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Plasma membrane of trout spermatozoa: I. Isolation and partial characterization

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Abstract

The plasma membrane from spermatozoa of rainbow trout was isolated by four techniques: sonication, hypotonic shock, mechanical homogenization after freeze-thawing, and nitrogen cavitation, in combination with continuous sucrose gradient centrifugation. Nitrogen cavitation (900 PSI, 20 min equilibration at 4°C) was the most effective technique.

Following nitrogen cavitation, four bands were recovered in the sucrose gradient at densities $\approx$ 1.03, 1.05, 1.09 and 1.15 g/ml. Electron microscopy revealed membrane vesicles of various sizes in bands 1 to 3, while enzyme analysis revealed a 3.9 to 5.5-fold enrichment in 5'-nucleotidase and little contamination by lactate dehydrogenase (cytosol) and succinic dehydrogenase (mitochondria). Lipid analysis of bands 1 and 2 indicated a 6 to 7-fold enrichment in cholesterol and a cholesterol: phospholipid ratio of 0.59–0.70. Seven classes of phospholipids were present in bands 1–3 with no significant differences observed among bands. These data indicate that the vesicles (in bands 1 and 2) obtained after nitrogen cavitation are primarily plasma membranes. Membranes in band 3 appear to be slightly contaminated with nuclear membranes.

Most of the plasma membrane proteins were acidic to neutral. The 2 main membrane proteins were 42 and 30 Kilodaltons.

Introduction

The sperm plasma membrane is an important participant in several steps leading to fertilization. However, several of the events preceeding the delivery of the paternal genome into the egg which occur in mammals, do not occur in salmonid fish. There is no epididymal maturation (although changes of the physicochemical parameters of seminal fluid along the genital tract might influence the spermatozoan metabolism in salmonids (Morisawa and Morisawa 1988)), capacitation or acrosome reaction. In salmonids, the critical step for mating success is the initiation and maintenance of sperm motility (Cosson *et al.* 1989). Flagellar beating is controlled by external K^+ concentration and by Ca^{2+} influx through the plasma membrane (Cosson *et al.* 1989). This suggests that the biochemical and biophysical properties of this cell organelle could influence the ability of fish sperm to fertilize eggs.

When stored in the male genital tract, sperm cells are subjected to large (up to 200 mOsmoles) and rapid changes in osmotic pressure (Maisse *et al.* 1988; Loir *et al.* 1990a). The ability of the plasma membrane from trout sperm to resist osmotic shock varies throughout the breeding period and between

males and is positively correlated with freezing tolerance (Malejac *et al.* 1990). Properties of the plasma membrane of fish spermatozoa also appear to determine cryopreservation success (Baynes and Scott 1982; Maisse *et al.* 1988).

Changes in body temperature initiate alterations in the composition of membranes which are responsible for "homeoviscous adaptation". Lipids are the only structural components of membranes modified as a consequence of thermal acclimation (Hazel 1984).

On the other hand, the distribution of fatty acids in the total lipid of trout sperm is greatly affected by diet (Watanabe *et al.* 1984). According to Baynes and Scott (1982), both the fatty acid composition and fertilizing capacity after cryopreservation differ between trout fed two different diets.

Our laboratory has taken the approach that detailed studies of 1) the molecular composition of the sperm surface, and 2) alterations induced by thermal adaptation and diet, would lead to an understanding of the mechanisms which are involved in the maintenance of motility and the freezing ability of salmonid spermatozoa. Before such studies can be attempted, it is necessary to develop a method for the isolation of plasma membranes in a highly purified form. Several methods have been used to detach the plasma membrane of sperm cells (McNamee 1989), including sonication, mechanical homogenization, hypotonic shock, and nitrogen cavitation. According to Gillis *et al.* (1978), the latter technique is the most effective. While our study was in progress, Lou *et al.* (1990) opted to prepare plasma membranes from salmon sperm by sonication. In this report, 1) preliminary experiments were carried out to identify the most suitable method of plasma membrane preparation from trout spermatozoa, and 2) we describe the nitrogen cavitation procedure we developed. Membrane preparations were characterized using electron microscopy, phospholipid analysis, marker enzyme analysis and electrophoretic analysis of proteins.

Materials and methods

Semen was collected throughout the spawning period, by hand stripping, from 1 to 3 year-old mature autumn-spawning and spring-spawning males of rainbow trout kept at 12–15°C in recycled freshwater, under natural photoperiod. Care was taken to avoid contamination by blood and feces (Maisse *et al.* 1988). Semen was stored on ice. Testicular sperm was collected in the following way. Testes were removed (after males had been anesthetized with 0.3% phenoxyethanol and killed by a blow to the head) and cut into pieces in seminal fluid mineral medium (SFMM, Billard and Jalabert 1974). The suspension was filtered through 150 μm nylon filters, then stored on ice.

All the following operations were carried out at 1–4°C.

Plasma membrane isolation using nitrogen cavitation

Semen or a sperm suspension obtained from several males, was centrifuged at 500 g for 15 min, followed by 1000 × g for 5 min. These conditions affected neither sperm motility nor morphology (Loir, unpublished results). Spermatozoa were resuspended (5 to 10 × 10^9 cells/ml) in 0.25 M sucrose in TM buffer (20 mM Tris-HCl buffer pH 7.8, 3 mM $MgCl_2$, 1 mM PMSF, 20 μg Soybean Trypsin inhibitor/ml, 0.1 μg pepstatin/ml). Thirty ml of sperm suspension was then submitted to nitrogen cavitation in a Parr cell disruption bomb (ref. 4639) packed in ice. Routinely, the cells were pressurized to 900 lb/in² (PSI; 62 bars) during a 20 min period, with continuous magnetic stirring. The cavitate was next homogenized by gentle strokes of a glass Dounce homogenizer. The homogenate was then layered over a 0.65 volume of 1 M sucrose in TM buffer, and centrifuged at 500 × g for 15 min followed by 1000 g for 5 min. The crude membrane fraction above the sucrose cushion was collected. Twenty ml of the crude preparation was next layered over 19 ml of a 0.25–1.8 M sucrose gradient. The gradients were made up in 9 steps (0.25, 0.375, 0.5, 0.75, 1 and 1.2 M: 2.5 ml; 1.4, 1.6 and 1.8 M: 1.5 ml) and rendered nearly continuous by storage for 20 h at room temperature. After centrifugation at 54,000 × g for 2 h (SW28 rotor, Beckman L8-55 ultracentrifuge), 4 bands were con-

sistently visible, at mean densities of 1.03 g/ml (Band 1), 1.05 g/ml (Band 2), 1.09 g/ml (Band 3) and 1.15 g/ml (Band 4). Bands were carefully removed (with a syringe and a bent needle), diluted 3-fold with TM buffer, and centrifuged at 100,000 g for 30 min. Pellets were suspended in 0.25 M sucrose in TM buffer (1−2 mg protein/ml) and either used immediately or stored at −70°C.

Membrane isolation by sonication

After resuspension (1.5 to 3×10^9 cells/ml) in SFMM pH 7.5, 1 mM PMSF, spermatozoa were sonicated in an ice-bath, 2 to 6 times for 30 sec (with 15 sec between treatments), using an MSE 150 W ultrasonic desintegrator at maximum power. Most of the nuclei and some (unbroken) flagella were removed by centrifugation (1,000 × g, 15 min). Sucrose was then added to a final concentration of 0.25 M, and the suspension was centrifuged through 0.375−1.8 M sucrose gradients, as indicated above.

Membrane isolation using hypotonic treatment

Washed and pelleted spermatozoa were resuspended in 20 volumes of 5 mM Tris-HCl buffer pH 8, 1 mM PMSF (1×10^9 cells/ml as a mean) for 15 min. Following centrifugation (1,000 × g, 20 min), the pellet was resuspended and homogenized by several strokes of a glass Dounce homogenizer. Sucrose was then added (0.25 M final concentration) and nuclei and flagellae were partly removed by centrifugation (1,000 × g, 15 min). The suspension was then applied to sucrose gradients as above.

Membrane isolation by freeze-thawing

Washed spermatozoa resuspended in TM buffer were frozen at −70°C. Slow thawing was carried out by storage overnight at 4°C. After addition of sucrose (0.25 M final concentration) the suspension was homogenized by several strokes of a glass

homogenizer and treated as described above for nitrogen cavitation.

Extraction of membrane proteins with Na deoxycholate

Membrane proteins were extracted according to Loir *et al.* (1990). Briefly, washed spermatozoa or fractions were treated for at least 45 min with 0.2% Na deoxycholate in 5 mM Hepes buffer pH 8. After centrifugation (1,000 × g, 20 min), proteins were precipitated from the supernatant by mixing with 9 volumes of ethanol. After sitting overnight at −20°C, proteins were collected by centrifugation (15,000 × g, 15 min).

Electron microscopy

Membrane material (obtained at the different steps after disruption of sperm cells by nitrogen cavitation and material obtained after sonication and hypotonic treatment) was pelleted by centrifugation, resuspended in 4% glutaraldehyde (in phosphate buffer, pH 7.5), washed in 0.2 M cacodylate buffer pH 7.4, and, finally post-fixed for 1 h in 1% osmium tetroxide (in cacodylate buffer containing 1.5% potassium ferricyanide). The material was embedded in 2% agar, dehydrated in acetone, and then embedded in Epon-Araldite. Sections were double-stained and observed with a Philips CM12 electron microscope.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide slab gel electrophoresis was performed according to Laemmli (1970) on 6−16% gradient gels. Pelleted material obtained at various steps of membrane isolation was resuspended either in sample buffer (0.062 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol and 5% β-mercaptoethanol) or in 0.2% Na deoxycholate. Proteins solubilised by deoxycholate were precipitated by ethanol (see above) and then resuspended in sample buffer. Before loading, all samples were

boiled for 5 min. Proteins were stained with 0.2% R250 Coomassie blue. Gels were scanned in a Vernon PHI5 densitometer.

Membrane proteins were also analysed by two-dimensional electrophoresis according to Dacheux *et al.* (1989). Briefly, the isoelectric focusing (IEF) separation was carried out according to O'Farrell (1975) in the presence of ampholytes (2–11 pH). The second-dimension electrophoresis was identical to the 1D system. The proteins were detected by silver staining according to Damerval *et al.* (1986).

Enzyme assays

Three enzymes were assayed in the membrane fractions. The method of Michell and Hawthorne (1965) was used for the determination of 5'-nucleotidase (EC 3.1.3.5; plasma-membrane marker) with the following modification. Fractions were incubated with 100 mM sodium glycerol 2-phosphate (phosphatase activities were saturated by this concentration), and either in the presence or absence of 5 mM adenosine 5'-monophosphate. The 5'-nucleotidase activity was estimated by the difference between the two values obtained. The incubation time was 1 h. All necessary controls were carried out. The amount of phosphate released was determined according to Lanzetta *et al.* (1979). Succinic dehydrogenase (EC 1.3.99.1; mitochondrial marker) was determined according to Green *et al.* (1955). Lactate dehydrogenase (EC 1.1.1.27; cytosolic marker) activity was determined using Boehringer kit (ref. 543039), after adaptation, to carry out multiple assays in 96-well plates, using a standard plate reader.

Lipid analysis

Lipids from sperm homogenates and from various sperm fractions obtained after cavitation were extracted according to Folch *et al.* (1957). The chloroform/methanol mixture (2:1, v/v) contained 0.02% Butyl Hydroxytoluene (BHT). The lower phase was carefully separated, dried and dissolved in 2-propanol. Total cholesterol was determined

after lipids were extracted, by an enzymatic method (Biochemica test combination, Boehringer) adapted to carry out assays in 96-well plates. Phospholipid phosphorus was determined after hydrolysis with sulfuric acid according to Bartlett (1959). Total phospholipid was calculated from the inorganic phosphate and expressed as μmol phosphate/mg protein. Phospholipids were collected according to Juaneda and Rocquelin (1985), the dried lower phase being dissolved in 0.5 ml chloroform then fractionated on Sep-Pak silica columns (Waters associates). Phospholipid classes were visualised by thin-layer chromatography (TLC) on silica gel plates (Merck, ref. 5626) in chloroform/methanol/acetic acid/petroleum ether (40/20/10/30, v/v) plus 1.8% (w/v) boric acid. Lipid bands were located using iodine vapor. Identification of the bands was by comparison to reference phospholipids (purchased from Sigma). Phospholipid classes were separated by high performance liquid chromatography (HPLC) using a Zorbax silica column (250 mm length, 4.6 mm internal diameter, 5 μm particle size) and an hexane/isopropanol/water solvent (3/2/.125 to .250, V/V). Phospholipids were assayed according to Bartlett (1959).

Other methods

Protein was usually determined according to Lowry *et al.* (1951). However, because of the interference of several molecules used, the method of Smith *et al.* (1985), using Pierce "BCA protein assay reagent" kit, was preferred for protein determinations related to lipid analysis. In both cases, bovine serum albumin was used as a standard. The mean difference between protein concentration values obtained for 22 samples by the 2 methods was equal to $\pm$ 5.8% of the mean value.

Results

Comparison of four methods for cell fractionation

Efficiency of sonication appeared to depend on the sperm concentration, the volume treated, and the
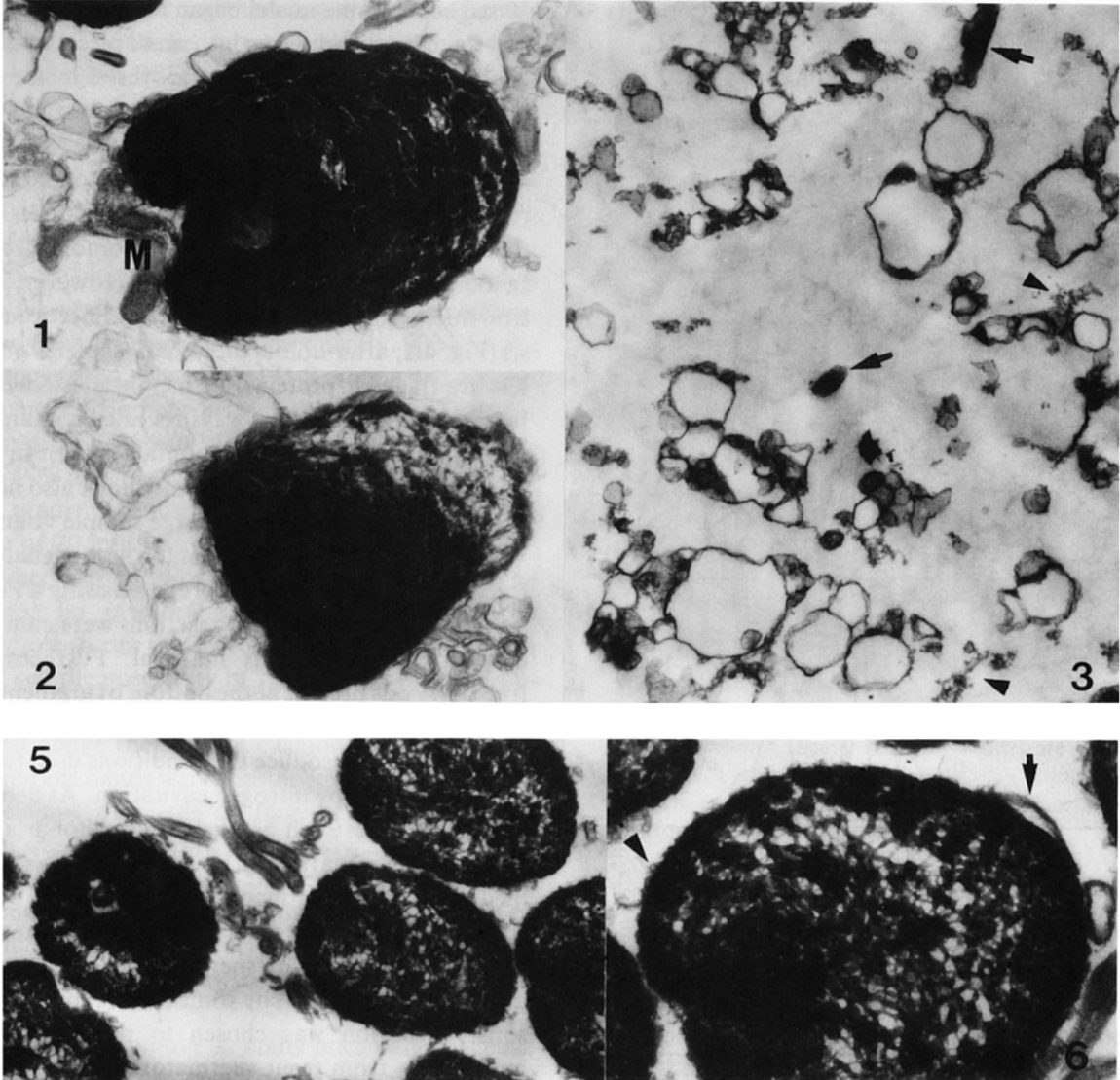
Fig. 1. A sperm head after sonication twice for 30 seconds. Plasma and nuclear membranes are only partly detached and chromatin is unchanged. M: mitochondria (×24,200).

Fig. 2. A sperm head after sonication 6 times for 30 seconds. Chromatin is decondensed and begins to disperse (×24,200).

Fig. 3. Membrane fraction collected from sucrose gradient (d ≈ 1.06 g/ml) after detachment by hypotonic treatment. Arrows: pieces of flagella. Arrowheads: non membranous material (×15,500).

Fig. 5. Sperm heads and flagellae in the homogenate obtained after nitrogen cavitation. Most of these organelles are devoid of plasma membrane (×14,200).

Fig. 6. Detail of a sperm head after nitrogen cavitation. Pieces of plasma membrane (short arrow) still adhere to the head. The nuclear membrane is hardly visible. Small membranous blebs (arrowhead) could correspond to the external layer of the nuclear membrane (×31,200).

geometry of the vessel, in addition to the power and frequency employed. The optimal duration time of sonication was difficult to determine. For instance, electron microscopy revealed that the plasma membrane was detached only partly from nuclei and flagellae by two 30 sec bursts (Fig. 1), while after six
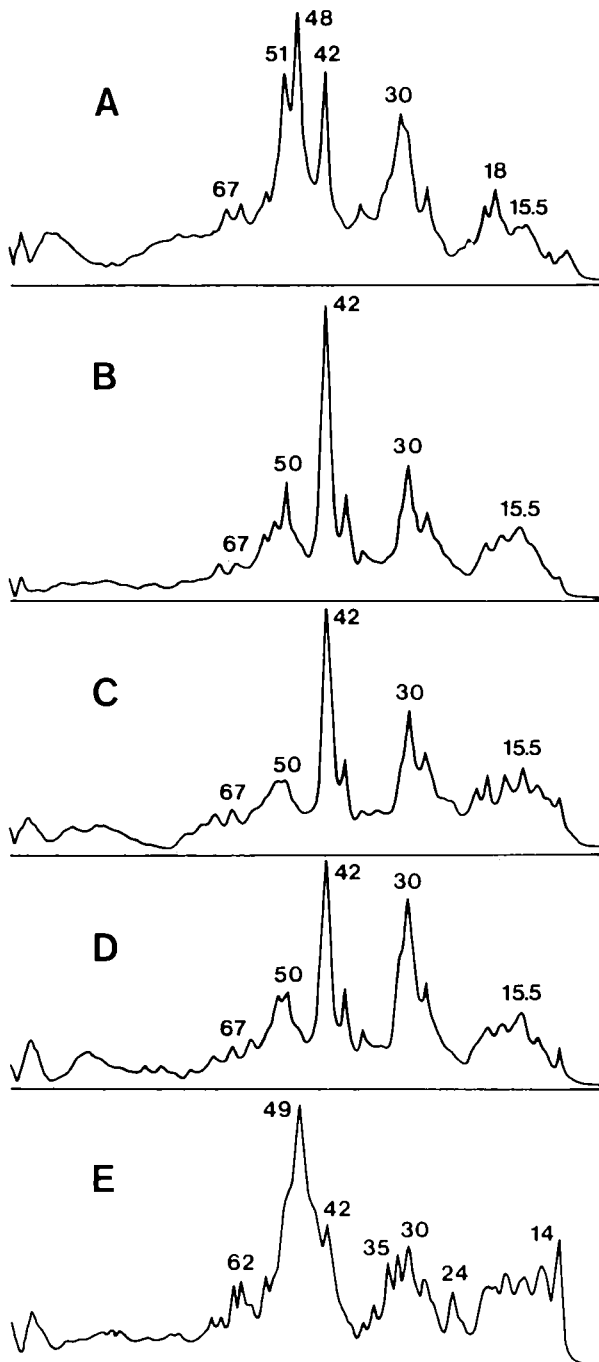
Fig. 4. Electrophoregrams (SDS gels) of proteins extracted from membrane vesicles obtained, A: after hypotonic treatment (protein 48 KD reveals flagellar contamination); B: after nitrogen cavitation, in band 1; C: idem, in band 2; D: idem, in band 3. E: proteins extracted from material obtained after nitrogen cavitation in band 4; proteins 24, 35, 48–49 (tubulin) and 62 KD correspond to the major flagellar proteins (Loir et al. 1990). Molecular weights of proteins are indicated in KD.

30 sec bursts some nuclei began to disperse (Fig. 2) and flagellae were broken into small pieces or disorganised so that membranes recovered in the gradient were contaminated by amorphous material and flagellar fragments.

Trout sperm subjected to hypotonic shock released large membrane ghosts which often contained a coiled flagellum. Potter homogenization favoured the removal of flagellae. However, electron microscopy (Fig. 3) and electrophoretic analysis (Fig. 4A; after Loir et al. (1990), proteins 48–51 KD are flagellar proteins) of membranes collected from sucrose gradients always revealed contamination by flagellae and other material (Fig. 3). In contrast to other methods, hypotonic shock also necessitated the centrifugation of large sample volumes.

Floculate material was present in homogenates of freeze-thawed spermatozoa prepared using a Potter homogenizer, and gradient fractions were contaminated by heterogeneous material. Furthermore, free DNA was present at the bottom of gradients indicating that nuclei had been destroyed.

It is easy to reproduce the conditions used to disrupt sperm cells by nitrogen cavitation. As detailed later, organelles such as nuclei, mitochondria and flagellae were unaffected by cavitation in isotonic medium while being inconstantly disrupted by the sonication and freeze-thawing techniques. In addition, large amounts of sperm can be processed in about the same time as by sonication. For these reasons, cavitation was chosen to prepare plasma membranes from trout spermatozoa.

Cell fractionation by nitrogen cavitation

To determine the optimal conditions for the disruption of sperm cells, we considered 1) the yield of deoxycholate-soluble (D-S) protein released into the supernatant collected above the sucrose cushion and 2) the yield of D-S protein collected from the gradient in bands 1 to 3, compared to the amount present in the cavitate.

The yields of D-S protein were not conspicuously different whether sperm cells were disrupted in hypotonic medium (TM buffer) or in 0.25 M sucrose in TM buffer. Thus, isotonic medium, which

minimised nuclear and mitochondrial damage, was retained. The applied pressure was varied between 600 and 1100 PSI. The yield of D-S protein obtained in the supernatant after cavitation at 600 PSI was lower (30−32%) than at 800 to 1100 PSI (35−46%). The yield of D-S protein after gradient centrifugation was 8.5−23% for 600 PSI, 18−26% for 800−850 PSI and 6−18.5% for 1100 PSI. Furthermore, when the pressure was 600 PSI, bands 2 and 3 were contaminated by only partly demembranated pieces of flagella. Consequently, 900 PSI was routinely used. The yield of D-S protein was not significantly different whether cavitation was for 20 or 40 min: 38−43% and 18−20% after 20 min versus 45−46% and 19−20% after 40 min, in supernatant and gradient bands respectively. Thus, the shorter time of 20 min was retained.

When numerous or large sperm samples are to be treated for membrane preparation, it would be desirable to run the sucrose gradients later. So, we checked the possibility of freezing the material obtained after cavitation. When the suspension collected above the sucrose cushion was frozen, the membrane bands were conspicuously contaminated by clumps of pieces of flagella aggregated with membranous material. Conversely, when the cavitate was frozen, then thawed and centrifuged through a 1 M sucrose cushion, the composition of the membrane bands obtained at subsequent steps appeared to be similar to those obtained without freezing.

In order to remove as many nuclei and flagellae as possible before gradient centrifugation, three procedures were tried. 1) the cavitate was centrifuged at 500 × g for 20 min; 2) the sucrose concentration of the cavitate was raised to 1.2 M prior to centrifugation at 500 × g for 20 min followed by 2,000 × g for 10 min; 3) the cavitate was centrifuged through a sucrose cushion (see Material and Methods). Whereas only 26% of the D-S protein was retrieved in the supernatant with the first method, 40−42% was retrieved with the 2 others. Electrophoresis and phase contrast microscopy were employed to show that the purity of the membrane fractions collected from the gradients was similar with the 3 techniques. Because it was easier to work with sucrose cushions, this method was routinely used.

Fig. 7. Material collected in band 4 in sucrose gradient after nitrogen cavitation. Some membrane vesicles are mixed with mitochondria (arrows) and pieces of flagellae, mostly devoid of plasma membrane. Arrowhead: section of flagellum surrounded by plasma membrane (× 24,200).

Morphological analysis

Electron microscopic analysis of the homogenate obtained after cavitation and homogenization revealed the following points. 1) Most of the flagella were detached from the heads. 2) About 80−95% of the flagellar membrane was detached (Figs. 5 and 7). 3) The classic organisation of the flagellar tubules was disrupted only in certain flagellae. 4) While in all the nuclei, the central chromatin was somewhat less compact than in untreated spermatozoa, the peripheral chromatin layer remained compact and homogeneous, displaying no sign of dispersion (Fig. 6). 5) Roughly 80−95% of the plasma membrane was removed from the heads. 6) Although it was difficult to distinguish the nuclear membrane, it seemed that at least 10−20% were unbroken and that an inestimable amount of the nuclear membrane, or of only its external layer, was broken into small vesicles either still adhering to the
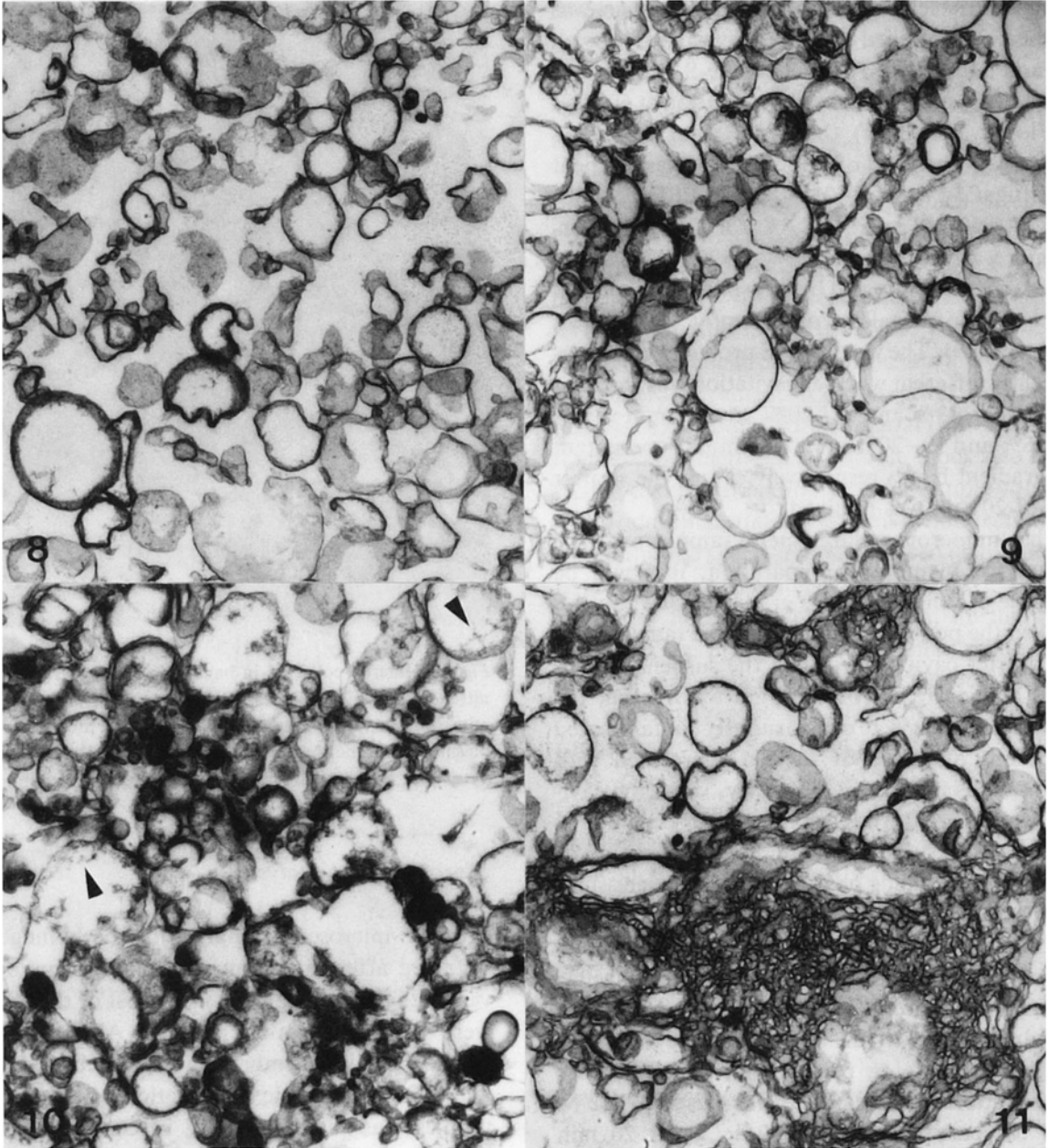
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Fig. 8, 9, 10 and *11*. Electron micrographs ($\times$24,200) of membranes obtained after nitrogen cavitation in, 8: band 1, 9 and 11: band 2, 10: band 3. In band 3, fibrous material (arrowheads) is attached to the inner side of vesicles. Two clusters of membrane sheets are shown in Fig. 11.

nuclei or not (Fig. 6). 7) All the mitochondria were detached from the heads.

Electron microscopy confirmed that the supernatant collected above the sucrose cushion contained flagellae mixed with membranous material and no nuclei.

Bands 1 (d $\approx$ 1.03 g/ml), 2 ($\approx$ 1.05 g/ml) and 3 ($\approx$ 1.09 g/ml) contained smooth vesicles of heterogeneous size (0.12–0.9 μm in diameter in band 1 and 0.15–0.75 μm in bands 2 and 3) and some membrane sheets (Figs. 8–10). At high magnification, orthogonal sections revealed the typical

Table 1. Specific activities of enzymes in cavitated trout spermatozoa and in sucrose gradient fractions

	Homogenate	B1	B2	B3	B4
Protein mg/10^{10} spzoa.	12.4 ± 1.9 (n = 16)	.068 ± .034 (n = 18)	.069 ± .020 (n = 18)	.113 ± .059 (n = 18)	.406 ± .222 (n = 4)
5'-nucleotidase (n = 3) nmoles Pi/min/mg	1.02 ± .17	4.16 ± .35 (×4.1)	5.64 ± .61 (×5.5)	3.95 ± 1.26 (×3.9)	1.68 ± .42
SDH (n = 2) nmoles/min/mg	475 ± 145		< 75		1609 ± 1209 (×3.4)
LDH (n = 3) U/mg protein	297 ± 120		84 ± 40		53 ± 23

Data are shown as Mean ± SD (number of experiments); at least 7 males were used for each experiment.
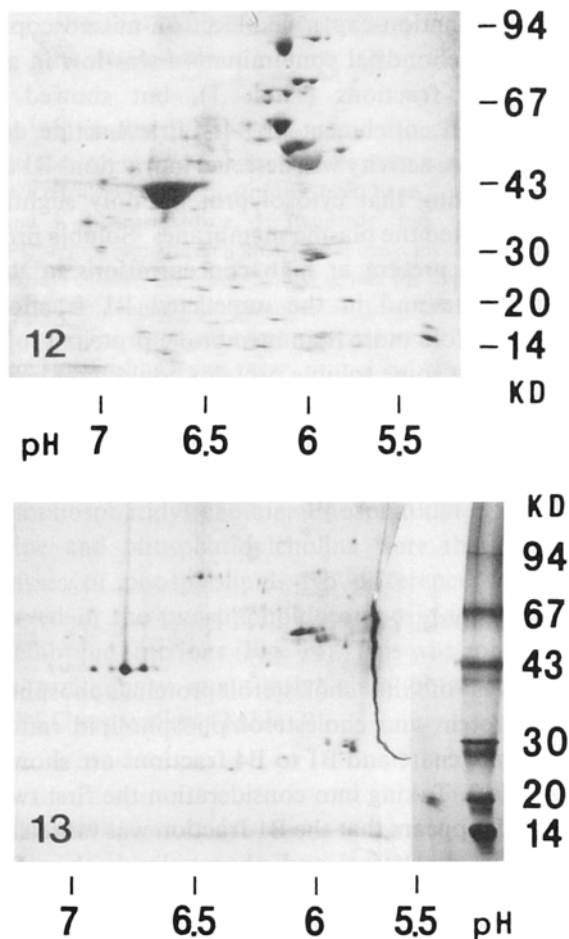

Fig. 12 and *13.* Two-dimensional electrophoresis of proteins, 12: extracted from spermatozoa by Na deoxycholate and 13: from membrane vesicles obtained in band 1 after nitrogen cavitation. Right: standard molecular weights.

trilaminar structure of biological membranes. Most of the vesicles present in fractions 1 and 2 did not contain electron-dense material. In band 3 (Fig. 10) and also in band 4 (Fig. 7), some vesicles showed a somewhat fibrous material adhering to the inner side of the membrane. Neither microtubules nor mitochondria were seen in fractions 1 to 3. In these 3 bands, some structures (0.4–2 μm mean diameter) composed of tightly packed membrane sheets (Fig. 11) were present while a very low proportion of vesicles were constituted of several concentrically-arranged membrane layers. Band 4 (Fig. 7) consisted of some membrane vesicles mixed with pieces of flagellae, some dispersed microtubules, well-preserved mitochondria and some amorphous material.

Protein analysis

After cavitation and centrifugation through a 1 M sucrose cushion, 35 to 39% (n = 3) of the total proteins present in the cavitate were retrieved in the suspension collected above the cushion. The relative amount of membrane protein present in bands 1, 2 and 3 was variable. The total recovery of sperm membrane proteins in the 3 bands varied around 2% (Table 1), *i.e.*, a mean of 250 μg membrane protein was obtained from 10^{10} spermatozoa. The yield could be improved (no quantitative estimation) by re-treating the material which had both

Table 2. Cholesterol and phospholipid content of cavitated trout spermatozoa and sucrose gradient fractions

	Homogenate (n = 3)	B1 (n = 3)	B2 (n = 5)	B3 (n = 4)	B4 (n = 2)
Chol./Prot. (nmoles/mg)	165 ± 67	1134 ± 598 (×6.9)	1041 ± 571 (×6.3)	573 ± 144 (×3.5)	173 ± 8
Phosp./Prot. (nmoles/mg)	354 ± 98	1920 ± 264 (×5.4)	1480 ± 382 (×4.2)	840 ± 276 (×2.4)	609 ± 109
Chol./Phosp.	.47 ± .08	.59 ± .19	.70 ± .23	.68 ± .29	.28 ± .04

Data are shown as Mean ± SD (number of experiments); at least 7 males were used for each experiment.

centrifuged into the sucrose cushion and pelleted under the cushion. The purity of the membrane fractions so obtained was similar to that obtained by the original procedure.

SDS-PAGE analysis revealed that membrane proteins and molecular weights ranging between 10.5 and 110 KD. The predominant proteins were 42 and 30 KD. Protein patterns were similar for fractions B1, B2 and B3 (Fig. 4). However, there was a tendency for the relative amount of 11−22 KD and especially 26−32 KD proteins to increase from B1 to B3, as compared to the 42 KD protein (Fig. 4B,C,D).

Membrane proteins were also present in B4, but they were mixed with proteins characteristic of flagellae, especially the main flagellar protein of 49 KD (Loir *et al.* 1990b), which predominated in this fraction (Fig. 4E). A similar pattern was observed in material obtained from the sucrose cushion.

Two-dimensional electrophoresis of membrane proteins from bands 1−3 revealed the presence of more than 50 proteins, the majority of which were acidic to neutral (Fig. 13). All of these proteins were also present in deoxycholate extracts of total washed spermatozoa, which also contained numerous additional proteins (Fig. 12). Several charge variants of apparently singular proteins were observed. Two minor proteins streaked across the gel map, suggesting they might be glycoproteins.

Enzyme analysis

Subcellular fractions were assayed for 5'-nucleotidase (Table 1). Specific activity of this enzyme was

3.9 to 5.5-fold greater in bands 1−3 than obtained in the homogenate. Measurements of succinic dehydrogenase activity, as a marker for mitochondrial contamination confirmed electron-miscroscopic data: mitochondrial contamination was low in all membrane fractions (Table 1), but showed a pronounced enrichment in B4. Little Lactate dehydrogenase activity was detected in fractions B1 to B3 suggesting that cytosol proteins only slightly contaminated the plasma membranes. Soluble proteins were present at high concentrations in the homogenate and in the unpelleted B1 fraction (about 30 fold more than membrane proteins) suggesting that some soluble proteins could have been trapped within membrane vesicles. However, sonication of the B1 fraction prior to pelleting the membranes, did not change significantly the protein pattern.

Lipid analysis

Estimates of the cholesterol/protein, phospholipid/protein and cholesterol/phospholipid ratios for homogenate and B1 to B4 fractions are shown in Table 2. Taking into consideration the first two ratios, it appears that the B1 fraction was more enriched in cholesterol and phospholipids than B2 and B3. Nevertheless, the cholesterol/phospholipid ratio was similar for the 3 fractions, being higher than in the homogenate or B4 fraction.

Seven classes of phospholipid were identified in membrane fractions by thin-layer chromatography (Fig. 14): diphosphatidyl glycerol, phosphatidyl
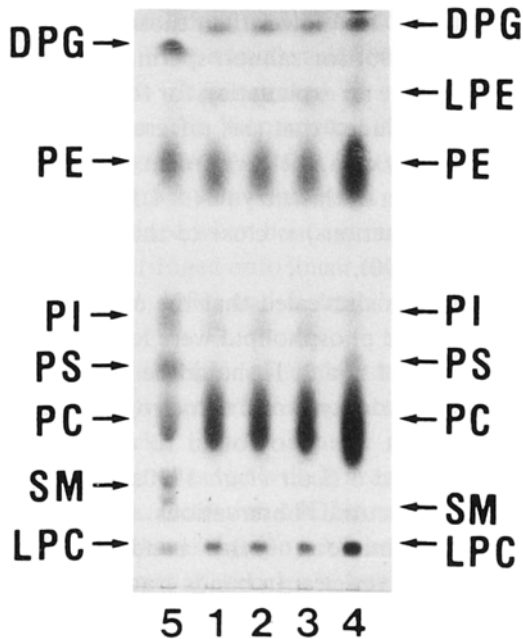
Fig. 14. Thin-layer chromatographic separation of phospholipids from, 1: membrane vesicles from band 1 obtained after nitrogen cavitation, 2: idem from band 2, 3: idem from band 3, 4: spermatozoa, 5: standards from bovine brain. LPC: lysophosphatidyl choline, SM: sphyngomyelin, PC: phosphatidyl choline, PS: phosphatidyl serine, PI: phosphatidyl inositol, PE: phophatidyl ethanolamine, LPE: lysophosphatidyl ethanolamine, DPG: diphosphatidyl glycerol. Phospholipids were visualized by iodine vapor.

Table 3. Phospholipid composition of the 3 membrane fractions. Distribution of the five major classes of phospholipid expressed as a percentage of total phospholipid, after HPLC separation

	B1	B2	B3
DPG	7 ± 5	10.5 ± 3.5	7.5 ± 3.5
PE	31.5 ± 2.5	30.5 ± 2.5	32.5 ± 2.5
PI	4 ± 1	2 ± 1	2.5 ± 0.5
PS	8.5 ± 3.5	7.5 ± 2.5	10.5 ± 2.5
PC	46.5 ± 2.5	47 ± 3	47 ± 1

Data are shown as means ± SD (n = 2). DPG: diphosphatidyl glycerol; PE: phosphatidyl ethanolamine; PI: phosphatidyl inositol; PS: phosphatidyl serine; PC: phosphatidyl choline.

ethanolamine, phosphatidyl inositol, phosphatidyl serine, phosphatidyl choline, sphyngomyelin and lysophosphatidyl choline. Phosphatidyl ethanolamine and phosphatidylcholine were the 2 main classes of phospholipid. No difference was observed in the phospholipid composition of the 3 membrane fractions (Fig. 14). This was confirmed by preliminary quantitative data obtained after HPLC separation (Table 3).

Discussion

The data presented in this study show both that nitrogen cavitation is an effective method for disruption of the plasma membrane from trout spermatozoa and that 3 subsets of membranes can be isolated using linear sucrose density gradient centrifugation.

Since the work of Gillis *et al.* (1978), nitrogen cavitation has been used to prepare plasma membrane from mammalian and invertebrate spermatozoa. This method overcomes the problems associated with the use of detergent, mechanical homogenization, hypotonic shock or sonication (McNamee 1989). The yield of membrane protein following release of the plasma membrane by nitrogen cavitation was always equal or greater than 1% of the total cell protein (Gillis *et al.* 1978: 1%; Mack *et al.* 1986: 1.6%), *i.e.*, according to Gillis *et al.* (1978) and Parks and Hammerstedt (1985) 20 to 30% of the membrane protein was obtained by this procedure.

Until the recent study of Lou *et al.* (1990), isolation of plasma membranes from fish spermatozoa had not been accomplished. Lou *et al.* (1990) prepared plasma membranes from salmon sperm by sonication, but, the recovery was only 0.6% of the total cell protein. Using nitrogen cavitation, we obtained a 3-fold higher yield for trout.

We have confirmed that nitrogen cavitation is the most effective of the methods tested. In agreement with Lou *et al.* (1990), we have also confirmed that it is difficult to both define the optimal conditions for sonication and to reproduce them, especially when using different sonicators.

To avoid contamination by the acrosomal membranes, a nitrogen pressure of 600–650 PSI has usually been used in the preparation of plasma membranes from mammalian spermatozoa (Noland *et al.* 1983). We have not examined the ultrastructural changes induced in trout sperm by

this pressure, but protein yields and fraction compositions indicate that detachment of membrane may not be as complete and the purity not as high at 600 compared to 900 PSI. Thus, we used 900 PSI for further experiments. However, under these conditions the nuclear membrane was also partly detached.

In the majority of previous studies, plasma membrane-enriched fractions were purified from spermatozoa by discontinuous sucrose density gradient centrifugation. However, this technique may lack the resolving power to distinguish between membranes possessing subtle differences. Lou *et al.* (1990) used discontinuous gradients and obtained only one band in preparations of salmon sperm plasma membranes. In contrast, we obtained 3 distinct membrane bands, the mean buoyant densities of which were 1.03, 1.05 and 1.09 g/ml respectively. These values are lower than those reported for membrane vesicles of mammalian spermatozoa (Agrawal *et al.* 1988, rat: 1.13 g/ml; Parks and Hammerstedt 1985, ram: 1.16 g/ml; Petersen *et al.* 1980, boar: 1.13−1.17 g/ml). Similarly, skeletal muscle plasma membranes from rainbow trout (Ablett and Selivonchick 1983) sediment at a lower density than the same membranes from rat. Ablett and Selivonchick (1983) attributed this behaviour to a high level of phospholipids which is a common feature of teleost membranes. In concordance with this, the phospholipid content of our membrane fractions was far higher (840 to 1920 nmoles/mg protein) than that recorded for membrane vesicles from human spermatozoa (Mack *et al.* 1986: 82 nmoles/mg protein). The same was also true for cholesterol: 573 to 1134 nmoles/mg protein versus 68. Furthermore, the molar ratio of cholesterol to phospholipid for trout muscle plasma membrane is half that of rat muscle plasma membranes. Similarly, for trout sperm plasma membrane we found this ratio to be 0.59−0.70 while it is 0.83 and 0.85 in plasma membranes of human (Mack *et al.* 1986) and ram sperm respectively (Holt and North 1985). On the other hand, cholesterol and phospholipid contents were higher in sperm, than muscle plasma membranes of trout. This may reflect tissue differences.

The activities we measured for 5'-nucleotidase

were around 7-fold lower than those measured by Lou *et al.* (1990) for salmon sperm plasma membrane. We have no explanation for this, although it cannot be excluded that this difference is species-specific. In any case, the enrichment we obtained in the distribution of this enzyme ($\times 4.5$ as a mean for the B1−B3 fractions) is close to that obtained by Lou *et al.* (1990).

Lipid analysis revealed that the enrichments of cholesterol and phospholipid were lower in band 3 than in bands 1 and 2. It should be noted that the specific 20β-hydroxysteroid dehydrogenase activity (EC 1.1.1.53.) was also found to decrease from band 1 to band 3 (Loir *et al.* 1990a). This agrees with ultrastructural observations demonstrating some contamination of this band by material trapped in the vesicles. In bands 1 and 2, there is a 6.3 to 6.9-fold enrichment of cholesterol and a 4.2 to 5.4-fold enrichment of phospholipid. These values and that for 5'-nucleotidase either approach those for mammals, or, are lower (Gillis *et al.* 1978; Mack *et al.* 1986). Lower values may result from the smaller size of trout compared to mammalian spermatozoa, which implies a higher membrane protein/total cell protein ratio in fish than in mammals (there is, for instance, 5 to 10 times more protein in human (Mack *et al.* 1987) than in salmon (Lou *et al.* 1990) or trout spermatozoa). Thus, for an identical purity of the membrane fractions, the enrichment of trout sperm membrane is necessarily lower.

The size of the membrane vesicles recovered in the 3 bands was not conspicuously different. In addition, the ultrastructural appearance of the vesicles in bands 1 and 2 was identical. After SDS-PAGE analysis, there is slight changes in the protein composition of membranes in the three bands. Among the proteins, the relative amount of which increases from B1 to B3, as compared to protein 42 KD, only 2 (14.5 and 29 KD) are seminal proteins adsorbed onto the gamete surface (Loir *et al.* 1990b). The quantitative changes of these 2 proteins in the 3 bands could be due to partial removal during membrane preparation. However, this interpretation is not applicable to the other 11 to 22 KD and 26 to 32 KD proteins. This suggests that membranes in the 3 bands have different characteristics which could reflect a lack of uniformity of

the plasma membrane in different regions of the sperm surface. Such a local variation of the plasma membrane was demonstrated in salmon sperm surface (Haruo and Yamamoto 1984): the membrane covering the tail differs in its physiological properties from that of the head. Several bands were also obtained when boar sperm plasma membrane was centrifuged onto linear sucrose gradients (Peterson *et al.* 1980) and a similar interpretation was proposed.

The possible origin of membranes present in band 3 is questionable. No biochemical or ultrastructural marker could identify sperm nuclear membrane, as in these cells there are neither nuclear pores nor RNA. Detection of DNA with propidium iodide was unsuccessful (unpublished data). However, the presence in vesicles in band 3 of fibrous material which looks like chromatin fibers, on one hand, and the fact that rat liver nuclear membranes have a buoyant density slightly higher than plasma membranes (McNamee 1989) on the other, suggest that pieces of nuclear membranes could have sedimented with plasma membranes in band 3, and maybe in band 4.

Acknowledgements

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