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Causes of Nondiffusing Lipid in the Plasma Membrane of Mammalian Spermatozoa†

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ABSTRACT: In the plasma membranes of most mammalian somatic cells, lipid is nearly completely free to diffuse laterally in the plane of the membrane. In mammalian spermatozoa and certain other highly polarized mammalian cells, a significant fraction of the plasma membrane lipid is not free to diffuse laterally. Using the technique of fluorescence recovery after photobleaching, we have demonstrated that a variety of fluorescent lipid analogues exhibit a nondiffusing fraction in the plasma membrane of the anterior region of the ram sperm head. The possible causes of this nondiffusing fraction were investigated. The nondiffusing lipid fraction is not the result of lipid oxidation during handling, and it is not released by extensive enzymatic digestion of the membrane surface proteins or the "blebbing" of the membrane by hypoosmotic shock. When lipid bilayers were prepared from protein-free lipid extracts of the plasma membranes of spermatozoa, most of the nondiffusing fraction was retained. These results suggest that the nondiffusing lipid fraction results from lipid factors such as lateral phase separations, which can cause such a nondiffusing fraction in model systems.

Fluorescence recovery after photobleaching (FPR)[1] measurements show that, in the plasma membrane of most somatic cells, lipid is nearly completely free to diffuse laterally in the plane of the membrane [for reviews, see Peters (1981) and Edidin (1981)]. In contrast, the plasma membranes of mammalian spermatozoa have large nondiffusing lipid fractions (Wolf & Voglmayr, 1984; Wolf et al., 1986a,c). This nondiffusing lipid pool develops during spermiogenesis (Wolf et al., 1986c) and becomes an increasingly larger portion of total plasma membrane lipid as the sperm further differentiate during epididymal maturation (Wolf & Voglmayr, 1984) and capacitation (Wolf et al., 1986a). The fraction of nondiffusing lipid in mature sperm can exceed 50%, which is far greater than the 10-15% observed in most somatic cell plasma membranes (Axelrod, 1979). This may be related to the highly polarized nature of the mammalian spermatozoa (Friend, 1982), since significant immobile lipid has been observed only in other highly polarized mammalian cells, such as those of epithelia (Weaver, 1985) and endothelia (Nakache et al., 1985).

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[1] Abbreviations: BSA, bovine serum albumin; $C_N$diI, 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (N = alkyl chain length); D, diffusion coefficient; FPR, fluorescence recovery after photobleaching; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NBD-PC(6), NBD acyl chain labeled phosphatidylcholine; NBD-PE, NBD amino labeled phosphatidylethanolamine; PBS, phosphate-buffered saline with 1.2 mM Ca^{2+} and 5 mM Mg^{2+}; % R, percent recovery; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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In this paper we report our investigation of the possible causes of this nondiffusing lipid fraction in the plasma membrane of ejaculated ram spermatozoa. The following specific questions were addressed:

(1) Because mammalian sperm plasma membranes contain large amounts of polyunsaturated fatty acids (Parks & Hammerstedt, 1985; Poulos et al., 1973; Scott et al., 1967), is the nondiffusing fraction an artifact of lipid oxidation during sperm preparation?

(2) Does the nondiffusing lipid fraction result from interaction with surface coat proteins which could be released by enzymatic digestion?

(3) Does the nondiffusing lipid fraction result from interaction with underlying cytoskeletal elements which could be released by hypoosmotic "blebbing" of the plasma membrane?

(4) Does the nondiffusing lipid fraction result from lipid–lipid interactions which would be retained in bilayers formed from plasma membrane lipid extracts?

Materials and Methods

FPR. The technique of FPR and the specifics of our instrument have been described in detail elsewhere (Axelrod et al., 1976; Koppel et al., 1976; Wolf & Ziomek, 1983). A 63 × 1.4 na Leitz Plan neofluor plan apochromat objective was used in these studies. The beam exp(−2) radius for this objective was determined to be 0.9 ± 0.1 μm (Schneider et al., 1983). Bleaching times were 20 ms at ~50 mW at 514.5 or 457.9 nm. Monitoring intensities were ~5 μW. Data were analyzed on an IBM PC computer according to algorithms described by Barisas and Leuther (1980) and Wolf and Edidin (1981). Measurements made on sperm heads or on reconstituted membranes were fitted to two-dimensional recovery (Axelrod et al., 1976), while those on sperm tails were fitted to one-dimensional recovery (Koppel, 1979). All measurements were made at room temperature.

Fluorescent Lipid Analogues. Two classes of fluorescent lipid analogues were employed in these studies: 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanines [$C_N$diI (alkyl chainlength = N)] (Molecular Probes, Eugene, OR) and 7-nitro-2,1,3-benzoxadiazol-4-yl- (NBD-) labeled phosphatidylethanolamine (NBD-PE) and phosphatidylcholine [NBD-PC(6)] (Avanti Biochemicals, Birmingham, AL). NBD-PE has the NBD group attached to the amine of the ethanolamine; NBD-PC(6) has the NBD attached to the amine on an aminocaproyl which replaces one of the lipid's acyl chains. All of these probes have been extensively used as fluorescent lipid analogues in FPR studies [for review, see Peters (1981)]. In the case of the $C_N$diI's, Wolf (1985) has shown that they label only the outer leaflet of ram sperm plasma membranes.

Labeling of Sperm with Lipid Analogues. Sperm were labeled with all of these lipid analogues by ethanol injection. Stock solutions of probe were prepared at 1 mg/mL in absolute ethanol; 10^7 sperm were added to 400 μL of phosphate-buffered saline containing 1.2 mM Ca^{2+}, 0.5 mM Mg^{2+}, and 5 mM glucose (PBS) with 0.4% w/v bovine serum albumin (BSA) and 0.2% NaN_3 plus probe and incubated. The amounts of probe used and incubation times were as follows: C_{10}diI, 1 μL, 8 min at 4 °C; C_{12}diI, 1 μL, 8 min at 4 °C; C_{14}diI, 1 μL, 8 min at 34 °C; C_{16}diI, 1 μL, 8 min at 34 °C; C_{18}diI, 1 μL, 8 min at 34 °C; NBD-PC(6), 2 μL, 12 min at 34 °C; NBD-PE, 2 μL, 12 min at 34 °C. The sperm suspension was then layered over 1 mL of PBS with 10% BSA and 0.2% w/v NaN_3 and centrifuged at 500g for 10 min. The pellet was resuspended. A fraction of it was placed on an acid-washed glass slide and covered with an acid-washed coverslip, the edges of which were sealed with nail polish for observation and FPR measurements.

Spermatozoa. Sperm used in these experiments were collected by either electroejaculation (Blackshaw, 1954) or use of an artificial vagina (Frank, 1950) from Corriedale rams aged 2–5.5 years. Rams were maintained under controlled conditions with 8.5 h of incandescent light per day. Collected sperm were protected from temperature shock. Sperm stored at room temperature remained viable for several hours after collection.

Immediately after collection, sperm were washed twice by centrifugation for 10 min at 500g in PBS and assessed for motility. They were then washed once in PBS + BSA + 0.2% NaN_3, the NaN_3 being added to immobilize them for FPR measurement. They were then counted on a hemacytometer.

Preparation of Oxygen-Depleted and Oxygen-Enriched Media. Oxygen-depleted PBS was prepared by aeration with nitrogen for 16 h. Oxygen-enriched medium was prepared by aeration with pure oxygen for 1 h.

Formation of Membrane "Blebs". Blebs of the sperm tail plasma membrane were formed by incubation of labeled spermatozoa in 1:3 PBS–water for 5 min. Data were collected from blebs that were exclusively associated with the spermatozoan tail. This blebbing technique has been used to distinguish osmotically viable sperm (Jeyendran et al., 1984).

Pronase Digestion. Immediately following labeling, some sperm were digested at 37 °C with 50 μg/mL Pronase (protease from *Streptomyces griseus*) (Calbiochem, LaJolla, CA) in 200 μL of PBS with 0.2% NaN_3 for 15 or 60 min.

Preparation of Plasma Membrane Fractions. Plasma membrane fractions were produced by a modification of the procedure of Parks and Hammerstedt (1985), which produces vesicles from the anterior head region of the plasma membrane. Ejaculates from approximately six rams were collected to yield about 8 mL. The pooled sperm were washed 3 times in PBS by centrifugation at 1200g for 20 min. The approximately 1-mL pellet was then gradually cooled to 4 °C and maintained at this temperature for the remainder of the procedure. The pellet was diluted 10 times with Hank's balanced salt solution without Ca^{2+} and Mg^{2+} to inhibit the acrosome reaction and counted (typical count = 6×10^8 sperm/mL). The sperm were diluted further, prior to nitrogen decompression, with Ca^{2+}- and Mg^{2+}-free Hank's. Nitrogen decompression was then carried out in a Parr Instrument Co. cell disruption bomb (Moline, IL) after equilibration at 750 psi for 10 min. Under these conditions, the plasma membrane over the anterior region of the head, which is most easily ruptured, is separated from the sperm. The suspension was then centrifuged at 6000g for 10 min at 4 °C. The supernatant was centrifuged at 100000g for 30 min at 4 °C. The supernatant from this high-speed spin was drawn off and discarded. The four pellets, the plasma membrane fractions, were each suspended in 1 mL of Hank's and pooled. The sample was then centrifuged for 10 min at 10000g to remove any remaining large materials. Specimens were taken at each step for subsequent assays.

Lipid Extraction. Lipids were extracted by a modification of the procedure of Bligh and Dyer (1959). A total of 3.75 mL of methanol–chloroform (2:1) was added per milliliter of suspended membrane. The sample was shaken intermittently over the course of 1–4 h and then centrifuged at 900g for 5 min to remove precipitates; 2.5 mL of 1:1 chloroform–H_2O was added per milliliter of original suspension. The sample was then spun at 10000g for 10 min at 4 °C. The bottom phase was collected, placed in a 25-mL round-bottomed, acid-washed flask, and dried in a rotary evaporator at 30 °C for 1 h. The lipids were then dissolved in 2 mL of chloroform.

Assays for Membrane Purity. As discussed above, our membrane fractionation procedure is a modification of that of Parks and Hammerstedt (1985). They have shown that this procedure produces plasma membrane vesicles from the anterior region of the sperm head which are largely free of acrosomal and mitochondrial contamination. To verify this observation, several tests were performed.

Membranes were checked for mitochondrial contamination with the cytochrome oxidase assay according to Wharton and Tzagoloff (1967). Total protein was assayed according to Lowry et al. (1951). Samples were found to contain less than 5% mitochondrial contamination.

Acrosomal contamination was assessed by assaying for acrosin with the fluorescent substrate CBZ-arginine–amino-(trifluoromethyl)coumarin (Enzymes Systems, Livermore, CA) according to modifications of the procedures of Smith et al. (1980) and Zaneveld (1973). A total of 100 μL of sample was added to 400 μL of a 50 mM $CaCl_2$ solution (pH 3.0); 50 μL of the sample in $CaCl_2$ solution was then added to 1.8 mL of TES buffer (pH 8.0) with 100 μL of a stock substrate solution (1 mg/mL) in dimethylformamide. The reaction was monitored on a Perkin-Elmer MPF-3 spectrofluorometer (excitation = 400 nm; emission = 505 nm). Enzyme activities were assayed from initial slopes and were normalized to a per sperm basis. Linear dependence of slope upon concentration was measured for a trypsin standard. Intact sperm were sonicated to assay total sperm acrosin activity. In comparison to sonication, nitrogen cavitation released no measurable acrosin activity (<2%), and negligible acrosin activity was detected in the membrane preparation (<0.5%).

Membrane purity was further assessed with two monoclonal antibodies against sperm antigens, ESA-152 and CFA-110. These antibodies were generously supplied by Dr. Grant Fairbanks. Their specificities have only partially been characterized. ESA-152 is a maturation-dependent sperm plasma membrane antigen. It is present on all regions of the sperm plasma membranes, being most intense on the posterior region of the head (Wolf et al., 1986b). CFA-110 is an acrosomal antigen. It is not present on plasma membranes. It appears to be associated with the inneracrosomal membrane; however, its precise location within the acrosome remains to be determined. These antibodies were employed to assess acrosomal contamination with a spot immunoassay (Hawks et al., 1982; Hawks, 1986). Sperm subcellular fractions were spotted as dilution series on a Type BA85 (0.45-μm pore) nitrocellulose membrane (Schleicher & Schuell, Keene, NH) on a Bio-Dot microfiltration apparatus (Bio-Rad, Richmond, CA). The blots were visualized by a horseradish peroxide conjugated goat anti-mouse IgG (Cooper Biomedical, Malvern, PA) developed with 4-chloro-1-naphthol and hydrogen peroxide. Titers of total sperm activity for each of these antigens were measured postcavitation and precentrifugation and were found to be 1:50 for ESA-152 and 1:200 for CFA-110. Titers for the final membrane pellet were found to be 1:1000 for ESA-152 and 1:40 for CFA-110. This represents a 100-fold enrichment for ESA-152 over CFA-110. This actually represents an underestimate of the enrichment because, as we show below, this procedure selects for the anterior region of the head plasma membrane. This region represents about one-third of the total plasma membrane (Peterson et al., 1980). In addition, the anterior head is the weakest region for ESA-152 expression. We can estimate using the prebleach intensity from FPR measurements of ESA-152 diffusion (Wolf et al., 1984) that the concentration ratio of ESA-152 in the anterior head to that of the posterior head is 0.43. Thus the overall enrichment of plasma membrane over CFA-110 is probably more like 1/1000. In any event, this estimate of plasma membrane purity is in the same range as that obtained by acrosin assay above.

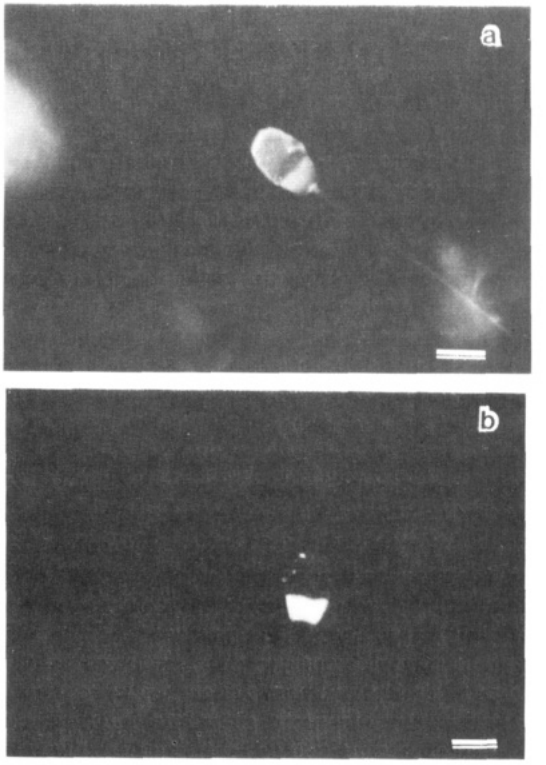

FIGURE 1: Fluorescence photomicrographs showing the selective removal of membrane from the anterior region of the ram sperm head by nitrogen cavitation. (a) Intact sperm labeled with rhodamine-succinylated concanavalin A. ConA labels all regions of the sperm surface. (b) Labeled sperm which were then cavitated. Sperm heads are severed from their tails. Label is largely removed by cavitation from the anterior but not the posterior region of the head. Bar = 10 μm.

Cavitation Is Selective for the Anterior Region of the Head Plasma Membrane. Parks and Hammerstedt (1985) report that this cavitation procedure is selective for the anterior region of the head plasma membrane. To confirm this result, sperm at 1×10^8 cells/mL were labeled for 20 min at room temperature in 25 μg/mL rhodamine-succinylated concanavalin A (ConA). The sample was then washed to remove unbound ConA. Figure 1a is a fluorescence photomicrograph showing ConA labeling of sperm. Both the anterior and posterior regions of the head, as well as the midpiece and tail plasma membrane, label intensely. The labeled sperm were then cavitated as described above. Figure 1b is a fluorescence micrograph showing ConA labeling of these cavitated sperm. Cavitation causes the tails to sever from the heads. However, the tails remain labeled with ConA (not shown). The fluorescence on the posterior region of the head remains unchanged. However, cavitation removes nearly all of the membrane fluorescence from the anterior region of the head.

Large Unilamellar Vesicles. Large unilamellar vesicles were prepared from lipid extracts to which $1{:}10^3$ lipid probe had been added as described previously (Klausner & Wolf, 1981). They were taken up in 100-μm path-length flat capillary tubes (Vitro Dynamics, Rockaway, NJ) for photobleaching measurements.

RESULTS

Generality of Nondiffusing Lipid Fraction. Previous experiments demonstrating the existence of nondiffusing lipid in the plasma membranes of mammalian spermatozoa have

Table I: Diffusion of Fluorescent Lipid Analogues on the Anterior Region of Ejaculated Ram Sperm Heads[a]

analogue	D ($\times 10^9$ s/cm^2)	% R
C_{10}diI	9.3 ± 1.3 (18)	65 ± 2 (18)
C_{12}diI	4.65 ± 0.40 (20)	65 ± 2 (20)
C_{14}diI	3.56 ± 0.28 (40)	63 ± 2 (40)
C_{16}diI	4.73 ± 0.71 (134)	62 ± 1 (134)
C_{18}diI	4.68 ± 0.49 (10)	75 ± 3 (10)
NBD-PE	6.0 ± 1.1 (15)	58 ± 2 (15)
NBD-PC(6)	3.28 ± 0.32 (10)	64 ± 2 (10)

[a] Data given are mean ± standard error of the mean. The number of measurements is given in parentheses.

used the fluorescent lipid analogue C_{16}diI (Wolf & Voglmayr, 1984; Wolf et al., 1986a,b). Since lipid probes selectively partition into a subset of membrane lipid domains (Wolf, 1988; Klausner & Wolf, 1980), we tested whether lipid analogues other than C_{16}diI also exhibited nondiffusing fractions. FPR measurements of the diffusibility of seven lipid analogues, C_{10}diI, C_{12}diI, C_{14}diI, C_{16}diI, C_{18}diI, NBD-PC(6), and NBD-PE, in the anterior region of ejaculated ram sperm heads were measured. The results of these measurements are given in Table I. Diffusion coefficients (D) range from $\sim 3 \times 10^{-9}$ to $\sim 9 \times 10^{-9}$ cm^2/s, consistent with values obtained in other mammalian cell plasma membranes (Peters, 1981; Edidin, 1981). All analogues show nondiffusing fractions. Percent recoveries (% R) range from 58% to 65%. Thus, all probes tested indicate approximately the same fraction of nondiffusing lipid.

Is the Nondiffusing Lipid Fraction an Artifact of Oxidation? Because the lipids of mammalian spermatozoa contain large amounts of polyunsaturated fatty acids, we were concerned with the possibility that the nondiffusing lipid fraction resulted from lipid oxidation during handling. Therefore, we measured the diffusion of C_{16}diI in the anterior region of the head, comparing sperm handled, following ejaculation, in deoxygenated medium under nitrogen to sperm handled in oxygen-enriched medium. Results of these experiments are given in the top row of Table II. We see no difference in either D or % R between these two media. Thus oxidation during handling does not cause the nondiffusing lipid fraction. These experiments do not rule out the possibility that oxidative agents play a role in creating nondiffusing fractions in vivo.

Does the Nondiffusing Lipid Fraction Result from Interaction with Surface Coat Proteins? To determine whether the nondiffusing lipid fraction results from interaction with surface coat proteins, sperm that had been labeled with C_{16}diI were incubated in 50 μg/mL Pronase for 15 or 60 min. Pronase was chosen for these studies because it is a highly nonspecific and potent protease. Treatment of ram spermatozoa with less specific proteases under similar conditions has been shown to remove iodinated surface proteins (Voglmayr et al., 1980). The action of Pronase on sperm is readily observed as it causes the sperm suspensions to flocculate. The results of these experiments are given in the second row of Table II. Data are given for untreated sperm and for sperm

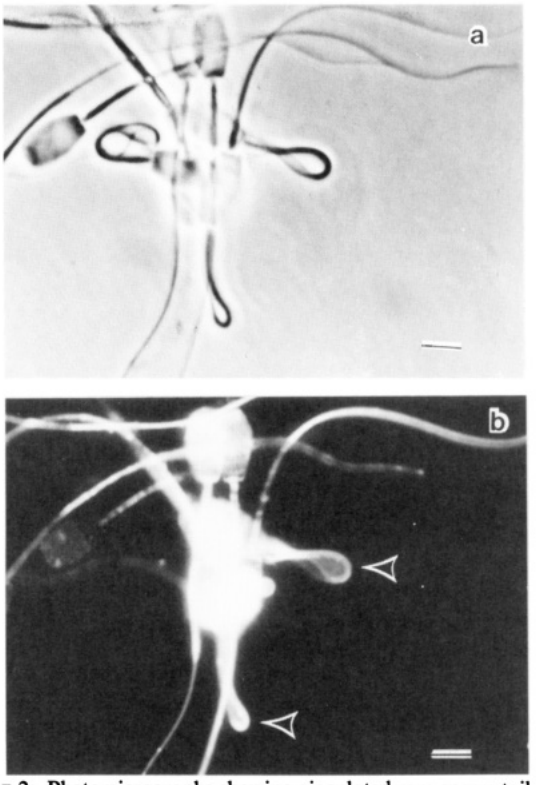

FIGURE 2: Photomicrographs showing ejaculated ram sperm tail blebs (arrows) labeled with C_{16}diI: (a) phase; (b) fluorescence. Tail tends to hook at the site of the bleb. The presence of membrane in this hook region is demonstrated by the C_{16}diI fluorescence staining. Bar = 10 μm.

treated with Pronase for 60 min. Again, measurements were made on the anterior region of the sperm head. Pronase did not significantly affect either D or % R.

Does the Nondiffusing Lipid Fraction Result from Interaction with Underlying Cytoskeletal Elements Which Could Be Released by Hypoosmotic Blebbing of the Plasma Membrane? Wu et al. (1982) and Tank et al. (1982) have shown that in somatic cells blebbing of the plasma membrane so that it becomes separated from underlying structures releases constraints to the lateral diffusion of protein and, to a lesser degree, of lipid. The nondiffusing fractions of membrane proteins can disappear, and their diffusion rates can increase by 2–3 orders of magnitude. While lipids, as discussed above, have little or no nondiffusing fraction in somatic cells, their diffusion rates can increase 2–3-fold as a result of membrane blebbing.

In light of these experiments, we ask whether blebbing would release the nondiffusing lipid of ram sperm membranes. After labeling with C_{16}diI, blebbing of the tail plasma membrane was induced by hypoosmotic shock. The tail or midpiece bends to accommodate the bleb, and as a result, the blebbed membrane fills in the concave side of the bend (Figure 2). Diffusion on normal sperm tails was compared to diffusion on tail

Table II: Experiments To Investigate the Cause of Nondiffusing Lipid Fraction in the Plasma Membrane of Ejaculated Ram Spermatozoa[a]

control condition	exptl condition	D ($\times 10^9$ s/cm^2)		% R	
		control	exptl	control	exptl
oxygen-depleted medium, AH	oxygen-enriched medium, AH	6.1 ± 0.5 (42)	4.9 ± 0.5 (42)	72 ± 1 (42)	78 ± 1 (42)
untreated AH	pronase-treated, AH	12.3 ± 0.7[b] (39)	11.4 ± 0.1 (22)	73 ± 1 (39)	75 ± 1 (22)
untreated, T	blebbed, T	5.2 ± 1.4 (31)	5.0 ± 0.6 (31)	60 ± 3 (31)	64 ± 2 (31)
AH	large unilamellar vesicles from AH lipid extracts	4.6 ± 0.3 (109)	3.0 ± 0.3 (89)	62 ± 1 (109)	72 ± 2 (89)

[a] Values given are mean ± standard error of the mean. Number of measurements is given in parentheses. AH = anterior region of the head. T = tail. [b] Control values given are not composite but rather represent data paired with the particular experimental set. Such a comparison avoids animal and daily variations.

membrane blebs. Tail data were fitted to one-dimensional recovery while bleb data were fitted to two-dimensional recovery. The results are given in the third row of Table II. We find that blebbing affects neither the diffusion rate nor the diffusing fraction of C_{16}diI.

Does the Nondiffusing Lipid Fraction Result from Lipid–Lipid Interactions Which Would Be Retained in Bilayers Formed from Plasma Membrane Lipid Extracts? We next asked whether the nondiffusing lipid fraction could result from purely lipid effects, such as lateral phase segregations. To answer this question, vesicles from the plasma membrane of the anterior region of the ram sperm head were prepared. Lipid was extracted from these preparations, and large unilamellar lipid vesicles were prepared from these extracts mixed in the presence of $1:10^3$ C_{16}diI. In the bottom row of Table II, diffusion in these vesicles is compared to diffusion in the intact plasma membrane of the anterior region of the ram sperm head. We find that most of the nondiffusing lipid fraction is retained in these lipid extract membranes.

Discussion

The presence of nondiffusing fractions in lipid-extract membranes raises the question: What kind of lipid–lipid interactions may result in such nondiffusing fractions? Diffusion studies in model systems indicate the most likely cause (Fahey & Webb, 1978; Derzko & Jacobson, 1980; Klausner & Wolf, 1980). In homogeneous fluid-phase bilayers, 100% recovery is observed. However, in mixed-phase membranes, such as those formed from a one-to-one mixture of dilauryl- and dipalmitoylphosphatidylcholines at 10 °C (Klausner & Wolf, 1980), large nondiffusing fractions are observed. These model systems contain coexistent gel and fluid lipid phases. The results of our experiments suggest that such lateral-phase segregations may contribute to the nondiffusing lipid fractions in mammalian sperm.

If lipid factors are the cause of nondiffusing lipid fractions, then the next question is: What distinguishes sperm lipids from those of somatic cells? Sperm are peculiar in their lipid composition in that they contain large amounts of plasmalogens and other ether-linked phospholipids (Evans et al., 1980; Horrocks & Sharma, 1982) as well as large proportions of long, polyunsaturated aliphatic chains (Evans et al., 1980; Poulos et al., 1973; Scott et al., 1967). In the plasma membrane of the anterior head region of ram sperm, for instance (Parks & Hammerstedt, 1985), choline phospholipids account for almost $^2/_3$ of all phospholipids; ~50% of these have an ether linkage. Of this 50%, $^2/_3$ are plasmalogens (i.e., have one alkenyl ether linkage), and the rest have an *O*-alkyl component. The ether linkage is more resistant to enzyme degradation (Lands & Hart, 1965). Of all phospholipids from this membrane, ~47% of the acyl chains are 22:6. It is also possible that sperm-specific glycolipids could control lateral domain organization (Thompson & Tillach, 1985).

The ultimate question is, of course: What is the physiological significance of this immobile lipid to sperm function and to fertilization? While excessive conjecture about this issue is certainly premature, one important point should be made. In many biophysical studies of membrane lipid lateral-phase separation, the parameter varied to control such separations is temperature. Temperature is not, however, a *physiologically* significant parameter in most *mammalian* cell systems. Mammalian spermatozoa are a major exception to this rule. In sheep, for instance, there is a 5 °C difference between the testis, where sperm are stored, and the oviduct, where fertilization occurs. Peculiarities in lipid composition could result in lateral-phase separations and/or nondiffusing lipid fractions. These could represent specializations that enable spermatozoa to respond to this temperature gradient. It should be noted that nondiffusing lipid fractions are common in poikilothermic cells (Wolf et al., 1981; Weaver, 1985; Treistman et al., 1987). In the case of sperm, temperature, by virtue of regulating membrane domain organization, could play a regulatory role in fertilization.

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Lipid Requirement of the Branched-Chain Amino Acid Transport System of *Streptococcus cremoris*†

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ABSTRACT: The role of the membrane lipid composition on the transport protein of branched-chain amino acids of the homofermentative lactic acid bacterium *Streptococcus cremoris* has been investigated. The major membrane lipid species identified in *S. cremoris* were acidic phospholipids (phosphatidylglycerol and cardiolipin), glycolipids, and glycerophosphoglycolipids. Phosphatidylethanolamine (PE) was completely absent. Protonmotive force-driven and counterflow transport of leucine was assayed in fused membranes of *S. cremoris* membrane vesicles and liposomes composed of different lipids obtained by the freeze/thaw–sonication technique. High transport activities were observed with natural *S. cremoris* and *Escherichia coli* lipids, as well as with mixtures of phosphatidylcholine (PC) with PE or phosphatidylserine. High transport activities were also observed with mixtures of PC with monogalactosyl diglyceride, digalactosyl diglyceride, or a neutral glycolipid fraction isolated from *S. cremoris*. PC or mixtures of PC with phosphatidylglycerol, phosphatidic acid, or cardiolipin showed low activities. In mixtures of PC and methylated derivatives of PE, both counterflow and protonmotive force-driven transport activities decreased with increasing degree of methylation of PE. The decreased transport activity in membranes containing PC could be restored by refusion with PE-containing liposomes. These results demonstrate that both aminophospholipids and glycolipids can be activators of the leucine transport system from *S. cremoris*. It is proposed that aminophospholipids in Gram-negative bacteria and glycolipids in Gram-positive bacteria have similar functions with respect to solute transport.

Membrane transport proteins catalyze the highly specific vectorial transfer of solutes across the membrane. Most studies on the effects of the environment on the functional properties of these proteins have focused on the water phase. Especially the effects of pH, ion composition, etc. have been studied. However, only a fraction of a membrane protein is exposed to the water phase. In order to function efficiently, a large part of the protein must be embedded in the lipid bilayer. As such, the activity of these proteins is expected to be modulated by the surrounding lipid bilayer. A number of bilayer features may be considered, such as viscosity (or lipid packing density) and charge at the lipid–water interface, parameters which are determined by the structures of individual lipids and their mutual interactions.

A limited number of reports address the question of how alterations in the lipid environment of a membrane protein affect the activities of these enzymes, and information about

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