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REGIONAL DIFFERENTIATION IN BULL SPERM PLASMA MEMBRANES

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SUMMARY. Bull sperm heads and tails have been separated by proteolytic digestion (trypsin) and plasma membranes have been isolated, using discontinuous sucrose density gradient centrifugation. Plasma membrane bound Ca^{2+}-ATPase is shown to be associated mostly with the tail membranes. Pyrene excimer fluorescence and diphenylhexatriene fluorescence polarization experiments indicate a more fluid lipid phase in the tail region. Differences in surface charge distribution have been found, using 1-anilinonaphthalene-8-sulfonate and Tb^{3+} as fluorescent probes.

INTRODUCTION

The mammalian spermatozoan is a highly differentiated cell of sophisticated architecture, consisting of structurally different head and tail regions. The plasma membrane of the head domain, selectively fuses and vesiculates with the outer acrosomal membranes during the exocytotic acrosome reaction (1). The coordination of flagellar motion is closely linked to the plasma membrane components of the tail (2). Studies on the regional asymmetry of the plasma membranes of the male gamete, might therefore, provide some insight into the phenomena of acrosome reaction and motility.

We have earlier detected the presence of a Ca^{2+}-ATPase in the isolated plasma membranes of bull sperm (3). In this communication, we report that the ATPase is almost exclusively localised in the tail region. Marked differences in the lipid phase mobility of the head and tail membranes

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has been observed, using pyrene excimer fluorescence and DPH^+ fluorescence polarization as probes. Fluorescence studies of regional differences in the surface charge distribution and calcium binding sites, using ANS and Tb^{3+} are also presented.

MATERIALS AND METHODS

Isolation of sperm heads and tails

Bull semen was diluted with two volumes of 0.15 M NaCl-5mM HEPES, pH 7.0. Sperm cells were separated from diluted semen by centrifugation at 6500 rpm and washed twice by the same procedure. Proteolytic cleavage of sperm into heads and tails was carried out using trypsin, essentially as described by Calvin (4). The cleaved cells were collected by centrifugation at low speed, washed and resuspended in buffered saline (0-5°C). From this mixture, heads and tails were separated, on a discontinuous sucrose density gradient consisting of 1.8 M-2.05 M-2.20 M sucrose, by centrifugation at 91,000 g for 1 hour. Tails separate at the 1.8 M/2.05 M interface, while the undecapitated cells band at the 2.05 M/2.20 M interface. The heads pellet down at the bottom of the tube. Tails and heads thus collected were centrifuged and washed at $\sim$ 7000 rpm. Further purification of the tail fraction was carried out by recentrifugation for 90 minutes, using the same sucrose gradient. The resolution of heads and tails by this method is $> 95\%$.

Preparation of plasma membranes

The isolated heads and tails were homogenized and plasma membranes were prepared as described earlier, for intact bull sperm (3,5). Protein and inorganic phosphate estimation (6,7) and Ca^{2+}-ATPase activity (8), were determined by standard procedures.

Fluorescence studies

Fluorescence spectra were recorded on a Perkin-Elmer MPF-44A fluorescence spectrophotometer, with 5 nm excitation and emission band pass. Pyrene and DPH were incorporated into membranes by rapid mixing of a stock solution (2 mM) in ethanol and THF respectively, such that the solvent concentration did not exceed 1% . For DPH polarization experiments, the membranes were incubated with the probe, for 15 minutes, at 37°C and measurements were made using λ (excitation) = 355 nm and λ (emission) = 430 nm. ANS was freshly recrystallised before use and $TbCl_3$ was used as an aqueous solution (5 mM stock). All solutions for fluorescence studies were prepared in 10 mM Tris-HCl, pH 7.4 buffer. A protein concentration of 100 μg/ml was used.

+Abbreviations : DPH, 1,6-Diphenyl-1,3,5-hexatriene ; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid ; ANS, 1-Anilinonaphthalene-8-sulfonate ; THF, Tetrahydrofuran.

RESULTS AND DISCUSSION

Mammalian spermatozoa can be cleaved into head and tail regions by a variety of physical and chemical techniques (9-11), with varying degrees of structural damage (11-13). The chemical dissection of sperm using trypsin has been reported to be the mildest procedure (12,14). We have applied this 'trypsin-mediated proteolytic cleavage' method to bull sperm and separated heads from tails as

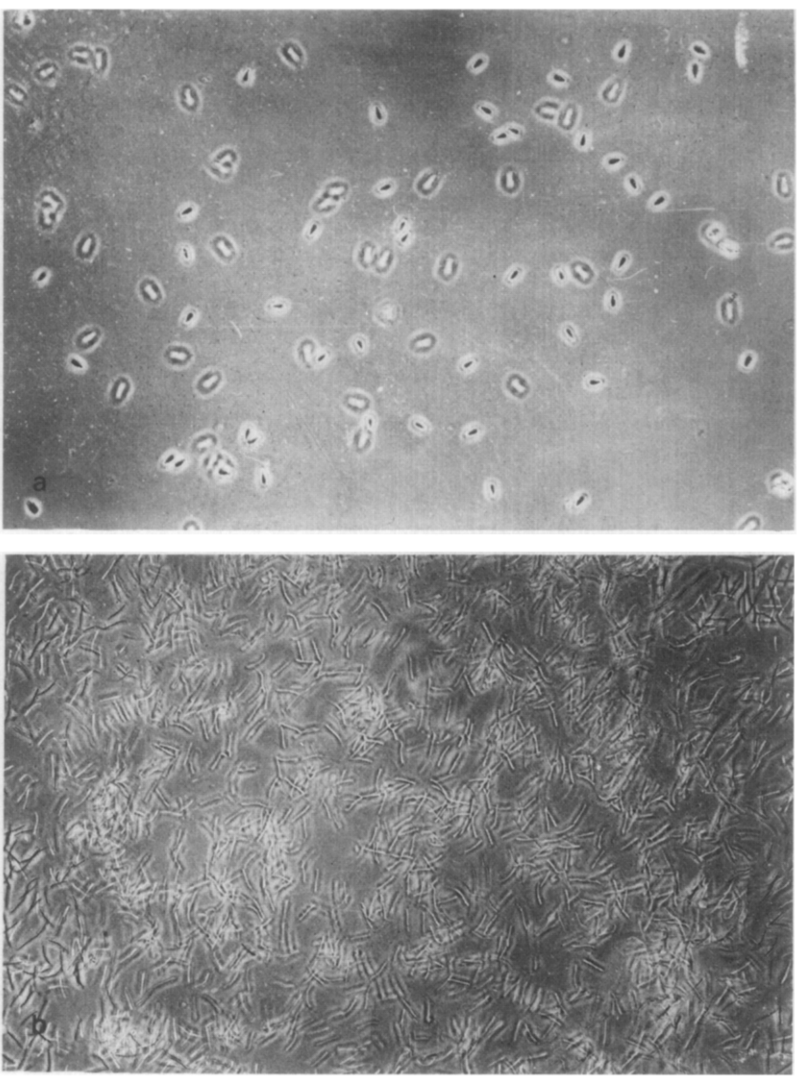

Fig.1. Phase contrast micrographs (X400) of (a) heads, (b) tails and (c) intact sperm.

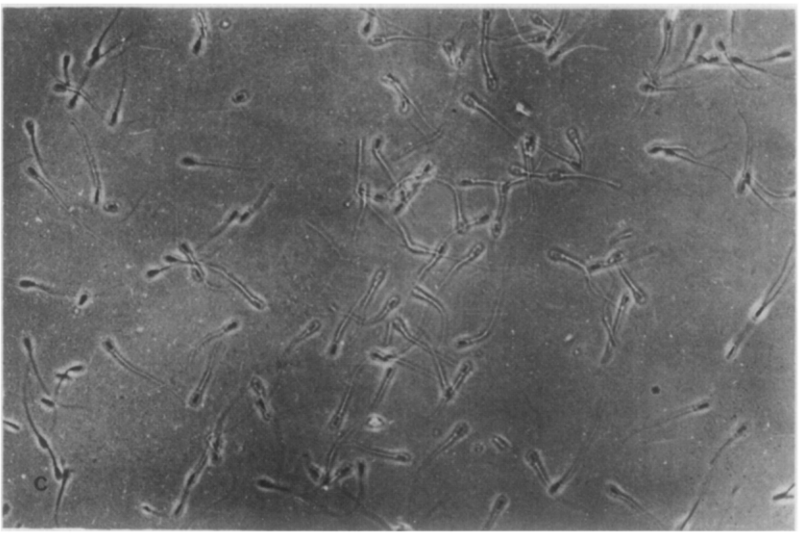

Fig. 1--Continued.

recommended by Calvin (9). The purity of isolated head and tail fractions appear to be > 99% (Fig.1). In view of the structural and functional diversity of the head and tail regions (15), plasma membranes of the head and tail fractions were prepared and characterised as described for intact bull sperm (3).

We have earlier identified the presence of a Ca^{2+}-ATPase, primarily confined to the plasma membranes in bull sperm and suggested that this enzyme could serve as a marker for the cell surface membranes (3). The results of the present study (Table 1) clearly show that, most of the ATPase activity is associated with the tail membranes. Interestingly, the control machinery for forward propagation is located in the spermatozoan tail (16). The localization of Ca^{2+}-ATPase in the tail region may be of considerable importance, since, ATP hydrolysing enzymes have been implicated in a number of functions related to the mechanochemical generation of energy, required for sperm motility and in the maintenance of ionic equilibria (2,17,18). The functional

TABLE 1

Plasma membrane Ca^{2+}-ATPase activity and emission parameters of fluorescent probes in bull sperm head and tail membranes[a].

		Heads	Tails
Ca^{2+}-ATPase[b]		3.1 (±0.7)	40.9 (±2.5)
DPH polarization 'p'		0.138 (±0.006)	0.104 (±0.005)
ANS	'n'	105 (±7)	180 (±10)
	K_D	1.1 (±0.5)	2.1 (±0.3)
Tb^{3+}	'n'	50 (±3)	84 (±6)
	K_D	0.7 (±0.2)	0.8 (±0.2)

[a] Results shown are the average of experiments with a minimum of three different membrane preparations.

[b] Activity expressed in μ moles of Pi liberated/mg. protein/hr.

significance of the low Ca^{2+}-ATPase activity found in the head membranes is not clear, though it may be of particular relevance, in view of the Ca^{2+}-transmembrane fluxes associated with the induction of acrosome reaction, in the head region (1).

The aromatic hydrocarbon pyrene, in a diffusion controlled process can form dimers in the excited state (excimers), with characteristic emission at 470 nm. The excimer fluorescence can readily be used to measure membrane fluidity, since the formation of excimers is related to the lateral mobility of pyrene molecules in the lipid phase (19,20). Fig.2a shows the emission spectra of pyrene dispersed in aqueous buffer and in the plasma membranes of the head and tail fractions. The excimer band intensity is relatively low in the head membranes. The dependence of the dimer/monomer (D/M) ratio, on pyrene concentration,

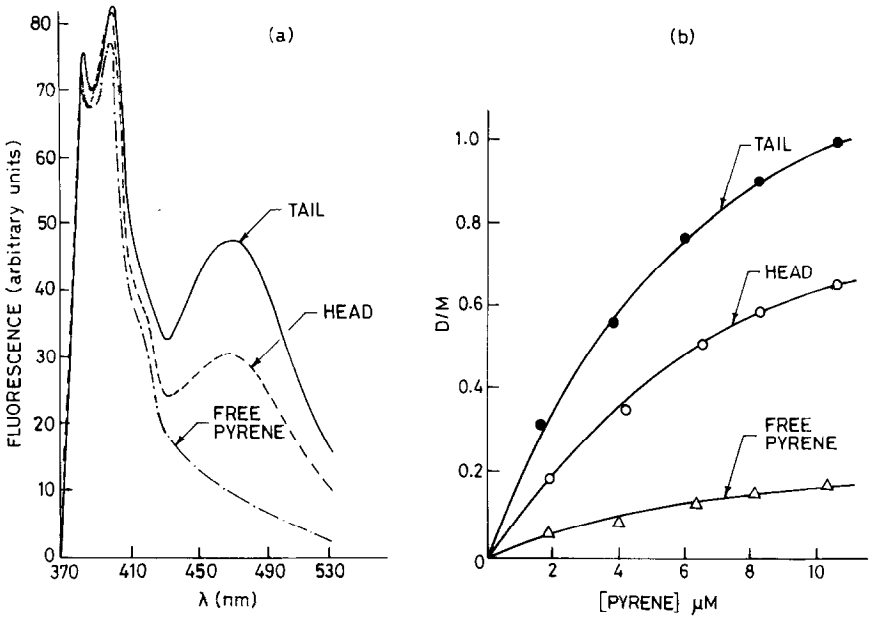

Fig.2. (a) Uncorrected emission spectra of pyrene (4 μM) incorporated into isolated head and tail membranes of bull sperm. Protein concentration: 100 μg/ml.
(b) D/M intensity ratio for pyrene incorporated into membranes, as a function of pyrene concentration. Protein concentration 100 μg/ml.

for the two membranes is shown in Fig.2b. Over the concentration range studied, a large population of excimers is readily formed in the membranes of the tail fraction. These results suggest that the plasma membrane lipid phase is more fluid in the tail region.

Fluorescence polarization of DPH incorporated into membranes reflects the rotational mobility of the probe in the lipid layer (21). Polarization values ('p') for the membranes of the head and tail fractions are summarized in Table 1. The significantly lower 'p' value obtained for the tail membranes correlates well with the relatively higher lipid phase mobility observed in that fraction, using pyrene excimer fluorescence.

The extent of binding of the anionic fluorescent probe ANS is determined by membrane surface charge (22-24)

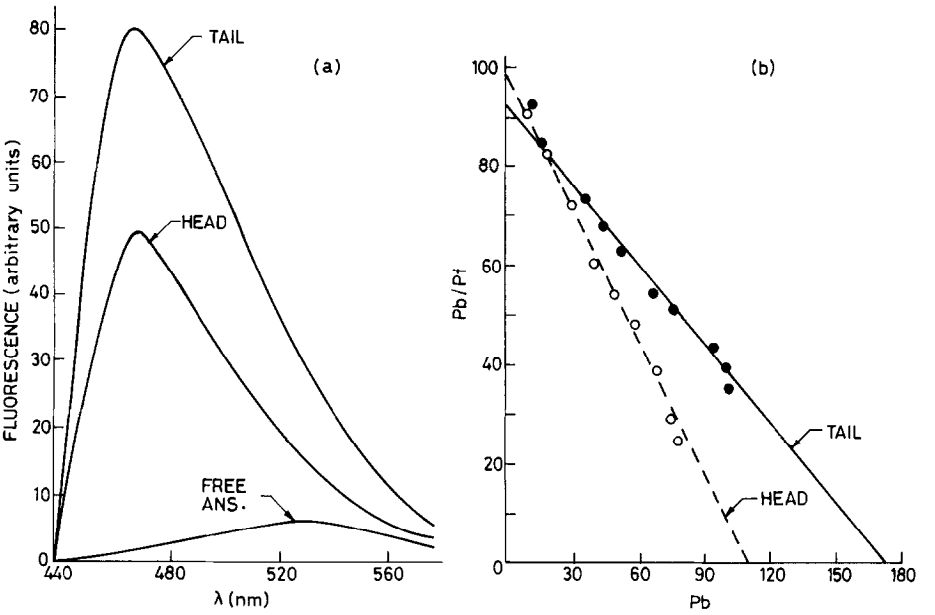

Fig.3. (a) Fluorescence spectra of ANS in the presence of isolated head and tail membranes of bull sperm. λ excitation = 370 nm. ANS concentration : 20 μM. Protein concentration: 100 μg/ml.
(b) Representative Scatchard plots for ANS binding to bull sperm head and tail membranes. λ emission = 472 nm. Pb and Pf are concentrations of bound and free probe, respectively.

and the degree of lipid disorder (25). Fig.3a shows the emission spectra of ANS in the presence of head and tail membranes. Probe binding is accompanied by large intensity enhancements and the emission peak is blue shifted to 472 nm. Fluorescence titrations were carried out and conventional Scatchard analysis (Fig.3b) was adopted for data treatment (25). The results of the experiments (Table 1) clearly establish large differences in the regional distribution of surface charge and lipid organization in the plasma membranes of head and tail regions. The greater binding of ANS to tail membranes is in good agreement with the observation that the plasma membrane of the tail is relatively more fluid, since ANS binding reflects both

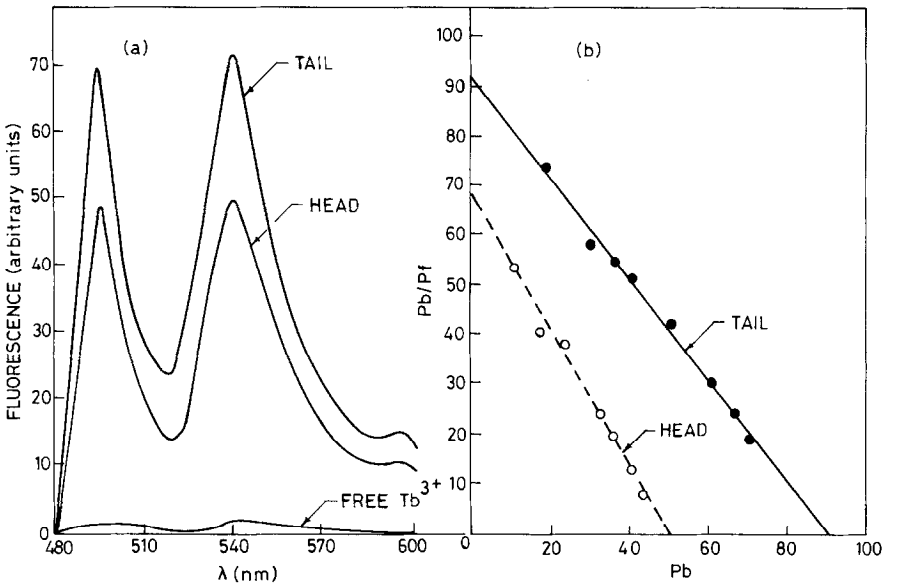

Fig.4. (a) Fluorescence spectra of Tb^{3+} in the presence of isolated head and tail membranes of bull sperm. $\lambda_{excitation}$ = 295 nm. Tb^{3+} concentration 25 μM; Protein concentration 150 μg/ml.
(b) Representative Scatchard plots for Tb^{3+} binding to bull sperm head and tail membranes. $\lambda_{excitation}$ = 295 nm. $\lambda_{emission}$ = 546 nm. Pb and Pf are concentrations of bound and free probe, respectively.

charge and packing effects; more disordered membranes accommodating larger amount of probe (25).

Terbium fluorescence has been widely employed to probe Ca^{2+}-binding sites in the studies of proteins (27,28) and biological membranes (29,30). Emission spectra of Tb^{3+}-fluorescence, in the presence and absence of head and tail membranes are shown in Fig.4a. The fluorescence of Tb^{3+} is considerably enhanced at 546 nm, on binding to membranes. Fluorescence titrations were carried out; Scatchard plots have been constructed (Fig.4b) and the binding parameters are presented in Table 1. The significantly higher binding of Tb^{3+} by the plasma membranes of the tail as compared to that of the head region suggests a larger number of Ca^{2+}-binding sites on the tail surface.

The observed differences in the lipid phase fluidity, surface charge density including Ca^{2+}-binding sites and the distribution of membrane-bound Ca^{2+}-ATPase, between the head and tail regions, constitute clear evidences for the regionally differentiated state of sperm plasma membranes. The precise physiological relevance of regional specialization of the spermatozoan surface, remains to be established.

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