CHAPTER 1

Introduction
Section A. The Spermatozoon: Its Fate in Female Reproductive Tract

1.1 Definition of Capacitation

Testicular spermatozoa are morphologically differentiated, but are neither progressively motile nor fertilization-competent. The progressive motility is acquired during their transit from the caput to the cauda region of the epididymis when the immature, immotile testicular spermatozoa undergo a number of physiochemical and morphological changes collectively referred to as maturation (Bedford, 1975; Austin, 1985; Eddy, 1988). Epididymal maturation transforms an immature and immotile spermatozoon to a mature and motile spermatozoon. But, such mature epididymal spermatozoa are still unable to fertilize the oocyte and they need to undergo another maturation process in the female reproductive tract termed 'capacitation'. Capacitation was discovered by Austin (1951, 1952) and Chang (1951, 1955) and it was defined as the finite and obligate period of residence of the spermatozoon in the female reproductive tract during which it acquires the ability to fertilize the oocyte. This period of residence varies from species to species and may range from 1-2 h as in sheep and rat to as much as 10-12 h in ferret (Table 1) (Austin, 1970).

Capacitation is an event which is absolutely essential for successful completion of fertilization and therefore, there is a need to understand this process. During the last 50 years attempts have been made to understand this important process which in \textit{in vivo} occurs in the oviduct or the uterus (Yanagimachi, 1994). These studies have indicated that during capacitation spermatozoa undergo two distinct physiological changes namely 'hyperactivation' and 'acrosome reaction' which are manifestations of
the process of capacitation. Hyperactivation and acrosome reaction could thus be considered as the upstream endpoints of capacitation and precede fertilization, the benchmark endpoint of the capacitated spermatozoon.

Table 1. Time required for *in vitro* capacitation of spermatozoa from different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Time (h)</th>
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<tbody>
<tr>
<td>Rabbit</td>
<td>~ 5</td>
</tr>
<tr>
<td>Pig</td>
<td>3-6</td>
</tr>
<tr>
<td>Rat</td>
<td>2-3</td>
</tr>
<tr>
<td>Hamster</td>
<td>2-4</td>
</tr>
<tr>
<td>Sheep</td>
<td>1.5</td>
</tr>
<tr>
<td>Ferret</td>
<td>3.5-11.5</td>
</tr>
<tr>
<td>Monkey</td>
<td>5-6</td>
</tr>
<tr>
<td>Human</td>
<td>5-6</td>
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</table>

1.2 Hyperactivation

Yanagimachi (1969a and b, 1970) was the first to notice that shortly before the acrosome reaction was initiated, hamster spermatozoa exhibited a change in their motility pattern such that the sperm trajectory changed from a linear progressive trajectory to a less linear trajectory with the spermatozoa exhibiting greater degree of lateral head displacement, higher degree of bending in the proximal region of the flagellum and increase in tail beat frequency and curvilinear velocity. This type of motility was characteristic of capacitated spermatozoa and was referred to as
'activation' but later it was changed to 'hyperactivation' so as to distinguish it from the motility pattern of the spermatozoa in fresh ejaculates. Hyperactivation of sperm motility has now been described for every mammalian species examined (Gwatkin, 1969; Yanagimachi, 1969a, 1970; Katz et al., 1989) and hyperactivated spermatozoa have been observed to exhibit various motility patterns distinct from non-hyperactivated spermatozoa.

1.2.1 Motility Pattern and Motility Parameters of Hyperactivated Spermatozoa

Hyperactivation transforms the linear progressive type of motility of non-hyperactivated spermatozoa to a non-progressive type of motility with whiplash like beatings of the tail (Yanagimachi, 1981) resulting in motility trajectories such as circular, helical, figure of eight, whiplash, hatchet, wriggling, dancing, star-spin, thrashing etc (Suarez, 1996; Yanagimachi, 1994; Burkman, 1990, Shivaji et al., 1995; Jayaprakash et al., 1997) (Fig. 1). It was also observed that the kind of trajectory hyperactivated spermatozoa follow also depends on the depth of the chamber used (Suarez et al., 1990; Shivaji et al., 1995). For instance, when a slide chamber (130 μm depth) or a cannula (200 μm depth) were used, hyperactivated hamster spermatozoa exhibited planar, circular, hatchet, helical and wriggling type of motility. But, when the Makler's chamber was used only planar, circular, and hatchet patterns were visible (Shivaji et al., 1995). This was probably due to space constraint in the Makler's chamber wherein the depth is less than the size of the hamster spermatozoon.

Visually hyperactivated spermatozoa could be differentiated from non-hyperactivated spermatozoa based on their trajectories (Fig. 1). But, more recently with the advent of the computer-aided semen analysis (CASA) system it has been possible
to objectively discriminate non-hyperactivated from hyperactivated spermatozoa based on various motility parameters such as VAP (average path velocity), VSL (straight linear velocity), VCL (curvilinear velocity), LIN (linearity), STR (straightness) and ALH (amplitude of lateral head displacement) (Fig. 2). Using these parameters it has been possible to sort hyperactivated from non-hyperactivated spermatozoa of human (Burkman, 1991; Peedicayil et al., 1997), hamster (Suarez, 1988; Shivaji et al., 1995), boar (Holt et al., 1997), bull (Farrell et al., 1998), rabbit (Perez-Sanchez et al., 1996), domestic cat (Stachecki et al., 1993), rat (Cancel et al., 2000), ram (Mortimer and Maxwell, 1999) etc (Table 2).

Fig. 1 Sequential tracing of a single hamster caudal spermatozoon (starting from position 1) showing (A) planar, (B) circular, (C) helical, (D) hatchet, and (E) wriggling motility pattern. The tail beating pattern and the orientation of the head of the spermatozoon was traced frame by frame from the visual monitor of the analyzer using the playback facility. Figure from Shivaji et al (1995).
Fig. 2  Diagrammatic representation of motility parameters of a spermatozoon trajectory determined using CASA. The solid circles denote the head positions of the spermatozoon. The solid line, dotted line and the broken line denote the actual path, mathematical average path (visually appraised) and the net displacement respectively. The single and double arrows show the initial and the final positions of the spermatozoon respectively. VAP, VSL and VCL are calculated from the actual path, average path and the net displacement respectively.

Table 2.  Motility parameters of non-hyperactivated and hyperactivated caudal epididymal spermatozoa of hamster incubated in TALP

<table>
<thead>
<tr>
<th>Motility parameters</th>
<th>Non-hyperactivated spermatozoa</th>
<th>Hyperactivated (circular) spermatozoa</th>
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<tbody>
<tr>
<td>VAP (μm/s)</td>
<td>132.3</td>
<td>256.3</td>
</tr>
<tr>
<td>VCL (μm/s)</td>
<td>323.7</td>
<td>459.9</td>
</tr>
<tr>
<td>VSL (μm/s)</td>
<td>101.6</td>
<td>44.6</td>
</tr>
<tr>
<td>STR (%)</td>
<td>77</td>
<td>17</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>ALH (μm)</td>
<td>17.8</td>
<td>28.8</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>25</td>
<td>19.1</td>
</tr>
</tbody>
</table>
1.2.2 Physiological Significance of Hyperactivation

Hyperactivation is probably required to facilitate the spermatozoal movement through the viscous oviductal fluid (Suarez et al., 1990) and the cumulus oophorus and to penetrate the zona pellucida (ZP) (Fraser, 1981). It has also been proposed that hyperactivation helps spermatozoa in escaping from the pockets and grooves in the oviductal mucosa and increase their chances to encounter the oocytes (Suarez and Osman, 1987) by virtue of their frequent directional changes. Finally, hyperactivation may help the spermatozoa in gaining enough thrust which in combination with the acrosomal enzymes may help in the penetration of the oocyte for successful fertilization.

It has also been suggested that hyperactivation may facilitate chemotactic response in mammalian spermatozoa (Suarez et al., 1993). This is an attractive hypothesis considering that bovine (Vijayasarathy et al., 1980; Iqbal et al., 1980) and human spermatozoa (Gnessi et al., 1985; Ralt et al., 1991; Shivaji et al., 1993) exhibit chemotaxis.

1.2.3 Regulation of Hyperactivation

Comparatively little is known about the molecular mechanism of hyperactivation except that it requires exogenous calcium (Yanagimachi, 1982; Fraser, 1987) and that hyperactivated spermatozoa accumulate greater concentration of calcium compared to the non-hyperactivated spermatozoa (Suarez et al., 1993). Furthermore, the increase in calcium ion concentration was more in the flagella than in the head of the spermatozoa (Suarez and Dai, 1995) indicating that the temporal rise in calcium ion during hyperactivation is a phenomenon that is more or less completely associated with the
flagella of the spermatozoa. This increase in intracellular calcium level during hyperactivation is brought about by the opening of calcium channels in the plasma membrane (Stauss et al., 1995). However, how the opening of such channels is regulated is yet unknown. In vivo, chemotactic factors such as progesterone (Mbizvo et al., 1990) present in the human follicular fluid (Ralt et al., 1994) can induce hyperactivation and this process is regulated by the ability of progesterone to induce calcium ion influx in spermatozoa (Thomas and Meizel, 1989; Meizel and Turner, 1991). Finally, the observation that the number of hyperactivated spermatozoa in the oviduct increases near the time of ovulation further indicates that there are probably other physiological factors which regulate this process in vivo.

1.2.4 Hyperactivation and Capacitation

It appears that hyperactivation and capacitation are linked to each other. However, Olds-Clarke (1989) demonstrated that in mouse spermatozoa carrying 't' haplotype hyperactivation occurred prematurely in vitro, but capacitation occurred later at the normal time indicating that the two are independent of each other. Furthermore, it was demonstrated that sub-optimal concentrations of NaHCO₃ in the medium inhibited hyperactivation while capacitation was normal (Boatman and Robbins, 1991; Stauss et al., 1995). De Mott et al (1995) also showed that in a specific medium completion of hyperactivation was preceded by an hour of capacitation in hamster spermatozoa. Therefore, as of now it is very difficult to conclude that capacitation and hyperactivation are closely linked to each other (Visconti et al., 1998).
1.3 Acrosome Reaction

Although it was known for many years that the acrosome of the spermatozoon is altered prior to capacitation, it was Dan (1952, 1956) who first recognized the acrosome reaction as an essential step of normal fertilization in a wide variety of animals. Capacitated spermatozoa after passing through the oocyte's vestments namely the cumulus oophorus come in contact with the zona pellucida and undergo the acrosome reaction. Acrosome reaction is basically an exocytotic event that involves the fusion and vesiculation of the outer acrosomal membrane and the surrounding plasma membrane, and culminates in the dispersal and release of the acrosomal contents (Fig. 3). It is an obligate requirement for successful fertilization to occur since spermatozoa are unable to penetrate the zona by force alone that is generated by hyperactivated motility (Green, 1988; Yanagimachi, 1994).

Fig. 3 Diagram showing the progress of acrosome reaction in spermatozoa starting with point fusions of the outer acrosomal membrane and the overlying plasma membrane. Point fusions coalesce leaving hybrid vesicles and releasing acrosomal contents. The equatorial segment remains intact. Figure from Curry, M.R. and Watson, P.F. (1995).
1.3.1 Mechanism and Control of Acrosome Reaction

Acrosome reaction is a regulated exocytosis process involving agonist-receptor interaction and intracellular signaling. Over the years a number of agonists and receptors have been identified and attempts have also been made to understand the molecular basis of acrosome reaction with respect to transmembrane signaling and the role of second messengers.

1.3.1.1 Agonist-Receptor Interaction

A number of agonists or physiological inducers of acrosome reaction and their receptors have been reported from different species. However, the two main agonists are - zona pellucida (Bleil and Wassarman, 1980, 1983) and progesterone (Meizel et al., 1990) which is trapped in the cumulus oophorus and/or may be produced by cumulus cells themselves (Yanagimachi, 1994; Schuetz and Dubin, 1981). Studies have also demonstrated that progesterone is a very efficient membrane perturbing agent capable of aggregating membrane vesicles and inducing fusion as in sperm acrosome reaction (Shivaji and Jagannadham, 1992). Other agonists which have been proposed to induce the acrosome reaction are epidermal growth factor or EGF (Naz and Ahmad, 1992; Lax et al., 1994; Murase and Roldan, 1995), atrial natriuretic peptide or ANP (Anderson et al., 1994; Zamir et al., 1995), prolactin (Mori et al., 1989), interleukin 6 (Naz and Kaplan, 1994), c-kit - a stem cell factor (Feng et al., 1997,1998) and γ-aminobutyric acid or GABA (Shi and Roldan, 1995b; Shi et al., 1997). Thus, many inducers of acrosome reaction have been identified including ZP3 which is the most probable physiological inducer.
The identity and function of sperm-associated ZP3 binding receptor has been controversial (Kopf and Gerton, 1991; Ward and Kopf, 1993; Wassarman, 1995; Tulsiani et al., 1997). The three most important ZP3 binding receptors which have been identified so far are 'sp56' (Bookbinder et al., 1995), 'p95' (Leyton and Saling, 1989) and β-galactosyltransferase (Dubois and Shur, 1995). As of now, the role of 'sp56' (Bookbinder et al., 1995; Foster et al., 1997) and 'p95' - two proteins from mouse sperm as the zona binding receptor, is still uncertain. But, a specific form of β-galactosyltransferase (GalTase) also from mouse spermatozoa is likely to be the receptor for ZP3 (Dubois and Shur, 1995). However, targeted over-expression of this protein in spermatozoa showed that such spermatozoa had reduced ability to bind to the ZP in contrast to the anticipation that such spermatozoa will show enhanced ability to bind to the ZP. On the other hand, GalTase gene null mice were fertile but were unable to undergo ZP3-induced acrosome reaction (Lu and Shur, 1997). Thus, the data suggests that GalTase may be important for sperm-egg binding but not required for fertility absolutely.

1.3.1.2 Transmembrane Signaling and Second Messengers in Acrosome Reaction

It appears that two such second messenger pathways are functional in the acrosome reaction (Fig. 4A, B). One pathway involves the activation of adenylyl cyclase and the formation of cAMP, probably coupled with the inhibition of cAMP-phosphodiesterase which hydrolyses cAMP to 5'-AMP (Zaneveld and De Jonge, 1991) (Fig. 4A, B). cAMP activates protein kinase A (PKA) which brings about the changes in protein phosphorylation (Baldi et al., 1996). Results obtained using reagents that alter the generation and catabolism of cAMP, permeable analogs of cAMP that increase
endogenous levels of cAMP and the inhibitors of PKA strongly suggests that this pathway is involved in the acrosomal exocytosis (Baldi et al., 1996; Naor and Breitbart, 1997). Very recently, it has also been shown that acrosome reaction involves, cyclic guanosine monophosphate (cGMP) dependent protein kinase (PKG) which is activated by nitric oxide (Revelli et al., 2001).

Fig. 4 Proposed second messenger pathway in acrosome reaction (A) cAMP-dependent pathway and (B) phosphoinositide-dependent pathway. Figure from Zaneveld et al (1991).
The other pathway involves the formation of diacylglycerol (DAG) and inositol triphosphate (InsP$_3$) by the activation of a phospholipase C which hydrolyses phosphatidylinositol biphosphate (PIP$_2$) (Zaneveld and De Jonge, 1991; Naor and Breitbart, 1997) (Fig. 4B). DAG and Ca$^{2+}$ activate protein kinase C which, in turn, brings about changes in protein phosphorylation (O'Toole et al., 1996). However, it is not clear at present what is the exact role of cAMP/PKA or PKC pathway during exocytosis in spermatozoa, since the targets for these pathways for membrane fusion have not yet been identified. It is possible that cAMP targets an early step in the sequence underlying exocytosis, such as cyclic nucleotide-gated Ca$^{2+}$ channels (Weyand et al., 1994). Other second messengers like lysophosphatidylcholine and arachidonic acid (Roldan and Fragio, 1993a, b) have also been implicated in the acrosomal exocytosis.

### 1.3.2 Role of Calcium in Acrosome Reaction

One of the very early responses generated upon agonist-receptor interaction during spermatozoa-zona binding is the activation of ion fluxes. Among these, Ca$^{2+}$ plays a very important role and has been shown to be essential for the acrosomal exocytosis (Yanagimachi, 1994). Ca$^{2+}$ is necessary for the activation of intracellular enzymes and for the actual fusion of the membranes (Shivaji and Jagannadham, 1992; Baldi et al., 1996; Yanagimachi, 1994; Roldan and Fragio, 1993a, b).

### 1.4 Physiological Regulators of Capacitation

Capacitation of spermatozoa is a prerequisite for successful fertilization and therefore, this phenomenon needs to be regulated such that fertilization-competent spermatozoa are available at the site of fertilization. It is possible that in *in vivo* both
positive and negative regulators of capacitation exists in the female reproductive tract. For instance, it is possible that regulation of capacitation lies in the derepression of inhibitory modulators of capacitation through the removal of decapacitating factors (Hunter and Nornes, 1969; Yanagimachi, 1994). Chang (1957) observed that the seminal plasma of a variety of species prevented capacitated rabbit spermatozoa from penetrating homologous eggs following deposition in the oviducts and called this process 'decapacitation'. Subsequently, the decapacitation factor from rabbit seminal plasma, which also showed antifertility activity (Hunter and Nornes, 1969) was purified and shown to be a glycoprotein of molecular weight 170 kDa (Reyes et al., 1975). Zaneveld and Williams (1970) proposed that this factor could inhibit the activity of corona-penetrating enzyme needed by the spermatozoa for penetrating the corona-radiata during fertilization. Seminalplasmin a protein (Shivaji, 1984; Shivaji and Bhargava, 1987; Shivaji et al., 1990) isolated from bovine seminal plasma which prevented the accumulation of Ca\(^{2+}\) by bovine epididymal spermatozoa (Rufo et al., 1982) was also identified as a decapacitation factor. These investigators suggested that this protein present on the sperm plasma membrane, is removed during capacitation which in turn leads to an uptake of calcium ions and induction of acrosome reaction. Fraser et al (1990) demonstrated that a 40 kDa protein in mouse epididymal spermatozoa acts as the decapacitation factor by regulating Ca\(^{2+}\)-ATPase activity. More recently, an N-glycosidically linked oligomannosidic glycopeptide (MGP) has been shown as the decapacitation factor present in human seminal plasma (Lopes et al., 1998).
1.5 Molecular Basis of Capacitation

Over the years the definition of capacitation has changed from a mere temporal event in the female reproductive tract to a more detailed definition reflecting all the physiological and molecular changes the spermatozoa undergo in the female reproductive tract so as to become fertilization-competent. Therefore, in order to attain a complete understanding of this complex and poorly understood phenomenon, it is necessary to consider events occurring both in the head (acrosome reaction) and in the tail (motility changes) (Visconti and Kopf, 1998). This has now become possible with the ability to capacitate spermatozoa in vitro and the added ability to monitor the endpoints of capacitation (namely hyperactivation, acrosome reaction and/or in vitro fertilization) and by simultaneously monitoring changes at the molecular level. But, considering that mammalian spermatozoa are terminally differentiated and devoid of DNA replication, transcription and translation, it would be logical to assume that post translational modifications of proteins such as phosphorylation and dephosphorylation are likely to play an important role with respect to sperm function. In fact, phosphoproteins (Huacuja et al., 1977; Chulavatnatol et al., 1982; Noland et al., 1984; Horowitz et al., 1985; Nikolopoulou et al., 1986; Wooten et al., 1987; Berruti & Martegani, 1989; Mitra & Majumder, 1991; Haldar & Majumder, 1996; Naz et al., 1991; Uma Devi et al., 1996, 1997), protein kinases which phosphorylate proteins (Leyton & Saling, 1989; Leyton et al., 1995; Kalab et al., 1994; Burks et al., 1995; Visconti et al., 1995a; Luconi et al., 1996; Uma Devi et al., 1997) and protein phosphatases which dephosphorylate proteins (Tang & Hoskins, 1975; Vijayaraghavan et al., 1996; Barua et al., 1985; Tash et al., 1988; Ahmad et al., 1995; Ashizawa et al., 1995, 1997; Smith et al., 1996; Uma Devi et
al., 1999) have been detected in the spermatozoa of mammals and have been implicated in sperm motility acquisition, capacitation, acrosome reaction and fertilizing ability. Thus, since all the components of the signal transduction pathway are associated with spermatozoa, it is tempting and likely that the molecular basis of capacitation centers around a cross-talk between the various components of the pathway involving second messengers (Ca$^{2+}$ and cAMP), enzymes (protein kinases and protein phosphatases) and the substrates (proteins).

1.5.1 Protein Tyrosine Phosphorylation during Capacitation

Visconti et al (1995a, b) were the first to find a correlation between the capacitation state and protein tyrosine phosphorylation in mouse spermatozoa. It was observed that cauda epididymal spermatozoa of mouse, following incubation under conditions conducive to capacitation, exhibit a time-dependent increase in protein tyrosine phosphorylation of a subset of proteins of mol. wt. 40-120 kDa which correlated with the capacitation state of the spermatozoa as judged by the sperm to undergo the acrosome reaction and fertilize ZP intact cells in vitro. Further, they also observed that caput spermatozoa which do not possess the ability to undergo capacitation and fertilize eggs (Yanagimachi, 1994) do not exhibit the changes in protein tyrosine phosphorylation as observed for the cauda spermatozoa. Ever since these pioneering studies by Visconti et al (1995a, b) correlation between protein tyrosine phosphorylation and capacitation was confirmed for human (Leclerc et al., 1996; Osheroff et al., 1999), bovine (Galantino-homer et al., 1997) boar (Kalab et al., 1998) and hamster (Kulanand and Shivaji, 2001). The data obtained from these studies also indicated that the increase in protein tyrosine phosphorylation associated with capacitation is species-
dependent because constituents of the medium, such as calcium, BSA and sodium bicarbonate, had different effects in different species. For instance, mouse spermatozoa require calcium, BSA, and bicarbonate for capacitation and the associated protein tyrosine phosphorylation (Visconti et al., 1995a), whereas human spermatozoa required BSA and bicarbonate but not calcium (Carrera et al., 1996; Luconi et al., 1996). In fact in the presence of calcium, protein tyrosine phosphorylation is down-regulated in human spermatozoa. The increase in protein tyrosine phosphorylation during capacitation in mouse (Visconti et al., 1995a), bovine (Galantino-Homer et al., 1997), human (Leclerc et al., 1996; Osheroff et al., 1999), boar (Kalab et al., 1998) and hamster (Visconti et al., 1999c) has been shown to be regulated by a c-AMP-dependent pathway that involves a protein kinase A (PKA). This signaling pathway involving protein tyrosine phosphorylation and cAMP is unique to spermatozoa and has been confirmed by the fact that inhibitors of PKA are able to inhibit protein tyrosine phosphorylation observed during capacitation. It is important to note that being a serine/threonine kinase, PKA cannot influence the substrate for tyrosine phosphorylation directly. This necessitates the role of protein tyrosine kinase or protein tyrosine phosphatase or both. It is possible that the activity of such enzymes is influenced directly by PKA. However, another possibility is that the substrates for tyrosine phosphorylation are primed by first getting phosphorylated at serine/threonine residue(s). Thus, signaling events during sperm capacitation are unique in the sense that PKA and protein tyrosine phosphorylation/dephosphorylation appear to be coupled in this process. But, the temporal sequence of these signaling events is yet to be established. Further, the identification of the enzymes responsible for the tyrosine phosphorylation pathway(s)
will definitely add to our knowledge of the signaling events regulating capacitation. It would also be important to identify the phosphotyrosine proteins which are specifically phosphorylated during capacitation and to understand their function.

1.5.2 Protein Serine/Threonine Phosphorylation during Capacitation

Although, serine/threonine kinases such as protein kinase A (PKA) and protein kinase C (PKC) have been implicated in sperm-capacitation, little is known about the proteins which get differentially phosphorylated during capacitation when compared to tyrosine phosphorylation. Therefore, there is a need to identify and characterize phosphoproteins which get differentially phosphorylated (if any) at serine/threonine residues. Furthermore, their cross talk with the other components of the signaling cascade will certainly help understand the signaling event during capacitation in a better way. Recently, Naz (1999) using specific antibodies against serine and threonine phosphorylated proteins has shown that a set of 8-9 proteins in the range of mol. wt. from 15-200 kDa in human spermatozoa undergo serine or threonine (or both) phosphorylation, and the phosphorylation intensity increases with the time of incubation during capacitation. One of these proteins was identified as fertilization antigen-1 (FA-1) which was earlier shown to be tyrosine phosphorylated during capacitation and implicated in human sperm capacitation and zona pellucida binding (Naz and Ahmad, 1994). Further, a shift in the subcellular localization of these proteins was also observed. These findings indicate that serine/threonine phosphorylation may have a physiological role in sperm capacitation and/or acrosome reaction and it deserves further intensive investigation.
1.6 Identification of Phosphoproteins Involved in Capacitation

As of now, it is not known whether increase in protein tyrosine phosphorylation is due to upregulation of protein tyrosine kinase, inhibition of protein tyrosine phosphatase or both. But, what is clear is that the phosphotyrosine proteins which are the endpoints of the signaling pathway probably influence sperm capacitation and therefore, need to be characterized. As of now, only two phosphotyrosine proteins from mammalian spermatozoa involved in capacitation have been characterized. These proteins were identified as a A-kinase anchoring protein (AKAP) and designated as AKAP82 (or, AKAP 4) and FSP95 (also called AKAP3 or, AKAP110) (Carrera et al., 1996; Mandal et al., 1999; Vijayaraghavan et al., 1999; Turner et al., 2001).

The role of AKAPs is to tether PKA via its regulatory (RII) subunit and to bring PKA in close proximity to its substrate (Rubin, 1994) and thus, regulate phosphorylation of the substrate and amplify the biological effects of cAMP signaling (Fig. 5). AKAPs normally consist of two conserved structural modules - a targeting domain that serves as a scaffold and anchor, and tethering domain that interacts with PKA regulatory subunit. Although AKAPs have been identified on the basis of their interaction with PKA, they also bind other signaling molecules, mainly phosphatase and other kinases. For example, AKAP79 which is found in the brain binds PKC and calcineurin, a Ca^{2+}-calmodulin dependent protein phosphatase (Coghlan et al., 1995; Klauck et al., 1996).
AKAP82 (also called as AKAP4 or FSC1) was the first AKAP that was identified and observed to be the major fibrous sheath protein of the axoneme and was localized to the principal piece of the tail in mouse spermatozoa (Fig. 6) (Carrera et al., 1994; Johnson et al., 1997; Fulcher et al., 1995; Turner et al., 2001). It was synthesized as a precursor protein (pro-AKAP82) which following proteolytic cleavage yielded AKAP82. The mature AKAP82 could bind to the RII subunit of PKA. Subsequently, the homologues of AKAP82 and its precursor were identified in the spermatozoa of rat.
(Brito et al., 1989), bovine (Moss et al., 1999) and human (Carrera et al., 1996) based on their amino acid sequence, and ability to bind the RII subunit of PKA. Some of the AKAPs were also shown to be localized to the fibrous sheath as observed in the case of the rat testis specific AKAP (TAKAP) (Mei et al., 1997).

Fig. 6 Diagrammatic representation of the tail elements at the level of the principal piece of the spermatozoon. Figure from Curry, M.R. and Watson, P.F. (1995).

FSP95 (Fibrous Sheath Protein, mol. wt. 95), also referred to as AKAP 3 or AKAP 110, is the other testis specific AKAP (Turner et al., 2001; Mandal et al., 1999; Vijayaraghavan et al., 1999) demonstrated to be associated with the fibrous sheath of the sperm flagellum (Mandal et al., 1999) and the acrosome region of the sperm head (Vijayaraghavan et al., 1999). More interesting is the observation that another AKAP from human called hAKAP220 (Reinton et al., 2000) shows differential localization during gametogenesis and migrates from the cytoplasm in premeiotic pachytene
spermatocytes to the centrosome of post meiotic germ cells and finally to the midpiece of spermatocytes and mature sperm. Thus, based on their localization and ability to interact with RII type PKA (Moss et al., 1999; Carrera et al., 1994) or with both RI and RII type of PKA (Reinton et al., 2000; Vijayaraghavan et al., 1999), they have been implicated in motility and capacitation of spermatozoa. In fact, recently it has been demonstrated that addition of a cell-permeable anchoring inhibitor peptide (S-Ht31) that has the sequence required for RII binding, dramatically inhibited sperm motility in a time- and a dose-dependent manner (Vijayaraghavan et al., 1997). Further, it is interesting to note that the AKAPs associated with the flagellar sheath undergo phosphorylation changes during sperm maturational event such as capacitation and this varies from species to species (Carrera et al., 1996; Johnson et al., 1997; Brito et al., 1989). For instance, human AKAP82 and FSP95 are phosphorylated at tyrosine during capacitation, whereas in mouse and rat they undergo phosphorylation at serine/threonine residue(s). Thus, from these studies it appears that AKAP proteins are important regulatory proteins for a number of sperm functions and accordingly they have differential properties with respect to their localization, phosphorylation and affinity for a particular type of PKA. FSP95 has high homology to mouse AKAP82 and human AKAP82. Interestingly, the two potential intracellular anchoring domains of mouse sperm pro-AKAP82 are conserved in both human pro-AKAP82 and FSP95 cDNA (Carrera et al., 1994; Turner et al., 1998), however, the RII binding domains of human and mouse AKAP82 are not conserved in FSP95.
1.7 Other Modulations of Capacitation

1.7.1 Ion-Fluxes

Capacitation is correlated with change in intracellular concentrations of ions such as, Na\(^+\), K\(^+\), and HCO\(_3\)\(^-\). For instance, intracellular K\(^+\) concentration decreases while HCO\(_3\)\(^-\) and Na\(^+\) concentrations increase during capacitation (Brooks, 1983; Setchell et al., 1994; Yanagimachi, 1994). Such changes bring about the hyperpolarization of the sperm plasma membrane (Arnoult et al., 1996) which in turn activates Ca\(^{2+}\) channels and thus, intracellular Ca\(^{2+}\) level goes up (Florman et al., 1998). This is consistent with the presence of low voltage activated Ca\(^{2+}\) channels in spermatozoa (Arnoult et al., 1996). However, the mechanism by which hyperpolarisation regulates capacitation is poorly understood.

1.7.2 Nitric Oxide

Nitric oxide and other free radicals have recently been implicated in sperm functions such as hyperactivation, capacitation and acrosome reaction (De Lamirande and Gagnon, 1993). It has been observed that such free radicals upregulate protein tyrosine phosphorylation in human spermatozoa (Leclerc et al., 1997). It can be hypothesized on the basis of such preliminary data that nitric oxide modulates capacitation by regulating the protein tyrosine phosphorylation through cAMP-dependent pathway.

1.8 The Working Model of Capacitation

The working model of capacitation needs to reflect at the molecular level the physiological changes that the spermatozoa undergo during capacitation (Fig. 7). At the molecular level capacitation of spermatozoa could be essentially visualized as a
transmembrane and intracellular signaling pathway involving second messengers, protein kinases, protein phosphatases and the phosphorylated proteins, like any other signal transduction pathway.

Fig. 7 Working model demonstrating the signaling pathways hypothesized to regulate sperm capacitation (Visconti et al., 1998). (-) and (+) indicate negative and positive regulation respectively. Abbreviations used: BSA, bovine serum albumin; chol, cholesterol; HBP, heparin binding protein; PTK, protein tyrosine kinase; pTyr Ptase, phosphotyrosine phosphatase; PDE, phosphodiesterase; PK-A, protein kinase A.

This pathway in spermatozoa is unique in that it is probably the only cell system known where capacitation-dependent protein tyrosine phosphorylation is regulated by a cAMP-dependent pathway that involves protein kinase A (Visconti et al., 1995b, Galantino-Homer et al., 1997; Leclerc et al., 1996; Osheroff et al., 1999; Kalab et al.,
Evidence for this molecular model for capacitation involving transmembrane and intracellular signaling pathways have come from a number of studies involving the effect of various components (such as serum albumin, Ca$^{2+}$, NaHCO$_3$, cAMP etc.) which modulate capacitation either by stimulating or inhibiting the process and simultaneously manifesting its effect at the level of protein phosphorylation. Therefore, for a better understanding of the model proposed by Visconti et al (1998) it would be absolutely essential to establish as to how the effects of the modulators of capacitation are coupled to the transmembrane and intracellular signaling events.

1.8.1 Role of Serum Albumin in Capacitation

Serum albumin (usually BSA) is an essential component of in vitro capacitation media for mammalian spermatozoa absence of which is known to inhibit capacitation in mouse, hamster and human spermatozoa (Visconti et al., 1995a, b; Visconti et al., 1999a, b; Emiliozzi and Fenichel, 1997; Carrera et al., 1996). Although the exact function is yet to be established for BSA, it is believed to facilitate capacitation by acting as a sink for cholesterol which it removes from the sperm plasma membrane (Cross, 1996; Cross, 1998; Davis, 1981; Davis et al., 1979; Go and Wolf, 1985; Langais and Roberts, 1985; Suzuki and Yanagimachi, 1989). This is further strengthened by the observation that capacitation is inhibited by the addition of cholesterol and/or analogues to the capacitation medium (Visconti et al., 1999a, b). Human semen contains cholesterol and other sterol which can account for the inhibitory effect of seminal plasma on human sperm capacitation (Cross, 1998), presumably by preventing cholesterol efflux from the sperm plasma membrane and serum albumin can be
substituted in *in vitro* capacitation medium with cholesterol binding compounds such as high density lipoproteins (Therien et al., 1997; Therien and Manjunath, 1996; Visconti et al., 1999a, b) and β-cyclodextrins (Choi and Toyoda, 1998; Cross, 1999 Osheroff et al., 1999; Visconti et al., 1999a). It is also important to consider what component of the female reproductive tract fluid might serve as a cholesterol acceptor *in vivo*. Albumin-like proteins present in the female tract fluid may be important in this context. The identity of such acceptors *in vivo* remains to be clarified.

The mechanism of cholesterol removal and how this initiates intracellular signaling is not known. However, it is postulated that removal of cholesterol brings about decrease in the membrane fluidity (Wolf and Cardullo, 1991; Wolf et al., 1986), which may cause opening of ion-channels and entry of Ca\(^{2+}\) and HCO\(_3^-\) and activate adenylyl cyclase.

### 1.8.2 Role of Calcium and Bicarbonate in Capacitation

Numerous studies have demonstrated that capacitation is calcium-dependent (Das Gupta et al., 1993; Visconti et al., 1995a). The regulation of capacitation by calcium occurs via different targets, some of which are mediated through cAMP. Ca\(^{2+}\) would modulate the cAMP level in a spermatozoon by two ways - by stimulating adenylyl cyclase (Gross et al., 1987) or by inhibiting cAMP-phosphodiesterase (Wasco and Orr, 1984). Thus Ca\(^{2+}\) could influence cAMP-dependent tyrosine phosphorylation in spermatozoa. However, the effect of Ca\(^{2+}\) in different species is not the same. For instance, Ca\(^{2+}\) has a positive effect on protein tyrosine phosphorylation in mouse spermatozoa (Visconti et al., 1995a) whereas it inhibits protein tyrosine phosphorylation
in human spermatozoa (Carrera et al., 1996; Luconi et al., 1996). An increase in intracellular sperm Ca\textsuperscript{2+} during capacitation has been observed by some investigators, whereas others have shown no changes during this maturational event (Yanagimachi, 1994). However, the action of Ca\textsuperscript{2+} at the level of effector enzymes involved in the sperm signal transduction suggests that this divalent cation is likely to play an important role in capacitation.

The requirement of NaHCO\textsubscript{3} for capacitation is well established in the mouse (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Shi and Roldan, 1995a; Visconti et al., 1995a) and in hamster (Boatman and Robbins, 1991; Visconti et al., 1999c). The influx of bicarbonate ions in the spermatozoon could be responsible for the known increase in intracellular pH that is observed during capacitation (Uguz et al., 1994; Zeng et al., 1996). An additional target for the action of this anion could be the regulation of sperm cAMP metabolism, since the synthesis of cAMP by mammalian sperm adenylyl cyclase is markedly stimulated by bicarbonate ions (Garty and Saloman, 1987; Okamura et al., 1985; Visconti et al., 1990). Thus, it appears that bicarbonate ions affect capacitation by regulating the protein tyrosine phosphorylation via cAMP. In fact, it has been shown that specific channel blockers of bicarbonate ions inhibit protein tyrosine phosphorylation also and this reverts back to normal when cAMP analogues are used along with the channel blockers. Recently Chen et al. (2000) have demonstrated that a soluble form of adenylyl cyclase is present in the cytosol of the spermatozoon which gets stimulated by direct interaction with bicarbonate ions. This enzyme remains unaffected by forskolin, a specific modulator for the transmembrane adenylyl cyclase. Interestingly, the sequence of the catalytic domain of this cyclase has sequence
homology to cyanobacterial adenylyl cyclase and the cyanobacterial cyclase is also bicarbonate ion-dependent. It will be interesting to find out if this soluble form of adenylyl cyclase can be stimulated by other ions like Ca$^{2+}$. In addition to these modulators heparin and nitric oxide have also been shown to positively regulate capacitation mediated through their effects on cAMP, Ca$^{2+}$ and protein tyrosine phosphorylation.

The temporal sequence of events in the working model of capacitation has not been established. But, it is logical to assume that events occur initially at the membrane level and subsequently the signal is transduced intracellularly. It is possible that changes in the fluidity of the spermatozoal plasma membrane and subsequent redistribution of membrane proteins due to cholesterol efflux (by BSA) (Yanagimachi, 1994) stimulate uptake of HCO$_3^-$ and Ca$^{2+}$ (Visconti et al., 1995a) which act as positive regulators of adenylyl cyclase, thus resulting in an increase in intracellular cAMP (Visconti et al., 1995b). Increase in intracellular calcium and cAMP could also occur due to heparin (Parrish et al., 1994; Galantino-Homer et al., 1997). Ca$^{2+}$ regulates cAMP levels through phosphodiesterase and it is also required for acrosome reaction, whereas, cAMP positively regulates protein kinase A. PKA in spermatozoa regulates capacitation-dependent protein tyrosine phosphorylation by either stimulating protein tyrosine kinase (PTK) or inhibiting protein tyrosine phosphatase (PTP). Recently, it has also been shown that reactive oxygen species and nitric oxide upregulate protein tyrosine phosphorylation (Leclerc et al., 1997; Aitken et al., 1995) and membrane hyperpolarization due to enhanced K$^+$ permeability and thus, positively regulate capacitation (Zeng et al., 1995; Arnoult et al., 1996).
1.9 Conclusions

From the foregoing information it could be concluded that capacitation of mammalian spermatozoa is an obligate event occurring prior to fertilization. This phenomenon is a temporal event which occurs in the female reproductive tract (but could be induced *in vitro*) and appears to be linked to hyperactivation and acrosome reaction at least in some species. At the molecular level it correlates positively with protein tyrosine phosphorylation mediated through a cAMP-dependent PKA, thus making the signaling pathway unique. Further, though capacitation is a phenomenon which occurs in spermatozoa of all mammals, differences have been observed with respect to various aspects of capacitation such as:

(a) its linkage with hyperactivation and acrosome reaction

(b) variations in the effects of modulators of capacitation at the physiological and molecular levels, and

(c) difference in the identity of the phosphorylated proteins.

Therefore, in order to get a better insight into this complex and poorly understood phenomenon studies need to be extended to acquire inputs related to the above aspects of capacitation.
Section B. Objectives

The objectives of the present thesis are to get more inputs on the molecular basis of capacitation so as to be able to understand this complex phenomenon. With this in view, studies were designed to understand the modulation of capacitation, using known positive and negative modulators of capacitation, at the level of hyperactivation, acrosome reaction, and the signaling pathway, especially with respect to protein phosphorylation using hamster spermatozoa as the model system. The specific objectives of the present thesis are:

1. To establish whether protein phosphorylation (at serine, threonine and tyrosine residue) is capacitation associated.
2. To establish linkages, if any, between hyperactivation, acrosome reaction and protein tyrosine phosphorylation using modulators of capacitation such as BSA, PVA, sodium bicarbonate, calcium and specific inhibitors of the signal transduction pathway.
3. To identify the major phosphorylated protein and to establish its sequence, localization and probable function.
4. To propose a model for hamster sperm capacitation.