CHAPTER 4

DISCUSSION
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"Fertilization is a unique event in the life of an organism and results from requisite anc
reciprocal cell-induced sperm and egg activation events mediated by unique cellular anc
environmental cues associated either with the gametes or the reproductive tract / environment".

Ward and Kopf, 1993

Thus apart from the gametes which are the two cellular components involved in
fertilization the success of the process depends also on their environment - the male and the
female reproductive tracts. It is in this context that the male gamete, the spermatozoon, is unique
in that it is under the influence of the male tract during spermatogenesis, maturation and during
ejaculation but, following deposition in the vagina or the cervix it is subjected to the environment
of the female reproductive tract. In the female reproductive tract the spermatozoa have to remain
viable, motile and also undergo capacitation, hyperactivation and the acrosome reaction. Thus
components of the female reproductive tract such as those present in the luminal fluid of the
uterus and the oviduct may ultimately influence the fertilizing ability of the spermatozoa. A
knowledge of these components and their influence on spermatozoa would help us to understand
the molecular basis of gamete interaction.

4.1 SECRETORY PROTEINS OF THE FEMALE REPRODUCTIVE TRACT

The female reproductive tract undergoes cyclic morphological, biochemical and
physiological alterations that are hormone-dependent. Earlier studies have demonstrated a
consistent increase in the uterine weight of mouse (Edgren and Calhoun, 1957), rat (Tantayaporn
et al., 1974) and hamster (Hall et al., 1977) under the influence of estradiol such as during
proestrus / estrus phase or in ovariectomised animals injected with estradiol. The present results
confirm the above observations and also demonstrate that testosterone and progesterone do not
Induce any increase in uterine weight as demonstrated earlier by Hall et al., (1977). The results also indicate an increase in the weight of the cervix and oviduct in the adult estrus female, in the estradiol-treated OVX female and in immature animals injected with estradiol. These morphological changes are probably a manifestation of the various biochemical changes that occur such as increase in the biosynthesis of proteins in uterus, oviduct and cervix under the influence of estradiol.

4.1.1 Secretory proteins of the uterus

$^{35}$S-methionine incorporation studies clearly indicate that the hamster uterus under in vitro conditions synthesizes and secretes a number of proteins into the medium. The increase in protein synthesis was time dependent and estradiol increased the synthesis above the control level. Similar increase in protein synthesis following estradiol-treatment has been demonstrated in rat (Surani, 1977a, 1977b; Komm et al., 1986; Takeda, 1988; Takeda et al, 1988), guinea-pig (Abdi-Dezfull and Poyser, 1993), mouse (Altken, 1977) and baboon (Fazleabas et al., 1988); but in dog (Buhi et al., 1992) and sheep (Murray and Sower, 1992) no significant increase was observed probably due to the small number of animals used in these experiments.

SDS-PAGE analysis of the $^{35}$S-methionine labelled media proteins secreted by the uterine horns of immature animals indicated the presence of about 35 distinct bands following Coomassie Blue staining. The number of proteins and the intensity of the bands was not altered irrespective of whether the animals were treated with estradiol, progesterone or testosterone. Further, the protein patterns was similar to that of hamster serum proteins implying that these proteins are serum contaminants carried over by the tissue during dissection. However, since ample care had been taken to avoid such a contamination it is also possible that these proteins are serum transudates in the uterus which are released during the experiment (Beier, 1974). Autoradiography of the above gels further confirmed that the bulk of the media proteins were not
labelled and hence were not synthesized and secreted by the uterus and were thus of serum origin.

The uterine horns of immature hamsters synthesized and secreted a number of proteins irrespective of the hormonal status of the animal. A few of the prominent proteins had a molecular mass of 72 kDa, 43 kDa, 38 kDa, 14 kDa and a group of proteins with a mol. wt. > 94000. Besides these proteins it was observed that estradiol induced the synthesis of a 60 kDa protein and suppressed the synthesis of a 30 kDa protein apart from suppressing to varying degrees the 14 kDa, the 72 kDa and few of the high mol. wt. proteins. The above effects of estradiol were also obvious in the two dimensional gels. Testosterone and progesterone did not bring about any such changes and the protein profile were similar to that observed in control animals. That the above changes are indeed estradiol-specific was further confirmed by similar results in OVX-hamsters treated with estradiol, in adult hamsters in the estrus stage and in the in vivo experiment with immature animals. In this experiment, immature female hamsters were injected with estradiol for 3 days and on the fourth day they were anaesthetised and 35S-methionine was injected into their uterine horns after ligations at both the ovarian and cervical ends. After 6 hours the fluid in the uterine horns was collected by aspiration and analyzed by SDS-PAGE. The synthesis of the 60 kDa protein was increased whereas the synthesis of the 30 kDa was suppressed in the estradiol-treated animals when compared to the controls (data not shown).

Uterine secretory proteins whose syntheses are regulated by estradiol have been described in several mammals such as in rat (Komm et al., 1986; Takeda, 1988; Takeda et al., 1988), mouse (Teng et al., 1986; Pentecost and Teng, 1987), rabbit (Beier, 1968; Krishnan and Daniel, 1967); sheep (Murray and Sower, 1992), cat (Bareither and Verhage, 1980; Verhage and Fazleabas, 1988; Verhage et al., 1988, 1989), baboon (Fazleabas and Verhage, 1987) and in human beings (Bell, 1986; Fay and Grudzinskas, 1991). However, even in these earlier studies very few proteins were amenable to a detailed characterization probably due to the minute
amounts which were secreted. Extensive studies have indicated that the major estradiol-induced protein in the uterus of rat was (structurally related to) complement component C3 (Komm et al., 1986; Kuivanen et al., 1989; Sundstrom et al., 1989a, 1989b) and in mouse it was lactotransferrin (Teng et al., 1986; Pentecost and Teng, 1987). In human beings the secretory proteins of uterus which are induced by estradiol include prolactin (Bell, 1986; Randolph et al., 1990), lipocortin (Gurpide et al., 1986), CA 125 (Weintraub et al., 1990) and secretory component (Sullivan and Wira, 1983; Sullivan et al., 1984). The estradiol-induced 60 kDa protein secreted by the hamster uterus is different from the above reported proteins with respect to its mol. wt. which is close to that of the 65 kDa protein in rat (Komm et al., 1986) and the 57 kDa protein in sheep (Murray and Sower, 1992). But like other estradiol-induced proteins which were not induced by testosterone or progesterone as in rat (Komm et al., 1986; Brown et al., 1990; Wheeler et al., 1987), sheep (Murray and Sower, 1992), baboon (Fazleabas et al., 1988), guinea pig (Ogilvie et al., 1991; Abdi-Dezfulli and Poyser, 1993) and dog (Buhi et al., 1992) the 60 kDa protein of hamster was also not induced thus confirming that its induction is estradiol-specific. The physiological function of these estradiol-induced proteins is not known though they may be involved in the maintenance of a sterile environment (Brown et al., 1990), in transport of iron (Pentecost and Teng, 1987) or influence spermatozoal motility and capacitation (Boomsma and Verhage, 1987).

In hamster, estradiol was also observed to suppress the synthesis and secretion of a 30 kDa protein by the uterus which was normally present in control animals and animals treated either with progesterone or testosterone. An acidic protein cP1 (mol. wt. 37000) in dog uterus (Buhi et al., 1992) and a 40000 mol. wt. protein in mouse (Quarmby and Korach, 1984) were also shown to be suppressed by estradiol. Further, unlike in rat (Sharpe et al., 1991), guinea pig (Abdi-Dezfulli and Poyser, 1993), rabbit (Beier, 1968; Krishnan and Daniel, 1967), cat (Murray et al., 1985; Verhage et al., 1989; Boomsma and Verhage, 1987), sheep (Murray and Sower, 1992), pig (Roberts and Bazer, 1984, 1988), baboon (Fazleabas et al., 1988) and man (Bell, 1986;
Seppala et al., 1987), progesterone did not induce the synthesis of any secretory protein in the uterus of the hamster.

4.1.2 Secretory proteins of the cervix

The efficiency of sperm transport from the vagina to the oviduct is dependent on the nature of the mucus secreted by the cervix the consistency of which varies depending on the levels of estradiol and progesterone (Harper, 1988). The cervical mucus consists of proteins and other low mol. wt. compounds (sugars, peptides, lipids etc.) which are dispersed in the aqueous phase of the mucous gel. Despite its important role hardly any studies have been directed towards characterizing the secretory proteins of the cervix. In hamster, the cervix i.e., the region extending from the junction of the uterine horns upto the vagina secreted a number of proteins and were similar to that secreted by the uterine horns. Like in the uterine horns a protein of mol. wt. 60000 was also induced in the presence of estradiol in addition to two other proteins (mol. wt. 38000 and 56000 respectively). Further, the 30 kDa protein was not suppressed by estradiol. Progesterone and testosterone did not induce or suppress the synthesis of any proteins. In rat the proteins secreted by the uterine horns and cervix following estradiol treatment were identical (Komm et al., 1986) though differences were evident in hamster.

4.1.3 Secretory proteins of the oviduct

The oviduct is a fluid-filled compartment which provides a conducive environment for fertilisation and early development. The volume of fluid and its contents varies depending on the concentration of circulating steroid hormones (Hunter, 1980). The main components of the oviductal fluid are proteins (Aitken, 1979), amino acids (Thibault, 1972), electrolytes (Borland et al., 1980) and various energy substrates (Hamner, 1973). The bulk of the proteins in the tubal fluid were identified as serum transudates though specific proteins were also synthesized and secreted depending on the estrous cycle or the hormonal status of the animal as in mouse (Kapur
and Johnson, 1985), hamster (Robitaille et al., 1988), rabbit (Feigelson and Kay, 1972; Beier, 1974; Hyde and Black, 1986), sheep (Sutton et al., 1984; Buhi et al., 1990, 1991; Roberts et al., 1976), cow (Gerena and Killian, 1990; Roberts et al., 1975), baboon (Fazleabas and Verhage, 1986; Verhage and Fazleabas, 1988), monkey (Mastroianni et al., 1970) and woman (Moghissi, 1970; Verhage et al., 1988; Lippes and Wagh, 1981).

The present results also indicate that the hamster oviduct secretes a number of proteins which constitute only a small proportion of the total proteins and hence were not detectable on SDS-PAGE gels following Coomassie Blue staining. Further, like in the uterus and cervix, the 60 kDa protein was estradiol-induced. Robitaille et al. (1988), in their study on hamster oviducts, did not investigate for the presence of secretory proteins with respect to various hormone treatments. Hence, a comparison of results would not be correct. However, their results did indicate a prominent 43 kDa protein and a protein with a molecular mass of 160 to 250 kDa which was termed 'oviductin' (Kan et al., 1989). A number of proteins in the high mol. wt. range (> 115 kDa) have also been detected in the hamster, mouse (Kapur and Johnson, 1985), sheep (Buhi et al., 1991), pig (Buhi et al., 1990) and baboon (Fazleabas and Verhage, 1986). Apart from estradiol-induced proteins the oviduct of mammals are also known to secrete proteins which are estradiol-suppressed such as the 46 kDa protein in sheep (Buhi et al., 1991) or progesterone-induced such as the 60 kDa protein in sheep (Buhi et al., 1991). However, in the hamster none of the secretory proteins of the oviduct were either estradiol-suppressed or progesterone-induced.

The exact function of oviductal secretory proteins is still unknown but they are likely to be involved in various functions of the gametes such as the acrosome reaction (Parrish et al., 1989a and b) of spermatozoa. Oviductal secretory proteins of high mol. wt. range in rabbit, mouse, hamster, baboon and sheep are capable of associating with the zona pellucida of the ovulated oocytes and have thus been implicated in gamete interaction (Gandolfi et al., 1989; Kapur and Johnson, 1985; Leveille et al., 1987; Robitaille et al., 1988) and early development.
4.1.4 Proteins of the uterine luminal fluid

In many mammals accumulation of fluid in the uterus at estrus or following estrogen stimulation (Long and Evans, 1922; Blandeau, 1945; Odor and Blandeau, 1949) is well documented. Though threshold levels of estrogen are known to be required for the formation, retention (Armstrong, 1968; Kennedy, 1974) and increase in the intraluminal protein content (Hasegawa et al., 1973) it is yet not clearly understood as to what regulates the differential passage of various serum proteins into the uterine lumen at various stages of the estrous cycle (Hall et al., 1977; Beier, 1974). This fluid is rich in proteinaceous material and includes selective serum transudates (Beier, 1974) and proteins which are synthesized in the uterus as demonstrated in the present thesis.

Uterine fluid is generally collected by any one of the following methods: flushing or washing out with isotonic saline, absorption by filter paper, aspiration under negative pressure, ligating the distended uterus prior to recovering the fluid, by cannulating the uterus and by the use of Intrauterine chambers (Kulangara, 1976; Surani, 1977a and b; Teng et al., 1986; Hall et al., 1977; Murray and Sower, 1992; Davis, 1982; Liu and Godkin, 1992; Verhage et al., 1989). However, in hamster, none of the above methods was suitable since it accumulated very little fluid (about 5 µl) and also preliminary attempts to procure fluids by flushing with PBS did not yield substantial volume of proteins. Hence a simple method was resorted to wherein the cervical ends of the uterine horns were ligated and the accumulated fluid aspirated out. By this method the volume of fluid recovered was comparable to that reported for rat (Surani, 1977b) and mouse (Teng et al., 1986) and the protein concentration was about 5 mg / ml. Further, variation in the volume of fluid collected and its protein concentration was observed depending on the steroid hormone administered. But, the protein profile on SDS-PAGE was the same for all animals and a 67 kDa protein was present in maximum amount. This protein was not labelled in the
autoradiograms of the secretory proteins of uterine horns thus indicating that it is probably a selective transudate into the uterine lumen from the serum.

The 67 kDa protein was purified to homogeneity from the uterine fluid and its N-terminal analysis revealed a striking similarity to rat and hamster serum albumins. Antibodies against the 67 kDa protein of the hamster uterine fluid specifically cross-reacted with the 67 kDa protein present in the uterine fluids of rats and mice as well as with that present in the sera of mouse, rat and hamster and BSA. These data give proof that the major protein present in the hamster uterine fluid is albumin and thus confirms previous reports of albumin being a major component in mammalian uterine fluids (Beier, 1974; Hall et al., 1977) as in hamster (Hall et al., 1977), rat (Surani, 1977b; Teng et al., 1986), rabbit (Davis, 1982) and ewes (Voglmayr and Sawyer, 1986) etc. Earlier studies in hamster have indicated that uterine fluid proteins exhibit variations depending on the estrous cycle thus reflecting a hormonal dependence. In fact, studies with ovariectomised hamsters have indicated that albumin and transferrin are the major proteins in the uterine fluid and maximum albumin was observed in OVX animals treated either with progesterone or progesterone plus estradiol (Hall et al., 1977). The present study confirms the observations of Hall et al. (1977) regarding the hormonal dependence of serum albumin in uterine fluid. These proteins in the uterine fluid have been investigated due to their possible role in two processes, namely transport of spermatozoa in the female reproductive tract and nurture of early foetus (Toner and Adler, 1985; Beier, 1974).

4.2 ANALYSIS OF SPERMATOZOAAL MOTILITY DURING MATURATION AND HYPERACTIVATION

Computer assisted sperm analysis (CASA) system was used in this work for objective analysis of spermatozoa. Although the development of this technique is relatively recent, the computer aided sperm motility analysis system (CASA) has now been extensively applied for
human semen analysis (Vantman et al., 1989; Tesarik et al., 1992; Lewis et al., 1993) and has been found to be more discriminatory, rapid and easier than the routine semen analysis (Mathur et al., 1986). However, these studies have clearly highlighted the importance of standardization with respect to the parameter settings of the instrument (Knuth et al., 1987) and standardization of measurement conditions (Mack et al., 1989) so as to enable the possibility of sperm analysis in multicentre studies without any significant differences (Davis et al., 1992).

Compared to the work done on human spermatozoa, the number of investigations carried out in other mammalian species using CASA is rather small. The evaluation of spermatozoal motility using this technique has been done in bull (Stephens et al., 1988), rat (Slott et al., 1991; Yeung et al., 1992) and rabbit (Young et al., 1992). Although high-speed videomicrographic analysis of hamster sperm motility has been carried out (Drobnis et al., 1988; Katz et al., 1986; Suarez, 1988) data on the quantitation of the motility parameters of hamster spermatozoa is limited. In the present investigation, this technique has been used to study the motility of hamster spermatozoa during maturation and capacitation.

4.2.1 Motility characteristics of hamster caput, corpus and cauda epididymal spermatozoa

The instrument (HTM-S Motility Analyzer, Version 7.2) had to be standardized to identify properly the motile and non-motile spermatozoa of hamster. Since the size and morphology of spermatozoa vary from species to species, each of the parameters for analyzing motility had to be set with respect to hamster. Subsequently, spermatozoal motility and their maintenance at different stages of sperm maturation and capacitation were analyzed. Maturation studies were done using spermatozoa collected from different tissues of the male reproductive tract. Maintenance of hyperactivated motility was investigated using BSA and the synthetic polymer PVA as well as the standard medium for hamster spermatozoa, namely Tyrode’s Lactate Pyruvate (TLP).
Only one type of motility pattern was observed in caput spermatozoa in which the spermhead, along with the midpiece, kept bending backwards and coming back, thereby assuming an 'U' shape, with very little progression. This was similar to that described by Yanagimachi et al. (1983) for caput spermatozoa of Chinese hamster. Our results also confirmed that most caput spermatozoa are immotile and those that move do so at an extremely sluggish rate (Mohri and Yanagimachi, 1980) and their flagella beat with a low amplitude and frequency (Ishijima and Mohri, 1985). This pattern of motility was not analyzed quantitatively earlier probably due to the inability of these spermatozoa to survive for long periods of time (Suarez, 1988) as observed (as in this thesis). But, using the HTM-S analyzer it was possible to analyze their motility. In this type of movement, the velocities were very low but the linearity and straightness were fairly high, indicating lack of circular trajectory. The reason for the lack of sustenance of the motility of the caput spermatozoa is not yet clear. The motility status may be influenced by the availability of energy sources and other metabolites like cAMP and ATP (Hoskins et al., 1975; Acott et al., 1983; Ishijima and Mohri, 1985; Ishida et al., 1987) or the presence of a protein factor in the epididymal fluids (Turner and Giles, 1982; Usselman and Cone, 1983; Turner and Reich, 1985). Previous studies had demonstrated that the motility of hamster caput spermatozoa could be maintained by manipulating the in vitro conditions (Cornwall et al., 1986).

The corpus spermatozoa, in addition to exhibiting a motility pattern similar to the caput spermatozoa also exhibited rapid wriggling movements resulting in greater VAP and progression. These spermatozoa resembled the darting pattern of hamster sperm movement described by Corselli and Talbot (1986). Thus, there seems to be a transition from sluggishness to more rapid forward movement as the spermatozoa enter the corpus epididymis. But these spermatozoa lack the ability to sustain this movement. In rat, the acquisition of progressive motility in the corpus
epididymal spermatozoa is more distinct (Hinton et al., 1979) and also coincides with the acquisition of fertilizing ability (Dyson and Orgebin-Crist, 1973).

The majority of the spermatozoa collected from the proximal cauda epididymis moved in a curved path and within 1 hour majority of them had attained the fast circular pattern of movement which persisted for about 4 h. Thus a dramatic increase in maintenance of motility and a change in pattern of motility was seen in proximal cauda spermatozoa compared to spermatozoa from the caput and corpus. These changes are probably influenced by the components of the male reproductive tract secretions as in bull, where a forward motility protein added to the spermatozoa during epididymal transit aids in the development of progressive motility (Acott and Hoskins, 1981; Acott et al., 1979).

Thus in hamster, as in other mammals spermatozoa gradually acquire beating capacity during maturation in the epididymis (Moore, 1983; Cooper, 1986; Eddy, 1988) probably brought about by biochemical modification of the axoneme and/or by alteration of the intracellular environment in which the axoneme functions (Ishijima, 1990). In order to ascertain whether the axoneme itself (which is the motile machinery of the spermatozoon) had developed its functional capacity before the spermatozoon has acquired its motility in the epididymis, the method of demembranation and reactivation was employed with golden hamster and ram spermatozoa (Ishijima and Mohri, 1985, 1986; Ishijima et al., 1986; Mohri and Yanagimachi, 1980). The results obtained from these investigations indicated that the motility machinery was already functionally assembled in the immotile caput or feebly motile testicular spermatozoa as well as in actively motile cauda epididymal spermatozoa. Thus the inability of the immature spermatozoa to exhibit motility with intact plasma membranes seems to be due to some other reason related to inhibition of active motility. Experimental evidence obtained by Ishijima (1990) suggest that the beat frequency of reactivated spermatozoa depends on the concentration of MgATP$^-$ and that Ca$^{++}$
and cAMP are able to regulate the amplitude of bending waves concomitant with an increase in flexibility of the flagellum at the midpiece region.

Spermatozoa from the distal cauda epididymis resembled the freshly ejaculated spermatozoa in their movement characteristics (Horan and Bedford, 1972) and exhibited relatively high values for trackspeed, linearity and straightness and lower ALH values. Thus the distal cauda spermatozoa exhibited a progressive motility more linear than that of proximal cauda. In hamster, as in other mammals, the spermatozoa are known to acquire the ability for circular movement first and only subsequently start to swim progressively (Gaddum, 1968; Fray et al., 1972; Acott et al., 1983). Hence, the later acquisition of hyperactivated circular movement is probably an induction of a preexisting potential rather than an acquisition of a new motility pattern (Suarez, 1988).

4.2.2 Motility characteristics of hyperactivated spermatozoa

Mammalian spermatozoa in vitro following incubation under appropriate conditions are known to undergo a distinct change in motility termed as hyperactivation (Yanagimachi, 1969a and b, 1970, 1981). Since acquisition of hyperactivated motility is a morphological indicator of capacitation (Yanagimachi, 1969a and b, 1970), efforts were made to characterize the patterns of hyperactivated motility and to distinguish them from non-hyperactivated spermatozoa in human semen samples (Robertson et al., 1988; Burkman, 1991). Morphologically normal spermatozoa exhibited a significant change in their movement pattern, i.e., hyperactivation, after six hours of incubation in capacitating medium in vitro (Morales et al., 1988). This change was reflected in the spermatozoa swimming trajectories, which become less straight (lower linearity), exhibit greater degree of lateral head displacement about the average path, higher degree of bending in the proximal flagellum and an increase in beat amplitude and curvilinear velocity. In general, the swimming trajectories of human spermatozoa during in vitro capacitation indicated a tendency for
increasing curvature in the track (Burkman, 1984) and lateral motion of the head along its path (Aitken et al., 1982; Mortimer et al., 1984). In hamster, hyperactivation of the spermatozoa is normally seen after 1 to 2 hours and reaches a maximum by 4 hours (Llanos and Meizel, 1983; Katz et al., 1986; Cummins and Yanagimachi, 1986; Cherr et al., 1986; Suarez et al., 1984; 1986; Suarez, 1988).

In the present investigation hyperactivated spermatozoa characterized by their distinct circular movement were first observed after 1 h and by 2 h of incubation, about 70% of the spermatozoa were moving in small circles. Earlier studies had also indicated that in hamster spermatozoa hyperactivation transforms the linear progressive type of motility to a nonprogressive type of motility with whiplash like beatings of the tail (Yanagimachi, 1981) resulting in spermatozoa that exhibit various motility patterns such as 'circular', 'helical', 'darting' and 'figure-of-eight' (Yanagimachi, 1969a and b; 1981; Suarez et al., 1984; Suarez, 1988; Drobnis et al., 1988; Gwatkin and Anderson, 1969). In the present study it has been observed that the hyperactivated spermatozoa are predominantly of two distinct types, the SCM and the FCM spermatozoa. The FCM spermatozoa, like the helical spermatozoa, exhibited rotation of the head to an extent of 90\(^{\circ}\) (but not 180\(^{\circ}\)) and also resembled the hyperactivated spermatozoa of hamster with respect to the average path velocity (Suarez, 1988). The variations observed by us may be either due to the fact that the earlier data was obtained at 60 frames/sec using a videomicrographic method compared to 25 frames/sec which was the upper limit using the present automatic computer aided semen analysis system or because the media used were different. Human spermatozoa following hyperactivation were also extremely vigorous with high curvature flagellar bends but were less progressive and their motility was described as 'crawling', 'serpentine', 'whiplash', 'circling', 'helical', 'thrashing' and 'starspin' (Burkman, 1991); 80% of all hyperactivated spermatozoa exhibited the circling pattern (Burkman, 1984). There has also been some discrepancy with regard to 'invigoration' or increase in beat frequency of hamster spermatozoa occurring during capacitation (Katz et al., 1986; Suarez, 1988).
The two classes of hyperactivated spermatozoa (SCM and FCM) in the present experiment could be differentiated from one another and the nonhyperactivated progressively motile spermatozoa based on a number of motility parameters because of distinct differences in velocity, linearity, straightness and amplitude of lateral head displacement. This has become possible because apart from VAP, VSL and STR for hamster hyperactivated spermatozoa which have been manually analyzed earlier (Suarez, 1988), the present study has also acquired additional information with respect to VCL, LIN, BCF and ALH. Linearity, velocity and head displacement have been used earlier to differentiate and sort-hyperactivated human spermatozoa from nonhyperactivated spermatozoa (Burkman, 1991). Normally hyperactivated spermatozoa which move in circles are less progressive and thus have a low VSL and LIN. In the present study the FCM, SCM and the hatchet spermatozoa exhibited significant reduction in VSL, LIN and STR compared to the nonhyperactivated spermatozoa, thus confirming earlier studies. The LIN of hyperactivated human spermatozoa is also low (Morales et al., 1988). Further, an interesting observation has been the increased ALH (> 8 μm / sec) seen in hyperactivated spermatozoa and this single parameter could also be considered as a characteristic feature of FCM spermatozoa since all these spermatozoa (unlike the nonhyperactivated and the SCM spermatozoa) exhibited ALH > 8 μm / sec. The hyperactivated spermatozoa in hamster and human are similar in that they have high VCL, low VSL and high ALH (Tesarik et al., 1990; Morales et al., 1988). Similar changes with respect to increased ALH and reduction in progressive motility has been observed during hyperactivation of spermatozoa from guinea-pigs (Katz et al., 1978 a and b; Katz and Yanagimachi, 1981), mice (Fraser, 1977), monkeys (Behboodi et al., 1987), rabbits (Johnson et al., 1981; Suarez et al., 1983), rams (Cummins, 1982) and also in hamsters (Katz et al., 1978a and b; 1986; Suarez et al., 1984). Increased ALH in hyperactivated spermatozoa is a significant functional change and has been correlated with the fertilizing ability of human spermatozoa (Aitken et al., 1985; Jeulin et al., 1986; Mortimer et al., 1986). The third motility pattern observed was that of spermatozoa exhibiting a hatchet-like
stroke of the head which was very rare. Such spermatozoa were observed in the cumulus matrix by Drobnis et al. (1988) who suggested that the extreme bending of the midpiece during the hatchet strokes generates high-amplitude movement of the sperm head (like the high ALH of FCM) which may mechanically weaken and cut through the cumulus and facilitate spermatozoal movement (Drobnis et al., 1988).

Hyperactivation of spermatozoa may be an absolute necessity so that the spermatozoa successfully encounter the various barriers prior to binding and fusing with the oocyte. The hyperactivated motility patterns observed were 'figure of eight' and 'darting' in the oocyte-cumulus complexes (Corselli and Talbot, 1986), the hatchet type in the cumulus matrix (Drobnis et al., 1988) and spermatozoa that beat their tail with high beat frequency and acute bending as in the zona pellucida (Katz et al., 1986). Further, there is evidence to indicate that hyperactivation is beneficial to spermatozoa for detaching from the oviductal mucosa, for penetrating viscoelastic substances in the oviduct and for penetrating the zona pellucida (Suarez et al., 1993). More recently it has also been considered that hyperactivation may be a type of chemotactic response in mammalian spermatozoa (Suarez et al., 1993). In fact, studies have indicated 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. However, comparatively little is known about the molecular mechanism of hyperactivation except that it could be induced by the calcium ionophore A23187 (Suarez et al., 1992), that intracellular calcium increases in hyperactivated spermatozoa and that the Ca$^{2+}$ levels oscillate with the flagellar beat cycle (Suarez et al., 1993).

4.2.3 Media for use with hamster spermatozoa

Spermatozoa collected from the distal cauda epididymis represent those which have undergone the maturation processes in the male reproductive tract and have attained the potential to fertilize eggs (Horan and Bedford, 1972). Hence, mature swim up spermatozoa from
the distal cauda were used for all the studies involving interaction with female tract proteins. Preliminary, studies were carried out initially to ascertain the suitability of different media (TLP, TALP, TLP-PVA and TALP-PVA) with respect to spermatozoal motility (Table 11). The percentage of motile spermatozoa and the sustenance of motility was very poor in TLP but was best maintained (50% by the end of 6 hours) in TLP-PVA and TALP-PVA. TALP in the absence of PVA was not as efficient (37%). Earlier studies had indicated that BSA is necessary for maintaining the quantity and quality of spermatozoal motion in vitro (Bavister, 1973; Miyamoto and Chang, 1973), though the actual mechanism of its action has not yet been elucidated. Bavister (1981a and b) had demonstrated that the synthetic polymer PVA could substitute for BSA in maintaining the motility of the spermatozoa but differed from BSA in that it could not induce acrosome reaction in spermatozoa. In fact the present results confirm the beneficial role of PVA in sustenance of spermatozoal motility.

The swim up spermatozoa collected in TLP, on dilution with the respective medium, initially showed a reduction in motility but subsequently the spermatozoa started moving uniformly in small circles with lower track speed and ALH typical of hyperactivated SCM spermatozoa. The flagellar beat was also not vigorous. The SCM spermatozoa were a transient population visible only for a few minutes exclusively in media supplemented with BSA or PVA. However, by two hours almost all the motile spermatozoa started moving fast in a circular path and the diameter of the circular path was similar both in the SCM and FCM spermatozoa. Majority of the FCM spermatozoa exhibited high track speed, high amplitude of lateral head displacement and vigorous beating of flagellum with principal and reverse bends (Woolley, 1977; Woolley and Osborn, 1984) typical of hyperactivated spermatozoa. Thus hyperactivation was observed in all media and no significant difference was observed (Table 11).
4.3 INTERACTION OF PROTEINS OF THE FEMALE REPRODUCTIVE TRACT WITH HAMSTER SPERMATOZOA

Proteins of the female reproductive tract may interact with spermatozoa and thus influence processes associated with fertilization like sperm transport, motility and capacitation. For this purpose an in vitro method was adopted in which the effects of female reproductive tract components were checked on various parameters of spermatozoa. In hamster fertilization is noticed approximately 4 to 6 h after insemination in vivo (Cummins and Yanagimachi, 1982) and therefore observations regarding motility, hyperactivation and acrosome reaction were made up to 6 h of in vitro incubation.

4.3.1 Influence of uterine fluid proteins on sustenance of motility, hyperactivation and acrosome reaction of spermatozoa

Based on their observations on the initiation of spermatozoal motility after mating in the rat and the hamster, Bedford and Yanagimachi (1992) concluded that the uterine fluid is a primary activating agent of spermatozoal motility in the rat, whereas in the hamster active motility developed only in the oviduct. The fluid retained in the rat uterus under natural conditions is known to be secreted by the endometrium and oviduct (Coutinho, 1974) and is retained in the uterus at estrus by the high degree of muscle tone at the cervix (Blandeau, 1945) and the uterotubal junction (Gaddum-Rosse, 1981). In hamster, though fluid secretion is increased manifold in estrus (Hall et al., 1977), its retention in the uterus is poor (present work) and hence the recovery by aspiration was poor. But, substantial volumes of uterine fluid could be obtained by the method of cervical ligation. Fluid thus obtained has been evaluated for its effects on hamster spermatozoa.

Total uterine fluid of hamster could sustain the motility and induce hyperactivation and acrosome reaction of hamster spermatozoa in a concentration dependent manner (Table 12).
Results with 1 mg UF/ml of TLP were comparable to results obtained with TALP (containing 3 mg BSA/ml of medium); thus clearly indicating that uterine fluid could be used as a substitute for BSA in the medium. However, uterine fluid devoid of the Blue Sepharose bound proteins (which would include many serum proteins) was not as effective as total uterine fluid. This would clearly imply that the Blue Sepharose bound proteins which includes the 67 kDa protein may have an important role to play.

The 67 kDa protein purified from uterine luminal fluid, and identified as hamster serum albumin, however, was able to maintain motility and induce hyperactivation only upto 4 h after which there was a decrease; but acrosome reaction induction was as observed in TALP. BSA and other fluids like sera from blood and follicular fluid are known to aid in capacitation, acrosome reaction and fertilization in vitro in various species (Barros and Garavagno, 1970; Miyamoto and Chang, 1973; Lui et al., 1977). For instance, serum albumin of various species could induce acrosome reaction in mouse spermatozoa (Miyamoto and Chang, 1973). Albumin was identified as the bovine follicular fluid protein involved in the induction of acrosome reaction in vitro, in hamster spermatozoa (Lui et al., 1977).

The exact mechanism by which albumin influences motility, capacitation and acrosome reaction of spermatozoa is not clearly known. Serum albumin has been identified as the uterine sterol acceptor for binding cholesterol from spermatozoa of rat (Davis et al., 1979), rabbit (Davis, 1982) and mouse (Go and Wolf, 1985) under capacitating conditions, thereby modulating the lipid levels in spermatozoa which is a biochemical event occurring during capacitation. It has been proposed that such modulations would lead to a decreased cholesterol/phospholipid ratio in the plasma membrane of spermatozoa, which in turn would facilitate a Ca^{2+} influx and induce the acrosome reaction (Davis, 1981). Dow and Bavister (1989) showed that direct contact was required between BSA and hamster spermatozoa for capacitation in vitro. Apart from serum
albumin, Ravink et al. (1992) have identified a 64 kDa lipid transfer protein from human follicular fluid that supports capacitation of human spermatozoa and proposed (Ravinik et al., 1993) a concerted action of this protein with albumin in aiding capacitation.

All these results taken together point to the following deductions. The observation that the uterine fluid could maintain motility, hyperactivation and induce acrosome reaction like TALP and the 67 kDa protein purified from the uterine fluid also could act in vitro in a similar manner, showed that this protein must be the one which is responsible to a large extent for the maintenance of motility, capacitation processes and transport of spermatozoa through the uterus of female hamsters. The fact that the flow through fraction, comprising a mixture of the rest of the proteins of the uterine fluid, cannot maintain motility and hyperactivation by itself, gives additional proof for the requirement of the protein albumin in the processes leading to fertilization in the in vivo environment of the female reproductive tract.

4.3.2 Influence of uterine fluid proteins on motility characteristics and motility patterns of spermatozoa

The parameters of spermatozoal movement which could be analyzed and quantitated by the HTM-S include the velocity, linearity, straightness, flagellar beat frequency and amplitude of lateral head displacement. These parameters assume importance due to the fact that a relationship may exist between these and the final fertilizing ability of spermatozoa (Holt et al., 1985; Mortimer et al., 1986; Burkman, 1984). The motility parameters of the non-hyperactivated spermatozoa and the hyperactivated spermatozoa were not altered irrespective of the medium used (TALP, TLP plus uterine fluid, TLP plus 67 kDa and TLP plus uterine fluid - Blue Sepharose bound proteins). The hyperactivated spermatozoa swam in circles and showed the characteristic decrease in linearity and increase in ALH. These changes were observed even at low concentrations of UF (300 µg/ml). The spermatozoa treated with 67 kDa protein showed an
increased mean ALH of 16 μm compared to 14 μm for spermatozoa incubated in TALP and other uterine fluid treatments. This increase, although small, may be significant since high ALH is correlated with increasing fertilizing ability in spermatozoa (Mortimer et al., 1986; Jeulin et al., 1986).

Spermatozoa of hamster following hyperactivation in TALP exhibited predominantly two motility patterns viz., slow circular movement (SCM) and fast circular movement (FCM); but very rarely, spermatozoa moving progressively along a straight path or in a circular path and exhibiting hatchet like strokes of the head were also observed. During the 6 h of incubation some spermatozoa which were moving at a high speed appeared to switch from one pattern of movement to another (e.g. circular to linear to circular etc). This may correspond to the multiphasic phenomenon described for human spermatozoa by Burkman (1991) as erratic switching of one pattern to another. Quantitative data analysis of these with the existing software was not possible.

In media containing uterine fluid also, the circular moving spermatozoa (SCM and FCM) were the most common hyperactivated spermatozoa but the hatchet pattern was present in more numbers compared to TALP and media containing 67 kDa protein. The proportion of hatchet type to circular in the media containing uterine fluid was about 30%. Further, SCM spermatozoa persisted for a longer time in the presence of uterine fluids (upto half an hour) compared to other media. Although visually the path taken by these SCM and FCM spermatozoa appeared circular, in reality they may be taking helical turns as suggested by Suarez (1988). The observations of a particular type of motility in specific treatments is not uncommon. Phillips (1972) observed crawling or snakelike motility of mouse spermatozoa recovered from the uterus or oviduct after insemination. Detailed studies clearly demonstrate that pattern of motility of spermatozoa vary depending on the in vivo site (such as the cervical mucus, uterine fluid, oviductal fluid, cumulus oophorus and zona pellucida) and on the composition of the medium under in vitro conditions.
For instance, human spermatozoa on exposure to solubilized cumulus intercellular matrix, started moving in a pattern characterized by very high curvilinear and progressive velocities (Tesarik et al., 1990). Very recently Suarez et al. (1991, 1992) demonstrated that physicochemical properties of the fluids such as viscosity could cause the change in patterns of movement. This could also account for the heterogeneity of patterns observed in uterine fluids from various animals.

The mammalian spermatozoa has to pass through the cervical mucus, cumulus oophorus and the zona pellucida in the female reproductive tract before it could fuse with the egg. For this the spermatozoa would need to push or thrust their way against these materials and this thrust may vary, depending on the component of the female tract to be traversed (Green, 1988; Katz et al., 1978a and b). Thus mechanics of spermatozoal movement would involve a balance between active sperm forces (generated by sperm flagellar undulations and thrust of sperm head) and their consequent resistances from the secretions of the female tract and ovum vestments (Katz and Drobnis, 1990; Katz et al., 1989). The passage of spermatozoa from the site of insemination to the site of fertilization is achieved by the active swimming of the spermatozoa as well as by the contractions and ciliary activity of the female reproductive tract (Overstreet, 1983; Katz et al., 1989). In rats, the uterine fluid is known to aid in the contraction of the uterus (Toner and Adler, 1985). The efficiency of spermatozoal transport in the female will therefore depend on the movement potential of the spermatozoon and the environment of the female reproductive tract. To cite a few examples, the cervical mucus is most easily penetrated by the spermatozoa at estrus (Green, 1988) and the flagellar beat of spermatozoa in mucus is different from that in semen (Katz et al., 1981, 1982) which is supposed to be due to the viscoelastic properties of the mucus. Hyperactivated mouse spermatozoa could penetrate viscoelastic media more efficiently than fresh spermatozoa and therefore may be more efficient at penetrating oviductal mucus and cumulus matrix in vivo (Suarez and Dai, 1992; Suarez et al., 1992).
4.3.3 Influence of other female tract proteins

Now it remains to be determined whether other proteins of the uterine fluid or the oviductal fluid have any role to play, in the processes leading to fertilization. Earlier studies have indicated that capacitation of ram spermatozoa is influenced by a polypeptide in uterine fluid (Voglmayr and Sawyer, 1986). More recently Schnitzer (1992) and Schnitzer et al. (1992) have reported the existence of glycoproteins of molecular weights 60 kDa, 30 kDa and 18 kDa in various tissues of mice which have the ability to bind albumin and have postulated a role for the 60 kDa protein in transcytosis of albumin along the continuous endothelia of body organs (Schnitzer, 1992). The 60 kDa and 30 kDa proteins of the hamster female reproductive tract whose synthesis is estradiol-dependent, could be any of the above proteins described by Schnitzer (1992).

Previous workers have observed that as spermatozoa pass through the oviduct some of them transiently attach to the oviductal epithelium periodically before resuming to move again (Katz and Yanagimachi, 1980; Smith and Yanagimachi, 1989) thus hinting at the probable role of structural proteins of the uterine endothelium lining the lumen in spermatozoal transport. In hamster, the movement of spermatozoa in the oviduct involved episodes of whip-lash like flagellar bending punctuated by periods of more progressive swimming (Katz and Yanagimachi, 1980). The trajectories of these spermatozoa seemed to be influenced by the epithelial surfaces of the oviduct, suggesting that the interaction between the spermatozoa and these surfaces could play a role in oviductal sperm transport. The stage of the reproductive cycle also seems to matter in oviductal sperm transport as the spermatozoa resident in the oviductal isthmus prior to ovulation could be stimulated to undergo activated motility while those remaining in the isthmus were no longer able to respond (Overstreet et al., 1980). Thus the proteins of the female reproductive tract could be affecting the number of spermatozoa finally reaching the egg, as also aiding them in attaining the type of motility most fit for transport through that particular region of the female reproductive tract.
4.3.4 Some unanswered questions

Millions of spermatozoa are deposited in the female tract of mammals during insemination. Katz et al. (1981, 1982) have analyzed the movement characteristics of bull and human spermatozoa in penetrating cervical mucus at estrus and identified a subpopulation of initial or "vanguard" spermatozoa which swam more rapidly and efficiently than the remaining spermatozoa. The authors proposed that this difference in propulsive efficiency could be due to an alteration in local mucus properties resulting from sustained permeation of spermatozoa. Similarly, in the spermatozoa which are transported through the other secretions of the female tract also, various subpopulations having different motility patterns could be formed depending on the respective resistances offered. The in vitro observations have to be correlated with experiments in vivo before any deductions regarding the function could be made conclusively.

Another question which arises from studies on maturation and capacitation is whether successful fertilization requires a particular sequence of motility. Did the spermatozoa that reach their final destination, namely the ovum, score over the others due to some coordination in time and space? That is, did these spermatozoa have the advantage of undergoing the specific type of motility required at a specific region of the reproductive tract, eg. hatchet-like instead of circular, in the uterus?

Our studies indicate that the proteins of the uterus play an active role in spermatozoal transport. Exactly what happens under in vivo conditions is yet to be determined.