The Development of Regionalized Lipid Diffusibility in the Germ Cell Plasma Membrane during Spermatogenesis in the Mouse

David E. Wolf,* B. Keyes Scott,‡ and Clarke F. Millette‡

*Endocrine Reproductive Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545;
‡Department of Anatomy and Cellular Biology, Laboratory of Human Reproduction and Reproductive Biology,
Harvard Medical School, Boston, Massachusetts 02115.

Abstract. The lipids and proteins of sperm cells are highly regionalized in their lateral distribution. Fluorescence recovery after photobleaching studies of sperm membrane component lateral diffusibility have shown that the sperm plasma membrane is also highly regionalized in the extents and rates of diffusion of its surface components. These studies have also shown that regionalized changes in lateral diffusibility occur during the differentiative processes of epididymal maturation and capacitation. Unlike mammalian somatic cells, sperm cells exhibit large nondiffusing lipid fractions. In this paper, we will show that both regionalized lipid diffusibility and nondiffusing lipid fractions develop with the morphogenesis of cell shape during spermatogenesis in the mouse. Pachytene spermatocytes and round spermatids show diffusion rates and the nearly complete recoveries (80–90%) typical of mammalian somatic cells. In contrast, stage 10–11 condensing spermatids, testicular spermatozoa, cauda epididymal spermatozoa, as well as the anucleate structures associated with these later stages of spermatogenesis (residual bodies and the cytoplasmic droplets of condensing spermatids and testicular spermatozoa), exhibit large nondiffusing fractions. Both the diffusion rates and diffusing fractions observed on the anterior and posterior regions of the head of stage 10–11 condensing spermatids are the same as the values obtained for these regions on testicular spermatozoa. Possible mechanisms of lipid immobilization and possible physiological implications of this nondiffusing lipid are discussed.

MATURE spermatozoa are highly differentiated cells, whose surface components, both protein and lipid, are highly regionalized in their lateral distributions (4, 14, 18, 21, 25, 26, 30, 32, 33, 44, 45). These surface regionalizations, which presumably reflect the specialized functions of specific surface regions, evolve with the further differentiation of the spermatozoa during epididymal maturation and capacitation (4, 32, 35, 37). A critical unanswered question is how spermatozoa restrict the free random diffusion of their membrane components to effect lateral surface regionalization. To address this question, several recent studies have used the technique of fluorescence recovery after photobleaching (FRAP)[1] to measure the lateral diffusion rates of both proteins and lipids within or between distinct surface regions of the plasma membrane of mammalian spermatozoa (31, 55, 58, 59). In the context of the present study, these experiments have provided at least three noteworthy observations.

First, lipids are not completely free to diffuse laterally in the plasma membrane of mammalian spermatozoa. That is, a significant fraction of the lipid is immobile and does not recover in a FRAP measurement. This has been observed by Wolf and Voglmayr (55) in ram spermatozoa for the fluorescent lipid analogue 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ($C_{16}diI$), by Wolf et al. (58) in mouse spermatozoa for $C_{16}diI$, and by D. E. Koppel (personal communication) in guinea pig spermatozoa for $C_{16}diI$ and nitrobenzoxadiazole-labeled phospholipids. In contrast, the lipids of most homeothermic cells show complete or nearly complete diffusibility (12, 37, 53). Thus, these large nondiffusing fractions appear to be a trademark of mammalian spermatozoa. This in turn raises the questions: What is the cause of these nondiffusing fractions? Is this cause related to the mechanisms of surface regionalization? Do nondiffusing lipids play a role in sperm physiology?

Second, Wolf and Voglmayr (55) have shown in ram and Wolf et al. (58) in mouse that the lateral diffusibility of $C_{16}diI$ differs over the morphologically distinct regions of the sperm surface. Significant differences between regions are observed for both lipid probe diffusion coefficient (D)

1. *Abbreviations used in this paper:* $C_{16}diI$, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; D, diffusion coefficient; FRAP, fluorescence recovery after photobleaching; %R, percent recovery.

and diffusible fraction (percent recovery) (%R). Therefore, the sperm plasma membrane can not be described in terms of a bulk membrane viscosity.

Third, changes in lipid diffusibility during the differentiative processes of epididymal maturation (55) and capacitation (58) are also regionalized. The patterns of these regionalizations are consistent with expected physiological changes.

In this paper, we extend these studies of lipid diffusibility and sperm differentiation to the later stages of spermatogenesis. Spermatogenesis is the process by which spermatozoa form in the testes. For a complete description of spermatogenesis see references 5 and 16. Briefly, during late spermatogenesis each large round tetraploid pachytene cell undergoes two meiotic reduction divisions to form four round haploid spermatids. These round spermatids then undergo spermiogenesis where they develop the characteristic sperm morphology (34, 39). Spermiogenesis does not involve further cell division, and can be divided into three separate phases. The first phase is known as the Golgi phase since it is characterized by the presence of robust Golgi apparatuses. During the Golgi phase the acrosomal vesicle and granule begin to develop. Elongation of the flagellum also begins. The second phase is the acrosomal phase, which is marked by the flattening of the acrosome and the formation of an acrosomal cap. Flagella elongation continues. Toward the end of this phase, nuclear condensation begins. A microtubular cage structure known as the manchette forms which may be involved in sperm nuclear condensation and/or the initiation of head shape change. The final phase of spermiogenesis is the maturation phase when the sperm takes on its characteristic species-specific shape, concomitant with the extrusion of excess cellular material in the form of cytoplasmic droplets and residual bodies.

In this paper we will show that lipid diffusion in the plasma membranes of pachytene spermatocytes and round spermatids is typical of that observed on other mammalian cells. That is, nearly complete recovery with diffusion coefficients in the range of 10^{-9} to 10^{-8} cm²/s are observed. However, at the beginning of the maturation phase, specifically stage 10–11 condensing spermatids, the earliest stage during which one can reliably distinguish between the anterior and posterior regions of the head, a significant nondiffusing lipid fraction is observed. Furthermore, both the diffusion coefficient and diffusing fraction on these two regions is identical to that observed on structurally mature testicular spermatozoa.

Materials and Methods

Animals and Cell Preparation

Adult CD-1 mice aged 60–120 d were obtained from Charles River Breeding Laboratories (Wilmington, MA). Mixed seminiferous cell suspensions were prepared by testicular decapsulation and manual teasing of the seminiferous tubules, without exposing the cells to any exogenous enzymes. For purposes of FRAP measurements, mixed suspensions were used directly and the cell being measured was typed using phase-contrast microscopy. For some of the photomicroscopy, purified cell preparations (6) were used. Identification of particular cell types was conducted using well-established morphological criteria (6, 42).

Carbocyanine Dyes and Cell Labeling

C_{16}diI was obtained as a perchlorate from Molecular Probes, Inc. (Junction City, OR). This probe was chosen because it has been shown to behave as a "typical" membrane lipid in mammalian cells (12, 37, 53–55, 57, 58). Furthermore, Wolf et al. (58) have shown that in mouse spermatozoa this probe does not label internal membranes, and is in fact confined to the outer leaflet of the sperm plasma membrane (52).

200 µl of a suspension of ~10^6 cell/ml were incubated with 5 µg/ml of C_{16}diI and 0.5% ethanol for 8 min at room temperature. This suspension was seated on a 1-ml cushion of Hanks' balanced salt solution (HBSS) containing 50 mg/ml bovine serum albumin and centrifuged at 400 g_{max} for 10 min. The pellet was resuspended in ~100 µl HBSS for FRAP measurement or Krebs' Ringer Phosphate for photomicroscopy.

Photomicroscopy

Photomicrographs were made on a Zeiss Universal microscope using an M35 camera system, rhodamine epi-illumination optics, and either a 63 × 1.4 NA plan apochromat or a 25 × 0.8 NA plan neofluor oil immersion objective. Photomicrographs were made on Kodak Tri-X film developed in Edwal F7 to an effective ISO of 1,000.

FRAP Measurement

The technique of FRAP has been described in detail elsewhere (2, 27, 54). FRAP provides two measures of lateral diffusion in the plane of the membrane: the fraction of the component that is free to diffuse (%R), and the diffusion coefficient (D) of that fraction. Typical lipid diffusion coefficients range from 10^{-9} to 10^{-8} cm²/s, which means that they diffuse 1 µm in ~1 s or less. The specifics of our instrument, which is similar to other published designs (2, 27), have been described elsewhere (54, 56). The beam exp (−2) radius was determined (47) to be 0.9 ± 0.1 µm. Bleaching times were ~5 ms at ~10 mW at 514.5 nm. Monitoring intensities were ~1 µW. The counting interval was 25 ms. Data were fitted using nonlinear least squares programs after Bevington (7) to algorithms described by Barisas and Leuther (3) and Wolf and Edidin (54).

Results

C_{16}diI Labeling of Sperm and Germ Cells

Fig. 1 shows low magnification photomicrographs (phase-contrast and fluorescence) of pachytene (a and b), round spermatids (c and d), residual bodies (e and f), and testicular mouse spermatozoa (g and h) labeled with C_{16}diI. In all cases, diffuse "ring" staining indicative of plasma membrane labeling is observed. Fig. 2 shows stage 10–11 condensing spermatids at higher magnification. For clarity, the photomicrographs in Figs. 1 and 2 were made using purified or enriched cell populations. However, identical labeling patterns were observed for each stage in mechanically prepared testicular cell suspensions.

The results of FRAP measurements of lipid analogue lateral diffusibility in the plane of the plasma membrane of these various cell types and structures are shown in Tables I and II. Diffusion coefficients range from 1.08 to 5.7 × 10^{-9} cm²/s, which is typical of lipid diffusion rates observed on other mammalian cells (12, 37, 52, 54–56, 58). Since the time to diffuse a distance varies as the square of the distance, such diffusion rates mean that a lipid molecule can diffuse distances of 1 µm in ~1 s, but to diffuse the entire ~100 µm length of the sperm would require ~3 h. In Table I, we compare C_{16}diI diffusibility on pachytene spermatocytes, round spermatids, stage 10–11 condensing spermatids, and testicular spermatozoa. For comparison purposes we also include in Table I results on cauda epididymal mouse spermatozoa from Wolf et al. (58). We observe that on the round cells nearly all of the lipid is free to diffuse; 80–90 %R is observed. These values of %R are typical of mammalian cells. However, with the development of the sperm head (condens-
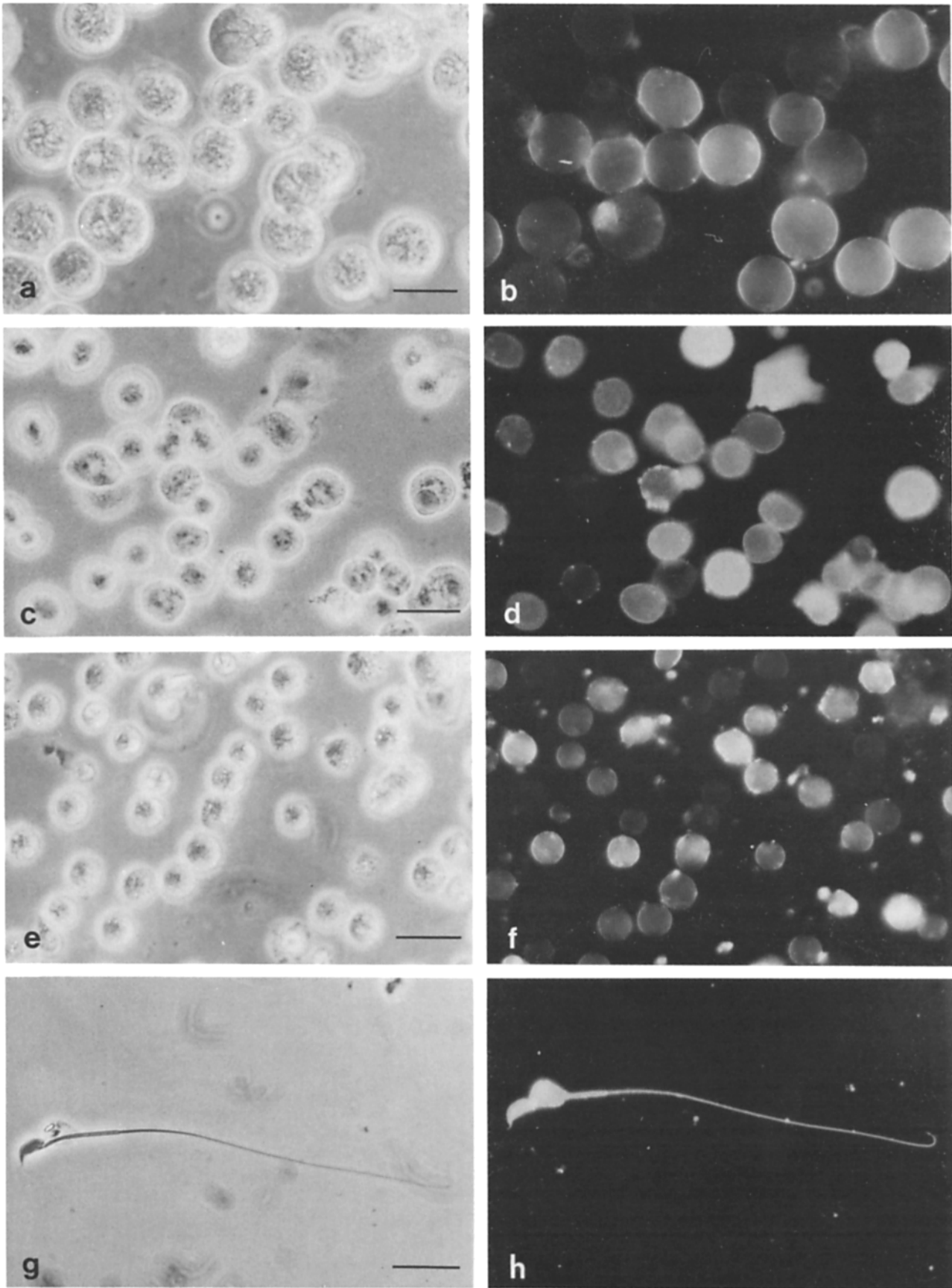
Figure 1. Spermatogenic cells labeled with C_{16}diI. Labeling procedure is described in text. Phase-contrast (*left*) and fluorescence (*right*). (*a* and *b*) Pachytene spermatocytes; (*c* and *d*) round spermatids; (*e* and *f*) residual bodies; (*g* and *h*) testicular spermatozoon. Bar, 20 μm.
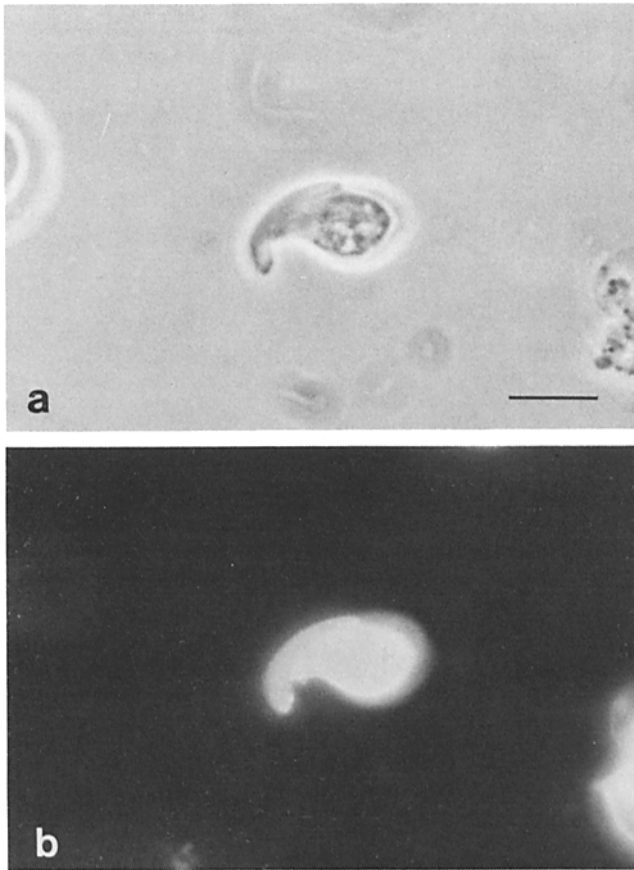
Figure 2. Stage 10–11 condensing spermatid labeled with C_{16}diI. Labeling procedure is described in the text. (a) Phase-contrast; (b) fluorescence. At this stage of spermiogenesis, the anterior and posterior regions of the head are clearly distinguishable. Bar, 10 μm.

Table II. C_{16}diI Diffusion in the Plasma Membrane of the Anucleate Structures of Mouse Spermatogenic Cells

Structure	$D \times 10^9$ s/cm^2	%R
Residual bodies	2.41 ± 0.30 (23)	54 ± 4 (23)
Cytoplasmic droplets of condensing spermatids	5.7 ± 1.7 (23)	64 ± 5 (13)
Cytoplasmic droplets of testicular sperm	2.3 ± 0.8 (3)	70 ± 17 (3)

Data shown is mean ± SEM. Beside each value the number of measurements is given in parentheses.

ing spermatids), we observe the appearance of a large immobile lipid fraction, which is characteristic (55, 58) of mammalian spermatozoa. Indeed, both D and %R on stage 10–11 condensing spermatids are found to be statistically identical to values obtained on testicular spermatozoa. For comparative purposes, Table II shows lipid diffusibility values obtained on anucleate structures (residual bodies, the cytoplasmic droplets of stage 10–11 condensing spermatids, and a limited population of the cytoplasmic droplets of testicular spermatozoa). The diffusion rates are again found to be in the range typical of mammalian cells. However, significant

immobile lipid fractions, characteristic of mammalian spermatozoa are observed. In conclusion, the immobile lipid fraction characteristic of mammalian spermatozoa develops concurrent with archetypal sperm morphology.

Discussion

As discussed above, it has previously been shown that sperm plasma membrane lipid lateral diffusibility is regionalized and that it changes during the differentiative processes of epididymal maturation (55) and hyperactivation/capacitation (58). This paper extends this observation to the later phases of spermatogenesis. Most striking is the observation that the large nondiffusing lipid fractions which appear to be characteristic of mammalian spermatozoa develop concurrent with the development of characteristic sperm morphology.

Lipids are generally reported to recover completely in most mammalian cell plasma membranes. A closer look at available data however, shows that this "complete" recovery often means 80–100% (1, 12, 37, 53, 54, 57). A notable exception to this generalization has been reported on intestinal epithelial cells, which are also highly polarized (51). On poikilothermic membranes, large nondiffusing fractions are often observed (11, 38, 51, 60). These results should be compared to model membrane studies. On homogeneous fluid phase synthetic membranes, 100% recoveries are observed (10, 17, 24, 61). On gel phase synthetic membranes 0% recoveries are observed (17, 46, 61). On mixed phase synthetic membranes, large nondiffusing lipid fractions can occur (24).

It is, therefore, not completely accurate to state that lipids are always completely free to recover on mammalian cell

Table I. C_{16}diI Diffusion in the Germ Cell Plasma Membrane during Mouse Spermatogenesis

Stage	Anterior region of head		Posterior region of head	
	$D \times 10^9$ s/cm^2	%R	$D \times 10^9$ s/cm^2	%R
Pachytene* spermatocyte	1.77 ± 0.18 (32)	87 ± 3 (32)	–	–
Round spermatid*	2.17 ± 0.19 (27)	81 ± 2 (27)	–	–
Condensing spermatid Stage 10–11	2.39 ± 0.26 (20)	59 ± 3 (20)	3.26 ± 0.45 (21)	58 ± 4 (21)
Testicular sperm	2.85 ± 0.33 (11)	65 ± 4 (11)	3.31 ± 0.71 (12)	65 ± 3 (12)
Cauda epididymal sperm‡	2.11 ± 0.21 (25)	61 ± 2 (31)	1.91 ± 0.17 (26)	57 ± 3 (31)

Data shown is mean ± SEM. Below each value the number of measurements is given in parentheses.
*Diffusion data for pachytene spermatocytes and round spermatids is listed in the anterior head column.
‡Data for cauda epididymal spermatozoa is from Wolf et al. (58).

plasma membranes. The 10–20% nondiffusing fractions observed on some somatic cell types and observed here on pachytene spermatocytes and round spermatids may reflect a physiological phenomenon. Clearly, however, this immobilization phenomenon becomes exaggerated on condensing germ cells and spermatozoa.

A key question is, of course, what causes a major portion of the lipid to be nondiffusing in sperm cell plasma membranes. Wolf et al. (58) have shown that only the outer leaflet of the mouse sperm plasma membrane labels with C_{16}diI. Thus, one may rule out this immobile fraction being due to internalized probe or to probe intercalated into endomembranes which are closely opposed to the plasma membrane. Several alternative possible causes come to mind: interaction of probe with nondiffusing or relatively slowly diffusing membrane proteins, interaction of the bilayer with underlying cytoskeletal structures, and the presence of gel phase domains within the bilayer such as those observed in model membranes composed of mixtures of phospholipids (for review see reference 53). We are currently actively investigating each of these possibilities. Preliminary results indicate that some immobile lipid may be present in protein free lipid bilayers prepared from sperm plasma membrane lipids, suggesting that phase separations may indeed play a role in causing immobile lipid in vivo.

Sperm are peculiar in their lipid composition in that they contain large amounts of plasmalogens and other ether-linked phospholipids (15, 22, 49), as well as 22-carbon fatty acid chains with six double bonds (15, 36, 40, 48, 49). These anomalous compositions could account for both the regionalization and the observed nondiffusing fractions of sperm membrane lipids. In this respect, it will be of interest to determine the changes in plasma membrane lipid composition during spermatogenic cell differentiation.

It is tempting to suggest a direct causal relationship between sperm shape and nondiffusing lipid fraction. Round germ cells, pachytene spermatocytes, and round spermatids, show "normal" recovery, whereas later, polarized germ cells show low recovery. The appearance of relatively low recovery on residual bodies and cytoplasmic droplets, both of which are round, argue against such a simplistic relationship. Indeed, the droplet and residual body data support the suggestion that the appearance of nondiffusing lipid is developmentally stage specific. More significantly, since the presence of immobile lipid is common to all regions of the sperm and to both cytoplasmic droplets and residual bodies, the cause(s) of this nondiffusing fraction is most probably a structure or factor common to all of these cell regions or fractions.

The relative uniqueness of this nondiffusing plasma membrane lipid among mammalian cells raises the question of the role(s) which this immobile lipid plays in sperm physiology. The sperm cell plasma membrane is highly regionalized, both in function and in the distribution of its surface components. Immobilization of some of the lipid pool may play a role in effecting this regionalization. Sperm cell plasma membranes are also relatively unique among mammalian cells in that they are limited in their ability to synthesize membrane lipids and proteins (8, 19, 20, 23, 29, 40, 41, 48, 49). Rather, spermatozoa appear to be modified from without as they come in contact with the luminal fluids of the epididymis and the female reproductive tract (9, 28, 43, 50) as they prepare for fertilization (13). It has also been proposed that

lipids are an expendable metabolite for spermatozoa (28). Immobile lipid could be sequestered into a readily available pool for catabolism. Conversely, this pool could be protected from catabolism. Consistent with this latter hypothesis is the observation that %R decreases with both epididymal maturation (55) and capacitation (58). The unusual lipid compositions and the resulting nondiffusing lipid fractions may reflect a modification which enables spermatozoa to survive and undergo transformation during the long journey from testis to oviduct.

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