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Functional and Biochemical Characteristics of Human Prostasomes

Minireview based on a doctoral thesis

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INTRODUCTION

Reproduction and semen composition. Spermatozoa were identified as constituents of human semen in the seventeenth century by the Dutch scientist Anton van Leeuwenhoek. Spermatozoa are highly differentiated cells the purpose of which is to reach the site of fertilisation and fuse with the oocyte in order to form a diploid zygote. Spermatozoa develop within the testis in a hormonally regulated process denoted "spermatogenesis" but they acquire the motility and fertilising capacity during the epididymal transit. The process ultimately yields a population of potentially fertile cells that are stored in the cauda epididymis. Freshly released spermatozoa from the male reproductive tract are not immediately fertile but must undergo a maturation process which is termed capacitation (10, 20). Once capacitated, sperm cells can undergo acrosomal exocytosis, "the acrosome reaction", and penetrate the zona pellucida and fuse with the oocyte plasma membrane (165).

At the moment of ejaculation the spermatozoa are suspended in the seminal plasma which is a complex mixture of fluids secreted by, besides the testis and epididymis, the sex accessory gland. Accessory glands of human male reproductive tract are the following: the bulbo-urethral or Cowper's gland, the urethral or Littre's gland, the seminal vesicles and the prostate gland. The location of accessory glands along the male reproductive tract is shown in Fig. 1. The fluids are ejaculated in a specific time sequence. The first secretions to appear are those from the Cowper's and Littre's glands. This is followed by secretion from the prostate gland which is mixed with the bulk of spermatozoa. The last portion to be emitted is that from the seminal vesicles. This makes possible to study the glandular origin of different components by splitting the ejaculate in separated parts (152). As many as six different fractions can be collected from one ejaculate and analysed separately for the components of interest (32). The accessory secretions exhibit marked species-dependent and individual variations in respect of both quantity and composition. In man the prostatic fluid is slightly acid, rich in zinc, acid phosphatase and citric acid and it contributes to about 1/3 of the ejaculate volume. The two seminal vesicles produce together about 2/3 of the total ejaculate with a fluid rich in basic proteins, prostaglandins

and fructose. The spermatozoa and epididymal, ampullary and Cowper's gland secretions comprise only approximately 5% of the volume of the ejaculate (166).

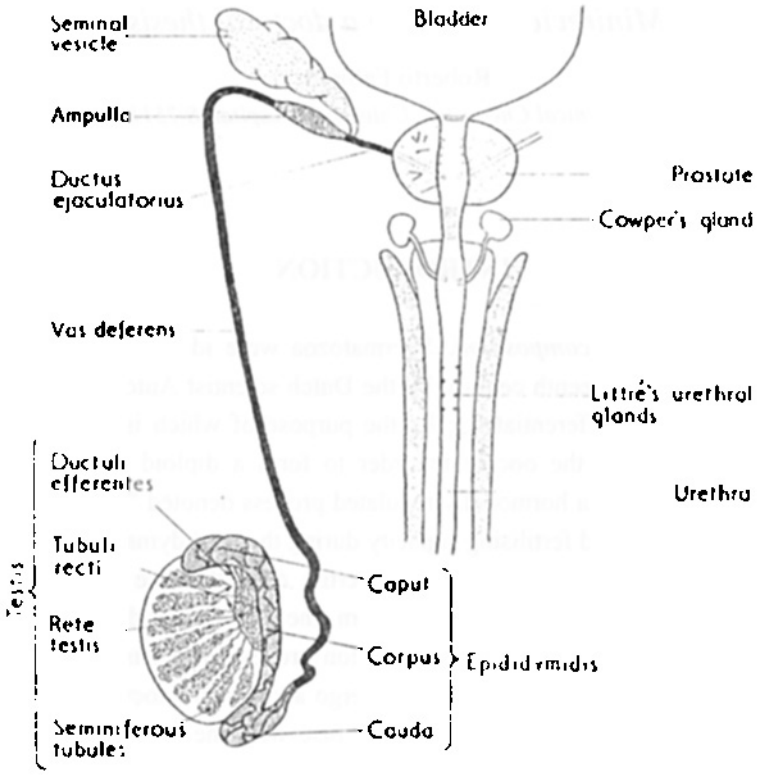


Figure 1. Accessory organs along the male reproductive tract (modified from Mann and Lutwark-Mann, 1981).

Comparison between cauda epididymal and ejaculated spermatozoa in animals has shown that both cell types are capable to fertilise the oocyte when placed in the female tract but they display large differences in membrane features, ion transport capacity and metabolism (51). Although no differences in percentage of motile spermatozoa were noted the mean velocity was lower for caudal epididymal spermatozoa than ejaculated spermatozoa. These differences may be due to the interaction between sperm cells and some components of seminal plasma. Much less is known about human epididymal spermatozoa, especially in healthy normal men (161) making it difficult to describe the role that the accessory glands may play on the physiology of the ejaculated spermatozoa.

The prostate gland: morphology and secretory function. Unlike other organs, the detailed anatomy of the human prostate gland has not been described until recently. Studying the

well-defined lobes of rat prostate, Tisell and Salander were the first to dissect the human prostate and to distinguish three pair of lobes: two dorsal, two lateral and two medial lobes (156). Viewed from the dorsal aspect, the prostate appears externally fairly homogeneous and identification of the boundaries between the lobes is not easy (5). In a transverse section of the prostate the lobes are arranged in an onion pattern, with the medial lobes centrally around the ejaculatory ducts and the lateral and dorsal lobes forming the outer lobes (39).

Histologically the prostate is composed of a large series of independent branching ducts, all of which enter the prostatic urethra. Secretion of compounds by the prostate gland depends on the synthesising activity of its epithelial cells and transudation from blood serum (166). The epithelial cells, like other cells of the exocrine gland, contain an abundance of rough endoplasmic reticulum and a large Golgi apparatus (125).

Most of the data concerning the prostatic origin of the various seminal components come from studies either made on prostatic fluid obtained by massage of the gland or by analysis of the various fractions of the split ejaculate. Human prostatic fluid contains high amounts of monovalent and divalent cations such as sodium, potassium, calcium, magnesium and zinc. Zinc may play an essential role for the stabilisation of the quaternary structure of the condensed sperm chromatin (61). Prostatic fluid is rich in a number of different enzymes, among which some are involved in the carbohydrate metabolism while others are proteolytic enzymes. Most of the spermine, spermidine, cholesterol and all of the citric acid of human semen originate in the prostate gland. Other specific compounds such as prostatic antibacterial factor, prostatic specific antigen and prostate binding protein are secreted by the prostate gland (116).

Besides these soluble substances the prostate gland secretes a particulate fraction organised in well defined organelles the properties of which will be described in the next section.

The prostasome

Ultrastructure and secretion. The presence of granules and vesicles in human prostatic fluid and seminal plasma was first described by Ronquist et al. in 1978 (123). In that original study the prostatic fluid and the split-ejaculate fractions were centrifuged at 1,500 x g in order to remove cells and cell fragments (pellet I). The resultant supernatant was again centrifuged at 40,000 x g for 12 hours, the pellet obtained (pellet II) was fixed and processed for electron microscopy.

Pellet II from prostatic fluid consisted of granules and vesicles bounded by a unit membrane. Sometimes two or more unit membranes were present forming small myelin figures. Similar granules and vesicles were also observed in pellet II from split ejaculate fractions together with an amorphous substance. Six fractions were collected from each ejaculate and analysed. The first fraction which originated from the prostate gland contained only granules and vesicles. The last two fractions originating from the seminal

vesicles contained mainly the amorphous substance (123). Pellet II obtained after ultracentrifugation of whole ejaculate contained both vesicles and amorphous substance and they could be separated by gel filtration on Sephadex G 200 (146).
Secretory granules and vesicles with a size and ultrastructure similar to those isolated from prostatic fluid and seminal plasma were demonstrated within the epithelial cells of the human prostate gland and consequently designed "prostasomes" (17). The diameter of prostasomes regardless of whether they were observed intracellularly or extracellularly was in the range 30-500 nm (15). A recent size analysis in a fluorescence activated sorter showed a distribution where the majority of the particles were below 800 nm in diameter with a median size between 100 and 200 nm (60). Prostasomes have a density of 1.03 when analysed by continuous silica density gradient centrifugation (123), they do not contain any cytosol but they may contain small spherical particles of approximately 15 nm in diameter (60). In the prostate epithelial cells prostasomes are mostly enveloped by bigger storage vesicles together with electron dense material (125). Two mechanisms appear to be responsible for the translocation of prostasomes from the interior to the exterior of the cell (125). One is a normal exocytosis in which the membrane of the storage vesicle fuse with the cell plasma membrane and the content is discharged into the acinar lumen. In the other mechanism the storage vesicle is displaced *in toto* from the interior to the acinar lumen with perforation of the apical plasma membrane. This process has been called "diacytosis" (17). A reduced amount of prostasomes in seminal plasma was observed in a patient with Klinefelter's disease who had the serum testosterone level reduced by 50% (16). In another patient, with a well differentiated carcinoma of the prostate, the secretion of prostasomes was reduced by 85% after 2 weeks of treatment with an antiandrogenic drug (Flutamide) (124). These observations suggest a role for testosterone in the secretion of prostasomes but definitive conclusions need further studies.

Lipid composition. Prostasomes are mostly surrounded by a typical trilaminar membrane structure the composition of which has been determined regarding phospholipids, cholesterol and fatty acids. The phospholipid and cholesterol content referring to protein content was 0.27 μmol/mg and 0.54 μmol/mg, respectively (8). This gives rise to a cholesterol-to-phospholipid molar ratio of about 2.0, which is very high in comparison with an expected value of 0.8 - 1.3 reported for cholesterol-rich membranes, including myelin (30). The phospholipid composition of prostasomes revealed a predominance of sphingomyelin, representing nearly half of the total phospholipid content. Phosphatidylethanolamine, phosphatidylserine and lysophospholipids were also abundant while phosphatidylcholine and phosphatidylinositol were present at a low level. Saturated and monounsaturated fatty acids were predominant in both glycerophospholipids and sphingomyelin. All these properties made the prostasome membrane highly ordered and minimally perturbed in presence of the membrane proteins (8).
There is no definitive indication to what extent -if any- the unusual lipid composition of prostasomes is due to adsorption of constituents from the seminal plasma. In this regard it

is important to point out that the plasma membrane of the spermatozoa, which are bathed in the same medium as prostasomes, has a very different lipid composition. In human spermatozoa the plasma membrane contains, in comparison with prostasomes, less sphingomyelin and more phosphatidylcholine (114) resulting in a cholesterol/phospho-lipid ratio which is only 0.83 (87).

Prostasome membrane-bound enzymes

1) Mg^{2+}- and Ca^{2+} dependent ATPase. There are membrane-linked enzyme systems that mediate the vectorial transport of ions across biological membranes against ion gradients. The energy for this type of reactions is derived from ATP and the enzyme is an ATPase. ATPases exist in different subclasses.

a) P-type ATPases which include the Na^+-K^+ ATPase and Ca^{2+} ATPase of both the plasma membrane and the sarcoplasmic reticulum. They are characterised by active site phosphorylation, which is inhibited by vanadate, during catalysis (112, 113).

b) F-type ATPase present in the mitochondria, the function of which is to synthesise ATP in a reverse ATPase reaction at the expense of a proton gradient, "chemi-osmotic coupling" (35, 100).

c) V-type ATPase characteristic of vesicles (e.g. lysosomes) is responsible for pumping protons from the cytoplasm to the interior of the organelle maintaining a pH gradient (101).

Prostasomes contain a rather high ATP splitting activity which is linked to their membrane (123). Detergents (deoxycholate, Triton x 100 and dodecanylsulphate) are potent inhibitors, 0.1 % of deoxycholate inhibits 86% of the activity. The ATPase is dependent on Mg^{2+} and/or Ca^{2+} for its activity whereas Zn^{2+} is a competitive inhibitor. The enzymatic activity is not affected by Na^+, K^+ and ouabain, ruling out a Na^+-K^+ ATPase (123). The best substrate is ATP but other nucleoside triphosphates are also hydrolysed in the following order of efficiency: ITP, CTP, UTP and GTP. The apparent Km for ATP is 0.38 and 0.71 mmol/L in presence of Ca^{2+} and Mg^{2+}, respectively (123). Prostasome ATPase shares some inhibitory characteristics with Ca^{2+}-transport ATPase of other tissues (Table I). La^{3+} is a specific inhibitor of the Mg^{2+}- and Ca^{2+} dependent ATPase in erythrocytes and also inhibits the prostasome ATPase. Calmodulin modulates the transport of Ca^{2+} in human erythrocytes (86, 137) whereas Ca^{2+} transport ATPase of sarcoplasmic reticulum is not regulated directly by calmodulin (19, 67). Prostasome ATPase is not primarily activated by calmodulin, but there is indeed a stimulation after the prostasomes first have been treated with calmidazolium, a specific antagonist to calmodulin (127). This suggests the presence of endogenous calmodulin in the prostasome membrane. Differences also exist between prostasome ATPase and sarcoplasmic reticulum ATPase regarding the effect of quercetin and oleate (Table I). From these results it has been proposed that the prostasome ATPase system may be responsible for the vectorial transport of Ca^{2+} through the membrane into the prostasomes (127). This view is supported by the finding that Ca^{2+}

occurs in greater concentration in the prostasomes than in the surrounding seminal plasma (147). More recently a zinc dependent ATPase activity has been described in prostasomes, it is inhibited by vanadate and has an apparent Km of 0.43 mmol/L (128). However, it is not known whether this is a specific ATPase or is a part of the Mg^{2+}-and Ca^{2+} dependent ATPase.

Table 1.

	Ca^{2+} transport ATPase		**Mg^{2+} and Ca^{2+} ATPase**
	Sarcoplasmic reticulum	**Erythrocytes**	**Prostasomes**
La^{3+}	---	Inhib.	Inhib.
Calmodulin	No	Stim.	No
Calmidazolium	---	Inhib.	Inhib.
Quercetin	Inhib.	---	No
Oleate	Stim.	---	Inhib.

Abbreviations: Inhib. = inhibition; stim. = stimulation; no = no effect; --- = not examined.

*2) **Aminopeptidase.*** A hydrolytic activity on the synthetic substrate succinyl(alanine)$_3$-paranitroanilide (Suc(Ala)$_3$pNA) is also linked to the prostasome membrane (64). The enzyme does not penetrate the agarose gel, is recovered in the void volume after gel filtration and is solubilized in an active form by Triton X100. The enzyme activity is completely inhibited by 150 mmol/L of O-phenanthroline and can be restored by zinc ions whereas protease inhibitors do not reduce the hydrolytic activity (64). Owing to its alkaline pH optimum of 7.8 the enzyme is probably not active in the prostatic fluid but becomes active upon mixing with the secretions of the other glands. Indeed it seems that the physiological substrates for the enzyme are proteins secreted from the seminal vesicles which participate in the coagulation process of human seminal plasma. Furthermore it has been shown that addition of O-phenanthroline to the coagulated semen inhibits the liquefaction of the gel. This inhibition can be reversed by zinc ions (73), hence prostasomes with their Zn^{2+}-dependent aminopeptidase may participate in the liquefaction of human semen.

*3) **Protein kinase.*** When prostasomes are incubated in presence of [γ^{32}P]ATP the terminal phosphoryl group is transferred from ATP to the hydroxy group of serine and threonine residues of endogenous acceptor proteins. Serine is most actively phosphorylated in the presence of Mg^{2+} (Ser P/Thr P ratio 3.5) while in the presence of Zn^{2+} the phosphothreonine is the predominant reaction product (Ser P/Thr P ratio 0.19)

(148). This finding indicates the presence of at least two or more isoenzymes. Ca^{2+} and adenosine are inhibitors to both kinase reactions and cAMP stimulates the activity by 23% and 26%, respectively. Low endogenous protein kinase activity is present on the surface of sperm cells (148). Although the plasma membrane of bovine epididymal spermatozoa possesses protein kinase activities, the active site of the enzyme is probably oriented towards the cytosol of the sperm cell (104). The low protein kinase activity found on outer surface of the human spermatozoa agree with studies on rat caudal epididymal spermatozoa (47) and is in accord with the view that the cAMP-dependent phosphorylation takes place in the cytosol of the cells. Incubation of prostasomes and spermatozoa together resulted in a 10-fold increase of protein phosphorylation supporting the view that sperm proteins are substrates for the prostasome enzyme (148). The function of these protein kinases on the membrane of prostasomes is not known. However, it has been shown that the thickness of prostasome membrane increases upon incubation of the organelles with ATP under optimal phosphorylation conditions. This phenomenon has been interpreted as a consequence of conformational changes of the phosphorylated proteins (148). At this point it would be interesting to identify the acceptor proteins both in prostasomes and in the spermatozoa.

4) γ-Glutamyltransferase. Human seminal plasma contains an abundance of γ-glutamyltransferase (GGT) activity which is 200-500 times higher than in normal serum (132). The prostate secretion is the major source of the enzyme (159) and most of it is bound to the membrane of prostasomes (70). A part of GGT is also present in the semen as a water-soluble protein which may originate from the membrane bound enzyme of prostasomes as a consequence of proteolytic digestion of the hydrophobic domain (70).

5) Other enzymes. *Phospholipase A_2.* Phospholipases are enzymes that catalyse the hydrolysis of phospholipids and are classified according to the bond involved in cleavage: phospholipase A_1 and A_2 selectively remove fatty acids from the sn-1 and sn-2 position respectively; phospholipase B is a lysophospholipase; phospholipase C cleaves the bond between glycerol and a phosphate; and phospholipase D hydrolyses the amino alcohol moiety from a phospholipid. Phospholipase A_2 is an ubiquitous enzyme existing in both membrane-bound and soluble forms (57). This enzymatic activity has been investigated in human semen using a labelled ^{14}C-oleate substrate (74). The enzyme shows a broad pH-optimum range with two peaks at pH 8.0 and 10.5. The activity is 8-9 times higher at pH 10.5 than 8.0 and is Ca^{2+}-dependent. More than 50% of the total activity can be precipitated by ultracentrifugation of seminal plasma with an enrichment of 3 times in specific activity in the pellet II containing prostasomes (74).
There is no correlation between prostasome-associated phospholipase A_2 activity and either semen volume or sperm concentration. The same high activity is also present in prostasomes isolated from seminal plasma of azoospermic men (74).

Whether prostasomes contain to any appreciable extent other types of phospholipases is not known.

Lactate dehydrogenase. Although this enzyme is believed to be free in the cytosol of the cells it has also been found associated with the prostasome membranes (110). The isoenzyme distribution in the prostasomes is different from that of seminal plasma showing a higher amount of LD_1 in the prostasome preparation (110). However, the question still remains whether the prostasome activity is due to adsorption of the enzyme from the seminal plasma, or the enzyme is located inside the organelle being not necessary membrane-bound.

Angiotensin converting enzyme, **ACE.** The prostate gland is the main source of ACE in human seminal plasma and most of the enzyme is associated to the prostasome membranes (63). A comparison between ACE from blood plasma and seminal plasma has shown small differences in the kinetics parameters and inhibition by peptidase inhibitors and active peptides. Zincov ^{TM}, which is an inhibitor of Zn-containing metalloproteases, inhibits only ACE from blood plasma (63) which indicates another isoform in the prostasome membrane. The functional role of the enzyme in seminal plasma is not known.

The membrane of prostasomes has a complex protein pattern showing more than 75 protein entities when analysed by two dimensional polyacrylamide gel electrophoresis (74). This emphasises the possibility that many other enzymes may be linked to the prostasome membrane. For instance, two forms of oxytocinase activity have been reported to exist in human seminal plasma by gel filtration chromatography, the most abundant of which is eluted in the void volume of the column (134) suggesting a link to the prostasome membrane.

Prostasome membrane-bound proteins

1) CD59, CD55, CDw52. The membrane attack complex (MAC) inhibitory protein CD59 has been identified in human seminal plasma by Western blotting using a monoclonal antibody. The reactive band has a molecular weight of 20 kD and is apparently identical to the erythrocyte CD59 in terms of molecular mass, antigenicity, and amino-terminal amino acid sequence. It binds to the membrane of guinea pig erythrocytes, murine fibroblasts and human spermatozoa (130). Incubation of erythrocytes with either seminal plasma or CD59 purified from seminal plasma makes the cells more resistant to lysis by human MAC. These properties are reduced by pretreatment of CD59 with phosphatidylinositol specific phospholipase C (PIPLC) supporting the view that the glycosylphosphatidylinositol (GPI) anchor is necessary for the biological function of this protein. The GPI anchor of membrane proteins will be further discussed below. More than 90% of the CD59 precipitates upon ultracentrifugation of cell-free seminal plasma and it has been shown by

immunoelectron microscopy that all CD59 is bound to the prostasome membrane. It has been proposed that prostasomes represent a pool of CD59 which may be transferred from the prostasome membrane to the membrane of spermatozoa protecting them from functionating complement in the female reproductive tract (130).
Two other GPI-linked proteins are carried by prostasomes, decay accelerating factor (DAF; CD55) and CDw52. DAF is present in seminal plasma at a concentration of 0.5 μ g/L, 30% of which is prostasome-associated. CDw52 is a lymphocyte antigen of uncertain function and at least 90% of it is prostasome-bound in seminal plasma (131).

*2) **Granulophysin.*** This protein has first been identified as a 40 kD integral membrane glycoprotein in platelet dense granules (41). It was later shown that granulophysin is also present in many other tissues having a secretory function (44). Purified prostasomes specifically react with a monoclonal antibody (D545) specific for granulophysin. 60% to 85% of prostasome particles are positive as determined by an indirect immuno-fluorescence method (144). The reactivity of D545 antibody examined by Western blotting shows a broad band of 32 to 37 KD. Strong positive staining of epithelial cells and secretions have been shown by immunohistochemical staining of the prostate tissue, staining was also seen in the epididymis but less strong than in the prostate (144). The function of this protein is so far not known.

Other constituents

An enrichment of some components has been reported in prostasomes compared to seminal plasma. The most striking finding was the presence of ADP (81 nmol/g protein) and GDP (71 nmol/g protein) in prostasome preparation while only trace amount of these nucleotides were present in the prostasome-free seminal plasma (126). In this regard prostasomes were compared with human platelet dense granules which also contain adenine nucleotides, ADP and ATP, and Ca^{2+}. It has been hypothesised that the nucleotides form a macromolecular structure held together by divalent cations through the nucleotide phosphate groups, giving the prostasomes their typical electron-dense pattern (126).
Double-stranded DNA is also present on the prostasome membrane surface as shown by digestion with restriction enzyme (*Hinf I*) and agarose gel electrophoresis (109). The origin of this DNA is not known but sperm cells, blood cells and bacteria do not seem to be the main source. However, contamination of the prostasome sample with virus nucleic acid was not excluded.

Biological functions

Sperm motility. The first indications that prostatic fluid had an effect on human sperm motility were reported in 1972 (76, 77). In those studies it was shown that vesicular fluid

contained one or more factors which depressed sperm motility and reduced their survival. Prostatic fluid not only maintained good motility but also protected spermatozoa from the effects of vesicular fluid. In a later study (78) it was found that washing the ejaculated spermatozoa with protein-free buffered salt solution significantly decreased their forward motility. Subsequent addition of albumin, seminal plasma or different fractions of split ejaculate immediately restored a good progressive motility. At low concentration, fluid from fractions three and four of the split ejaculate were the most effective while at higher concentration fluid from the first fraction had the greatest effect on the progressive motility. It was concluded that the prostate gland secreted one or more factors essential for sperm motility (78, 79). On the other hand, others have reported the presence of prostate-derived factors inhibitory to the survival of washed spermatozoa (136).

A stimulatory effect on sperm progressive motility of pellet II (obtained from ultracentrifugation of cell-free seminal plasma) was shown on human washed spermatozoa suspended in 155 mmol/L NaCl solution (145). Seminal plasma devoid of pellet II components instead displayed a reduced effect. The progressive motility of washed spermatozoa resuspended in a buffer supplemented with pellet II material was threefold higher than when ultracentrifuged seminal plasma was the suspending medium. By means of gel filtration it was established that prostasomes are responsible for such a promotive activity although other promotive factors may be present in the mainly prostasome-free seminal plasma (146).

Preincubation of prostasomes with either divalent cationophore A 23187 or deoxycholate 0.1% resulted in diminished promotive activity. Repeated freezing and thawing also reduced the effect by about 30%. From these results it was believed that prostasomes induced promotive activity by modulating the calcium ion composition in the microenviroment of spermatozoa by the vectorial transport of calcium across the membrane in which the ATPase system may play a central role (146).

Semen liquefaction. Human semen coagulates immediately after ejaculation, but the clot liquefies spontaneously within about 20 min. Components of the seminal vesicle secretion are considered to generate the coagulum matrix (7, 91). It has been reported that several basic proteins are released during liquefaction (71) as a consequence of degradation of the large vesicular protein entities into a series of minor proteins (72). The involvement of prostasome aminopeptidase in the liquefaction process has been suggested by the following considerations: like the semen liquefaction process this enzyme is a) not inhibited by EDTA or EGTA, b) inactivated by o-phenanthroline, c) reactivated by Zn^{2+}, and d) inhibited in the presence of non-chelated Zn^{2+} (73).

Immunosuppression. Human seminal plasma has a potent immunosuppressive activity (149) which may play an important role in reproductive physiology by preventing sensitisation of the cells of the immune system of the female reproductive tract to the alloantigenic proteins present on the sperm surface and in seminal plasma (52, 59).

Studies in which the immunosuppressive activity has been measured by suppression of natural killer (NK) cell activity have identified prostaglandins (PGs) as the major immunosuppressive components in human semen (157). Prostaglandins E (PGE1 and PGE2) are in fact present in high concentration in human semen, around 80 μmol/L, together with the 19-hydroxy PGE1 and 19-hydroxy PGE2, both present in about 300 μ mol/L (153). In other studies, in which the mitogen induced lymphoproliferation assay has been used, immunosuppressive activity has been detected in high molecular weight components (82, 92), but first recently identified as prostasomes (60). A suspension of prostasomes corresponding to 40% of that present in the original seminal plasma gave a 69% suppression of thymidine incorporation. This capability was not impaired by boiling the prostasome preparation. Prostasomes did not affect NK cell function but they inhibited the ability of macrophages (P338D1) to phagocytose latex beads (60).
Interaction of prostasomes with poly- and mononuclear cells was also investigated (143). Prostasomes bound to neutrophils, monocytes and lymphocytes in an energy-independent fashion. Following incubation at 37°C internalisation of prostasomes by neutrophils and monocytes (but not lymphocytes) took place. Preincubation of monocytes and neutrophils with prostasomes effectively reduced the uptake of latex particles. In contrast, this treatment was not effective in phagocytosis of opsonized bacteria (*St. aureus*). Prostasomes also inhibited superoxide anion generation in response to activation by phorbol myristate acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (FMLP).

Using free-zone electrophoresis it was demonstrated that prostasomes interacted with spermatozoa (129). Carbohydrates were not involved in the binding which probably was of hydrophobic nature. So far, the molecules responsible for such an interaction have not been identified. However, this phenomenon together with the immunosuppressive activity may help spermatozoa to ascend the female reproductive tract protecting them from deleterious effects of phagocytosing cells and consequently enhance the chance of conception (143).

Membrane vesicles in animal semen

Membrane vesicles have also been described in seminal plasma of different animals but they are quite different from human prostasomes in that they are not secreted by the prostate gland. Two classes of membrane vesicles were isolated from rabbit seminal plasma by sucrose discontinuous density gradient, the less dense vesicle fraction and the dense vesicle fraction (26). They originated from the epididymis and inhibited fertilisation (27, 28). Different vesicles were separated from rat epididymal fluid, they had an electron-lucent appearance and a diameter ranging from 0.3 to 3 μm (36). The vesicles were believed to arise from the epithelial cells of the epididymis. Two enzyme activities were associated with the vesicles: β-galactosidase which was located inside and N-acetylglucosaminidase which was bound to the external surface of the vesicles.

Membrane particles have also been reported in ram (15) and bovine (4) seminal plasma. Some enzyme actives in common with human prostasomes were reported to be associated with bovine vesicles such as Mg^{2+}-and Ca^{2+} activated ATPase and γ-glutamyl transpeptidase while other enzymes were absent (e.g. angiotensin converting enzyme and aminopeptidase). The vesicles were secreted by the seminal vesicle epithelium and hence they were named "vesiculosomes". Vesiculosemes isolated from bovine seminal vesicle secretion were claimed to promote forward motility of epididymal spermatozoa and to induce an acrosome reaction in 80% of the cells after 4 hours of incubation (4).
Whether the prostate gland of animals secretes membrane particles which can resemble human prostasomes in their structure, secretion and chemical composition is completely unknown.

Motility stimulating factors

Motility is a characteristic feature of the spermatozoa which is a prerequisite to ensure successful sperm migration from the vagina to the site of fertilisation. Various factors have been reported to affect sperm movement both in a positive and negative way. Motility stimulating factors can be divided into low and high molecular weight factors. Among the low molecular weight factors, cyclic AMP (cAMP) is well known to play an important role in both the initiation and maintenance of sperm motility (48, 90). Accordingly methylxanthines, which inhibit phosphodiesterase activity stimulate sperm motility and metabolism (22, 68).
Adenosine is a modulator of intracellular cAMP concentration by the interaction with two types of receptors: A_1 receptor which mediates an inhibition and A_2 receptor which mediates a stimulation of adenylyl cyclase activity (158). A stimulatory effect on human sperm motility has been shown by 2'deoxyadenosine (6, 50) and adenosine (140) supporting the presence of A_2 receptors on human spermatozoa (141).
Calcium ions are also involved in many aspects of sperm function such as maturation, capacitation, acrosome reaction and motility. Regarding the involvement of extracellular calcium in sperm motility there have been conflicting results. In some studies calcium has been reported to stimulate (34) whereas in another study a high level of calcium appears to inhibit sperm motility (75). In line with the inhibitory activity are also studies in which calcium chelators have been shown to stimulate sperm movement (46). Finally, it has been reported that addition of calcium to washed human spermatozoa did not affect the progressive motility (88). Other divalent cations may affect sperm motility to various degrees. A stimulatory influence of magnesium and manganese ions has been reported (83, 89) while zinc ions at a high concentration seem to be deleterious (119).
Prostaglandins, which are present in semen in a high concentration have been postulated to affect sperm function. With the use of time-exposure photomicrography it was found that addition of PGs to the incubation media increased sperm velocity and frequency of sperm

head rotation (5). A significant improvement in percent motility was also demonstrated for PGE_2 on washed spermatozoa at a concentration between 12.5 and 50 μg/mL (24).
Carnitine and acetylcarnitine are two other compounds which positively affect sperm motility, although it is believed that carnitine is converted to acetylcarnitine prior to its stimulatory action (150).
Among the high molecular weight factors a sperm forward motility protein has been described in bovine seminal plasma. The purified protein initiates forward motility of epididymal spermatozoa only in the presence of phosphodiesterase inhibitors (2).
Serum, which is often a component in the media used for intrauterine insemination and in vitro fertilisation, contains both high and low molecular weight motility stimulating factors (29). Fractionation of serum proteins by gel filtration showed that the stimulating factor was a macromolecular complex with a MW of about 200 kD (169). The protein complex was purified and it was shown that it was formed by an association between an IgG molecule and apolipoprotein A-I molecule (115).

GPI-anchored membrane proteins

Integral membrane proteins are characterised by a hydrophobic domain which interacts with the hydrophobic core of the lipid bilayer. Usually, the hydrophobic domain is constituted of one or more hydrophobic amino acid sequences of the polypeptide chain. An alternative mode of anchoring involves the covalent attachment of a glycolipid, glycosyl-phosphatidylinositol (GPI) to the C-terminal carboxyl group of the protein (25, 85). These proteins are translocated across the membrane of the endoplasmic reticulum (ER) in which they are initially anchored by a hydrophobic amino acid sequence. Immediately after synthesis, the hydrophobic sequence is cleaved on the luminal side of (ER) membrane (14), and the new carboxyl terminus is covalently linked to the preformed GPI (62, 97). One characteristic of most GPI-proteins is their conversion from membrane-bound to soluble hydrophilic forms by treatment with purified phosphatidylinositol-specific phospholipase C (PIPLC) and it was this phenomenon that eventually revealed the GPI anchor (49, 84). The ability of PIPLC to release proteins attached to the membrane in this fashion has permitted the identification of several GPI-proteins with disparate functions. This group of proteins includes distinct hydrolytic enzymes, complement regulatory proteins, neural and lymphocyte cell adhesion molecules, protozoal coat proteins, prions, tumour markers and a number of antigens with specific cellular distribution but with unknown function (85). The majority of GPI-anchored proteins described so far has a primary location at the cell surface. However, the major protein of the pancreatic zymogen granule, GP-2 (66) and two proteins in adrenal chromaffin granules (37) have been reported to be GPI-anchored. In addition, they are expressed exclusively on the apical membrane surface of polarised epithelial cell lines (80) suggesting that the GPI anchor may be a signal for targeting the attached protein selectively to that site (81). Another proposed role for the GPI anchor is that it facilitates

the release of the protein from the membrane by serving as a substrate for anchor-specific phospholipases (45).

The GPI anchors contain a linear ethanolamine (EthN-P-ManManManGlcN) core sequences that are variably decorated in different organisms and the biosynthesis of which has started to be elucidated (33), although the enzymes involved have not been purified yet.

Prostasomes, which contain at least three GPI-proteins (CD59; DAF; CDw52) have been considered to carry and to provide a reservoir of these proteins (131). In this context it could be anticipated that prostasomes may contain several other GPI proteins such as folate binding protein which is present in human seminal plasma with its GPI anchor (42).

MATERIALS AND METHODS

Semen samples and sperm preparation. For prostasome preparations, semen samples were obtained from different patients visiting the infertility clinic for investigation and classified as normozoo- (sperm density ≥20 million/mL) and azoo-spermic (sperm density = 0) based on spermiogram finding. Some samples were obtained from men subjected to vasectomy for the purpose of voluntary sterilisation. Prostatic fluids were obtained by massage of the prostate gland. For sperm preparations, ejaculates were obtained from normozoospermic men who were undergoing evaluation before entering the *in vitro* fertilisation programme.

Motile spermatozoa were selected by a "swim up" procedure using Earle's Balanced Salt Solution (EBSS), enriched with 1% human serum albumin, sodium pyruvate (1 mM) and penicillin G (100 IE/mL). After swim up, sperm cells were washed once with the EBSS medium (300 x g, 5 min) and three times with an isotonic 50 mmol/L Tris-HCl buffer, pH 7.32, containing 138 mmol/L NaCl and 1 mmol/L $MgCl_2$ (800 x g, 15 min).

Prostasomes and boiled-prostasomes were included in the swim up medium either containing albumin or lacking it. Different EBSS (E) media were obtained each containing one of the five additives: albumin (E+A), prostasomes (E+P), boiled prostasomes (E+BP), albumin plus prostasomes (E+A+P) and albumin plus boiled prostasomes (E+A+BP).

Prostasome preparation. Prostasomes were isolated from the ejaculates by means of differential centrifugation and chromatography on Sephadex G200. After liquefaction, semen samples were centrifuged (1,000 x g, 20 min) in order to remove cells and cell debris (pellet I), the supernatant was recovered and centrifuged again (100,000 x g, 2 h). The small pellet obtained (pellet II), which contained prostasomes and amorphous material (125) was in some experiments used as such, in other experiments it was further subjected to chromatography on Sephadex G200. The fractions, corresponding to the void volume of the column and containing prostasomes, were pooled and ultracentrifuged (100,000 x g, 2 h). The final pellet was dissolved in a convenient buffer and used for the experiments. This preparation was defined "pure prostasomes".

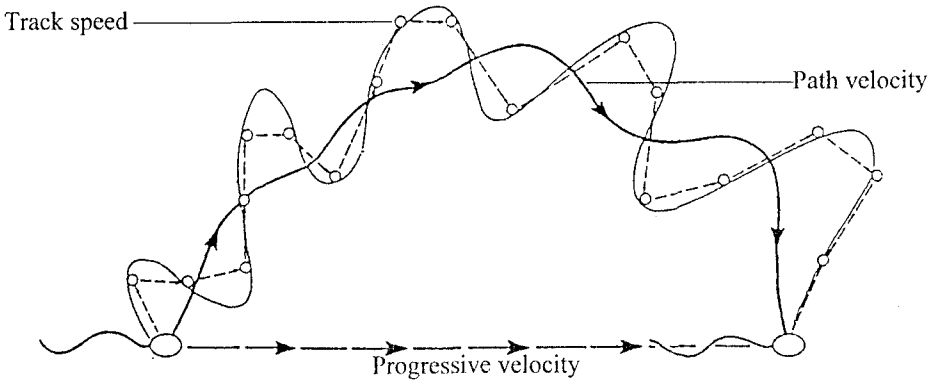
Evaluation of sperm motility parameters. Sperm motility parameters were evaluated by an HTM semi-automated motility analyser (Hamilton-Thorn Research, Inc., Danvers, MA, USA) using a micro cell chamber, depth 20 μm (Fertility Technologies, Inc., Natick, MA, USA).
The set-up of main analysis parameters and conditions of measurements are presented in Table 2.

Table 2.

Parameter set-up and conditions of measurement of the Hamilton-Thorn HTM image analysis system (according to HTM manual)	
Temperature (°C)	37.2
Diluent sample	0:1
Chamber	μ-Cell^{TM}
Image type	Phase contrast
Field selection	Auto
Calculate ALH	Yes
Beat frequency	Yes
Automatic sort	No
Frames at frame rate	20 at 25/s
Minimum contrast	8
Minimum size	6
Lo/Hi size gates	0.5 - 2.0
Lo/Hi intensity gates	0.5 - 2.0
Number of data points	7

At each time of measurement 150-200 spermatozoa were recorded for the evaluation of sperm movement characteristics. Motility parameters assessed in papers I, II, and III were: total cells (10^6/mL) include both motile and nonmotile sperm cells; motile cells (Velocity >10 μm/s; %) include cells moving with path velocity greater than 10 μm/s; progressive velocity (Velocity Straight Line VSL, μm/s) is the straight line distance between the beginning to the end of the track divided by the time taken Fig. 2; path velocity (path VEL μm/s) gives a smoothed cell velocity along its path; track speed (curvilinear velocity VCL, μm/s) represents an approximation to the average "curvilinear velocity" Fig. 2, and depends on the frame acquisition rate: at infinite acquisition rate track speed would be equal to mean curvilinear velocity, while at low acquisition rate track speed approximates the progressive velocity; the difference between track speed and path velocity is a measure of lateral head displacement (ALH, μm); linearity (LIN, %) is defined as the progressive (or straight-line) velocity of the cells divided by the track speed x 100, as indicated in Fig.

2; progressive motility (L >80, %) represents the percentage of sperm cells moving with linear index greater than 80, the linear index is the ratio of the progressive velocity and the path velocity Fig. 2; straightness (%); beat cross frequency (BCF, Hz); hyperactivity (%) represents the percentage of sperm cells moving with linearity ≤80%, ALH ≥5 μm and VCL ≥90 μm/s (167).



$$\text{Linear index} = \frac{\text{Progressive velocity}}{\text{Path velocity}} \qquad \text{Linearity} = \frac{\text{Progressive velocity}}{\text{Track speed}}$$

Figure 2. Parameters of sperm movement (according to HTM manual)

RESULTS

Highly motile spermatozoa were selected by a swim up procedure from normozoospermic samples and then washed three times with an isotonic Tris-HCl buffer containing 1 mM $MgCl_2$. After the third washing spermatozoa did not display any forward motility when analysed by either μ-Cell chamber or siliconized Bürker chamber. Instead about 2/3 of the cells were immobile whereas the other 1/3 were sticking to the glass wall showing on-spot motility but no progression.

Addition of pellet II material (pellet obtained after ultracentrifugation of sperm-free seminal plasma), "pure" prostasomes (first peak recovered after gel filtration of pellet II) and albumin restored progressive motility of the spermatozoa at various degrees while the second peak of the gel filtration (lacking prostasomes) did not affect the motility at all. Albumin is known to stimulate sperm motility (83), accordingly it is always included in media used for sperm preparations. On an equal protein basis we have shown, under defined experimental conditions, that prostasomes were superior in their stimulatory activity to albumin; for instance, at 0.25 g protein/L, albumin induced a motility which was 6±4% of the cells, 2±3% of which displayed a progressive motility and an ALH of

0.6±1 µm while the corresponding values for prostasomes were: motile cells, 41±6%; progressive motility, 14±2% and ALH, 2.7±0.5 µm. However, an additive effect was seen when prostasomes (0.25 g protein/L) were combined with albumin, 0.25 g/L. At optimum concentrations of albumin (2.3 g/L) and prostasomes (0.75 g protein/L) the motility restoring ability on spermatozoa was similar. Increasing concentrations of prostasomes up to 1.5 g protein/L did not improve the effect while higher concentrations of albumin displayed a reduced effect. No motility-stimulating activity was observed when the concentration of albumin was 14 g/L. Heating prostasomes at 80°C for 20 min or ultrasonication for 30 min did not affect their motility stimulating properties.
A common feature in all of the experiments performed was that the forward motility was initiated within minutes after the addition of prostasomes or albumin, it was higher within the first hour and was followed by a decline over time so that in most instances no motility was observed after 2-3 h of incubation at 37°C.

The reason why the motility faded away over time could be shortage of fuel since the suspending buffer did not contain any substrates for energy metabolism. To test this hypothesis different metabolites were included during the incubation of spermatozoa together with prostasomes. The results clearly showed that glucose, fructose and mannose sustained the prostasome-induced forward motility for more than 3 h whereas no motility was induced by the hexoses alone. Similar results were obtained when prostasomes were replaced by albumin. Accordingly, in the absence of a hexose a dose/response relationship between prostasomes and sperm motility was only possible for freshly incubated spermatozoa. On the contrary, in presence of 5 mM fructose, such a relationship could be evoked in spermatozoa that had been incubated for up 2 h. Other substrates such as galactose, pyruvate and lactate were without effect. The minimum concentration for a hexose to be effective varied from 5 mM for fructose to only 0.5 mM for glucose or mannose.
Many other substances were tested for promotive activity on sperm forward motility in combination with prostasomes without additional effect. Among them were ATP, creatine phosphate, xanthine, 2'deoxyadenosine, cAMP, dibuturylcAMP and inositoltrisphosphate. Calmidazolium (0.01 mM) and lithium (1.0 mM) were inhibitory and after 30 min of incubation sperm cells were completely immotile. After 15 min of incubation also EDTA was inhibitory while EGTA was without effect. An evidently positive effect on sperm motility parameters (progressive velocity and path velocity) was observed when adenine (2 mM) was present in the incubation medium together with prostasomes and glucose (5 mM) while the adenosine effect was weaker in this context. Increasing concentrations of adenine gave a curve profile indicating a dose/response relationship followed by a plateau at a concentration exceeding 2.5 mM.
In order to investigate the effect of divalent cations on sperm forward motility, the sperm cells were washed either in presence or in absence of $MgCl_2$ and then incubated with prostasomes for 15 min and increasing concentrations of either $ZnCl_2$ or $CaCl_2$. The

results showed that the presence of magnesium ions in the washing buffer was beneficial for sperm motility whereas zinc ions at concentrations lower than 0.1 mM did not show any evident effect opposite to the deleterious effects seen at higher concentrations. Calcium ions had only a slight if any effect.

In order to investigate further the positive influence of prostasomes on sperm forward motility and to find a possible clinical application, swim-up experiments were performed using different conventional media enriched with prostasomes or boiled prostasomes. Semen samples (0.2-0.5 mL) were layered under 0.5 mL of five different media (see materials and methods) and incubated for 1 h at 37°C under 5% CO_2. The upper layer of the medium (0.25 mL) was aspirated and the sperm motility parameters were measured at 0, 1, 3, 6 and 22 h of incubation.
The yield of motile spermatozoa at zero time in the media where albumin (10 mg/mL) was replaced by boiled prostasomes (1 mg protein/mL), was increased by 32% ($p<0.001$) while native prostasomes were less active in this regard. The combination of albumin with either prostasomes or boiled prostasomes showed improved progressive motility after 1 and 3 h of incubation. In contrast, after 22 h of incubation only the media containing boiled prostasomes displayed a good progressive motility while the media containing native prostasomes were deleterious for progressive motility. It was also observed that at 22 h of incubation albumin was necessary for sustaining the progressive motility and for maintaining a high degree of membrane intactness as shown by the release pattern of lactate dehydrogenase (LDH) from the sperm cells into the medium. About 35% of the total sperm LDH was released at 22 h of incubation in the medium lacking albumin while less than 10% of LDH was released in the media containing albumin. A positive effect of prostasomes and boiled prostasomes on sperm hyperactivity (%) and ALH was also evident during the first 6 h of incubation particularly in the media without albumin.

The detrimental effect of native prostasomes on sperm motility after a long period of incubation may be due to some enzymes associated with the prostasome membrane that have their target substrate on the surface of spermatozoa. Therefore, boiling prostasomes for 3 min may have prevented this effect. On the other hand the beneficial effect of prostasomes on motility as demonstrated during the first 6 h survived the boiling treatment. To exclude the possibility that prostaglandins were responsible for this phenomenon we measured the prostaglandin content in purified prostasomes. The total amount of the four E prostaglandins was only 0.018±0.011 μmol (n=3) per preparation of prostasomes obtained from four pooled ejaculates.
We have also shown that prostasomes converted arachidonic acid to 15(S)-hydroxyeicosatetraenoic acid. The reaction product was identified by reverse- and chiral-phase HPLC and gas chromatography-mass spectrometry. The enzyme involved in the conversion was an arachidonic acid 15-lipoxygenase which has previously been shown to be present on human washed spermatozoa (108).

Synaptophysin (Sy) is an integral membrane glycoprotein which has been used as a marker for endocrine, neuroendocrine and neural tissue (163). In neuronal and neuroendocrine cells, Sy is located in the neurotransmittor-containing small synaptic vesicles (163) and also in secretory granules of the pancreatic islet A and B cells (55). Sy was identified in human seminal plasma from normozoospermic as well as from azoospermic men. Since this protein is membrane-bound by spanning the lipid bilayer four times (54), it was of interest to study whether the protein was associated with the prostasome membrane. After the first centrifugation step from two normozoospermic ejaculates (1000 x g, 20 min) most of Sy was recovered in the supernatant (supernatant I). Pellet II obtained after ultracentrifugation of supernatant I contained about 27% of Sy while 53% was still in the new supernatant (supernatant II). Gel filtration of pellet II demonstrated that Sy was eluted in the void volume of the column together with aminopeptidase activity (a marker enzyme for prostasomes). The same experiment performed with supernatant II containing the 53% of Sy showed that most of Sy was eluted as a high molecular weight, aggregated from.
Other proteins, which are characteristic for neuroendocrine and endocrine tissues are the chromogranin (Cg) family, which consists of at least CgA, CgB (secretogranin I) and CgC (secretogranin II). These proteins are found in the peptide/hormone-containing large dense core vesicles (163). Prostasomes contained also high amounts of CgB (2,300 pmol/g protein), neuropeptide Y, NPY (3,000 pmol/g protein) and vasoactive intestinal polypeptide, VIP (2,500 pmol/g protein) while CgA was present in very low amounts and CgC was below the detection limit. The presence of Sy, CgB, NPY and VIP in the purified prostasome preparation was also demonstrated by immunoelectron microscopy. Morphological investigation of the purified prostasomes revealed intact organelles with size and characteristics similar to the ones reported earlier (17).

Human semen is rich in many hydrolytic enzymes the most studied of which is probably the prostatic acid phosphatase. The particularly high activity of this enzyme in human semen makes it difficult to study other phosphorolytic enzymes such as alkaline phosphatase and 5'nucleotidase. However, prostasomes may present a source of 5'nucleotidase in human semen since at least one form of the enzyme is membrane-bound (168).
A phosphohydrolase activity was indeed present on the purified prostasomes with either 5'AMP or p-nitrophenylphosphate (p-NPP) as substrate. The AMPase activity was very little inhibited by tartrate and levamisole (two specific inhibitors for acid and alkaline phosphatase, respectively) while the activity on p-NPP reached very low values at high concentrations of inhibitors (tartrate 10 mmol/L, levamisole 2 mmol/L). By including these two inhibitors in the assay mixture it was thus possible to study the effect of specific 5'nucleotidase inhibitors on the AMPase activity. A distinct inhibition was obtained by adenosine 5'(α, ß methylene) diphosphate at about 100 µmol/L, by Ni^{2+} at about 10 mmol/L and by concanavalin A at about 20 µg/mL. The substrates more effectively hydrolysed next to 5'AMP were 5'IMP and 5'GMP while 3' and 2'AMP were inefficient.

Ultrasonication of prostasomes for 20 min did not increase the 5'nucleotidase activity. Triton X-100 stimulated the activity by 50-60% at a concentration of 0.05%. The enzyme obeyed Michaelis-Menten kinetics and the apparent Km for 5'AMP was 11.2±2.1 μmol/L and Vmax 64.7±11.4 nmol/mg protein/min. In presence of Triton X-100 the Km value was reduced by 30% and the Vmax value was increased by 60%.

Prostasomes, purified from 12 semen samples and from expressed prostatic fluid of 2 patients, were examined for the protein content and alkaline phosphatase (ALP), 5'nucleotidase (5'NT) and alkaline phosphodiesterase I (APD) specific activities. The semen samples were subdivided in normozoospermic, azoospermic and vasectomised groups. The highest recovery of prostasomes (mg of protein) was obtained from semen samples of azoospermic men, 4.25 mg/mL, while the lowest value was from prostatic fluid, 1.12 mg/mL. The specific activities of ALP and APD were about the same in prostasomes isolated from seminal plasma regardless of subgroup. However, a higher activity of ALP and a lower activity of APD was observed on prostasomes isolated from prostatic fluid. The highest value for 5'NT was obtained in prostasomes purified from seminal plasma of normozoospermic men.
Treatment of prostasomes with PIPLC (2 U/mL) for 2.5 h at 37°C released about 50% of ALP, 30% of 5'NT and 12% of APD while aminopeptidase was not significantly released. After the PIPLC treatment the enzymes were recovered in the hydrophilic phase after repartition in Triton X-114. Different detergents and carbonate buffer (0.1 mol/L, pH 11.2) were tested for the ability to release the enzymes from the prostasome membranes. The most efficient detergent was octyl glycoside which released about 40% of the proteins and almost 100% of the enzymes. However, the carbonate buffer released about 50% of the proteins but very little enzymes. Both ALP and 5'NT were resistant to the treatment with Triton X-100 and Triton X-114.

DISCUSSION

Prostasomes and sperm motility. Several studies have indicated that the secretions of the accessory sex glands may affect sperm motility in a positive as well as a negative way. However, prolonged exposure of spermatozoa to seminal plasma results in a marked decline of both sperm motility and vitality, and consequently in a reduced, or totally inhibited fertilising capacity (56, 121). Accordingly, it is essential that spermatozoa to be used in assisted reproduction procedures, such as intrauterine insemination or *in vitro* fertilisation are separated from the seminal plasma constituents soon after ejaculation. Different culture media can be used for washing and resuspending sperm cells, and generally these media are supplemented with albumin or in some cases with heat-inactivated human serum (160).
Washing of spermatozoa in a protein-free isotonic buffer resulted in a gradual loss of forward motility. This was in line with previous studies performed on human (78) and

bovine spermatozoa (11). Hamster spermatozoa were particularly sensitive to washing or dilution (13). The mechanisms behind this phenomenon are not know but the most likely explanation is that some components are lost from the surface of spermatozoa during washing and perhaps it makes the sperm cells sticking to the glass slides (21). However, this inactivation was not irreversible because good progressive motility could be restored by addition of prostasomes or albumin. It is important to emphasise that prostasomes were more efficient than albumin when compared on an equal protein basis and differently from albumin, prostasomes retained the promotive activity after heat treatment. Since prostasomes are membrane-surrounded organelles only a small part of the total protein content is exposed to the external surface. This led us to believe that the effect of prostasomes was specific and probably not mediated by proteins. The heat stable factor could be a lipid molecule. It has been shown that liposomes may promote sperm motility (9) and the platelet-activating factor (an ether phospholipid) and its lyso-form as well as lysophosphatidylcholine could stimulate sperm linear and curvilinear velocity (53).
It should be noted that the immunosuppressive activity recently attributed to the prostasomes survived boiling (60) and it raises the question whether these two different functions have a common denominator. Prostaglandins of the E series are present in human seminal plasma in high concentrations. They are responsible for most of the immunosuppressive activity of semen (157) and they affect sperm motility pattern by increasing the velocity and the frequency of sperm head rotation (5). Therefore it was justified to investigate whether the prostasome preparation was contaminated by prostaglandins. We found only trace amounts of prostaglandins on purified prostasomes which could not affect sperm motility and function significantly. In addition, the relative amounts of the four E prostaglandins found in prostasomes were similar to those of seminal plasma, suggesting that they were produced by the seminal vesicles and adsorbed to some prostasome proteins.

Observation of sperm cells in the light microscope before and after addition of prostasomes showed that the effect on sperm forward motility by prostasomes was not only a simple anti-sticking phenomenon. Instead prostasomes, by interacting with spermatozoa (129), may affect some membrane properties such as ion permeability. A receptor mediated effect could also be considered. Although we cannot completely exclude the possibility that prostasomes prevent the adhesion of spermatozoa to the slides we believe that this event, if present, is a consequence of the stimulatory activity on forward motility. Similarly to prostasomes, it was shown that other organelles (isolated from bovine seminal plasma) originating from seminal vesicles induced sperm motility in bovine epididymal spermatozoa suspended in a modified Ringer medium (4). However, human seminal vesicle secretion contained only a sedimentable, amorphous substance (123) which was eliminated from the prostasome preparation by gel filtration and which was shown in the present study not to induce any forward motility.

Substrates as energy source for sperm movement. The motility evoked by prostasomes and albumin faded off with time so that after 2-3 h of incubation no motile cells were seen. Glycolytically metabolised sugars such as glucose, fructose, and mannose were all effective to sustain the prostasome induced sperm motility over time although the minimum concentration required was different for the hexoses. The higher concentration of fructose (5 mM) necessary to achieve the same effects as those of glucose and mannose (0.5 mM) may reflect different affinities of these substrates for the sperm hexokinase which is the first enzyme in the glycolytic pathway (91). Glucose was more effective in supporting in vitro penetration of zona-free hamster oocytes by human spermatozoa than fructose or mannose (122). This test is an indirect indicator of sperm capacitation and acrosome reaction (164).
Galactose, pyruvate and lactate were not useful in this context. It is possible that the galactose-glucose interconversion which involves four enzymatic reactions did not take place in human spermatozoa or, alternatively, these metabolites were not able to cross the sperm plasma membrane due to e.g. lack of appropriate carriers.

Adenine stimulates sperm motility. Among several different substances tested in concert with prostasomes and glucose, adenine was the only one to be efficacious in enhancing sperm motility parameters in presence of prostasomes and hexose while the effects of adenosine and 2-deoxyadenosine were weaker or practically absent. Progressive velocity and path velocity were the only parameters that were more positively affected by adenine when prostasomes were present in an optimum concentration (1 g protein/L) while a positive effect of adenine was also recorded regarding the per cent of motile cells when the concentration of prostasomes was reduced by 50%. The mechanism of action of adenine is obscure but is probably not mediated via prostasomes. Adenine enters cells by a simple diffusion process which is not saturable (31). The adenine molecule may be active in itself or after having been metabolised by adenine deaminase (EC 3.5.4.2) or adenine phosphoribosyltransferase (EC 2.4.2.7). The products of the first reaction are hypoxanthine and ammonium ion. However, we did not achieve any motility promoting effect by 0.5 mM hypoxanthine and xanthine, and addition of 1 mM of ammonia to the incubation buffer did not influence the pH. Accordingly, the metabolic pathway involving adenine deaminase being essential was ruled out. The product of the adenine phosphoribosyltransferase reaction is 5'AMP which can be used either for the building up of ATP or for the adenosine production by the cytosolic 5'nucleotidase (168). However, the effect of adenine was instantaneous after addition to the sperm cells negating a metabolic route being essential for such activity. In addition, these cells were saturated with fuel, since glucose was present at 5 mM. It has been shown that adenosine stimulates human sperm motility by interacting with surface located A_2 receptors (140, 141). Although we cannot exclude the possibility that adenine is a source for intracellular adenosine production it must be translocated from outside of the cell before being active. Furthermore, the adenosine effect was weaker during the first hour of incubation and

absent in the following hour compared with adenine. Instead, it is more likely that the adenine effect was mediated by a receptor which may be present on the surface of the spermatozoa or in the cytosol as well. This would also fit with the hyperbolic saturation curve obtained with increasing concentration of adenine. Whether adenine directly interacts with the adenosine receptor is not known.

Prostasome inclusion in the swim-up medium. The motility stimulating activity of prostasomes was evaluated on spermatozoa which were washed in an artificial isotonic buffer and subsequently resuspended in the same solution enriched with prostasomes under different conditions. As discussed above, sperm cells may have suffered some unknown modification during the washing procedures which makes it difficult to really estimate whether prostasomes may influence sperm motility under more physiological conditions. Therefore, prostasomes were included in the swim-up media and the motility parameters were recorded over time for up to 22 h of incubation. From the results of these experiments we may conclude that prostasomes possessed at least two distinct factors which had an effect on sperm motility: the first one demonstrated heat stability which resulted in a positive effect on progressive motility and induced sperm hyperactivity; the second one displayed heat lability which produced a deleterious effect on sperm motility after prolonged time of incubation.

Replacing albumin in the standard medium with prostasomes or boiled prostasomes resulted in also a higher recovery of motile spermatozoa. It is known that human spermatozoa incubated in capacitating media undergo motility changes resembling those observed in spermatozoa of some other species. These changes are characterised by the appearance of a population of hyperactive spermatozoa that displays a vigorous motility but little progression and large amplitudes of lateral head displacement (120). It is thought that this hyperactivity is typical of capacitated spermatozoa and precedes the acrosome reaction (120). Cholesterol may play an important role during capacitation of human spermatozoa (12). It has been shown that cholesterol concentration is reduced in human sperm plasma membrane during incubation under capacitating conditions (12). Cholesterol loss during capacitation may be an important step in preparing the sperm plasma membrane to the fusion events involved in the acrosome reaction (117). Prostasomes may induce a cholesterol efflux from the sperm plasma membrane since they contain a high amount of sphingomyelin and saturated phospholipid acyl chains (8) which are convenient moieties for cholesterol in membrane organisation.

The detrimental effect of native prostasomes on sperm motility could be mediated by some enzymatic activities which may act on the sperm or prostasome membrane lipids. Prostasomes contain phospholipase A_2 (74) and arachidonate 15(S)-lipoxygenase activities which may produce arachidonic acid and 15-hydroperoxyeicosatetraenoic acid, respectively. The latter may initiate lipid peroxidation and a gradual increase in the fusogenic properties of the sperm plasma membrane and consequently affect sperm function. 15-lipoxygenase might play a role in the acrosome reaction. It has been shown in

bovine spermatozoa that inhibition of this enzyme was associated with inhibition of acrosome reaction (65). Furthermore, *cis*-unsaturated fatty acids which could be released by phospholipase activity stimulated the acrosome reaction of hamster spermatozoa (95). Whether prostasomes induced a premature acrosome reaction was therefore of interest and studied further. The results are presented in figure 3.

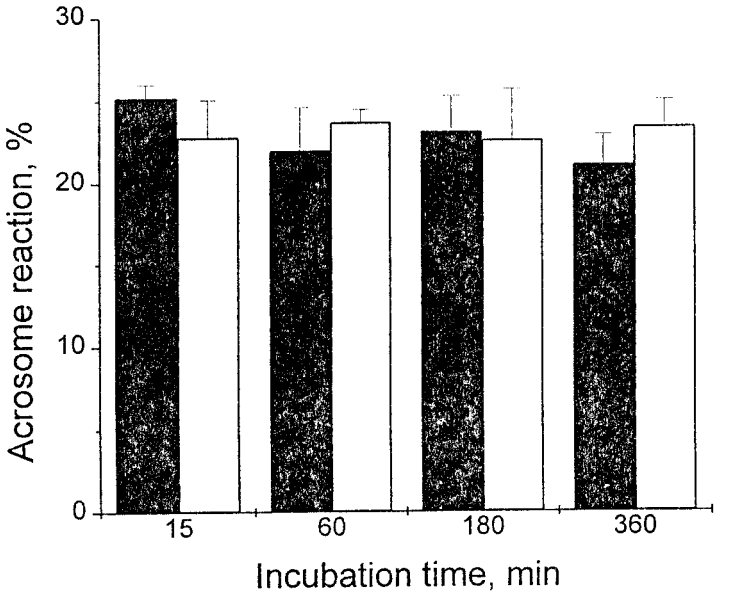


Figure 3. Percentage of acrosome reacted spermatozoa after swim-up and subsequent incubation in two different media: E + A (■) and E +A + P (□). Acrosome reaction was assessed by staining the spermatozoa with fluorescein isothiocyanate (FITC) labelled *Psium sativum* agglutinin (PSA) after permeabilization with methanol essentially as described by Medoza et al. (96). Only pattern B, characterised by the staining of the equatorial band was considered which gave the amount of recently reacted spermatozoa (154). Results are given as means ± SEM of four experiments performed with different semen samples. E = Earle's balanced salt solution; A = human serum albumin, 10 mg/mL; P = prostasomes, 1 mg protein/mL.

After swim-up in two different media (E+A; E+A+P) the sperm cells were incubated for 6 h and small aliquots were removed at each time for the acrosome staining. It is shown in the figure that about 20-25% of the cells were acrosome reacted regardless of the incubation time and presence of prostasomes in the media. Although these results represented the mean values of a small number of experiments (n=4) we may conclude that prostasomes did not affect significantly the acrosome reaction. From the physiological point of view this finding is not surprising, since the acrosome reaction must take place in proximity of the oocyte to allow sperm penetration through the egg vestments (38). The physiological mediators of acrosome reaction are probably associated with the cumulus cell mass and the zona pellucida (38). On the contrary, seminal plasma contains decapacitating factors which may prevent the acrosome reaction (56).

In conclusion, prostasomes induced a hyperactivated pattern on spermatozoa, probably by modifying the capacitation state of the sperm cell, although not being able to induce the acrosome reaction. In addition, native prostasomes were deleterious for sperm motility when incubated for a long time, but this effect could be avoided by boiling.
It has been shown that the incidence of hyperactivation was significantly reduced in subfertile men and it was positively correlated with sperm penetration into zona-free hamster oocyte and with the binding to the human zona pellucida (23). Furthermore, a positive correlation was revealed between the fertilisation rate and the total number of spermatozoa recovered after swim-up (155) and the sperm concentration at insemination (107).
Including boiled prostasomes in the swim-up media may improve the recovery of hyperactive spermatozoa from semen samples containing a reduced number of motile spermatozoa and consequently may increase the chances of fertilisation.

Biochemical findings

Synaptophysin, chromogranins, vasoactive intestinal polypeptide and neuropeptide Y.
We have established that CgB, NPY and VIP occurred in about equimolar amounts in human prostasomes whereas CgA and CgC concentrations were low or undetectable. These findings are consistent with recent studies where it was reported that some neuroendocrine markers like CgA, neurone specific enolase and serotonin were expressed in prostatic epithelial cells (3). The high amount of CgB compared to CgA detected in prostasomes has to our knowledge never been reported before. In this context it was interesting to note that CgB was the major component of the chromogranin protein family in prostatic carcinomas with neuroendocrine differentiation (138). Sy was also found to comigrate with prostasomes during the differential centrifugation and gel filtration. Although Sy is a well established immunocytochemical marker for neuroendocrine cells and neurones (162) a synaptophysin-like protein has also been detected in the serotonin-containing vesicles in platelets (18) and in granulocytes (1, 139), subsequently denoted granulophysin. It has recently been shown that prostasomes also contained granulophysin (144). Although the antibody used in this study probably cross-reacted with granulophysin we prefer to denote and identify the protein as synaptophysin since other neuroendocrine markers were present in the prostasomes as well. It is possible that Sy and granulophysin molecules are members of a family of proteins, maybe expressed in all cells that have a regulated release of granule content.
Since Sy in neurones is located in the small synaptic vesicles while chromogranins are present in the large dense core vesicles, it may be possible that prostasomes are a mixture of two kinds of vesicles bearing selectively different components. On the other hand, they may be a completely new kind of organelles with characteristics common to both types of vesicles.

Hydrolytic enzymes. 5'-nucleotidase (5'NT) is a ubiquitous enzyme which specifically catalyses the hydrolysis of the phosphate group from 5'-nucleotides. Various forms of the enzyme have been described and classified according to their location and molecular and kinetic properties, *viz.* one membrane-bound form and three soluble forms (168).
Non-specific phosphatases, which are abundant in human semen, also hydrolyse 5'-nucleotides (98). Therefore it was important in our study on prostasome 5'NT to find the conditions under which the unspecific phosphatase activities were inhibited. We have shown that inclusion of tartarte (10 mM) and levamisole (2 mM) in the assay mixture used for measuring 5'NT resulted in an almost complete inhibition of paranitrophenylphosphatase activity. Under this condition, it was possible to study the kinetics and inhibitory properties of 5'NT in prostasomes. According to the Km value, inhibition by adenosine 5' (α, ß methylene) diphosphate and concanavalin A, and the preference of 5'AMP as substrate (168), the 5'NT of prostasomes has been identified as the membrane-bound form. It is probably located at the outer surface of prostasomes since ultrasonication did not increase the enzyme activity and the substrate was available to the enzyme although being a charged molecule and therefore not able to penetrate the membrane bilayer.

The enzyme was characterised by a micromolar Km value for 5'AMP as substrate which was very similar to the purified 5'nucleotidase occurring in soluble form in human seminal plasma (99). Although this soluble enzyme preferentially hydrolysed 5'IMP, it might be derived from the prostasome-bound form as a consequence of either proteolytic digestion or GPI anchor removal by specific phospholipases (see below).
The biological function of the enzyme in human seminal plasma is not known, however, as discussed above, adenosine (one of the reaction products of the enzyme) may affect human sperm functions, particularly sperm motility via A_2 receptors (140, 141). In addition, adenosine may have its target site in the female genital tract, since it has been shown that adenosine was able to modulate the contractile activity of smooth muscles in the human fallopian tube (135).

Although the prostatic acid phosphatase has been the subject of many studies (111) the presence of alkaline phosphatase activity in human seminal plasma has received much less attention. In a study it was concluded that the alkaline phosphatase in human semen was identical to acid phosphatase (58). Human alkaline phosphatase (ALP) is expressed in four distinct isoenzymes which are encoded by different genes and can be distinguished on the basis of differential sensitivity to some inhibitors and its thermostability (43). These isoenzymes are: liver/bone/kidney or 'tissue non-specific' ALP; intestinal; placenta and placental-like or germ cell ALP. Germ cell ALP is present in normal testicular tissue (93) and is also secreted in appreciable amounts in human seminal plasma (94). The activity was related to the sperm count and dropped to undetectable levels in seminal plasma after vasectomy (69).

We have shown that purified prostasomes contained ALP as well as alkaline phosphodiesterase I (APD) activities. In table 3 the inhibitory characteristics and thermostability of prostasome ALP are shown in comparison with the known properties of the four isoenzymes. The similarity between prostasome ALP and the liver/bone/kidney isoenzyme regarding both the I_{50} (concentration of inhibitor required to produce 50% inhibition) and the T_{50} (time required to give 50% inactivation) clearly demonstrated that prostasome expressed a liver/bone/kidney ALP isoenzyme which was quite distinct from the germ cell ALP described previously.

Table 3.

	I50 (mM)				
	Prostasomes	L/K/B*	Placental*	Placental-like*	Intestinal*
Levamisole	0.016±0.001	0.03	1.7	2.7	6.8
L-Homoarginine	1.46±0.14	2.7	>50	36	40
Phenylalanine	32.4±2.7	31	1.1	0.8	0.8
Leucine	15.9±1.2	13.1	5.7	0.6	3.6
PheGlyGly	35.2±4	30.6	0.1	2.9	3.7
	T50 (min)				
56°C	6.5	7.4	---	---	>60
65°C	<1	1	>60	>60	6.5

*Data from Harris H. (43).
Abbreviations: L/K/B, liver/kidney/bone; PheGlyGly, L-Phenyalanylglycylglycine; I50, concentration of inhibitor reguired to produce 50% inhibition; T50, time required to give 50% inactivation.

PIPLC from *bacillus cereus* has been used to release ALP and 5'NT from the plasma membrane of different cells (84). We have shown that both ALP and 5'NT were released in substantial amounts from the prostasome membrane upon treatment with PIPLC. This suggests the existence of a GPI anchor which was removed by the PIPLC treatment converting the enzymes to a hydrophilic form. However, only partial release was achieved and this may have more than one reason. The GPI anchored proteins may be incompletely accessible to PIPLC, the GPI anchor may be chemically modified as is the case for the *ecto*enzyme acetylcholinesterase of human erythrocytes, or the enzymes are expressed in both GPI anchored and non-GPI anchored forms (133).
Although it is thought that GPI anchored proteins are mainly located at the plasma membrane surface some reports have shown the presence of such mode of anchoring in organellar membranes like the granules of chromaffin cells (37) and the pancreatic zymogen granules (66). A GPI anchored 5'NT was also demonstrated on the lysosome membranes (151).

A small but significant amount of APD was released by PIPLC treatment. However, this enzyme displayed a different behaviour regarding Triton X114 phase separation compared to 5'NT and ALP. After solubilization and phase separation of entire prostasomes, 65% of APD was recovered in the hydrophilic phase which may indicate that the protein had a lower hydrophobicity than 5'NT and ALP (80). However, carbonate buffer which is known to strip peripherally bound proteins (40) was not able to release APD from the prostasome membrane. Other studies regarding the presence of a GPI anchor on APD have produced conflicting results (84, 102, 103, 142). We may conclude from our data on human prostasomes that a minor part of APD contains a GPI anchor which can be released by PIPLC treatment while a major part of APD interacts with the prostasome membrane in an unknown way.

The physiological substrates for ALP and APD in semen are not known, this makes it difficult to anticipate a possible functional role of these enzymes in human seminal plasma. However, it has been shown that the plasma cell membrane glycoprotein PC-1, the function of which is not known, is identical to the enzyme APD and nucleotide pyrophosphatase (118). A recently identified ectoprotein Ser/Thr kinase (MAFP) has been purified from bovine liver and amino acid sequence analysis of the proteolytic peptides revealed 80-100% homology with PC-1 (105). It was demonstrated that MAFP in addition to the kinase activity possessed an alkaline phosphodiesterase activity as well (106). Hence, it might be possible that the protein kinase activity previously identified on prostasomes (148) and the APD activity reported in the present investigation are two expressions of the same protein. Further studies are necessary to validate this hypothesis.

In conclusion, prostasomes are extracellularly occurring organelles in semen which express different proteins and enzymes on their membrane surface. In addition, they may be considered as neuroendocrine-like vesicles having a neurotransmittor function the target cells of which are not known. However, the spermatozoa or the female reproductive organs are the most likely candidates.

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