Lipid remodelling during epididymal maturation of rat spermatozoa

Enrichment in plasmalogen choline containing long-chain polyenoic fatty acids of the $n-9$ series

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In their transit from the caput to the cauda segments of the epididymis, rat spermatozoa undergo significant modifications in lipid content and composition. The amount of lipid phosphorus per cell decreases, and most lipid classes show specific changes in their constituent fatty acids. A depletion of phosphatidylcholine and phosphatidylethanolamine, concomitant with a virtually unchanged amount of the corresponding plasmalogens, are the major alterations, plasmalogen choline thereby becoming the major phospholipid. Diphosphatidylglycerol, sphingomyelin and the phosphoinositides decrease to a lesser extent or do not change at all, also resulting in relative increases with sperm maturation. Concerning the fatty acids, the proportions of oleate ($C_{18:1, n-9}$) and linoleate ($C_{18:2, n-6}$) in most lipids decrease on movement of sperm from caput to cauda, augmenting in turn the proportions of longer-chain (C_{20} to C_{24}) and more unsaturated fatty acids. Docosapentaenoate ($C_{22:5, n-6}$) is a major acyl chain present in all lipids at both stages, but uncommon long-chain polyenoic fatty acids of the $n-9$ series are also present, being almost exclusively found in the choline glycerophospholipids. These fatty acids are found to undergo the most significant changes during sperm maturation. They are minor components of plasmalogen choline in immature spermatozoa, but increase severalfold on maturation, representing more than half of the acyl chains of this major lipid in cells from the cauda. The high concentration of $n-9$ polyenes in mature sperm plasmalogen choline raises intriguing questions on the possible role epididymal cells may play in providing spermatozoa with such an unusual phospholipid. These plasmalogen choline could contribute to the characteristic lipid domain organization of the mature spermatozoa plasma membrane.

INTRODUCTION

After leaving the testes and during their transit through the male reproductive tract, mammalian spermatozoa are known to undergo important morphological, physiological and biochemical modifications that contribute to their maturation [1], eventually leading to motile gametes potentially able to fertilize an egg. A key role in the accomplishment of such maturation is played by the epididymis. This highly convoluted duct displays regional variations [2] in the morphology and metabolic properties of its epithelium, which are relevant to the progressive development of mature sperm characteristics. The functional activity of the epididymis and sperm maturation are intimately related, the epididymis depending on testicular androgen for the maintenance of its epithelial cells in a differentiated state. Major modifications of spermatozoa during epididymal maturation arise from a dynamic process in which the sperm cells in part interact with the epididymal environment and in part undergo internal reorganizations; most of the alterations that take place in the epididymal transit are thought to contribute to the completion of sperm maturation, but the exact nature and relative importance of each of such contributions remain as challenging and still unanswered questions [3].

An important set of maturational changes of spermatozoa entail modification of their plasma membranes. Some, if not most, of these alterations of sperm surface are now known to

result from direct hormonally controlled epithelial cell action on the spermatozoa. For example, these epithelial cells synthesize proteins that are actually inserted into specific sperm plasma membrane domains (e.g. see [4,5]) and secrete enzymes such as the protease(s) that perform the final processing of certain proteins (as shown recently for the protease that acts on PH-30 [6], an endogenous sperm cell protein involved in sperm-egg fusion). Another important, although not so well defined, aspect of spermatozoal maturation involves changes in their lipid constituents. Research on the composition and metabolism of lipids at different stages of sperm maturation in a variety of mammals (e.g. see [7–16]) has shown that changes occur as sperm cells mature. Among the characteristics of fertile sperm lipids delineated in most of these studies, the presence of plasmalogens and other ether-linked phospholipids and the high degree of unsaturation of their phospholipid acyl chains, rich in C_{22} polyunsaturated fatty acids ($C_{22:5, n-6}$ and/or $C_{22:6, n-3}$ depending on the species) have long been acknowledged. The physiological significance of these characteristics of lipid molecules in terms of mature sperm functions, and the possible relationships between functional maturation of spermatozoa within the epididymis and the concomitant changes in lipid components, remain to be established. The present study describes some as yet uncharacterized features of the phospholipids and fatty acids of rat spermatozoa, and the effects of epididymal maturation on such components. The most conspicuous changes in lipids are shown

Abbreviations used: CGP and EGP are used to denote the (diradyl)-choline and -ethanolamine glycerophospholipids respectively; the terms phosphatidyl-, plasmalogen- and plasmalogen- are reserved respectively for the diacyl-, alk-1-enyl-2-acyl- and alkyl-2-acyl- subclasses of the corresponding glycerophospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; DPG, diphosphatidylglycerol; FAME, fatty acid methyl esters.

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to affect the choline glycerophospholipids (CGP), with plasmenylcholine becoming the major phospholipid of mature sperm. The most prominent effect on fatty acids is shown to consist of an enrichment in uncommon long-chain polyenes of the $n-9$ series specifically in this plasmenylcholine.

MATERIALS AND METHODS

Albino Wistar rats fed on a Purina lab diet and water *ad libitum* were kept at $24 \pm 2^\circ\text{C}$ with a photoperiod of 14 h light/10 h dark from birth. Males weighing 350–370 g were killed by decapitation and their epididymes were rapidly removed. After carefully trimming adventitious tissues, epididymes were cut into three sections: proximal (caput), medial (corpus) and distal (cauda), corresponding to regions defined in [2]. To obtain spermatozoa from the caput and cauda sections, these were cut into small pieces and suspended by gentle stirring in a pH 7.4 medium containing 113 mM-KCl, 15 mM- NaHCO_3 , 3 mM- MgCl_2 , 1 mM-EDTA and 20 mM-Tris. The suspension was filtered through a fine mesh silk cloth and centrifuged at room temperature for 10 min (800 g). The sperm cells were washed once and resuspended in the above medium. Aliquots of this suspension were conveniently diluted with 0.15 M-NaCl in 0.2% formaldehyde and used for counting of sperm in an haemocytometer. The remainder was centrifuged at 13000 g for 30 min at 4°C .

Lipid extracts were prepared from the pellets, partitioned, and washed according to Folch *et al.* [17]. The residues were further treated with acidified chloroform/methanol (2:1, v/v) for polyphosphoinositide extraction and quantification [18]. Lipid phosphorus was determined as described by Rouser *et al.* [19]. Cholesterol was estimated using an enzymic/colorimetric procedure [20]. Some of the lipids (diacylglycerols, triacylglycerols etc.) were quantified by including an internal standard in the g.l.c. analysis of their fatty acids.

Phospholipid were separated into classes by two-dimensional t.l.c. [19]. Lipid spots were located by exposing the plates to I_2 vapour (for phosphorus measurements) or under u.v. light after spraying with 0.05% dichlorofluorescein in methanol (for fatty acid analyses).

To study the levels and fatty acid compositions of their major subclasses, choline and ethanolamine glycerophospholipids (CGP and EGP respectively) were eluted as described by Arvidson [21] and subjected to acid treatment, which resulted in the release of aldehydes from plasmalogens, giving rise to a lysophospholipid spot (from the plasmenyl subclass) that was easily separable from the corresponding unreacted phosphatidyl plus plasmanyl phospholipid by t.l.c. (in chloroform/acetone/methanol/acetic acid/water, 40:15:13:12:7.5, by vol.). The acid treatment consisted of vortex-mixing the lipid samples for 1 min with 100–500 μl (depending on sample size) of 0.5 M-HCl in chloroform/methanol (2:1, v/v) (or other volatile solvent), followed by rapid evaporation of the solvent mixture under N_2 and spotting of the sample on t.l.c. plates.

Fatty acid methyl esters (FAME) for g.l.c. analyses were prepared from lipids by means of 14% BF_3 in methanol [22]. G.l.c. analyses were performed using 2 m $\times$ 2 mm internal diam. glass columns packed with 15% OV-275 on Chromosorb WAW, 80/120 mesh (Varian, Sunnyvale, CA, U.S.A.), and N_2 as the carrier gas. The initial and final column oven temperatures were 160 and 220°C respectively, and a linear temperature programme was applied (usually $5^\circ\text{C}/\text{min}$). Injector and flame ionization detector temperatures were 220 and 230°C respectively, and the electrometer was operated in the dual-differential mode. Peak quantification was performed by electronic integration.

The unusual long-chain fatty acids appearing as the major components of CGP were identified as $n-9$ polyenes by analytical chromatographic procedures [23] essentially similar to those described elsewhere for identification of a novel group of fatty acids in retina [24]. Briefly, the fatty acids (as FAME) were first subjected to argentation t.l.c. to separate them according to their degree of unsaturation. Their behaviour in comparison with standards enabled the number of double bonds to be established, since they migrated as trienoic, tetraenoic and pentaenoic FAME. These same fractions were then subjected to catalytic hydrogenation, which allowed us to establish the number of carbon atoms present in the fatty acids (20, 22 or 24). They were also subjected to oxidative ozonolysis [25] in order to establish the positions of their double bonds.

The food pellets routinely fed to the rats were also analysed by g.l.c. Nearly 40% of their fatty acids were in the form of $\text{C}_{18:2, n-6}$, the rest being 24.4% $\text{C}_{18:1, n-9}$, 18.5% $\text{C}_{16:0}$, 2.2% $\text{C}_{16:1}$, 5.4% $\text{C}_{18:0}$ and 4.6% $\text{C}_{18:3, n-3}$, showing that the dietary supply of essential fatty acids was within normal ranges. The sperm lipid analyses were repeated on several occasions using adult rats of different ages and from different sources (at least three samples per analysis). Each sample contained the epididymal spermatozoa from several rats (usually four).

RESULTS

Lipids of rat spermatozoa during epididymal maturation

The lipids of rat spermatozoa underwent profound qualitative and quantitative modifications during the epididymal transit (Table 1). The amount of phospholipids per cell decreased by over half (54%) from the caput to the cauda epididymidis, but some of the phospholipid classes and subclasses decreased more than others and, as a result, some of the components were enriched relative to others. A considerable fall in the concentration of the phosphatidyl, and a virtually unchanged level of the plasmenyl, subclasses within the two major phospholipid classes, CGP and EGP, were the hallmarks of epididymal maturation. As a consequence of these changes, the plasmalogens became the major lipid components. The proportion of plasmenylcholine increased from about one-third ($28.8 \pm 6.8\%$) to more than one-half ($52.1 \pm 4.0\%$) of the total CGP, and that of plasmenylethanolamine from about one-quarter ($18.6 \pm 3.7\%$) to $42.1 \pm 6.0\%$ of the total EGP. Adding up both plasmalogens, they accounted for a relative increase from $\sim 19\%$ of the total phospholipids of spermatozoa in the caput to 38% in the cauda, with plasmenylcholine becoming the major phospholipid (27%, Table 1) of caudal spermatozoa.

Other phospholipids whose proportions increased during epididymal maturation due to relatively larger changes in other components were diphosphatidylglycerol (DPG) and sphingomyelin. The serine glycerophospholipids in turn were decreased the most, becoming a minor phospholipid class in spermatozoa from cauda, while the proportion of phosphatidylinositol (PI) remained unchanged. Interestingly, the polyphosphoinositides were the only lipids to remain at the same levels in caput and cauda spermatozoa, thus increasing their proportion significantly. The levels of non-esterified fatty acids were surprisingly high in caput spermatozoa, and this fraction underwent the greatest decrease in the progression from caput to cauda (81.8 ± 6.7 to $30.5 \pm 5.2 \mu\text{mol}/100 \mu\text{mol}$ of total lipid phosphorus; or 15.1 ± 1.2 to $2.6 \pm 0.4 \mu\text{mol}/10^8$ cells). Even though part of this considerable amount may have been produced artefactually by the activity of endogenous enzymes during preparation and handling of spermatozoa, the differences between caput and cauda are noteworthy, suggesting differences in the activity of

Table 1. Phospholipids of rat epididymal spermatozoa

Results are given as means $\pm$ S.D. from six samples. The total amount of lipid phosphorus per 10^9 cells was 18.4 ± 2.1 and 8.5 ± 1.0 μ mol for caput and cauda spermatozoa respectively. These values were used to estimate the contents given in the right hand columns. Significant differences are indicated (* $P < 0.05$). CP and EP are used to denote plasmacylcholine and plasmacylethanolamine respectively.

	Phospholipid content			
	(% of total lipid phosphorus)		(μ mol/ 10^9 cells)	
	Caput	Cauda	Caput	Cauda
CGP { PC	46.2 ± 1.2 {	32.8 ± 3.1	$52.0^* \pm 1.8$ {	$24.9 \pm 2.1^*$
CP				
EGP { PE	30.6 ± 1.2 {	24.9 ± 1.1	$26.9^* \pm 0.8$ {	$15.6 \pm 1.6^*$
EP				
Lyso-PC	6.0 ± 0.8	$4.0 \pm 0.7^*$	1.10	0.34^*
Lyso-PE	2.2 ± 0.4	2.3 ± 0.3	0.40	0.20^*
PS	4.0 ± 0.7	$1.5 \pm 0.3^*$	0.74	0.13^*
DPG	3.8 ± 0.4	$4.9 \pm 0.4^*$	0.70	0.42^*
PA	1.2 ± 0.3	1.1 ± 0.2	0.22	0.09^*
PI	4.3 ± 1.0	4.3 ± 1.0	0.79	0.37^*
PI phosphate	0.2 ± 0.02	$0.3 \pm 0.06^*$	0.03	0.03
PI bisphosphate	0.2 ± 0.01	$0.3 \pm 0.04^*$	0.03	0.03
Sphingomyelin	1.3 ± 0.4	$2.4 \pm 0.6^*$	0.24	0.20

such enzymes. The same pattern of decreasing levels was observed for the lysophospholipids. In contrast, triacyl- and diacyl-glycerols were minor components of sperm (2.5 ± 0.4 and 1.3 ± 0.4 μ mol/ 100 μ mol of lipid phosphorus respectively in cauda, and similar concentrations in caput sperm). The ratio of total phospholipid/total cholesterol was virtually unchanged (1.7 ± 0.2 and 1.9 ± 0.1 in caput and cauda respectively), indicating that both membrane components decreased to similar extents during the epididymal passage.

Changes in the fatty acids of CGP and EGP

Concomitant with its effects on phospholipids, epididymal maturation resulted in significant alterations in the proportions of major fatty acids in spermatozoa. The most prominent change consisted of a decrease in $C_{18:1, n-9}$ and a several-fold increase (both relative and absolute) in a group of trienoic and tetraenoic fatty acids of the $n-9$ series. Of all phospholipid classes, the uncommon polyenes in question were found to be most concentrated in the major CGP (Table 2). When the subclasses of CGP were separated, the $n-9$ polyenes were found to be very largely concentrated in the choline plasmalogen.

The $n-9$ polyenes added up to $\sim 6.0\%$ of the fatty acids of the total lipid of caput sperm, but increased to account for about 20% of the lipid of cauda sperm. Since the mass of phospholipid decreased by ~ 2 -fold, this ~ 3 -fold increase in $n-9$ polyenes was not only accounted for by relative decreases in other fatty acids (e.g. $C_{18:1, n-9}$) but represented a net enrichment. The lipid mainly responsible for these changes was plasmacylcholine, in which the fatty acids in question increased from 13.7 to 62% (~ 4.5 -fold) from caput to cauda sperm, in the presence of virtually unchanged levels of the phospholipid. As a result, the sum of $n-9$ polyenes increased from 6.0 to 30.4% of the fatty acids in total CGP.

The distribution of fatty acids in the major subclasses of CGP and EGP in spermatozoa from epididymal cauda showed a considerable specificity (Table 2). While $C_{22:5, n-6}$ was a major component of plasmacylethanolamine, the uncommon $C_{22, n-9}$ polyenes were highly concentrated in plasmacylcholine. They were also present, although in smaller proportions, in plasmacylcholine (results not shown) and phosphatidylcholine (PC), and

were even less common in the corresponding glycerophospholipids of ethanolamine. Arachidonic acid was a minor polyene in PC, but the major one in phosphatidylethanolamine (PE). Saturates such as $C_{16:0}$ and $C_{18:0}$ occurred in high proportions only in the phosphatidyl subclasses. They were minor components of the plasmalogens, where 50% of the hydrophobic chains were fatty aldehydes, almost entirely hexa- and octa-decanal (as observed by g.l.c.). The ratios between these two aldehydes were 26:74 in plasmacylcholine and 44:56 in plasmacylethanolamine from cauda spermatozoa (results not shown).

Long-chain C_{22} polyenes of the $n-9$ series were not detectable at all in the lipids of cauda epididymal boar spermatozoa analysed for comparison (results not shown). Docosahexaenoate ($C_{22:6, n-3}$) (virtually absent from rat sperm) and docosapentaenoate ($C_{22:5, n-6}$) (common to both) were the major polyenoic constituents of boar sperm lipids, in agreement with previously published data [16].

Fatty acid constituents of other lipids

The lipid classes displayed considerable specificity with respect both to their fatty acyl compositions and to the remodelling of this composition during epididymal maturation (Table 3). PI was unusual in that it contained the highest percentage of $C_{20:3, n-9}$ of all rat sperm constituents examined. Significant decreases in $C_{18:2, n-6}$ and $C_{20:3, n-6}$, concomitant with increases in $C_{22:3, n-9}$ and $C_{22:4, n-9}$, were observed in PI during sperm maturation. Besides the abundance of $n-9$ polyenes, rat sperm PI was also unusual in that it contained larger amounts of $C_{22:5, n-6}$ than of arachidonate ($C_{20:4, n-6}$). The minor serine glycerophospholipids had $C_{22:5, n-6}$ as their predominant acyl chain, followed by $C_{18:0}$, $C_{16:0}$, $C_{18:1}$ and $C_{20:4, n-6}$, all other fatty acids being lower than 2%. DPG characteristically had high levels (close to 80%) of mono- and di-enoic fatty acids, and low levels of saturates. The percentage of $C_{18:2, n-6}$ in DPG increased with sperm maturation to about the same degree as that of $C_{18:1, n-9}$ decreased. Some of the minor components of DPG, $C_{20:3, n-6}$, $C_{20:4, n-6}$ and $C_{22:4, n-6}$, also increased significantly. Oleate was the major component of the non-esterified fatty acid pool at both maturation stages. Both lyso-PC and lyso-PE had higher proportions of saturated fatty acids in sperm from caput than those from cauda epididymidis.

Table 2. Fatty acids of total lipids and of CGP and EGP from rat spermatozoa isolated from caput (A) and cauda (B) epididymis

Results are given as relative proportions (%), by weight) of the fatty acids shown (aldehydes excluded), and represent mean values from at least three separate groups of animals. Significant differences between A and B are indicated (* $P < 0.05$).

Fatty acid	Content (% by wt.)													
	Total lipids		EGP		PE†		Plasmenyl-ethanolamine		CGP		PC†		Plasmenyl-choline	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
C _{16:0}	13.5	10.4	15.1	14.5	16.6	18.3	2.3	4.0	13.5	9.7*	15.1	14.2	5.9	1.4
C _{16:1}	0.9	0.4*	0.5	0.5	0.5	0.6	0.3	0.2	0.8	0.2*	0.9	0.2	0.1	0.1
C _{18:0}	18.6	14.9	15.4	13.2	16.9	16.7	2.6	3.2	19.5	15.2*	22.0	22.8	7.5	1.1*
C _{18:1}	22.7	13.8*	17.6	6.4*	18.8	7.1*	7.0	4.6*	24.2	15.2*	25.6	18.7*	17.4	2.9*
C _{18:2, n-6}	4.5	5.3	1.7	0.9*	1.8	0.9	0.9	1.0	2.4	0.5*	2.5	0.7*	1.8	0.1*
C _{20:3, n-9}	0.7	0.7	0.7	1.0	0.5	0.6	2.2	2.1	0.4	0.3	0.5	0.4	0.1	0.1
C _{20:3, n-6}	0.5	0.5	0.2	—	0.2	—	0.4	—	0.2	—	0.2	—	0.3	0.1
C _{20:4, n-6}	9.9	7.9	21.6	23.3	22.0	27.2	17.8	12.6*	5.3	1.5*	5.1	1.9*	6.4	0.8*
C _{22:3, n-9}	0.8	2.1*	1.2	1.0	0.7	0.3	5.1	2.9*	0.8	3.1*	0.6	1.8*	1.6	5.5*
C _{22:4, n-9}	2.5	15.1*	2.3	3.6	1.6	1.5	8.8	9.5	3.2	25.2*	2.3	9.7*	8.0	53.6*
C _{22:4, n-6}	1.3	1.7	1.7	1.9	1.4	2.0	4.3	1.6*	1.4	2.0	1.0	1.6	3.2	2.8
C _{22:5, n-6}	21.7	24.5	19.5	30.8*	16.8	22.3*	43.5	54.3*	25.6	26.5	21.8	26.1	43.5	27.6*
C _{24:4, n-9}	1.9	2.0	2.3	2.3	2.0	2.1	4.4	3.0	2.0	1.8	1.6	1.3	4.0	2.8*
C _{24:5, n-6}	0.5	0.7	0.2	0.6	0.2	0.4	0.4	1.0	0.7	0.8	0.8	0.6	0.2	1.1

† The phosphatidyl lipids contained the minor plasmanyl subclasses in both cases.

Table 3. Fatty acids of lipids of rat spermatozoa isolated from caput (A) and cauda (B) epididymis

Results are presented as in Table 2. Abbreviations: DG, diacylglycerols; TG, triacylglycerols; NEFA, non-esterified fatty acids. Results are means ($n \geq 3$); significant differences between A and B are indicated (* $P < 0.05$).

Fatty acid	Content (% _o , by wt.)																	
	PI		PS		DPG		PA		DG		TG		NEFA		Lyso-PE		Lyso-PC	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
C _{16:0}	9.8	4.6*	11.0	16.0*	6.4	5.5	13.7	6.3*	5.2	3.9	15.5	18.8*	8.0	8.6	17.5	9.9*	17.5	6.8*
C _{16:1}	0.8	0.4	—	—	3.5	4.3	3.2	1.9*	1.5	1.6	7.3	7.9	1.7	1.8	1.7	1.8	2.1	1.2
C _{18:0}	33.5	39.6*	28.4	25.8*	3.1	2.6	26.2	28.3	19.7	22.5	5.2	5.4	14.9	25.2*	30.0	24.5*	30.6	22.2*
C _{18:1}	14.4	13.5	14.8	11.8*	45.7	30.1*	48.6	59.0*	35.7	48.3*	33.1	35.0	37.0	38.3	18.1	13.4*	19.6	26.7*
C _{18:2, n-6}	4.0	1.7*	1.0	0.5*	33.4	46.3*	1.8	1.1*	3.2	1.3*	32.2	25.2*	11.0	5.6*	3.1	1.2*	1.2	1.6
C _{20:3, n-9}	7.8	5.2*	0.1	0.2	—	—	0.4	0.1	—	—	0.4	0.2*	2.4	2.1	5.2	3.1	0.2	0.9
C _{20:3, n-6}	2.0	0.7*	0.3	0.5	2.5	4.4*	0.1	—	—	—	0.4	0.1*	0.6	0.2*	0.7	0.2*	0.2	—
C _{20:4, n-6}	8.9	8.0	6.0	4.2*	1.4	2.0*	2.1	0.6*	4.3	2.3	1.2	1.0	7.3	5.7	8.3	19.4*	2.9	3.8
C _{22:3, n-9}	1.9	4.0*	0.2	0.4	—	—	0.3	0.2	5.2	4.1	—	0.9	1.0	1.3	0.5	0.3	—	0.7
C _{22:4, n-9}	3.6	7.8*	0.4	0.5	—	—	0.3	0.2	6.7	8.5	1.2	1.9	3.5	3.5	3.3	3.2	2.7	10.9*
C _{22:4, n-6}	0.8	1.6*	1.2	1.4	—	0.1	—	—	0.4	0.7	—	0.4	0.3	0.2	0.5	0.3	1.4	2.0
C _{22:5, n-6}	11.7	11.5	34.8	36.9	1.5	1.8	3.2	2.1	16.8	5.4*	3.0	2.3	11.6	6.3*	10.3	21.6*	20.0	21.1
C _{24:4, n-9}	0.6	0.9	1.3	1.3	2.5	2.9	0.1	0.2	0.5	0.6	0.2	0.3	0.5	1.0	0.6	0.8	0.7	1.2
C _{24:5, n-6}	0.2	0.5	0.5	0.5	—	—	—	—	0.8	0.8	0.3	0.6	0.2	0.2	0.2	0.3	0.9	0.9

Arachidonate and docosapentaenoate increased significantly in lyso-PE (nearly 2-fold), whereas lyso-PC displayed a 3-fold relative enrichment in C_{22:4, n-9} with sperm maturation. Of all phospholipids examined, phosphatidate (PA) was the richest in oleate (~60%) and the poorest in polyunsaturated fatty acids. Diacylglycerols had a fatty acid pattern similar to that of PA, except for a somewhat higher proportion of polyenes. Triacylglycerols in turn were characterized by high percentages of oleate and linoleate, and very low levels of long-chain polyenes. Sphingomyelin did not undergo changes in its fatty acid composition upon rat sperm maturation. In both caput and cauda spermatozoa, the major components of sphingomyelin (results not shown) were C_{16:0} and C_{18:0} (each 23%), followed by C_{18:1}

(5.1%) and C_{20:0} (4.6%). A series of C₂₂ to C₂₅ saturated and monoenoic fatty acids (each about 4%) and some unidentified very-long-chain polyenes (all together, ~6% of the fatty acids) were also present in this lipid.

DISCUSSION

The results presented here show that, during their journey through the epididymis, rat spermatozoa undergo considerable lipid remodelling. The most dramatic of such changes involves a component that eventually becomes a major phospholipid, plasmenylcholine. This lipid, as well as plasmenylethanolamine, are clearly modified during the epididymal transit, since their

total amounts remain virtually constant while their fatty acid compositions change remarkably. In addition, most diacyl phospholipid classes, including major and minor ones, are shown to undergo both a decrease in their levels and a change in their fatty acid compositions. The decrease in lipid content per cell indicates that part of the membranes of the original spermatozoa is lost in the caput to cauda progression, and the changes in fatty acid composition suggest that such a loss is selective. One of the best known modifications selectively affecting epididymal sperm membranes involves the loss of a cytoplasmic droplet which, apart from plasma membrane, contains endoplasmic reticulum, lysosomes and peroxisomes. Such a loss of membrane is consistent with the parallel decrease in cholesterol (non-oxidizable) and phospholipid observed, and may also correlate with the changes in fatty acid composition.

Earlier work had shown both that ram and bull spermatozoa lose 25–50 % of their phospholipid and that the percentage of choline plasmalogen increases during the epididymal transit [9,12]. In those studies, there were marked differences in phospholipid and total fatty acid compositions between testicular and cauda epididymal spermatozoa [9,12], but not between the latter and ejaculated spermatozoa [12], indicating that the lipid pattern of caudal spermatozoa reflects that of sperm cells at their functional maturity. The presence of $C_{22:4, n-9}$ and other polyenoic fatty acids of the $n-9$ series in plasmalogen shown in the present study, and the several-fold increase in such fatty acids in this lipid on sperm maturation, are specific characteristics of rat sperm. Analysis of the fatty acids of individual lipids show that epididymal spermatozoa of bull [15] and boar [16] lack this type of fatty acid altogether, being instead rich in long-chain polyenoic fatty acids of the $n-6$ and $n-3$ series ($C_{22:5, n-6}$ and $C_{22:6, n-3}$ predominate, in particular in the ether-linked phospholipids). Since these two polyunsaturated fatty acids of epididymal sperm are qualitatively the same as abound in testes, in these animals only quantitative aspects of epididymal changes can be seen from lipid compositional studies. It is therefore a fortunate circumstance that, in the rat, a different class of polyunsaturated acyl group is incorporated into spermatozoa during maturation. This kind of fatty acid marker clearly shows, both quantitatively and qualitatively, that the epididymal passage plays an indispensable role in determining the final lipid composition of mature fertile spermatozoa.

Investigations to determine the relative contributions of the epididymal epithelial lining and the spermatozoa themselves to the changes in lipid content and composition of spermatozoa as they mature, and the metabolic pathways by which these changes take place, are still necessary. Concerning the mechanisms involved, both enzymic and non-enzymic processes are feasible. Removal of phospholipids from the surface of progressing spermatozoa, or even replacement of intact molecular species (e.g. a $C_{18:1}$ -rich for a $C_{22:4}$ -rich molecule), between spermatozoa and epididymal epithelial cells may take place, facilitated by phospholipid transfer proteins. Such proteins have been demonstrated to occur in the reproductive tract of rabbit, ram and boar, and one of their functions is to remove lipid droplets from epididymal spermatozoa [26]. In addition to this type of lipid traffic, enzymes produced in certain regions of the epididymal tract (made by specific epithelial cells or activated in sperm cells as they pass through such areas) may effect changes in lipids. The fact that the phospholipid classes that persist in spermatozoa after the epididymal passage differ from the original ones in fatty acid composition, i.e. in containing increasingly higher proportions of more elaborate (longer and more highly unsaturated) acyl groups as they mature, indicates that there are several distinct enzymic activities involved in the 'retailoring' of lipids, i.e. replacements of moieties in pre-existing lipid molecules.

These two mechanisms of membrane lipid turnover, protein-mediated replacement or removal of intact molecules and enzyme-controlled degradation or rearrangement of molecular moieties in pre-existing lipids, are not mutually exclusive, and may in fact co-exist.

Considering the type of enzymes involved in the remodelling of complex lipids in cells after their synthesis *de novo* in the endoplasmic reticulum, it is known that, in general, an important role is played by phospholipases and acyltransferases. Such enzymes handle fatty acyl groups, which are in turn products of desaturases (mostly microsomal) and elongases (microsomal and mitochondrial). Many unanswered questions arise when trying to apply these concepts to maturing sperm cells, since maturation entails the progressive loss, rather than the gain, of the capacity to synthesize molecular components. In the particular case of rat spermatozoa, besides the relative increase in plasmalogens, a selective modification of the acyl groups of these lipids occurs with maturation: whereas plasmalogencholines become rich in uncommon long-chain polyenes of the $n-9$ series during the caput to cauda progression, $n-6$ polyenes tend to be concentrated even more in plasmalogenethanolamine. Since the initial steps of plasmalogen synthesis are known to be located in peroxisomes [27], several intriguing problems need still to be resolved concerning how, where, when and why these lipids are synthesized. For example, it is still not known (1) in what cells such plasmalogens of spermatozoa are initially made; (2) what cells contain the enzymes needed to remodel the final acyl moieties of these lipids, introducing long-chain polyenoic fatty acids in place of shorter ones; (3) where along the male genital tract the peculiar long-chain polyenes that increase in spermatozoa with their maturation (whether $n-9$, $n-6$ or $n-3$ polyenes) are synthesized; and (4) in the case of rat sperm, what is the reason for long-chain polyenes of the $n-9$ series being so specifically concentrated in just one of the plasmalogens, suggesting either a remarkable degree of enzymic discrimination in the remodelling of this lipid or an unusually active turnover of its polyunsaturated acyl moieties.

Besides their characterization, the cellular (and subcellular) origin of the enzymes that modify the phospholipids of sperm within the epididymal tract also remains to be determined. Some of the enzymes of lipid metabolism mentioned previously may be endogenous for, and persist in, spermatozoa during the course of their maturation, while others must be provided by the epididymal epithelial lining. Despite being simple, highly specialized cells which, consistent with their function, have very few biosynthetic capabilities (they lack rough endoplasmic reticulum, a lysosomal system and peroxisomes), mature mammalian spermatozoa contain active phospholipase A_2 [28] and lyso-PC-acyltransferase [29]. Sperm from guinea pig cauda can use [^{32}P]P_i phosphate to label part of their lipids [30], and even after ejaculation, washed spermatozoa from bull show incorporation of ^{14}C -labelled fatty acids into neutral and polar lipids [31]. The very low concentration of $n-9$ polyenoic fatty acids we observed in rat testicular lipids and the several-fold increase these fatty acids displayed in the total lipid of spermatozoa with their epididymal maturation is consistent with the idea that a major part of the fatty acid constituents of sperm are synthesized within the epididymis.

The finding of considerable amounts of long-chain $n-9$ polyenes in the common laboratory rat ([23]; present results) contradicts the long-held belief that long-chain $n-9$ polyenoic fatty acids can only be observed in lipids of tissues or cells from animals with essential fatty acid deficiency. Our rats had the following characteristics: (1) they grew on diets that contained plenty of $C_{18:2, n-6}$ and $C_{18:3, n-3}$ fatty acids; (2) they were perfectly able to synthesize large amounts of $C_{22:5, n-6}$ (the major polyene

present in most membrane lipids of the mammalian reproductive tract, from the testes to the spermatozoa themselves) and $C_{22:6, n-3}$ (e.g. in retina [32] and brain); and (3) they were normally fertile, healthy and long-lived on such a diet, maintaining remarkably constant lipid and fatty acid compositions in their spermatozoa and epididymis from an early (2 months) to a very advanced age (27 months and more) (N. P. Rotstein, M. I. Avelaño & N. T. Vermouth, unpublished work). It is still possible that, since in the male rat the process of spermatogenesis is very active and continuous throughout its life, a partial deficiency of $n-3$ and $n-6$ precursors could occur within the epididymal tract, giving rise to a physiological increase in the production of $n-9$ polyenes. Besides being non-renewable through synthesis *in situ*, $C_{18:2, n-6}$ could in part be unavailable because of being esterified in certain lipids (it is the major component of sperm DPG, an important structural lipid), or merely because of being consumed in the synthesis of higher $n-6$ polyenes, such as $C_{22:5, n-6}$. The possibility that the lipid metabolism in the epididymis is quite demanding, thus partially exhausting the $n-6$ and $n-3$ polyenoic precursors, is also consistent with the unusual fatty acid composition of PI; instead of $C_{20:4, n-6}$ being predominant, as is typical of most mammalian tissues, significant amounts of $C_{20:3, n-9}$ and its C_{22} homologues were present in rat sperm in this phospholipid, which is known to display a high turnover rate of its polar and fatty acyl moieties in all vertebrate cells. The present findings thus serve to confirm the long-chain $n-9$ polyenoic fatty acids as normal, physiological products of metabolism.

One phospholipid component that virtually lacked long-chain polyenoic fatty acids of the $n-9$ series at both stages of epididymal maturation was DPG. The relative increase in this lipid (Table 1) and the significant changes it displayed in fatty acid composition (a remarkable enrichment in $C_{18:2, n-6}$) with sperm maturation (Table 3) were also interesting, and previously unreported, features of rat spermatozoa. DPG (2 mol of phosphorus/mol) was the fourth most abundant phospholipid on a molar basis, but on a weight basis it was the third most abundant, as 1 mol of DPG has two phosphatidyl moieties and hence the molecule is about twice as heavy and much bulkier than that of any other phospholipid. Even though DPG is a characteristic component of mitochondrial membranes, it has been reported to occur in high proportions in plasma membranes isolated from epididymal boar [16] and ram [28] spermatozoa. In ejaculated ram spermatozoa, PI and DPG on the one hand, and most of the EGP and CGP (mostly plasmalogen) on the other, appear to be located in the internal and the external monolayer of the plasma membrane respectively [28]. It has been hypothesized that anionic lipids such as DPG and PA could participate in the preparation of mammalian spermatozoa for the acrosomal reaction: such lipids tend to concentrate in areas of the membrane bilayer where fusion occurs during the acrosomal reaction as well as during capacitation [30]. The fusogenic properties of these lipids [33] have been ascribed to the fact that they remain segregated in fluid lipid domains. Even when the acrosome of a mature spermatozoon within the epididymis is very stable, this organization into domains could contribute later on (e.g. at fertilization) to form destabilizing areas which induce the above-mentioned, and perhaps other, physiological events mediated by membrane fusion.

Interspersed with the fluid domains, mammalian spermatozoa also exhibit large areas of stable, non-diffusing, lipid domains which develop during spermiogenesis and constitute an increasingly large proportion of the plasma membrane as the sperm matures [34,35]. Even though these studies were not done in the rat, it is tempting to speculate that an important component of the non-diffusing areas could be the highly unsaturated ether-linked phospholipids, such as the plasmalogens described here.

On the one hand, these lipids have distinctive physical properties, since plasmalogen spontaneously forms physical arrays which differ considerably from those of PC in model membranes [36]. On the other hand, it has been demonstrated that the above-mentioned non-diffusible membrane regions of spermatozoa are created by lipid-lipid interactions alone, and not by interactions with surface or cytoskeletal proteins [35]. Moreover, such stable lipid areas tend to increase as the spermatozoon further differentiates along the epididymal transit [34], as does the proportion of plasmalogens.

Work correlating changes in chemical composition and physical properties of spermatozoal membranes, and studies on how both are connected with sperm cell functional properties, have progressed slowly. The present study suggests that the relative enrichment that spermatozoa undergo during their epididymal transit in particular phospholipids, and the selective gain in specific polyunsaturated fatty acids most lipids display in that process, are physiologically significant for sperm functional maturity. Characterization of the intrinsic and extrinsic factors that affect the formation and turnover of the peculiar lipids of sperm will contribute to our understanding of this process.

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