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# ON SPERM NUCLEAR ZINC AND CHROMATIN DECONDENSATION

An in vitro study on the physiology of the ejaculated human spermatozoon

By

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# ERRATA

Page; Col=column; Par=Paragraph; Line; (<0 = from bottom)

## GENERAL

All "Huajacuja" should read as "Huacuja".

## THESIS

| Page | Col | Par | Line | Should read as: |
|---|---|---|---|---|
| 2 | 1 | 2 | 5 | **capacity** for human sperm chromatin decondensation. |
| | | 6 | 2 | The human sperm **nucleus** takes up zinc ... |
| | | | 3 | ... J Androl 9 (1): **77-80** |
| | | 8 | 2 | Dithiothreitol. |
| 5 | 1 | 2 | 7 | ... where the **spermatocytes,** spermatids and ... |
| | 2 | 2 | -6 | ... Lung **1974** ... |
| 7 | 2 | 2 | 7 | ... cf Orgebin-Crist **et al** 1975). |
| | | | 25 | ... Tesh & Glover **1969** ... |
| 11 | 2 | 1 | 5 | ... Gunn & Gould **1958** ... |
| 17 | 1 | 2 | -6 | ... (**VI**: Table 2). |
| | 2 | 2 | 16 | ... and **thereby possibly** competing ... |
| 23 | 1 | 3 | -1 | **preserved** in spermatozoa supplemented with zinc ... |
| 25 | 1 | 2 | 2 | [**new paragraph** should begin at]: **ARVER S. 1982**... |
| | | 6 | 1 | ... MACAPINLAC M.P. **1968**... |
| 27 | 1 | -3 | 1 | [reference missing]: **GUNN S.A. & GOULD T.C. 1958. Role of zinc in fertility and fecundity in the rat. Am J Physiol 193 (3): 505-508.** |
| 29 | 1 | 3 | 1 | [reference missing]: **KVIST U. 1982. Spermatozoal disulfide interaction: a possible event underlying physiological sperm nuclear chromatin decondensation. Acta Physiol Scand 115: 503-505.** |
| 32 | 1 | 4 | 1 | [reference missing]: **TESH J.M. 1969. Effects of the ageing of rabbit spermatozoa in utero on fertilization and prenatal development. J Reprod Fert 20: 299-306.** |

## PAPER II

| Page | Col | Par | Line | Should read as: |
|---|---|---|---|---|
| 196 | 2 | 1-2 | | [all parentheses refer to Fig. 2] |
| 200 | 1 | 2 | 4 | ... (grant **85-04X-07153-01A**) |

## PAPER III

| Page | Col | Par | Line | Should read as: |
|---|---|---|---|---|
| 483 | 1 | 2 | -4 | 6.5 x **$10^{-15}$** kg ... |

## PAPER IV

| Page | Col | Par | Line | Should read as: |
|---|---|---|---|---|
| 300 | 2 | 3 | 5-6 | ... (Lung **1974**) ... |
| 301 | 1 | 1 | 2 | 6.4x$10^{-15}$ **l** ... |
| | Table 1 | | | ... 6.5x$10^{-15}$ **kg** has been used (Lung 1974) |
| 302 | 1 | 6 | 1 | CHANDLER J.A. & BATTERSBY S. **1976a**... |

## PAPER V

| Page | Col | Par | Line | Should read as: |
|---|---|---|---|---|
| 78 | 1 | 2 | 2 | ... (Kvist et al **1985b**) ... |
| | | 4 | 4 | ... **p>0.05**)... |
| 80 | 1 | 8 | 2 | Scand 120: **52 (P61).** |

This thesis is based on experimental work reported in the following publications, which will be referred to in the text by their Roman numerals:

I BJöRNDAHL L & KVIST U. 1985. Loss of an intrinsic mechanism for human sperm chromatin decondensation. Acta Physiol Scand 124: 189-194.

II KVIST U & BJöRNDAHL L. 1985. Zinc preserves an inherent capacity for human sperm chromatin decondensation. Acta Physiol Scand 124: 195-200.

III ROOMANS GM, LUNDEVALL E, BJöRNDAHL L & KVIST U. 1982. Removal of zinc from subcellular regions of human spermatozoa by EDTA treatment studied by X-ray microanalysis. Int J Androl 5 (5): 478-486.

IV KVIST U, BJöRNDAHL L, ROOMANS GM & LINDHOLMER C. 1985. Nuclear zinc in human epididymal and ejaculated spermatozoa. Acta Physiol Scand 125: 297-303.

V BJöRNDAHL L, KJELLBERG S, ROOMANS GM & KVIST U. 1986. The human sperm takes up zinc at ejaculation. Int J Androl 9 (1): in press.

VI BJöRNDAHL L & KVIST U. 1986. Sperm number and vesicular fluid limit sperm nuclear zinc uptake in split ejaculates. Int J Androl, submitted for publication.

**Abbreviations commonly used in the thesis:**

| | |
|---|---|
| BSS | Buffered Salt Solution (composition: page 12) |
| DTT | Dithithreitol. |
| EDTA | Disodium ethylene diamine tetraacetate. |
| H+I% | Percentage zinc bound to HMW and IMW. |
| HMW | High-molecular-weight zinc-ligands (from seminal vesicles). |
| IMW | Intermediate-molecular-weight zinc-ligands (from seminal vesicles). |
| LMW | Low-molecular-weight zinc-ligands (from the prostate). |
| SDS | Sodium Dodecyl Sulfate or Sodium Lauryl Sulfate. |
| Zn/S | A measure of sperm head zinc concentration: 1000 x zinc-to-sulphur counts/s-ratio (X-ray microanalysis). |
| Zn/P | A measure of sperm head zinc concentration: 1000 x zinc-to-phosphorus counts/s-ratio (X-ray microanalysis). |

# CONTENTS

# I. INTRODUCTION

## 1. Historical survey

When Leeuwenhoek in 1677 reported the discovery of "small animals" (animalcules or later spermatozoa) in semen of men and several other species, an era of speculations on the significance of these "organisms" was initiated. The semen itself had already been found necessary for the reproduction of animals (Harvey 1651: cf Tyler 1967) and the testis was considered to be the source of the fertilizing semen (De Graaf 1668: cf Jocelyn & Setchell 1972). Some scientists were convinced that the animalcules were parasites and not associated with fertilization, while Leeuwenhoek postulated that the small animals were seeds of complete individuals which only had to be implanted in the uterus to start developing into new beings. In 1775 Spallanzani showed that amphibian ova also need contact with semen to start developing into a new individual. Since diluted semen, without visible spermatozoa, could also fertilize, he assumed erroneously that it was the seminal plasma without "spermatic worms" that initiated embryonic development. It was not until 50 years later that Prévost & Dumas repeated Spallanzanis experiments and concluded that filtered semen could not fertilize ova (cf Tyler 1967). Kölliker (1841) found spermatozoa in semen from most of the animals studied and concluded that the sperm shape is similar within genera, although it varies between different species with no individual having more than one type of spermatozoa. He also substantiated Leeuwenhoek's observations that spermatozoa are formed in the testis (cf Roosen-Runge 1977). Hertwig suggested (1876) that fertilization is based on the fusion of the nuclei of sperm and ovum, and a few years later Fol was first to report observations of spermatozoa actually penetrating the ovum (Kempers 1976; Schatten & Schatten 1983). Thereafter, seminal plasma was regarded, for several years, mainly as a vehicle and temporary nutrient during the transfer of the spermatozoon (cf Rodger 1975). However, Orgebin-Crist (1967, 1968) obtained results indicating that spermatozoa which had been in contact with the seminal plasma were more effective in fertilizing and giving rise to normal zygote development than were "mature" epididymal spermatozoa. The means by which contact with seminal plasma secures a fertilizing potential of a spermatozoon is not known. However, appropriate contact with prostatic fluid at ejaculation has been reported to sustain both sperm motility and sperm survival in human (Hotchkiss 1945; Rozijn 1961; Amelar & Hotchkiss 1965; Eliasson 1970; Eliasson & Lindholmer 1972; Lindholmer 1973). Furthermore, appropriate contact with zinc of prostatic origin seems to render the nucleus a specific stability and a potential for rapid unpacking of the condensed chromatin (Kvist 1980a).

The fundamental function of the spermatozoon is to convey the paternal genetic contribution to the ovum, thereby participating in the creation of a new individual with a unique set of genes. Although this function is universal in sexual reproduction, there is some variation in the means by which the transfer of genetic material is accomplished in different species. The topic of this thesis is primarely human reproduction. Some important variations between different species will, however, also be discussed.

## 2. Formation of spermatozoa in the testis

### Early spermatogenesis

The formation of spermatozoa takes place in the seminiferous tubules of the testis. "Stem" cells (spermatogonia), together with Sertoli cells, line the tubular walls. Spermatogonia multiply by mitotic divisions, a process which occur perpetually during the fertile life of the male. Some of the spermatogonia diverge from the mitotic cycle for further development into spermatozoa. At this point in the process, cell divisions become incomplete, in the sense that the cell membrane do not divide the

cytoplasm completely. This incomplete cell division results in a "clone" consisting of hundreds of connected daughter cells which, when they enter meiosis, are named primary spermatocytes (Setchell 1978). In the primary spermatocyte the genetic material is duplicated, homologous chromosomes form pairs, and a recombination of genetic material between homologous chromatids takes place (crossing-over). The primary spermatocyte then divides into two daughter cells called secondary spermatocytes, each having a haploid set of chromosomes. A second division follows immediately and results in two spermatids from each of the secondary spermatocyte. All spermatids thus have a haploid genome (a single set of chromatids) with a unique combination of genes (cf Setchell 1978).

Sertoli cells are somatic cells which reach from the walls of the seminiferous tubules to the lumen. They divide the intraluminal space into two compartments: the basal compartment, where the spermatogonia are located; and the adluminal compartment, where the spermatids and testicular spermatozoa are to be found. The branched Sertoli cells (Sertoli 1865; von Ebner 1871; Nicander 1967) themselves, and the tight cell-to-cell junctions between them, constitute one part of the Blood-Testis-Barrier (Kormano 1967; Setchell et al 1969). This barrier possibly creates the optimal environment for spermatogenesis and, in particular, for meiosis (Setchell 1978), and could also play a role in protecting the spermatocytes and subsequent germ cells, which express surface antigens different from those in somatic cells, from the body's immune system (Tung & Fritz 1978). The Sertoli cells are target cells for follicle stimulating hormone (FSH) and testosterone, and excrete androgen-binding-protein (ABP), by which testosterone, produced by Leydig cells, is trapped and transported into the seminiferous tubules and the post-testicular excurrent ducts (Purvis & Hansson 1981). The spermatogonia diverging from the mitotic cycle leave the basal compartment of the seminiferous tubules and begin meiotic division. During these divisions, the tight cell-to-cell contacts between the Sertoli cells temporarily untighten, allowing the spermatocytes enter the adluminal compartment (cf Plöen & Ritzén 1984). Here the newly formed spermatids become embedded in invaginations in the luminal surface of the Sertoli cells (Elftman 1950).

**Spermiogenesis and condensation of the sperm chromatin**

The differentiation of round spermatids into testicular spermatozoa is called the spermiogenesis or spermateleosis. The differentiating spermatids cannot utilize glucose and are supplied with lactate and pyruvate by the Sertoli cells (Jutte et al 1983). Redundant cytoplasm is phagocytized by Sertoli cells at spermiation, i.e. when the spermatozoa are released into the seminiferous tubules (Bugge & Plöen 1986). The differentiation is androgen-dependent and includes inactivation and condensation of the chromatin, formation of acrosome and tail, and, possibly, development of regional specializations of the cellmembrane (Plöen 1971; Klint 1985; Setchell 1978). Transcription of DNA ceases in the condensed chromatin (Monesi 1971; Monesi et al 1978), and no unscheduled DNA synthesis occurs. This indicates that damages in the DNA are not repaired until the fertilizing spermatozoon has penetrated the ovum (Matsuda et al 1985; Generoso et al 1981). The condensation of the sperm chromatin is achieved when somatic histones are exhanged first for transitional proteins and finally for sperm specific proteins (Chevaillier 1983; cf Bellvé & O'Brien 1983; Courtens 1983). DNA-nucleoprotein fibers are tied together side-by-side in a band-like manner. These bands are folded, giving the chromatin a semi-crystalline to crystalline-like structure (Koehler 1966; Lung 1972; Sipski & Wagner 1977; Kvist et al 1980; Subirana 1983; Livolant 1984). The composition of the sperm-specific proteins varies between different phyla from histone-like nucleoproteins to basic sperm-specific

proteins rich in arginine (Bloch 1969, Subirana et al 1973, Coelingh & Rozijn 1975, Bellvé & Moss 1983). Protein type seems to be related to the degree and strength of condensation, but is apparently not related to the shape of the condensed chromatin (Chevaillier 1983, Bellvé 1979). In placental mammals, sperm specific proteins contain, besides arginine, a relatively large proportion of sulphur-containing cysteine (Coelingh et al 1972; cf Bellvé 1979). In studies on bulls, stallions, rams, boars, and rats, one type of sperm-specific proteins was isolated for each. In mouse and human, two types of sperm-specific protein have been characterized for each species. One type, in each species, was also rich in histidine (Coelingh & Rozijn 1975; cf Bellvé 1979). The condensed state of the chromatin, achieved when somatic histones are exchanged, is characterized by (1) an increased stability (i.e. increased resistance to experimentally induced decondensation), (2) increased resistance to denaturation of DNA at high temperatures, and (3) reduced affinity for basic dyes and actinomycin D (Gledhill 1971). In studying ram spermatogenesis, Loir et al (1983) found that early spermatids developed a resistance to decondensation of chromatin first to heparin, and in later stages progressively to EDTA, sonication, DNase and trypsin. The chromatin of late spermatids in ram decondensed in vitro only in the presence of reducing compounds such as DTT (Di-thio-threitol), but were stable in the detergent Sodium Dodecyl Sulfate (SDS) alone (Loir et al 1983). Furthermore, soon after incorporation of thiol-containing basic sperm-specific proteins there was a sharp reduction in the amount of detectable thiol-groups and an increase in disulfide-bridges, as judged by histochemical methods (Loir & Courtens 1979). Apparently, the quarternary structure of the chromatin in testicular spermatozoa can also develop a disulfide-bridge dependent resistance to SDS, at least when studied in vitro. Others have suggested that this type of stability in the chromatin develops in spermatozoa of eutherian mammals during transfer through the epididymis (Calvin & Bedford 1970, 1971; cf Bedford 1975). In contrast, the chromatin of marsupial spermatozoa, which lacks cysteine residues in chromatin sperm-specific proteinsl does not develop a resistance to decondensation in SDS (Temple-Smith & Bedford 1976).

## 3. Spermatozoa in the epididymis

When the spermatids are released from the Sertoli cells they have aquired most of the morphological attributes associated with mature spermatozoa, and are renamed testicular spermatozoa (Setchell 1978). The testicular spermatozoa, together with the fluid produced by the Sertoli cells, are transported from the seminiferous tubules to the rete testis by means of contractions in the myoid cells in the tubular walls and in the testicular capsule (Risley 1963). The still immotile spermatozoa are further transported through the ciliated efferent ducts to the single duct of the epididymis. The transfer through the epididymal duct (initial, middle, and terminal segments: Glover & Nicander 1971) is achieved mainly by contractions in smooth muscle cells in the wall of the duct (Bedford 1975; Sjöstrand 1981; Jaakola & Talo 1983). The epithelium of the epididymal duct is apparently largly influenced by androgens, partly supplied by intraluminal androgens bound to ABP (androgen binding protein) (cf Hansson et al 1975).

### Sperm "maturation"

Spermatozoa taken from the rete testis cannot fertilize the ovum in species with highly developed initial and middle epididymal segments, while testicular spermatozoa of teleost fishes and some amphibians can. Thus, the need for epididymal "maturation" could be related to "new" demands on sperm survival created by the female genital tract in species with internal fertilization (cf Bedford 1979). In mammals, spermatozoa are both motile and capable of fertilizing by the time they reach the terminal segment of the epididymis (Benoit 1926; Glover & Nicander 1971; Bedford 1975; Orgebin-

Crist et al 1975). Although testicular spermatozoa can achieve capacity for forward motility, when retained in the rete testis by ligation (Young 1931), they cannot achieve full fertilizing capacity by storage there (Cooper & Orgebin-Crist 1977). However, spermatozoa can develop a fertilizing ability if they by ligation remain in the initial segment of the epididymis (Bedford 1967, Igboeli & Foote 1969). The power for fertilizing is lower in "young epididymal spermatozoa" compared to those entering the terminal segment as judged from the proportion of fertilized ova and observations of the delay in cleavage of ova after fertilization by these "young" spermatozoa (Orgebin-Crist & Jahad 1977).

Although epididymal spermatozoa apparently can start a normal zygote development, ejaculated spermatozoa have a higher rate of success. Thus, rabbit ova fertilized by spermatozoa from the terminal segment of the epididymis more often die before implantation compared to ova fertilized by ejaculated spermatozoa (Orgebin-Crist 1967, 1968). It is not settled whether this reflects a continuing "maturation" after ejaculation, or if contact with seminal plasma preserves an original capacity for fertilization during transfer to the ovum.

**Sperm storage**

The transit time of spermatozoa through the initial and middle segments of the epididymis is similar between species (2-6 days), whereas storage time in the terminal segment varies with the patterns of sexual acitivity in different species (cf Amann et al 1976; Foote 1969). Domestic animals can store spermatozoa for several ejaculates in the epididymis (cf Amann et al 1976; cf Mann & Lutwak-Mann 1981). A ram has epididymal reserves for 80 ejaculates in five days (cf Foote 1969), and the reserves in bulls can last for about thirty ejaculates under a period of three days (cf Mann & Lutwak-Mann 1981; Freund 1963). In contrast, the demands for sperm storage is apparently lower in man, since a frequence of five or more ejaculates in a week renders no accumulation of spermatozoa in the terminal segment of the epididymis (Freund 1963). Thus, different species have different demands for epididymal storage of spermatozoa.

The fertilizing ability of epididymal spermatozoa declines within a few days after castration, but systemic supplementation with androgens can counteract the loss of fertilizing ability (Benoit 1926; Moore & McGee 1928; cf Orgebin-Crist 1975). In addition, in uncastrated animals the fertilizing capacity decreases during prolonged epididymal sperm storage (2-3 weeks). This decline is accentuated (within 6-10 days) when ligatures are applied to prevent a continuos admixture of younger spermatozoa (cf Orgebin-Crist et al 1975; Martin-Deleon & Boice 1982). Stored spermatozoa lose the capacity to fertilize long before motility decreases; "aged" but still motile spermatozoa (1) have a lower fertilization rate, (2) cause an increased frequency of preimplantation losses, and (3) cause several different chromsome anomalies in the fertilized ova (Young 1931; Martin-DeLeon et al 1973; Tesh & Glover 1y79; Martin-DeLeon & Boice 1982). Martin-DeLeon & Boice (1985) found that male mice who had had sexual rest for two weeks, in comparison with mice mating every third day, gave rise to a significantly higher frequency of zygotes with various chromosome anomalies. The authors, however, only discussed this as a matter of post-meiotic selection or favoring during aging of spermatozoa with an unbalanced set of chromosomes, and did not discuss the possibility that the male genome could have been affected upon storage.

The conditions responsible for "maturation" of spermatozoa and for the preservation of a once achieved fertilizing capacity are not known. It could, although, be of significance that the composition of the luminal fluid varies extensively along the testicular excurrent ducts, rete testis, efferent ducts, and the different segments of the epididymis (Crabo 1965; Einarsson 1971; Howards et al 1979).

What changes occur within the sperm chromatin during passage through the epididymis? In rats, the resistance to chromatin decondensation in vitro (in the detergent Sodium Dodecyl Sulfate) increases with passage from the initial segment of the epididymis to the terminal segment, concomitant with a decrease in the amount of **detectable** free thiol-groups (Calvin et al 1973). Resistance to decondensation in SDS with the thiol containing compound DTT (Di-thio-threitol) also increases during the passage through the epididymis. Calvin & Bedford (1970, 1971) therefore suggested that the stability of sperm chromatin in eutherian mammals studied (rat, mouse, guinea-pig, bull, boar, cat, ferret, shrew, and man), and the decrease in detectable free thiol-groups is due to formation of cross-linking disulfide-bridges. However, Kvist & Björndahl (1984) found that the structural stability in SDS of rat and hamster spermatozoa from the initial epididymal segment was dependent on some divalent cation. Furthermore, these spermatozoa could achieve full structural stability (in head, head-tail connection, and tail) against SDS by a short exposure to zinc or thiol blocking agents. These are substances that normally prevent formation of disulfide-bridges (Chester 1978). Furthermore, when epididymal spermatozoa were stored in distilled water, sperm heads from the initial segment became more resistant to SDS with DTT than spermatozoa from the terminal segment (Calvin & Bedford 1970). This indicates that thiol groups in spermatozoa from the terminal segment are blocked and thereby hindered to take part in formation of surplus disulfide crosslinks during storage. Calvin & Bedford (1970) suggested that these thiols could be blocked by comittment into mixed disulfides with cystine or oxidized glutathione. The results of Kvist (1980a) and Kvist & Björndahl (1984) open the possibility that in chromatin stabilized by zinc, thiol-groups may concomitantly be hindered from taking part in oxidative super-stabilization during storage in vitro. That zinc is bound to thiol-groups in spermatozoa is indicated by the observation that ejaculated human spermatozoa treated with DTT or cysteine selectively lose zinc (Kvist & Eliasson 1978). Release of zinc induced by EDTA is accentuated in mature epididymal rat spermatozoa treated with thiol-blocking agents (Calvin & Bleau 1974). In addition, release of zinc doubles the amount of detectable thiol-groups in mature epididymal rat sperm heads (Calvin et al 1975). Thus, zinc's ability to chelate between sulfur in thiol-groups of adjacent nucleoprotein fibers could explain the development of nuclear stability towards decondensation in SDS, and the reduction in detectable amounts of thiol-groups. The affinity of zinc for thiol-groups could also explain the reduced effect of the thiol-compound DTT on "mature" spermatozoa. The suggestion that zinc can contribute to the stability of the chromatin already in the epididymal rat spermatozoon is supported by the earlier observations of Birnbaum et al (1961), who found higher contents of zinc in mature epididymal rat spermatozoa than in testicular spermatozoa. In contrast, Saito et al (1967) and Janick et al (1971) found high contents of zinc only in the ejaculated spermatozoa of dog and man.

## 4. Spermatozoa and seminal plasma

The major human male accessory sex glands are the ampulla of the vas deferens, the seminal vesicles, the prostate, and the bulbo-urethral glands (or Cowpers' glands). Other species have glands with similar names, but their respective functions are not always similar (Mann 1964; Mann & Lutwak-Mann 1981). I will here concentrate on the human prostate and seminal vesicles.

The human prostate produces normally an acidic fluid (pH <7: Huggins 1947; Blacklock & Beavis 1974; Kavanagh & Darby 1982), rich in citrate and zinc (cf Mann & Lutwak-Mann 1981; Arver 1982). Zinc was first discovered in semen by Bertrand & Vladesco (1921) and its prostatic origin was shown by Mawson & Fischer (1953). The concentration of zinc in prostatic fluid is several

hundred times higher than the concentration in blood plasma (cf Mann & Lutwak-Mann 1981). The prostate in dog seems to share these properties with the human prostate (Bartlett 1962).

Citrate is a zinc ligand with a pH-dependent capacity for binding zinc ($pK_{am}$=3.1; $pK_{am}$=4.8; $pK_{am}$=6.4). At low pH fewer binding sites are available, but at pH 7.4 more than 90% of citrate will be saturated with zinc (cf Sillén & Martell 1971). Thus, at low pH, as in the human prostatic fluid, citrate exposes fewer binding sites for zinc and the amount of "free" zinc available for uptake into spermatozoa would be increased. The more alkaline vesicular fluid is rich in fructose (cf Mann 1964), and in zinc-ligands of high- and intermediate-molecular-weight (HMW and IMW) (Arver & Eliasson 1982). Vesicular ligands have a high affinity for zinc and cause a post-ejaculatory redistribution of zinc from ligands of prostatic origin (mainly citrate) to ligands of vesicular origin, further reducing the concentration of free zinc available for possible interaction with e.g. spermatozoa (Arver 1982).

In most men, the majority of spermatozoa are expelled in the first fractions of the ejaculate together with mainly prostatic fluid (Lundqvist 1949, Mann & Lutwak-Mann 1981). The last fractions, which contain fewer spermatozoa, are dominated by seminal vesicular fluid. It seems, then, that most spermatozoa at ejaculation encounter an environment with high availability for zinc. In the normal course of events, however, spermatozoa are likely to leave the ejaculated fluid within a short period of time and enter the cervical mucus (Sobrero & MacLeod 1962). This indicates that any interaction of physiological importance between spermatozoa and seminal plasma would have to occur during a limited period of time.

## 5. Fertilization

Fertilization is completed when the intact paternal genome becomes available for development of the zygote. In mammals, fertilization normally occurs in the ampullary region of the Fallopian tube (or the oviduct) (cf Hunter 1975). In most species spermatozoa obtain the capacity to penetrate the ovum in the female genital tract. The mechanism for this "capacitation" is still obscure (Chang 1951; Austin 1951; Rogers 1978). One result of capacitation is the acrosome reaction, where the plasma membrane and the outer acrosomal membrane fuse and enzymes from the acrosome are released. These enzymes probably facilitate sperm passage through the cumulus oophorus surrounding the ovum (Austin 1969; Pikó 1969; Thibault 1971; Soupart 1976). The inner acrosomal membrane makes contact with glycoprotein layers of the zona pellucida. However, the biochemical nature of the mechanism for penetration through the thick zona pellucida in eutherian mammals is not known. Both enzymatic and nonenzymatic processes have been discussed (cf Bedford & Cross 1978; Bedford 1979; Klint 1985). The sperm head attach to the ovum with the equatorial region (Soupart 1976; Bedford et al 1979). The plasma mebranes fuse and the sperm core, i.e. nucleus and tail, sinks into the ooplasm. The nuclear envelope of the spermatozoon dissolves, and the detached tail and sperm mitochondria disintegrate (Soupart 1976). The sperm chromatin starts to decondense in its posterior region, i.e. the DNA-nucleoprotein fibers start unravelling (Szollosi & Ris 1961; Bedford 1972; cf Yanagimachi 1978). The mechanism of physiological decondensation is not known, but it depends on conditions created in the mature ooplasm and does not need the karyoplasm of the ovum (Tarkowski 1983). In vitro studies have shown that a high number of human spermatozoa can decondense simultaneously in one single ovum (Soupart & Strong 1975; Yanagimachi 1984). Disruption of stabilizing forces can, in vitro, be achieved with either the addition of **exogenous** thiols, which supposedly cleave disulfide-bridges (cf Mahi & Yanagimachi 1975), or by removal of zinc from spermatozoa (Kvist 1980a). Thus, removal of zinc from the sperm nucleus could facilitate decondensation

in the ovum and decondensation might, in fact, be initiated through the activities of those thiols which are inherent to the ovum (Perreault et al 1984) by cleaving crucial disulfide-bridge crosslinks, or by removing zinc from the sperm chromatin. The spreading of chromatin fibers has been suggested to be facilitated by repulsive forces generated through phosphorylation of the sperm-specific proteins (cf Young 1979).

A new, pronuclear, membrane is formed around the decondensing chromatin, and the male pronucleus develops from the contents inside this pronuclear membrane. Even if the sperm nucleus fails to decondense, a pronuclear membrane will form, but no development into pronucleus will occur (Yanagimachi & Noda 1970). For a normal pronuclear formation and normal embryonal development, a factor of the karyoplasm is essential: MPGF, mammalian pronuclear growth factor, the nature of which is unknown (Thibault 1969; Tarkowski 1983). More than five spermatozoa decondensed at the same time in one ovum will inhibit pronuclear formation (Yanagimachi 1982). The male pronucleus has a volume some 500 times that of the condensed sperm chromatin (Thibault 1971; Soupart 1976; Bedford 1972). During pronuclear formation, the sperm specific protamines are exchanged for somatic histones (Ecklund & Levine 1975). DNA repair takes places before the paternal DNA is duplicated to enable formation of metaphase chromsomes for the first mitotic division of the fertilized ovum (Generoso et al 1981). Syncronously with the formation of the male pronucleus, a female pronucleus duplicate the maternal DNA and form metaphase chromosomes. After the first mitotic division, the chromosomes of paternal and maternal origin will combine to form the nuclei of the daughter cells.

## 6. Chromatin decondensation in vitro

Decondensation of the sperm chromatin has been evoked in vitro with several different methods. Thiol-containing compounds, such as DTT, have been regarded necessary for decondensation of mature spermatozoa. However, the action of thiol-groups must be combined with either the action of detergents, proteases, or salts to obtain decondensation (cf Young 1979; cf Zirkin et al 1985). A proteolytic process intrinsic to the sperm head has been suggested (Marushige & Marushige 1975, 1978; Zirkin & Chang 1977; Zirkin et al 1985), but even these studies utilized exogenous thiol-groups, and Young (1979) has showed that the proteolytic action was caused by enzymes originating from the acrosome. Thus, these enzymes are likely to disappear before decondensation starts in the ovum.

Bedford et al (1973) found some men whose spermatozoa decondensed in the detergent Sodium Dodecyl Sulfate (SDS) without DTT, and Kvist & Eliasson (1978) found that zinc in similar cases could prevent spontaneous decondensation in SDS alone. Treatment with EDTA of spermatozoa, which had been supplemented with zinc, reversed the stability induced by zinc (Kvist 1980c). Kvist also found that treatment with EDTA could elicit decondensation of sperm chromatin in SDS even among "normal" men (Kvist 1980b,d; Kvist et al 1980), and that this capacity for decondensation depended on thiols intrinsic to the sperm chromatin (Kvist 1982). Regardless of the initial access to zinc, the capacity was decreased when the spermatozoa had been stored some hours (Kvist 1980a). Delgado et al (1980) found that the protamine heparin could induce chromatin decondensation in washed human spermatozoa. However, the buffer that they used contained EDTA (cf Peterson & Freund 1970). That decondensation evoked with heparin was also obtained by removal of zinc, was later communicated (Reyes et al 1983). Blazak & Overstreet (1982a,b) studied washed ejaculated spermatozoa and advocated that the normal chromatin stability of human ejaculated spermatozoa was not inherent to the chromatin, but that it was influenced through contamination by the seminal plasma. However, also these authors used a zinc-chelating medium for

washing the spermatozoa; it contained albumin, which is known to release zinc from spermatozoa and to facilitate decondensation in SDS (Kvist 1980d). The intrinsic mechanism for decondensation of sperm chromatin is neither influenced by $Mg^{2+}$ nor by $Ca^{2+}$, both of which are present in semen (Kvist 1980a). $Cu^{2+}$ can induce an irreversible, zinc-independent stability of the chromatin, indicating a formation of, for instance, disulfide-bridges (Kvist 1982). Furthermore, $Cd^{2+}$ has been found to interfere with the zinc-dependent chromatin decondensation of human spermatozoa. Spermatozoa exposed to $Cd^{2+}$ were more stable, than those exposed to the equivalent amount of exogenous $Zn^{2+}$, when decondensation in SDS was evoked with albumin (Casswall et al 1986).

## 7. Zinc and reproduction

Zinc is an essential trace element present in most organs in the body (Vallee 1959). Zinc stabilizes macromolecules and metalloenzymes, and participates in DNA-synthesis, RNA-transcription, and mitosis (cf Chester 1978). Therefore, zinc seems important for several steps in mammalian reproduction. A severe dietary deficiency of zinc can lead to dwarfism, late sexual development, decreased testicular function and oligospermia, and hypogonadism (cf Mann & Lutwak-Mann 1981). In rats with zinc deficiency, the first disturbance in the testis was an arrestment of late spermatid development (Barney et al 1969). In all examined species, mature spermatozoa contain significant amounts of zinc (cf Gunn & Gould 1970). The point of time and the anatomical location for this uptake of zinc has, however, not been completely uncovered, nor has the significance of spermatozoal zinc been thoroughly elucidated. However, in bats, the spermatozoa are stored in the female genital tract for months, and the genital tracts of both sexes contain considerable amounts of zinc (Chrichton et al 1982). This could indicate that a rich supply of zinc is beneficial to spermatozoa that are stored. In rats, the metabolism of zinc has been studied extensively: $^{65}Zn$ is recovered in the seminiferous tubules and follow the spermatozoa and the fluid to the epididymis (Wetterdal 1958; Timm & Schultz 1966; Gunn & Gould 1970). Birnbaum et al (1961) found higher contents of zinc in epididymal spermatozoa than in testicular spermatozoa, indicating that an uptake of zinc starts early in the post-testicular excurrent duct system in rats. Decrease in total content of zinc in spermatozoa, as assessed by atomic absorption spectrophotometry, has been reported to occur during sperm transfer from the initial to the terminal segment of the epididymis in rats and monkies (Calvin & Bleau 1974; Calvin et al 1975; Srivastava et al 1982, 1983). In contrast, Oliphant et al (1985), using X-ray microanalysis of rabbit spermatozoa, reported increased concentrations of zinc in whole spermatozoa and sperm heads from the terminal segment of the epididymis. The prostate in rats contains high amounts of zinc, but the importance of this prostatic zinc for uptake into spermatozoa is unclear. Epididymal and ejaculated spermatozoa are considered to contain equal concentrations of zinc in this species (Saito et al 1967). However, zinc derived from the prostate follows the spermatozoa into the oviduct (Gunn & Gould 1970). In dogs and in humans, spermatozoa seem to get a considerable contribution of zinc from semen (Saito et al 1967; Janick et al 1971). With X-ray microanalysis the human sperm head has been shown to contain significant amounts of zinc, and this zinc is most likely situated in the chromatin, since equivalent concentrations were found in washed preparations of whole sperm heads as in frozen and freeze-dried sections of unwashed spermatozoa (Chandler & Battersby 1976).

# II. AIM OF THE STUDY

The present study was initiated in light of earlier observations that the human spermatozoon seemed to have an intrinsic capacity for chromatin decondensation that is reversibly inhibited by zinc at ejaculation (Kvist 1980a). The aim was to further elucidate the importance of zinc for the stability of the sperm chromatin and for the preservation of this capacity for decondensation, and to investigate if the human sperm nucleus accumulates zinc at ejaculation. Furthermore, I aimed to shed light on possible factors regulating a physiological uptake of zinc into the sperm head at ejaculation.

# III. MATERIALS AND METHODS

In this section I will only give some general remarks on materials and methods used in the thesis, since all details are given in the separate publications (I-VI).

**Semen samples** were provided by volunteers: students at Karolinska Institutet (I-IV, VI) and men referred to the clinic of Obstetrics and Gynecology (University of Linköping) for sterilization (vasectomy) (V).

**Epididymal spermatozoa** were recovered from the most caudal part of the epididymis in preparations obtained immediately after orchidectomy.

**Vasal spermatozoa** were recovered from the prostatic portion of vas deferens during vasectomy. One prerequisite for recovery of vasal spermatozoa was that ejaculation had occurred in the morning of the day of surgery. Spermatozoa in these ejaculates were used for comparisons between zinc content in ejaculated and vasal sperm heads.

**Decondensation of the sperm chromatin** was studied (I-II) in ejaculated spermatozoa before or after wash in a buffered salt solution (BSS; mM: NaCl 123, KCl 5, HCl 24, TRIS 37; pH=8.0). One volume of sperm solution (spermatozoa in seminal plasma or BSS) was added to nine volumes of 1% Sodium Dodecyl Sulfate (SDS) in 0.05 M borate buffer (pH=9.0) with or without 6 mM EDTA. Incubation was performed at 37°C for 60 minutes and the reaction was stopped by addition of the equal amount of 2.5% glutaraldehyde in 0.05 M borate buffer (pH=9.0). Assessment of the degree of decondensation was done in phase contrast microscopy (400 X) by ranking 100 randomly selected sperm heads in each sample and treatment group: stable, moderately swollen, and grossly swollen sperm heads.

**Semen analyses** were only performed in investigations of the uptake of zinc into the sperm head (III-VI). **Zinc** was determined with atomic absorption spectrophotometry (Eliasson & Lindholmer 1971) both to record the concentration of zinc in semen per se, and to use this concentration as a marker for the secretory performance of the prostate. **Fructose** was determined with a colorimetric method (Karvonen & Malm 1955) and used as a marker for vesicular secretion.

**Sephadex gel chromatography** was performed (VI) to analyse the relative distribution of seminal zinc bound to different groups of ligands: high-molecular-weight zinc-ligands (HMW; Mr >80 000); intermediate-molecular-

weight zinc-ligands (IMW; Mr 3 000-80 000); low-molecular-weight zinc-ligands (LMW; Mr <3 000). HMW and IMW are considered to be secreted from the seminal vesicles, while LMW, probably mainly citrate, is of prostatic origin (Arver 1982).

**The principle for X-ray microanalysis** is that the electron beam generated in the electron microscope collides with inner shell electrons of atoms in the "target area" of the specimen. Electrons from outer shells, with higher levels of energy, will take the "empty places" in the inner shells thereby liberating the difference in energy as radiation of X-rays. The wave length (or energy) of this radiation is specific for the atom that was excited. The X-rays are detected by an energy-dispersive spectrometer system, consisting of a semiconductor detector and a multichannel analyzer. The amount of a specific atom is expressed by the intensity of radiation of a particular wave length (or energy). In thin sections (≈1-2 µm) the materia is excited at the point of inpact of the electron beam and in a cone with an angle of about 30° beneath this point. Therefore, most of the X-rays arise from the inner part of an intact sperm head, i.e. the chromatin. In a specimen with the size of a sperm head X-ray absorption and secondary fluorescence are negligible (Roomans 1982, 1983).

X-ray microanalysis was performed on spermatozoa washed once or twice with distilled water or, as in the case with epididymal and vasal spermatozoa, diluted in distilled water. Spermatozoa suspended in distilled water were placed on single-slot nickel-grids, covered by Formvar film (100 nm thick), and spermatozoa were left to air-dry. Thereafter, the specimens were coated with a thin layer of carbon to increase the resistance against thermal damage caused by the electron beam and to minimize electrical charging of the specimen during analysis. X-ray microanalysis was performed in the scanning transmission mode of the electron microscope (magnification 4000-5000 X), and the electron beam was directed to the postequatorial region of randomly chosen sperm heads. The results (spectrum of recorded X-rays of different energy) from each sperm head were computed to obtain values which were corrected with respect to background noise, intensity of the electron beam and detected radiation from the sperm heads. To avoid variations in zinc-levels due to variations in the total mass examined, all zinc concentrations are given in relation to sulfur or phosphorus. Those elements are supposedly incorporated into the nucleus during spermatogenesis: sulfur mainly in sperm specific protamines and phosphorus mainly in the nucleic acid of DNA. Thus, sperm nuclear zinc concentration was expressed as either 1000 x zinc-to-sulfurcounts/s-ratio, [Zn/S], or 1000 x zinc-to-phosphorus counts/s-ratio [Zn/P]. The choice between which of these two relations should be used did not seem to be of importance, and after the initial study (III), where only [Zn/P] was available, we preferred to use [Zn/S] due to the earlier observations that a connection between zinc and thiols in the nucleus could be of importance for chromatin decondensation.

**Statistics:** since the values for assessed degree of chromatin decondensation were not normally distributed, non-parametric statistical analysis (Matched Pair analysis) was used (I,II). Comparisons of distributions of zinc concentrations in sperm heads from different origins (epididymis, vas deferens, and ejaculate) were performed with the Mann-Whitney U-test, since values from non-ejaculated cells were not normally distributed (IV,V). In other cases it was likely that the distribution could equivalate a normal distribution, thus allowing the use of t-test (III), Pearson's correlation test, and multiple step-wise linear regression analysis (VI).

# IV. RESULTS AND COMMENTS

## 1. The capacity for chromatin decondensation evoked with zinc-chelators is a normal property of ejaculated spermatozoa

Human semen normally contains a small and variable proportion of spermatozoa that decondense chromatin in Sodium Dodecyl Sulfate (SDS) (Bedford et al 1973; Kvist 1980a). The proportion of decondensing spermatozoa increases when the spermatozoa are treated with zinc-chelators such as albumin or EDTA within a short timeframe to the treatment with SDS (Kvist 1980a). However, some spermatozoa, pretreated with zinc-chelators, fail to decondense in SDS. In addition, it was generally observed that the proportion of spermatozoa that decondensed spontaneously in SDS alone and the proportion that decondensed after treatment with zinc-chelators diminished with increasing intervals between ejaculation and exposure to SDS (Kvist 1980a). We therefore studied decondensation of spermatozoa five minutes after ejaculation. The nuclei of most of the spermatozoa exposed to SDS remained condensed whereas approximately 90% of spermatozoa decondensed in the SDS-EDTA (I: Fig. 3, squares). **Thus, the capacity for chromatin decondensation evoked with zinc-chelating compounds seems to be a normal property of freshly ejaculated spermatozoa.**

## 2. The capacity for decondensation is lost during sperm storage

Decondensation in SDS-EDTA was also studied among spermatozoa stored in seminal plasma and among spermatozoa separated from the same seminal plasma 20 minutes after ejaculation and thereafter washed and stored in a buffered salt solution (BSS). One hour after ejaculation nearly half of all spermatozoa failed to decondense, irrespective of in which solution spermatozoa were stored (I: Fig. 3, 60 min; Fig. 4, 1h). After the first hour, the rate by which spermatozoa lost the capacity to decondense was slower (I: Fig. 3 and 4). The rate for this loss was slowest among spermatozoa separated from the seminal plasma (I: Fig. 4). **It can be seen, then, that many spermatozoa lost the capacity for decondensation rapidly. If factors in seminal plasma influenced this loss, then this influence was mainly exerted during the first 20 minutes after ejaculation. Other spermatozoa lost the capacity at a slower rate. This "slow loss" was enhanced by some unknown factors present in the seminal plasma.**

## 3. Zinc preserves a capacity for chromatin decondensation

Questioning whether failure to decondense could be the result of the thiol-groups in the chromatin oxidizing and forming stabilizing disulfide-bridge crosslinks (Kvist 1982), and noting that zinc can protect thiols from oxidation (cf Chester 1978) impelled us to investigate whether zinc could prevent the loss of a capacity for decondensation during storage. We studied how exposure of spermatozoa to EDTA before storage and exposure to a buffer containing zinc (1.5 mM $ZnSO_4$) influenced the capacity for decondensation during storage. Almost all spermatozoa (91%) pretreated with a zinc-chelator before storage had lost the capacity for chromatin decondensation after 24 hours storage in BSS (II: Fig. 3 and 4: BSS-EDTA washed, stored 24 h). Treating the spermatozoa stored in BSS with supplements of zinc during storage prevented both the loss that was enhanced by treatment with EDTA and the "native" loss of capacity for chromatin decondensation (II: Fig. 2 and 4: + Zinc vs. washed without zinc). In other words, the capacity for decondensation, revealed in a certain proportion of spermatozoa before storage, was preserved throughout the whole period of storage in a saline solution supplemented with zinc. **In conclusion, the experiments presented in section 1-3 demonstrated (1) that the capacity for chromatin decondensation, evoked by a zinc-chelating medium (here EDTA) is a normal property of the freshly ejaculated spermatozoon; (2) that a variable fraction of spermatozoa rapidly**

**loses this capacity; (3) that seminal plasma can cause a further loss of the capacity for decondensation; (4) that a loss of the capacity can be enhanced experimentally by exposure of spermatozoa to a zinc-chelating medium; and (5) that zinc can preserve the capacity for decondensation during storage.**

## 4. The sperm head of the ejaculated spermatozoon contains zinc bound within the nuclear matrix

EDTA and albumin have here (I,II) and earlier (Kvist 1980a) been used as zinc-chelators to evoke decondensation in SDS. Both substances reduce the total zinc content of ejaculated spermatozoa in humans, as judged from zinc determinations in pellets of whole spermatozoa (Huajacuja et al 1973; Johnsen & Eliasson 1978). However, we lacked information on whether the content of zinc in the sperm nucleus was reduced following treatment which evokes decondensation in SDS. The elemental composition, e.g. zinc content, of subcellular regions could, with X-ray microanalysis, be determined in thin specimens like spermatozoa (III,IV,V). One special problem when studying zinc in ejaculated spermatozoa is the possibility of contamination by the high concentrations of zinc in seminal plasma. The observation that the concentration of zinc in sperm heads was not related to either the total concentration of zinc in seminal plasma or to concentrations of zinc bound to various groups of ligands (VI), ruled out that the measured zinc was mainly due to seminal plasma constituents dried on top of the sperm heads, or unspecific binding of subfractions of zinc to the sperm surface. Furthermore, the earlier studies of Chandler & Battersby (1976) showed that sections of frozen and freeze-dried sperm heads contained similar concentrations of zinc as did whole, air-dried sperm heads. These results indicate that the measured concentration of zinc mainly is a function of zinc bound within the sperm head, which is dominated by the nucleus. Moreover, the results of Chandler & Battersby (1976) ruled out the possibility that the amount of nuclear zinc is the result of an unspecific diffusion of zinc into the sperm nucleus during the preparation of spermatozoa for X-ray microanalysis, since free diffusion is hindered in a frozen specimen. The actual concentration of zinc in sperm head was calculated to be 3-4 times higher than that of seminal plasma (IV). Furthermore, sperm heads treated with SDS contained similar amounts of zinc as sperm heads washed in distilled water (IV). These two observations together, indicate that zinc in the nuclear matrix is retained by high affinity zinc-binding sites. **In conclusion, the present results indicate that the chromatin of the ejaculated human spermatozoon contains zinc.**

## 5. Zinc is released from the sperm nucleus of the freshly ejaculated spermatozoon exposed to zinc-chelating EDTA

EDTA-treatment reduced by 80-90% the concentration of zinc in sperm heads, [Zn/P], 20 minutes after ejaculation (III: Table 1). The size of this decrease was similar to that found by others in pellets of whole spermatozoa treated with albumin or EDTA (Johnsen & Eliasson 1978, 1981; Huacuja et al 1973). We found the highest concentrations of zinc in the junction between the sperm head and tail, and EDTA also released zinc from this subcellular region. This is worth noting since the earliest events of both the physiological decondensation and the decondensation evoked in vitro with zinc-chelators occur in this most posterior region of the sperm head (Björndahl & Kvist 1982). Decondensation when evoked with high concentrations of exogenous thiols, did not appear to initiate at the head-tail junction (Kvist et al 1980). **In conclusion, spermatozoa which have encountered a zinc-chelating medium lose zinc from the nucleus and from the head-tail junction. This depletion of zinc may explain why treatment with EDTA evokes a capacity for decondensation of the chromatin, but at the same time en-**

**hances the loss of this capacity during storage.**

## 6. Qualitative changes occur in sperm chromatin during storage

Whether the normally zinc-rich seminal plasma constitute a donator of zinc or a chelator of zinc vis-à-vis spermatozoa depends mainly on the zinc binding capacity of the various groups of ligands of prostatic and vesicular origin (Arver 1982, cf INTRODUCTION). It was of interest to study a possible loss of sperm zinc during storage of semen, since supplementation with zinc prevented the loss of the capacity for decondensation during storage in saline while the loss of the capacity was enhanced during storage in seminal plasma (II: Fig. 3 and 4). The sperm head [Zn/P] decreased significantly in the two cases with initially the highest [Zn/P], and increased in the other three. Battersby & Chandler (1977) studied three samples, and reported increased content of zinc in sperm nuclei after semen storage. Thus, we found no general decrease in total zinc content of sperm heads after 24 hours storage in seminal plasma. Furthermore, we could not exclude the possibility that the lower mean value for zinc concentrations in head-tail connection was due to chance. However, in aged spermatozoa a qualitative change in the structure of spermatozoa had occurred, since less zinc could be removed by treatment with EDTA (III: Table 1). A reduced extractability for zinc could contribute to the loss of capacity for decondensation, but it seems unlikely that this reduction would be the primary cause for this loss, since it is enhanced in the absence of zinc (II: Fig. 3). The reduced extractability could rather be secondary to sperm compaction due to oxidation of unprotected thiol-groups into stabilizing disulfide-bridges. Thus, formation of surplus disulfide-bridges occurs during storage in vitro of epididymal spermatozoa (rats: Calvin et al 1973), and storage in vitro also results in a further compaction of the epididymal sperm heads (rams: Nicolle et al 1985). Apparently, the diffusion of zinc from sperm heads to the surrounding buffer is normally facilitated by EDTA. Compaction in the boundaries of the nucleus could decrease the content of water in these regions and create a barrier for diffusion of zinc. Zinc could thereby become trapped within the nuclear matrix. **In conclusion, during storage in vitro, qualitative changes occur in the sperm chromatin, leading to a loss of the capacity for chromatin decondensation and to a reduced extractability of zinc. Both events could be due to compaction of the nucleus caused by formation of disulfide-bridge crosslinks.**

## 7. Zinc is taken up into the sperm nucleus at ejaculation

It was of interest to study a possible uptake of zinc into the sperm heads at ejaculation, since ejaculated spermatozoa contained measurable amounts of zinc (cf IV: Table 1) and since there are signs of an inhibition of the capacity for decondensation coinciding with ejaculation (Kvist 1980a). Saito et al (1967) and Janick et al (1971) had earlier studied the content of zinc in centrifuged pellets of epididymal and ejaculated spermatozoa. They concluded that zinc in ejaculated spermatozoa is derived from the prostate, both in man and in dog. However, we lacked information about the origin of zinc in the sperm nucleus. Epididymal spermatozoa, obtained from elderly men (age 61-75 years) undergoing orchidectomy, had lower zinc content in the heads than ejaculated spermatozoa from young men (22-31 years) (IV: Figs. 2, 3, and 4). However, since metabolism of zinc in the male genital tract is strongly dependent on androgens, there could be some doubt concerning the significance of the results. Nonetheless, vasal spermatozoa from another group of younger men (31-43 years) had similarly low contents of zinc in the sperm heads (V: Table 1) as the epididymal spermatozoa from the elderly men. Furthermore, we found significantly higher amounts of zinc in spermatozoa from ejaculates delivered by the same individuals (V: Table 1). In

**conclusion, the present results (IV-V) strengthen the assumption that zinc is taken up into the human sperm nucleus at ejaculation.**

## 8. Vesicular fluid hinders uptake of zinc into the sperm nucleus

The normal inhibition of decondensation of sperm chromatin in SDS was reduced by factors of seminal vesicular origin. Concentrations of zinc chich were sufficient in a buffered salt solution to completely inhibit decondensation, did not inhibit decondensation in vesicular fluid (Kvist 1980a). This indicates that zinc in seminal vesicular fluid was not available for inhibition of decondensation. Furthermore, Arver showed (1982) that the seminal vesicles secrete ligands with high affinity for zinc, and concluded that a redistribution of zinc from ligands of prostatic origin to ligands of vesicular fluid occurred after ejaculation. The concept that vesicular fluid hinders uptake of zinc into the sperm head was further supported by the observation that one man with a low sperm head zinc concentration also had a high contribution of vesicular fluid to the first, zinc-rich portion of the ejaculate (IV: Fig. 3). To identify possible factors of importance for uptake of zinc into the human sperm nucleus at ejaculation, spermatozoa were studied in different fractions of split-ejaculates, a technique which give variations both in number of spermatozoa and in the relative contribution of prostatic and vesicular fluids to each fraction. The earlier observation (IV) that vesicular fluid hinders uptake of zinc into the sperm head was verified since portions with sperm head [Zn/S] $<$ 80 had, compared to portions with [Zn/S] $\geq$ 80, encountered seminal plasma with similar prostatic contribution but with higher contribution from the seminal vesicles (IV: Table 2). All markers for vesicular fluid were negatively correlated to sperm head [Zn/S]. The markers were: for amount of vesicular fluid, the amount of fructose; for concentration of components of vesicular origin, the concentration of fructose; and for the zinc binding capacity of vesicular ligands, the proportion of total zinc bound to these ligands [H+I%]. **From these results, we infer that the vesicular fluid hinders uptake of zinc into the sperm head. The vesicular fluid could reduce the uptake of zinc into sperm heads by (1) binding of zinc to vesicular ligands, or (2) increasing the binding of zinc to citrate through a rise in pH of the seminal plasma (cf Sillén & Martell 1971).**

## 9. Evidence suggesting that uptake of zinc into the sperm nucleus is reduced when high numbers of spermatozoa are expelled

The concentration of zinc in sperm heads was lower in spermatozoa from portions with high total sperm numbers, in spite of that high sperm numbers occurred concomittantly with high concentrations of zinc in seminal plasma ($r_s$=0.48; t=1.98; p<0.05). Furthermore, sperm number showed a better negative correlation to sperm head [Zn/S] than did sperm concentration. Whereas sperm concentration is a function both of sperm number and of the volume of various fluids in which the spermatozoa are finally diluted, acutal sperm number is a better measure of how many spermatozoa are expelled, and thereby and possibly competing for prostatic zinc. A high sperm number could also mean (1) that the time for dissolution of the sperm pellet in prostatic fluid increases, and (2) that relatively more spermatozoa are expelled into the urethra later. In both cases, the period of time for appropriate contact between spermatozoa and prostatic fluid would be reduced. Nevertheless, that a high sperm number would limit sperm zinc uptake, implies that the uptake is discontinued, e.g. by the alkaline vesicular fluid, containing zinc ligands. Variations in sperm number and vesicular fluid marker fructose could explain 99.5% of variations in sperm head zinc concentration in the first fractions of the split-ejaculates. Reduced uptake of zinc into the sperm

nucleus, caused by reduced availability of zinc, may thus be one tentative explanation for the spontaneous chromatin decondensation in SDS among spermatozoa from men with low zinc-to-fructose molar-ratio in the seminal plasma, and also among spermatozoa expelled in parts of the ejaculate dominated by vesicular fluid (Kvist 1980a). No relation was found between zinc concentrations in sperm heads and total seminal zinc concentration, nor between zinc concentrations in sperm heads and concentration of zinc bound to low-, intermediate- or high-molecular-weight ligands. **In conclusion, the results reviewed in section 8 and 9 give further support to the concept that a physiological uptake of zinc into the sperm nucleus occurs at ejaculation, and that vesicular fluid and a high sperm number reduce this uptake.**

# V. GENERAL DISCUSSION

The nucleus of a fertilizing spermatozoon is liberated from the nuclear envelope after penetration into the ovum and the naked nucleus is surrounded by the ooplasm. Decondensation of the sperm chromatin starts immediately in the posterior region of the nucleus, where individual nucleoprotein fibers begin to unravel and separate (Soupart 1976; Bedford 1972). Decondensation has been studied extensively in vitro, using various detergents. A human sperm nucleus treated with SDS is demembranized (Kvist et al 1980), but the naked nucleus does not normally decondense. Thus, the chromatin is stabilized by bindings which preserve the quarternary structure of the chromatin and which resist the repulsion that SDS could induce between the nucleoprotein fibers. The nature of these connecting forces is, however, not completely known. It has earlier been shown (1) that normally only a small but variable proportion of ejaculated human spermatozoa decondense in SDS alone (Bedford et al 1973; Kvist 1980a); (2) that EDTA and albumin increase the proportion of spermatozoa decondensing in SDS (Kvist 1980a); and (3) that zinc, but no other divalent cation of prostatic origin, inhibits the decondensation evoked with EDTA (Kvist 1980a). Also decondensation evoked by zinc-chelators starts in the posterior region of the nucleus and results in unravelling of nucleoprotein fibers (Kvist et al 1980).

The results in the present study imply (1) that the stability of the chromatin in freshly ejaculated human spermatozoa mainly depends on nuclear zinc; (2) that release of zinc, for instance in the ooplasm, would facilitate a physiological decondensation; and (3) that the nuclear zinc of human spermatozoa is mainly taken up at ejaculation.

When spermatozoa were exposed to SDS with zinc-chelating EDTA soon after ejaculation, almost all spermatozoa decondensed. In contrast, most washed or unwashed spermatozoa remained stable in SDS alone. These results, and earlier results of studies on divalent cations and chromatin decondensation (cf INTRODUCTION), imply that the chromatin of the freshly ejaculated spermatozoon has a stability which is dependent on zinc in the chromatin. However, release of zinc enabled decondensation only if the release occurred concomitant with repulsion of the nucleoprotein fibers. This was evident when a majority of spermatozoa, exposed to zinc-chelating EDTA before storage, failed to decondense when exposed to SDS-EDTA after 24 hours storage in a buffer solution. In contrast, when washed spermatozoa were stored for 24 hours in a solution containing zinc, the initial capacity for decondensation could still be evoked by EDTA. If zinc was released without repulsion of chromatin fibers induced by SDS, another type of binding which stabilizes the chromatin, develops. In general, this happened to many spermatozoa within the first hour after ejaculation, regardless if they were washed and stored in a buffer solution. Other spermatozoa lost the capacity for decondensation in SDS-EDTA during prolonged storage, expecially if they were stored in seminal plasma. Thus, another type of stability than that predominating at ejaculation can develop during sperm storage. This development is enhanced in spermatozoa deprived of zinc and totally prevented when zinc is present. This "second type" of stability most probably involves oxidation of thiol-groups into disulfide-bridges. In general, free thiol-groups are susceptible to oxidation, e.g. into disulfide-bridges, in an environment lacking optimal redox-properties (cf Haugaard 1968; Shapiro 1972). Also, thiol-groups in spermatozoa are readily oxidized in vitro: Marushige & Marushige (1975) aimed to estimate the amount of free thiol-groups in the chromatin of epididymal rat spermatozoa. This was accomplished only if oxidation of thiol-groups was prevented; i.e. spermatozoa were recovered in darkness, at low temperature, and with thiol-blocking agents present. Furthermore, epididymal rat spermatozoa stored in vitro become "superstabilized" by disulfide-bridge crosslinks: they develop an increased resistance to decondensation in SDS with DTT. This kind of "superstabilization"

is retarded by thiol-blocking agents (Calvin & Bedford 1970, 1971).

How then to explain why zinc prevents the development of superstabilization, and that release of zinc enhances the development of superstabilization during storage? Zinc-ions have a high affinity for thiol-groups (Vallee et al 1961) and do not participate in redox-reactions in biological systems (cf Chester 1978). Hence, thiol-groups occupied by zinc are less susceptible for oxidation (Chvapil et al 1972; Chvapil 1973; Chester 1978).

One explanation for the observation that zinc preserves the capacity for decondensation would be that thiol-groups in the chromatin which bind zinc would be protected from oxidation, whereas unprotected thiol-groups readily would oxidize into disulfide-bridges. When zinc is removed from these thiol-groups without a concomitant separation of nucleoprotein fibers, thiol-groups in neighboring fibers most likely form crosslinking disulfide-bridges, thereby rendering the zinc-depleted chromatin a "second" type of stability. The rapid loss of capacity for chromatin decondensation during the first hour after ejaculation could, accordingly, be due to formation of crosslinking disulfide-bridges in spermatozoa with insufficient uptake of zinc at ejaculation. Seminal plasma can possibly enhance the loss of capacity for decondensation during storage by acting as a zinc-chelating medium (Arver & Eliasson 1982). The enhanced loss of capacity for decondensation could be explained by crosslinkage of a few crucial thiols, which have been freed from zinc by zinc binding to vesicular ligands. A limited zinc removal of this sort would not necessarily result in a detectable decrease in total content of zinc in the sperm nucleus. It is possible that disulfide crosslinks formed only in the border of the nucleus or in its posterior region, may totally impede the normal decondensation in SDS-EDTA. Crosslinkage of the outer parts of the nucleus could influence the capacity for diffusion in these parts and thereby be cause of the decrease in extractability of zinc in aged spermatozoa.

The way by which zinc stabilizes the freshly ejaculated human sperm chromatin remains a matter of speculations. However, zinc can contribute to the stability of polymeres of insulin and other macromolecules (cf Klostermeyer & Humbel 1966; Chester 1978). Therefore, it seems likely that zinc could stabilize a quarternary structure of nucleoprotein fibers by interchelating, for instance between free amino groups of arginine. The inclusion of thiol-groups in the basic sperm-specific proteins of spermatozoa in eutherian mammals creates the basis not only for disulfide-bridge crosslinks but also for stable complexes in which zinc could interchelate between free amino groups of e.g. arginine and free thiol-groups of cystein in adjacent fibers (cf Friedman 1973). If this were the case, release of zinc and repulsion of nucleoprotein fibers would alone result in a rapid decondensation. However, there are numerous reports advocating that the stability of the sperm chromatin in eutherian mammals is primarily due to disulfide-bridge crosslinks, since exogenous thiols, e.g. DTT, have been regarded indispensible for decondensation in detergents (cf Zirkin et al 1985). To what extent the chromatin of a spermatozoon is normally stabilized by disulfide-bridge crosslinks is difficult to elucidate since studies in vitro always involve the risk for oxidation of thiol-groups. It may be that disulfide-bridges are formed in vitro, creating a need for **exogenous**, disulfide-bridge cleaving thiols, as is the case in human spermatozoa aged in vitro (Kvist & Eliasson 1978). When spermatozoa are studied 1-2 hours after ejaculation, **endogenous** thiol-groups apparently are sufficient to cleave stabilizing disulfide-bridge crosslinks Kvist (1982). A progressive, oxidative loss of thiol-groups in the nucleus of the spermatozoon during storage in vitro would not only result in an increased amount of stabilizing disulfide-bridge crosslinks but also in a loss of potential disulfide-bridge cleavers. Furthermore, the reduced ability for extraction of zinc from the chromatin in "aged" spermatozoa could further reduce the amount of **endogenous** thiols avail-

able for disulfide cleavage. Thus, if a spermatozoon is laid open to oxidation in vitro, this creates the need for **exogenous** disulfide-bridge cleaving agents, e.g. thiols like DTT, in order to induce decondensation of its chromatin in vitro (Kvist & Eliasson 1978). However, it should also be noted that thiols are not only "disulfide-bridge cleaving agents", but also powerful zinc-chelators which selectively release zinc from spermatozoa (Kvist & Eliasson 1978).

The present results imply that the freshly ejaculated spermatozoon, when entering the cervical mucus, has a chromatin with a stability primarily dependent on chromatin zinc accumulated at ejaculation. Whether this kind of stability will be superseded by disulfide-bridge crosslinks in the fertilizing spermatozoon is not known. However, if crosslinkage occurs during the transition in the female genital tract, appropriate decondensation would take longer time or could even be incomplete. It is therefore of particular interest that ova fertilized by spermatozoa aged in utero show (1) failure of sperm chromatin decondensation and abnormal development of the male pronucleus (Thibault 1969, 1971); (2) reduced cleavage rate (Maurer et al 1969); and (3) increased pre- and postimplantation losses (Tesh 1969; Tesh & Glover 1969; Martin & Shaver 1972). In humans, a higher frequency of early spontaneousabortions have been reported to occur after sperm ageing in utero, i.e. when the interval between coitus and ovulation is increased (Guerrero & Rojas 1975). An increased frequency of embryonic death is also a general finding when spermatozoa are aged in vitro. This decline in fertilizing capacity has been suggested to be related to qualitative changes in the nucleus of the aged spermatozoa, changes which could be revealed as reduced staining of DNA with Feulgen (Salisbury & Hart 1970). This reduction in Feulgen-stainability during storage could be recovered by treatment with DTT (Esnault 1973), indicating that decreased Feulgen-stainability is a result of formation of disulfide-bridge crosslinks. Infertile men show a variable and significantly lower Feulgen-stainability of sperm nuclei than fertile men (cf Leuchtenberger 1957). Recently, Nicolle et al (1985) reported that, in rams, the decrease of Feulgen-stainability is most pronounced in epididymal spermatozoa stored in fluid collected from the uterus, compared to ejaculated spermatozoa stored in the same fluid. This decrease paralleled a reduction in the surface area of the sperm heads. From this it can be inferred that ejaculated spermatozoa are better protected from those qualitative changes in sperm chromatin which give a decreased Feulgen-stainability and compaction of the sperm head. Whether this is a function of nuclear zinc, accumulated at ejaculation, is not known. Spermatozoa in species other than human, are also provided with zinc at ejaculation. However, our knowledge about the origin of zinc in the sperm nucleus, of species other than man, is scarce (cf INTRODUCTION). It is here tempting to speculate that the various effects of zinc serve to extend the functional life span of the spermatozoon; i.e. that zinc (1) stabilizes the nucleus, (2) protects the nucleus from superstabilization, and (3) preserves a potential for rapid chromatin decondensation. The idea that nuclear zinc may help to preserve the fertilizing capacity of spermatozoa is further supported by (1) the finding of high contents of zinc in the oviduct in bat, where spermatozoa are stored for months (Crichton et al 1982), and (2) the recent observation that 5 of 10 men, living in marriages with hitherto unexplained, longlasting infertility, had low content of zinc in the sperm heads (Kvist et al 1985).

The results of my studies support the idea that the zinc stabilizing the chromatin of ejaculated human spermatozoa is taken up from the prostatic fluid at ejaculation. Moreover, when a high number of spermatozoa were expelled together, the average uptake of zinc was reduced. The uptake of zinc seems to be discontinued by the admixture of the alkaline vesicular fluid. Two mechanisms to explain this effect of vesiuclar fluid might be considered: (1) a rise in

pH, increasing binding of zinc to citrate of prostatic origin (cf Sillén & Martell 1971); and (2) the addition of other ligands with high affinity for zinc (Arver 1982). Both these actions would rapidly reduce the amount of zinc available for uptake into the spermatozoon.

## Clinical implications

The results of the present and earlier studies (Janick et al 1971; Kvist 1980a; Kvist et al 1985) imply that one physiological function of the human prostate is to provide the spermatozoa with an appropriate amount of zinc at ejaculation. This function seems to be counteracted by vesicular fluid and restricted when high numbers of spermatozoa are expelled.

Many spermatozoa from men with secretory dysfunction of the prostate (Kvist & Eliasson 1980) or with inflammatory reaction of the prostate (Milingos & Aravantinos 1985; Milingos & Eliasson 1985) show sign of impaired uptake of zinc, i.e. spontaneous decondensation in SDS. A significant rise in pH of the prostatic fluid, probably caused by a decrease in the amount secreted citrate, and decreased amounts of other substances originating from the prostate, e.g. zinc, are primary signs of reduced secretory function of the prostate (Blacklock & Beavis 1977; Kavanagh et al 1982). Admixture of a relatively abundant amount of vesicular fluid, acting as hydrogen-acceptor and zinc-chelator (Arver 1982) would rapidly decrease the amount of free zinc by a rapid binding of zinc both to citrate and to vesicular ligands. Thus, a reduced timeframe for appropriate uptake of zinc at ejaculation could be one cause of spontaneous chromatin decondensation of spermatozoa from men with inflammatory reaction of the prostate, although the seminal plasma concentrations of zinc in some cases could be regarded as normal (≥1.2 mM; cf Eliasson 1975; Abyholm et al 1981). It seems possible that spermatozoa from these men would be more susceptible do develop extensive disulfide-bridge crosslink stabilization of the chromatin during a prolonged transition in the female genital tract.

A high total number of spermatozoa (MacLeod & Gold 1957; Doepfmer 1962; Joël & Hayen 1971) and also a high number of spermatozoa expelled in fractions dominated by vesicular fluid (Amelar & Hotchkiss 1965) have both been reported as conditions which may cause male infertility. The present results open the possibility that a reduced uptake of zinc into the sperm nucleus could be one factor contributing to the reduced fertilizing capacity among these men.

The total concentration of zinc in the liquefied seminal plasma seems to be of no value to assess whether an appropriate uptake of zinc occurs or not, when evaluating male fertility. This function of the prostate would rather be evaluated with studies on spontaneous decondensation and loss of the capacity for decondensation.

# VI. SUMMARY AND CONCLUSIONS

Ejaculated human spermatozoa were studied in vitro with respect to their capacity to decondense the chromatin in sodium dodecyl sulfate (SDS). The content of zinc in sperm heads was studied in epididymal, vasal, and ejaculated human spermatozoa. These were the main results:

(1) Soon after ejaculation most spermatozoa decondensed the chromatin in SDS with zinc-chelating EDTA. Only few spermatozoa decondensed in SDS alone.

(2) During storage, many spermatozoa lost the capacity to decondense in SDS-EDTA. Half of all spermatozoa lost this capacity within the first hour after ejaculation whether they were washed and stored in a buffered salt solution (BSS) within 20 minutes after ejaculation, or simply stored in the original seminal plasma. During prolonged storage (24 hours) the capacity was better retained among spermatozoa stored in BSS, than in those stored in seminal plasma. Furthermore, among spermatozoa treated with EDTA before storage, the loss of capacity for decondensation during storage was enhanced. In contrast, the initial capacity for decondensation was completely prevented in spermatozoa supplemented with zinc during 24 h storage in BSS.

(3) The ejaculated human spermhead contained significant amounts of zinc bound within the nuclear matrix. With EDTA-treatment, 90% of sperm head zinc could be removed soon after ejaculation. After 24 h storage in seminal plasma significantly less zinc could be released by exposure to EDTA.

(4) Epididymal and vasal sperm heads had significantly lower contents of zinc than ejaculated sperm heads.

(5) The zinc content of ejaculated sperm heads from various portions of split-ejaculates was neither correlated to the total seminal plasma zinc concentration, nor to the concentrations of measured subfractions of zinc bound to various groups of zinc-ligands. However, most of the variations in sperm head zinc could be explained by variations in total sperm number and concentration of fructose secreted by the seminal vesicles.

The results seem to justify the conclusion that the human spermatozoon takes up zinc at ejaculation from the concomitantly expelled prostatic fluid, and that zinc subsequently acts as a reversible stabilizer of the sperm chromatin. The results also imply that inappropriate stabilization by zinc of the sperm chromatin is likely to occur in spermatozoa from men with prostatic dysfunction, men expelling the spermatozoa mainly in vesicular fluid, and men expelling high total numbers of spermatozoa. The possibility is discussed, that zinc stabilizes the quarternary structure of the sperm chromatin by chelating between e.g. amino- and thiol-groups of adjacent nucleoproteinfibers. Concomitantly, zinc would protect these thiol-groups from being comitted into superstabilizing disulfide-bridge crosslinks. Thereby would zinc preserve a potential of the chromatin for rapid decondensation in the ooplasm.

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