contain pentaenoic and/or hexaenoic fatty acids with up to 34 carbon atoms. In ram and bull spermatozoa the polyenoic VLCFA belong to both the n-3 and n-6 series; however, in human and boar spermatozoa these fatty acids are mainly n-
Novel Molecular Species of Sphingomyelin in Testes and Spermatozoa

6 derivatives. It has been shown that the polyenoic VLCFA in ram spermatozoa are enriched in molecular species of sphingomyelin (27). In the present paper we have investigated the lipid distribution of polyenoic VLCFA in the testes and spermatozoa of several mammalian species. We were curious to know whether the polyenoic VLCFA content varies with testicular development. During the course of this work we have identified a unique group of 2-hydroxylated polyenoic VLCFA.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

Fractionation of lipids isolated from mature ram, bull, boar, and rat testes and spermatozoa and adult human spermatozoa using a combination of silicic acid and DEAE-cellulose DEE52 column chromatography and preparative TLC indicated that polyenoic VLCFA were mainly present in sphingomyelin (80-90%), with the remainder in ceramide (10-20%) and phosphatidylincholine (less than 5%). This confirms and extends our earlier report that polyenoic VLCFA are concentrated in molecular species of sphingomyelin in ram spermatozoa (27). Sphingomyelin containing polyenoic VLCFA (designated polyenoic VLCFA-sphingomyelin) was found to be a minor component of seminal plasma and was undetectable in the accessory glands (prostate gland and/or seminal vesicles) of male reproductive tracts. This phospholipid was absent from the ovaries of female animals. It is noteworthy that brain and retina are the only other tissues reported to contain significant amounts of polyenoic VLCFA, but these compounds are localized in phosphatidylincholine, rather than sphingomyelin (33, 34, 39-41). Polyenoic VLCFA-sphingomyelin was found to be exclusively present in the seminiferous tubules of dissected adult rat testes. This suggests that the polyenoic VLCFA-sphingomyelin in spermatozoa originates from testicular germinal cells and/or Sertoli cells.

Polyenoic VLCFA represented approximately 15% of the total fatty acids in testicular and spermatozoan sphingomyelin of adult animals. The polyenoic VLCFA composition of sphingomyelin was similar in the testes and spermatozoa of a particular mammalian species, but there was a higher concentration of the polyenoic VLCFA-sphingolipid in spermatozoa. The polyenoic VLCFA composition of testicular and spermatozoan sphingomyelin varied between animal types. In human spermatozoa, the polyenoic VLCFA were mainly n-6 derivatives with two to four double bonds and even-carbon chain lengths up to 32. Polyenoic VLCFA belonging to the n-3 and n-6 series with four, five, and six double bonds and even-carbon chain lengths up to 34 predominated in ram and bull testes and spermatozoa. Rat and boar testes and spermatozoa contained principally n-6 polyenoic VLCFA with three to five double bonds and even- and odd-carbon chain lengths up to 34.

The total ion chromatograms produced by GLC-MS analysis of polyenoic VLCFA isolated from the sphingomyelin of rat and boar testes and spermatozoa, contained some additional, previously unreported, broad peaks. Their mass spectra contained ions at m/z 80, 91, and 150, which are characteristic of n-6 polyenoic VLCFA (36, 37). Each had a strong high mass ion which was smaller than that in the mass spectrum of the adjacent polyenoic VLCFA. From their mass spectra and their chromatographic behavior, these peaks were tentatively assigned as hydroxylated polyenoic VLCFA. Fig. 1 represents the partial total ion chromatograms of the fatty acid methyl esters obtained from the sphingomyelin of adult bull and boar testes, indicating the hydroxylated fatty acids.

Further confirmation that these broad peaks were hydroxylated polyenoic VLCFA was obtained after preparation of the PFP derivatives. Partial total ion chromatograms of derivatized polyenoic VLCFA methyl esters are presented in Fig. 2 for rat and boar testicular sphingomyelin. The PFP derivatives chromatographed as sharp peaks, and their mass spectra contained strong molecular ions as well as ions from the loss of pentafluoropropionic acid. These chromatograms show that for every polyenoic VLCFA with 28 or more carbon atoms there was a hydroxylated analogue. There were only minor differences in the composition of the fatty acids in the samples derived from the rat and boar.

The position of hydroxylation was not directly apparent from the mass spectra. Accordingly, a sample of the fatty acids from the sphingomyelin of rat testes was hydrogenated. Mass spectra were obtained of the hydroxylated C28 and C30 methyl esters that were produced. Fig. 3 shows the mass spectrum of hydroxylated C28:1 (m/z 74) methyl ester and its reduction product, hydroxylated C28:0 methyl ester. The McLafferty rearrangement ions of m/z 74 and 87, observed in the mass spectra of saturated methyl esters, were not present, but instead ions at m/z 90 and 103. These ions were used by Carballeira and Lopez (42) to identify 2-hydroxylated saturated fatty acids (as methyl esters) in marine sponges. The hydroxylated polyenoic VLCFA isolated from the sphingomyelin of rat and boar testes and spermatozoa were thus assigned as 2-hydroxylated. Unfortunately, the optical isomerism of the testicular and spermatozoan 2-hydroxylated polyenoic VLCFA could not be determined because the amount of sample available was too low. Naturally occurring 2-hydroxylated fatty acids have previously been found to possess the R configuration (42), and presumably this also applies to the 2-hydroxylated derivatives isolated in the present study.

To provide additional evidence that these 2-hydroxylated polyenoic VLCFA were associated with sphingomyelin, a FAB mass spectrum was obtained on a sample of the boar testicular sphingomyelin region after isolation by TLC. Protonated molecular ions of sphingomyelin containing saturated fatty acids were observed, but none for the hydroxylated polyenoic VLCFA species. The low mass region contained ions at m/z 125, 166, and 184 which are characteristic of a phosphocholine skeleton (38). Ions from other phospholipid skeletons were not detected in the spectrum. FAB-MS is often an unpredictable technique and there may be one of several reasons for the nonappearance of the molecular ions. The hydroxy group may inhibit the formation of protonated molecular ions, the protonated molecular ions may fragment before detection, or the protonated molecular ions of the saturated fatty acid species may suppress the formation of ions of other species present.

Hydrolysis of the boar testicular sphingomyelin fraction with sphingomyelinase, to remove the phosphocholine, gave a product with the same TLC relative retention as ceramide. A portion of the product was transmethylinated with pentafluoropropionic anhydride, and the fatty acid methyl esters analyzed by GLC-MS. 2-Hydroxylated fatty acids with zero to six double bonds and odd- and even-carbon chain lengths from 16 to 34 were identified. The remainder of the product was converted to a trimethylsilyl derivative and analyzed by GLC-MS. A series of ceramides containing fatty

---

Footnote:

2 Portions of this paper (including "Experimental Procedures," Figs. 1-4, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
acids such as C22:6, C24:0, and C26:0 were recognized by their molecular ions and characteristic fragmentation ions as described by Samuelsson and Samuelsson (43). Ceramides containing hydroxylated fatty acids including hydroxylated C24:0 (mass spectrum m/z 769 (M)+, 458, 283, 73), C24:1, C24:2, C23:2, and small amounts of C24:3,4,5,6 (mass spectrum m/z 785 (M-TMSOH)+, 592, 283, 73) and C25:0,1,6 (mass spectrum m/z 813 (M-TMSOH)+, 620, 283, 73) were identified. Unfortunately, the higher homologues above C26 were involatile, even with a short, lightly loaded capillary chromatography column operating at its temperature limit.

Acid hydrolysis of the boar testicular sphingomyelin gave a product with the same TLC mobility as lysosphingomyelin. The FAB mass spectrum (shown in Fig. 4) contained ions at m/z 437 and 465 which can be rationalized to belong to lysosphingomyelin containing two long chain bases, i.e. C18:1 and C18:2 (sphingosine).

Together these separate analyses confirm that the 2-hydro-xylated polyenoic VLCFA are indeed incorporated in molecular species of sphingomyelin found in rat and boar testes. To our knowledge, this is the first report that 2-hydroxylated polyenoic VLCFA exist in nature. 2-Hydroxylated VLCFA have been detected in the sphingolipids of mammalian brain (44, 45) and in the phospholipids of marine sponges (42, 46); however, these are saturated and monoenoic derivatives.

The polyenoic VLCFA content of sphingomyelin in rat testes varied from birth to sexual maturity, as depicted in Table 1. Polyenoic VLCFA-sphingomyelin was not present in the testes in 10- and 20-day-old animals. Sphingomyelin containing non-hydroxylated polyenoic VLCFA (C28:4,5,6, C29:5,6,7, and C30:5,6,7) was initially detected in rat testes 25 days after birth. At 30 days of postnatal life, 2-hydroxylated polyenoic VLCFA (C28:4,5,6, C29:5,6,7, and C30:5,6,7) were observed in addition to the non-hydroxylated derivatives in the sphingomyelin. From 35 to 50 days, the sphingomyelin contained non-hydroxylated and 2-hydroxylated polyenoic VLCFA which were n-3 hexaenoic and n-6 tetraenoic and pentaenoic derivatives with carbon chain lengths from 24 to 32. The non-hydroxylated and 2-hydroxylated n-3 hexaenoic VLCFA were no longer detectable in the testicular sphingomyelin of 55- and 60-day-old animals, unlike the corresponding n-6 tetraenoic and pentaenoic VLCFA. Table 1 indicates that the total amount of polyenoic VLCFA associated with sphingomyelin in rat testes increased approximately 19-fold from 25 to 40 days after birth (mainly due to an increase in non-hydroxylated and 2-hydroxylated C26:4,5,6 and C28:5,6,7) and then remained static to 60 days. The total amount of 2-hydroxylated polyenoic VLCFA relative to non-hydroxylated polyenoic VLCFA in the testicular sphingomyelin increased with maturation (Table 1). Differentiation of testicular germinal cells (spermatogonia → spermatocytes → round spermatids → condensing spermatids → spermatozoa) in rats is complete about 50 days after birth, with the formation of round and condensing spermatids at approximately 30 and 40 days, respectively (47, 48). The production of non-hydroxylated and 2-hydroxylated polyenoic VLCFA-sphingomyelin in rat testes after 25 days of life may be associated with spermatid development. It is also feasible that Sertoli cells have the capacity to produce polyenoic VLCFA-sphingomyelin at this time. There is evidence that polyenoic VLCFA are synthesized by chain elongation and desaturation of shorter-chain n-3 and n-6 precursors in rat testes (20, 22, 23, 25) and other tissues (49-52). Polyenoic VLCFA are presumably introduced into the sphingomyelin molecule via the direct transfer of ceramide to phosphocholine from phosphatidylcholine by phosphati- dylcholine-ceramide cholinephosphotransferase (53, 54). It is not known whether the polyenoic VLCFA become 2-hydroxylated in the unesterified form or after incorporation into ceramide or sphingomyelin. The synthesis of non-hydroxylated and 2-hydroxylated polyenoic VLCFA-sphingomyelin in the testes clearly warrants further investigation.

Sphingomyelin containing polyenoic VLCFA is likely to play a significant role in male reproductive physiology because of its exclusive presence in the testes and spermatozoa and its unique structure. This phospholipid may be crucial in maintaining membrane integrity of spermatozoa and facilitate their survival in the female reproductive tract. There is considerable evidence that sphingolipids and their breakdown products are important in cellular regulation (55-59). It is therefore tempting to speculate that polyenoic VLCFA-sphingomyelin and/or its polyenoic VLCFA-catabolism products (unesterified fatty acids and ceramide) may be metabolically active compounds and involved in the regulation of sperma- tozoan events such as motility, the acrosome reaction or membrane fusion during fertilization. The presence of 2-hydroxylated polyenoic VLCFA in the testicular and sper- matozoa sphingomyelin of some animal species is an additional complicating factor.

Acknowledgments.—We thank Peta L. Knappman for typing the manuscript. We are grateful to Professor B. P. Setchell of the Department of Animal Sciences, Waite Agricultural Research Institute, University of Adelaide, for helpful discussions and to Dr. C. Easton of the Department of Organic Chemistry, University of Adelaide, for the analysis of molecular species of sphingomyelin by MS.

REFERENCES
1. Carpenter, M. P. (1971) Biochim. Biophys. Acta 231, 52-79
2. Coniglio, J. G. (1977) in "The Testes" (Johnson, A. D., and Gomes, W. R., eds) Vol. 4, pp. 425-449, Academic Press, New York
3. Poulos, A., Darin-Bennett, A., and White, I. G. (1973) Comp. Biochem. Physiol. 46B, 541-549
4. Poulos, A., Brown-Woodman, P. D. C., White, I. G., and Cox, R. G. (1975) Biochim. Biophys. Acta 388, 12-18
5. Coniglio, J. G., Whorton, A. R., and Beckman, J. K. (1977) in "Function and Biosynthesis of Lipids" (Bazan, N. G., Brenner, R. G., and Giusto, N. M., eds) pp. 575-589, Plenum Publishing Co., New York
6. Marzouki, Z. M. H., and Coniglio, J. G. (1982) Biol. Reprod. 27, 312-319
7. Lewis, W. M., Northrop, C. A., Harrison, F. A., and Cox, R. W. (1983) J. Exp. Physiol. 68, 221-231
8. MacDonald, M. L., Rogers, Q. R., Morris, J. G., and Cupps, P. T. (1984) J. Nutr. 114, 719-726
9. Didolkar, A. K., and Roychowdhury, D. (1980) J. Reprod. Fertil. 65, 275-278
10. Dic, C. J., Habberfield, A. D., Sullivan, M. H., and Cooke, B. A. (1984) Biochem. J. 219, 529-537
11. Mizumura, K., Sato, J., and Kumazawa, T. (1987) Pflügers Arch. 408, 565-572
12. Bilsanska, B., and Wojtusiak, A. (1988) Folia Histochim. Cytobiol. 26, 53-59
13. Meisel, S., and Turner, K. O. (1984) J. Exp. Zool. 231, 283-288
14. Atten, R. J., and Kelly, R. W. (1986) J. Reprod. Fertil. 73, 139-145
15. Jackson, C. L., Nuzzo, N. A., Wilson, L., and Zaneveld, L. J. D. (1987) J. Androl. 8, 74-82
16. Oliw, E. H., and Sprecher, H. (1989) Biochim. Biophys. Acta 1002, 283-291
17. Beckman, J. K., Gray, M. E., and Coniglio, J. G. (1978) Biochim. Biophys. Acta 530, 267-274
18. Beckman, J. K., and Coniglio, J. G. (1979) Lipids 14, 282-287
19. Grogan, W. M., Farnham, W. F., and Szopiak, B. A. (1981) Lipids 16, 401-410
20. Bridges, R. B., and Coniglio, J. G. (1970) J. Biol. Chem. 245, 46-54
21. Beckman, J. K., and Coniglio, J. G. (1980) Lipids 15, 389-394
22. Grogan, W. M., and Lam, J. W. (1982) Lipids 17, 605-611
23. Grogan, W. M., and Huth, E. G. (1983) Lipids 18, 275-284
Novel Molecular Species of Sphingomyelin in Testes and Spermatozoa

EXPERIMENTAL PROCEDURES

Chemicals—Silicon acid (373 mg), 2,6-dim-1-butyl-4-methylyphenol, 2',4'-
di-butyldithiocarbamate and sphingomyelin (from Streptomyces sp.) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. DEAE-cellulose DE 52 was from Whatman Bio-
System Ltd., Maidstone, Kent, U.K. Silica gel 60 (40-63-μm mesh) was obtained from E. Merck, Darmstadt, Germany. The source of polar-tailed phosphodiester and N,N-dimethyltrifluoroacetamide was Procter Chemical Co., Rockford, Ill. U.S.A. Palladium on activated carbon (20%, w/w) was from Altech Chemical Co., Milpitas, Calif., U.S.A. and melittin and methyl ester (Eggpeptide) was from Alltech Associates Pty Ltd., Summer Hill, N.S.W., Australia. All other chemicals were of reagent grade, and solvents were distilled prior to use.

Collection of Testes and Spermatozoa—Neonatal male Porton rats were obtained from females of known gestational time and given access to a fasciating diet for 4 to 7 days. Whole animals were provided with a pellet diet (Cherstree, Aylesford, South Australia, Australia) and water ad lib. Rats (aged 10–15 days) were killed by carbon dioxide asphyxiation and the testes rapidly excised in saline in case, testes were mechanically dissected to separate seminiferous tubules from interstitial Leydig cells. Spermatocytes were collected from the cauda epididymis of mature rats (40–50 days) by in vivo stimulation with a 0.15 M NaCl wash (via the vas deferens). Adult rats, bulls and boar testes were obtained from the local abattoir. Semen was collected from adult rams by using an artificial vagina and from adult bulls and boars by electrical stimulation with a bipolar stereotaxic electrode. The semen was filtered to remove seminal gel and semen was obtained by massage from normal adult subjects. Spermatocytes were isolated by diluting semen with 5 volumes of 0.9% (w/v) NaCl and centrifuging at 700 g for 15 min at room temperature.

Extraction and Analysis of Lipids—Lipids were extracted from small amounts of testes (1–5 g) and spermatozoa (0.5 g) by the method of Folch et al. (33). For extraction of lipids from larger amounts of tissues (50 g), the procedure of Bligh and Dyer (32) was adopted, except for the substitution of 0.2 M HCI for water at the partitioning stage. Solvents used for extraction and subsequent analyses of lipids contained the antioxidant 2,4,6-tri-1-butyl-4-methylphenol (0.005%, w/v). The total lipid extracts were separated into neutral lipid, glycolipid and phospholipid fractions by silicic acid column chromatography and the phospholipids were resolved into non-esterified and acidic components by DEAE-cellulose DE 52 column chromatography as described previously (33,34). The neutral lipids and phospholipids were both further resolved by preparative TLC on 25 cm x 20 cm, 0.5% silica gel plates (200 μm) (Chromarods) in heptane/diethyl ether/acetic acid (40:10:1, by volume) or chloroform/methanol/water (90:10:1, by volume) for neutral lipids and chloroform/methanol/water (90:10:1, by volume) for phospholipids. The lipid zones were located under ultraviolet light after spraying the plates with 0.25% (w/v) rhodamine 6G in 95% (v/v) ethanol and were eluted from the silica gel with chloroform/methanol/acetone (35:15:2, by volume) as described by Arvidsson (35). The resulting extracts were partitioned with CHCl₃ to remove the CHCl₃ and then washed with water (1:1, v/v). Identification of the various lipids was based on a comparison of their TLC mobility with that of authentic standards.

Sphingomyelins hydrolysis of sphingomyelin was carried out by employing a commercial preparation of the enzyme (from Streptomyces sp.). The lipid sample (1 mg of water-saturated diethyl ether elution) was converted into mg of pentafluoropropanionic anhydride (50 μl). The lower phase (0.6 mg of potassium phosphate buffer (pH 7.4) containing 2 mg ml⁻¹ of N,O-bis(trimethylsilyl) trifluoroacetamide was removed by suction and washed with 1.75 ml of hexane. In some cases where less than 0.6 mg of water and 8 ml of chloroform/methanol (2:1, v/v) were added to afford partitioning. The lower phase was concentrated and applied to a silica gel 60 TLC plate, which was developed in chloroform/methanol/water (90:12:1, by volume). Derivatives were separated by liquid chromatography on a silica gel 60 TLC plate, which was developed in chloroform/methanol/water (90:12:1, by volume). Lipid products obtained were identified by thin-layer chromatography and unextracted fatty acids.

Portions of the lipid samples (1–10 μg) were transferred to 1 ml of 0.2 M NaOH in methanol at 75°C for 4 h and the labeled fatty acid methyl esters recovered after acidification (0.1 ml of 1 M HCl and 2 ml of hexane). In some cases 7 ml of anhydrous potassium chloride (25% by volume) and 1 ml of hexane were added and mixing vigorously under H₂ gas for 4 h at room temperature. The suspension was added to a 0.6 M NaOH solution and the phases were separated by liquid chromatography on a silica gel 60 TLC plate, which was developed in chloroform/methanol/water (90:12:1, by volume). The lipid products obtained were identified by thin-layer chromatography and unextracted fatty acids.

Brenton S. Robinson, David W. Johnson and AT Pouls

Supplementary Material

Novel Molecular Species of Sphingomyelin Containing 2-Hydroxylated Polyenylacyclic Long-Chain Fatty Acids in Neonatal Rats and Spermatozoa

Brenton S. Robinson, David W. Johnson and AT Pouls
**Novel Molecular Species of Sphingomyelin in Testes and Spermatozoa**

Mass spectrometry (MS) was conducted on a JEOL JMS-SX500 mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph, JMS-HSIOA fast-atom-bombardment (FAB) ion source and JEOL JMS-DX4030 data system. Electron-impact mass spectra of fatty acid methyl esters and ceramide trimethylsilyl derivatives were obtained using He gas as the carrier and an ionization voltage of 70 eV. The GLC conditions outlined above were employed to analyse fatty acid methyl esters. The n-series number of polyenic fatty acids was determined by the technique described previously (28,37). Analysis of ceramide trimethylsilyl derivatives was performed on a Scientific Glass Engineering fused-silica BP-1 column (0.53 mm i.d. x 30 m) at 300°C. The detector was maintained at 320°C for 50 min. The injector and ion-source temperatures were 320°C and 280°C respectively. FAB mass spectra of lyso-sphingomyelin were obtained in the positive-ion mode using He gas and a primary ion beam of 6 keV. The instrument was calibrated from m/z 100 to 1700 using ultramark. The lysosphingomyelin was dissipated in a strip of glycerol and spread on the ion target. Molecular species of sphingomyelin were analysed by collision-activation mass-analysed ion-mobility-energy spectroscopy of ions produced by FAB (38). For these studies mass spectra were measured as a known Generator JAF 240 mass spectrometer operating in the positive-ion FAB mode. He gas was used in the source with a primary beam energy of 6 keV.

**FIG. 1.** GLC-MS of sphingomyelin-bound polyenic VLCFA from bull and boar testes. Sphingomyelin isolated from adult bull (A) and boar (B) testes was transterfied and the fatty acid methyl esters analysed by GLC-MS as described under “Experimental Procedures”. Partial total ion chromatograms are shown. The polyenic VLCFA methyl esters shown in (B) are all n-6 isomers. For convenience the original labelling of the peaks has been retained; e.g. "32:6 n-3" means "(C32:6, n-3)." Abbreviation used: OH, hydroxylated.

**FIG. 2.** GLC-MS of sphingomyelin-bound polyenic VLCFA from rat and boar testes after derivatization. Adult rat (A) and boar (B) testicular sphingomyelin was transterfied and then treated with pentafluoropropionic anhydride. The FFA fatty acid methyl esters were analysed by GLC-MS as described under "Experimental Procedures". Partial total ion chromatograms are shown. The polyenic VLCFA methyl esters shown in (B) are all n-6 isomers unless labelled differently. For convenience the original labelling of the peaks has been retained; e.g. "32:6 n-3" means "(C32:6, n-3)." Abbreviation used: OPP, pentafluoropropionate.

**FIG. 3.** Electron-impact mass spectra of 2-hydroxylated polyenic VLCFA and its hydroxylated product from rat testicular sphingomyelin. Electron-impact mass spectra of 2-hydroxylated C28:4, n-6 methyl ester (A) and 2-hydroxylated C28:4 methyl ester (B) from rat testicular sphingomyelin were obtained as described under "Experimental Procedures".
Novel Molecular Species of Sphingomyelin in Testes and Spermatozoa

Fig. 4: FAB mass spectrum of boar testicular lysosphingomyelin. The FAB mass spectrum of lysosphingomyelin produced by acid hydrolysis of boar testicular sphingomyelin was obtained as described under "Experimental Procedures".

Table 1: Developmental changes in the acylglycero lysophospholipid content of spermatozoa at various stages of development.

| Stage | PL | GL | PS | PI | DPPC |
|-------|----|----|----|----|------|
| 5-day | 5.6 | 0.5 | 0.3 | 0.1 | 0.2  |
| 7-day | 4.8 | 0.7 | 0.4 | 0.2 | 0.3  |
| 9-day | 4.2 | 0.8 | 0.5 | 0.3 | 0.4  |

Footnotes: All data are expressed as mean ± SEM. *P < 0.05 compared with 5-day group.

The table shows the acylglycerol lysophospholipid content of spermatozoa at various stages of development. The content is expressed as mean ± SEM. Significant differences are indicated by *P < 0.05 compared with the 5-day group.