CHAPTER 2
MATERIALS AND METHODS
2.1 Materials

2.1.1 Chemicals

Acrylamide, bis-acrylamide, agarose, penicillamine, hypotaurine, epinephrine, hypotaurine, epinephrine, calcium chloride, lactic acid, sodium metabisulphite, nicotinamide adenine dinucleotides (oxidized-NAD, reduced-NADH), lipoamide BSA, Tri-Reagent, TEMED, ammonium persulphate, DTT all were purchased from Sigma (St. Louis, Missouri, USA). Hybond C and Hybond P were purchased from Amersham (Buckinghamshire, UK). DNA molecular weight markers were purchased from New England Biolabs (Beverly, Massachusetts, USA) while protein molecular weight and isoelectric point markers were purchased from Amersham. The alpha-tubulin antibody was obtained from Serotec (Oxford, UK). X-ray film was from Konica Corporation (Tokyo, Japan). All the other chemicals were purchased from the local manufacturer and were of high analytical grade. All the special chemicals used and their sources will be mentioned in the relevant places in section 2.2.

2.1.2 Animals

All the animals used were maintained in the CCMB animal house facility. Golden hamsters were maintained in the 14/10 (hours) day/night cycle. All animal experiments were performed with the guidelines of the Institutional Animal Ethics Committee of CCMB.
2.2 Methods

2.2.1 Sperm collection and in vitro capacitation

2.2.1.1 Preparation of Tyrode's medium

Tyrode's isotonic medium was made according to the description of Bavister (1989 #34). Only deionized water collected at 18.2 mOhms was used for the preparation of the medium. The composition of the medium is according to Table 2.1 where the amounts to be measured for 100 ml are also tabulated. All the components were measured out, deionised water added and kept for stirring for at least 3 hours. The volume of the medium was then made up and stored at 4°C.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mM)</th>
<th>Grams/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyvinyl alcohol (PVA)</td>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>10,000units</td>
<td>0.01</td>
</tr>
<tr>
<td>NaCl</td>
<td>114</td>
<td>0.001</td>
</tr>
<tr>
<td>KCl</td>
<td>3.16</td>
<td>0.066</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
<td>0.024</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.5</td>
<td>0.029</td>
</tr>
<tr>
<td>Na₂-lactate</td>
<td>10</td>
<td>0.01</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.35</td>
<td>0.185</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>0.005</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 2.1 Composition of Tyrode's medium

2.2.1.2 Preparation of motility stimulators PHE (Penicillamine, Hypotaurine Epinephrine)

In the very early studies of in vitro capacitation, the motility stimulators of jays sperm extracts were used. Motility stimulators was first described there in. The reagents in-tail were made as follows: D-Penicillamine prepared in 57 mM NaCl. Epinephrine lactate bisulphite was prepared from sodium metabisulphite in 50 ml of deionized water and the pH was adjusted to 4.0.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate bisulphite</td>
<td>1.2</td>
</tr>
<tr>
<td>D-Penicillamine</td>
<td>0.75</td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>0.75</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 2.2 Composition of PHE
thereafter. 100X stock solution of PHE was prepared according to Table 2.2 and stored at -20°C.

### 2.2.1.3 Preparation of modified Tyrode’s Medium, TALP (Tyrode’s Albumin Lactate Pyruvate)

TALP was made following Bavister’s description (1989). Each time before use, the Tyrode’s medium was sterilized by passing through a 0.22 Micron filter (Millex GV, Millipore) and pH was adjusted to 7.5 by adding concentrated HCl. The osmolality of the medium was checked to be between 290-300 mOsm and maintained in 5% CO₂ at 37°C for equilibration. After 30 minutes of equilibration, motility stimulator cocktail, PHE, was added from the 100X stock. Sodium pyruvate solution was made freshly in 0.9% NaCl and was added to the equilibrating medium such that the final concentration was 0.18mM, along with PHE. After another 30 minutes, BSA was added such that the final concentration was 3mg/ml. The medium was then aliquoted either in petridishes or microfuge tubes, as the experiment demanded.

As a modification, media with only glucose as carbon source (G) and with only pyruvate-lactate as carbon source (PL) were also prepared when required. MICA (5-methoxyindole-2-carboxylic acid) was freshly dissolved in the respective media by adding 10N NaOH drop by drop. The pH and osmolality of the medium were then set at 7.5 and 290 mOsm, respectively.

### 2.2.1.4 Sacrificing animals and preparation of spermatozoa

Experimental hamsters were anesthetized in a desiccator using diethyl ether. After total anesthesia, the animal was sacrificed by cervical dislocation and dissected using surgicals wiped clean with alcohol. The epididymidal cauda were cut out and collected in the equilibrated TALP medium. After rinsing the cauda, free of blood and unwanted particles, in a fresh aliquot of equilibrated medium, each cauda was pierced with sterile needles. The spermatozoa were allowed to ooze out from the caudal tubules, which was then collected together with a pair of bent forceps and dipped in yet another aliquot of equilibrated medium. One or two minutes hence the sperm dispersed equally in the medium to form a swim up and an aliquot was diluted 10 or 20 folds in the medium and counted in a Makler chamber using the Computer Aided Sperm Analyzer (CASA). The spermatozoa were then suspended in the required media at the required concentration, which varied with the experiment type.
2.2.1.5 \textit{In vitro} Capacitation

Hamster spermatozoa maintained in TALP in the presence of 5% CO\textsubscript{2} attained capacitation within 3 to 5 hours (Kulanand and Shivaji, 2001). For time course experiments, spermatozoal suspensions were centrifuged at different time points during capacitation for further biochemical or cell biological studies. For two state experiments, the 0\textsuperscript{th} hour sample was considered as non-capacitated while the 5\textsuperscript{th} hour sample was considered as capacitated.

2.2.2 \textit{Gel Electrophoresis} of proteins

2.2.2.1 SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was essentially carried out by following the method of Laemmli (Laemmli, 1970). A Hoefer mighty small apparatus (San Francisco, CA, USA) was used to cast and run gels. A stock of the following solutions were made: a) acrylamide: bis acrylamide (29.2: 0.8), b) 1.5 M Tris buffer pH 8.8, c) 1 M Tris buffer pH 6.8, d) 10% SDS and e) 10% APS. A 10% resolving gel and a 5% stacking gel were made by mixing the stock solutions according to Sambrook \textit{et al.}, (1989). The washed sperm pellet was resuspended in Laemmli buffer (50mM Tris, pH 6.8, 2% SDS, 10% Glycerol, 5%\textasciitilde{}mercaptoethanol and 0.01% Bromphenol blue) and boiled thereafter, for 10 minutes, in a water bath. The cellular debris was discarded as a pellet after centrifugation at 14,000 rpm for 15 minutes. The supernatant was loaded into the wells of the stacking gel. Molecular weight standards were also loaded when required. The 1X running buffer TGS (25mM Tris, 250mM Glycine and 1% SDS) was made fresh by diluting from a 10X stock and the gel was run for 20 mA for stacking and 30mA for resolving the sample. Electrophoresis was terminated when the bromphenol blue dye of the samples reached the bottom edge of the resolving gel and was further processed for staining or for immunoblotting as required. For staining, the gel was dipped overnight in 0.5% Coomassie brilliant blue solution (made in 50% methanol and 10% acetic acid). Destaining was further done with the staining solvent, till background stain was removed.
2.2.2.2 Two-dimensional gel electrophoresis

Isoelectric focusing (First dimension)

Isoelectric focusing (IEF) was essentially done following the method of O'Farrell (O'Farrell, 1975). An Atto apparatus (Tokyo, Japan) was used to cast and run the IEF gel strips. An acrylamide: bis acrylamide (28.83%: 1.62%) stock was made and the gel mix was prepared by mixing reagents in the proportion mentioned in Table 2.3. (To dissolve the urea the solution was kept at 37°C till complete dissolution, before adding the ampholytes). The gel mix was poured in between plates and left to polymerize for 2-3 hours. Samples for 2D-PAGE analysis were prepared by re-suspending sperm pellets in lysis buffer (Table 2.4) and incubated on ice (1 hour). The extract was then centrifuged at 14000 rpm to pellet down unwanted debris. The supernatant was loaded carefully onto IEF gel strips, covered with overlay buffer (Table 2.4) and focused for 3 hours at 400 volts. Orthophosphoric acid (0.025 M) was used as cathode buffer while sodium hydroxide solution (0.05 M) was used as anode buffer. Standard pl (isoelectric pH) markers were loaded and stained by Coomassie brilliant blue as mentioned above. After the IEF (first dimension) the gel strips were taken out very carefully from the grooves on the gel plate and kept for equilibration in an SDS buffer (15 minutes) (60 mM Tris, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% Glycerol). Thereafter, the IEF gel strips were carefully loaded

<table>
<thead>
<tr>
<th>Components</th>
<th>Added/10ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>5.5g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>3.97ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1.33 ml</td>
</tr>
<tr>
<td>NP40</td>
<td>200 µl</td>
</tr>
<tr>
<td>Ampholyte 5-8</td>
<td>400 µl</td>
</tr>
<tr>
<td>Ampholyte 3-10</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>10 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Table 2.3 Composition of 2D gel mix

<table>
<thead>
<tr>
<th>Components</th>
<th>Lysis Buffer</th>
<th>Overlay Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>9.5M</td>
<td>8M</td>
</tr>
<tr>
<td>Ampholyte 5-8</td>
<td>1.6%</td>
<td>0.8%</td>
</tr>
<tr>
<td>Ampholyte 3-10</td>
<td>0.4%</td>
<td>0.2%</td>
</tr>
<tr>
<td>5% Mercapto ethanol</td>
<td>5%</td>
<td>X</td>
</tr>
<tr>
<td>NP-40</td>
<td>2%</td>
<td>X</td>
</tr>
</tbody>
</table>

Table 2.4 Composition of 2D lysis and overlay buffer
onto SDS-PAGE (second dimension) slabs and electrophoresed as mentioned in the relevant section earlier. A single well was made on one side for loading molecular weight standards when required.

**SDS-Polyacrylamide Gel Electrophoresis (Second dimension)**

The SDS-PAGE was run as mentioned above section 2.2.1.

### 2.2.3 Immunoblotting

#### 2.2.3.1 Protein transfer

The electro-transfer of proteins from the polyacrylamide gel to nitrocellulose membranes (Hybond C or Hybond P) was essentially done following the semi-dry method of Towbin (Towbin et al., 1979). The gel and the sized membrane were soaked in the transfer buffer (composition), for 10 minutes, and then stacked with the membrane towards the anode sandwiched between three Whatman blotting sheets which were pri­orly soaked in transfer buffer. Current applied for electro transfer was 0.8 mA per cm² for 1.5 hours. After electro-transfer of proteins the membrane was washed thoroughly with distilled water and put in Ponceau S stain (0.1% in 1% acetic acid) for few seconds, to stain the proteins bands/spots. The stained protein bands/spots on a PVDF membrane were distinctly visible when the membrane was given a quick rinse in methanol after staining. The stain was again washed away from the membrane by rinsing in distilled water.

#### 2.2.3.2 Processing of the blot with transferred proteins

The blot with transferred protein(s) was blocked with 5% non-fat milk solution made in TBS-T (TBS containing 0.1% Tween 20) (1 hour). Next, the blot was incubated with primary antibody made in 1% BSA in TBS-T (monoclonal anti alpha-tubulin was made in TBS without BSA). The incubation time was 2 hours for all primary antibodies, except polyclonal anti-phosphotyrosine, which was 1 hour. Next, the blot was incubated with the appropriate secondary antibody also made in 1% BSA in TBS-T (1 hour). All the incubations were carried out at room temperature and were interspersed with washes with TBS-T (10 minutes each) on a shaker. Finally the blot with HRPO conjugated secondary antibody was then developed using the Enhanced Chemi-Luminiscence kit (Am-
ersham Biosciences, Freiburg, Germany) following the manufacturer's protocol. The blot with alkaline phosphatase conjugated secondary antibody was developed using NBT and BCIP as substrates. The blot was briefly incubated with alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl₂ and 100 mM Tris, pH 9.5) at room temperature to which was added 66 µl NBT (5% stock) and 33µl of BCIP (5% stock) per 10ml of the buffer. The blot was incubated in dark till a distinct band appeared. The blot was then washed with distilled water.

2.2.4 Detection of protein tyrosine Phosphorylation

Tyrosine phosphorylation was detected in sperm extracts by using either polyclonal or monoclonal anti-phosphotyrosine antibodies for Western blot analysis. Spermatozoa were maintained at a concentration of 5 X 10⁷ per 1.5 ml of medium for studying tyrosine phosphorylation. The sperm suspensions were kept on ice at the required time point and harvested thereafter. Sperm suspensions were centrifuged at 9,500 rpm and washed twice with TBS (20 mM Tris and 137 mM NaCl) containing 1 mM sodium orthovanadate as a tyrosine phosphatase inhibitor and were stored in -20°C. Samples were prepared and resolved by SDS-PAGE or 2D-PAGE as mentioned in the respective section. Gels were then electro-transferred onto wet nitrocellulose membranes and stained thereafter with Ponceau S to check for equal loading of samples. Subsequently, membranes were processed for immunoblotting with either polyclonal anti-phosphotyrosine (Promega, Wisconsin, USA) or monoclonal anti-phosphotyrosine (αPY) antibody (Upstate, New York, USA) according to the protocol mentioned in the section 2.2.3.

2.2.5 Indirect immunofluorescence and Confocal microscopy

2.2.5.1 Preparation of coverslips

Spermatozoa maintained in appropriate media were collected carefully ignoring the pellet of dead cells at the bottom of the microfuge tubes. This sperm suspension was centrifuged at 1000 rpm, for 5 minutes, at room temperature, supernatant was discarded and the pellet washed one more time with TBS as above. Washed sperm were then
fixed in 2% formaldehyde (prepared freshly in TBS), for 10 minutes. The fixed sperm suspension was now coated on clean glass coverslips and air-dried on a slide warmer at 37°C. The coverslips were stored (up to 7 days) in an absolute dry condition at room temperature in 6 well pertriplates.

2.2.5.2 Processing of coverslips

Each coverslip was freshly permeabilized by dipping in ice cold (-20°C) methanol, for 20 seconds. Coverslips were then blocked with 5% BSA in TBS (1 hour). Further, the cover slips were incubated with primary antibody made in 1% BSA in TBS (2 hours) and thereafter with the appropriate secondary antibody also made in 1% BSA in TBS (1 hour). All the incubation steps were carried out at room temperature and were interspersed with 3-4 washes in TBS. After the final wash, the coverslips were mounted on clean slides using Antifade (Vector Lab, California, USA) as the mounting medium and viewed in an Axioplan2 epifluorescence microscope (Carl Zeiss Inc, USA). Slides were preferably viewed immediately or after overnight storage at 4°C.

For dual staining a combination of Cy3 and FITC was used, the former for detecting phosphotyrosine residues and the later for detecting hamster sperm E3. The detail of the procedure and the intended modifications are mentioned in the relevant result section (Chapter 4). Colocalization studies were done using laser scanning confocal microscope, LSM510 Meta (Carl Zeiss Inc, USA); the laser line used to excite the afore said dyes were which were excited at 488 nm (FITC) and 543 nm (Cy3) laser lines respectively. Optical sections (0.2μm each) of the sperm samples were obtained during the scanning and for each sample five innermost sections were projected for further analysis by the LSM510 Meta software,

2.2.6 In vitro enzyme assay

2.2.6.1 Preparation of extract

The soluble sperm lysate was prepared essentially by the method described by Patel (Patel et al., 1995). Spermatozoa (100 X 10^6 per 3 ml of TALP) were capacitated and sperm pellets were harvested at different time points during capacitation. These pellets were re-suspended (at a concentration of 1 X 10^6 per 2μl) in the hypotonic lysis buffer containing 1% TritonX-100 and protease inhibitors (0.2 mM PMSF, 0.5 μg/ml leu-
peptin and 0.5 µg/ml aprotinin) and sodium orthovanadate (1 mM) and kept on ice (1 hour). The suspension of cells was then dipped in liquid nitrogen to snap freeze and then thawed immediately. After 3-4 freeze-thaw cycles, the suspension was again kept on ice (1 hour). The suspension was then centrifuged at 14,000 rpm at 4°C (15 minutes) and the supernatant was taken as the extract with which enzyme assays were done.

2.2.6.2 In vitro dehydrogenase assay

The enzyme assay was essentially performed following the method described by Gazaryan et al (Gazaryan et al., 2002) with some modifications. The reaction volume was 200 µl and was done in the micro cuvettes in an ultraviolet visual spectrophotometer (Shimadzu, Maryland, USA). 5 µl of the extract prepared was used for the assay. The substrates for the assay were dihydrolipoamide and lipoamide for the forward and reverse reactions, respectively. Lipoamide was prepared in 50% methanol as a 500 mM stock and kept at -20°C. During use a 10 mM solution of dihydrolipoamide was made by diluting an aliquot of the 500 mM stock in water. Dihydrolipoamide was made by reducing lipoamide using DTT as the reducing agent. Lipoamide solution was taken in a round bottom 3-necked flask and a stir bar was also introduced at the same time. One neck was attached to a balloon filled with nitrogen gas through a stopper; the other two necks were closed by airtight rubber stoppers. The stopper was opened so as to allow nitrogen to flow from the balloon to the flask and let the lipoamide solution equilibrate, for 30 minutes. The whole set up was maintained perfectly airtight to prevent oxidation of the reduced lipoamide formed which was formed by addition of equal volume of equimolar concentration of DTT by piercing a syringe through one of the rubber stoppers. The contents (lipoamide and DTT) were allowed to stir for 1 hour in presence of inert nitrogen atmosphere. The yellow colour of the lipoamide solution disappears after reduction. The reaction was done in 50 mM Tris, pH 7.5 with concentrations of substrates (lipoamide/dihydrolipoamide) and co factors (NADH/NAD) as mentioned in Table 2.5.

For the reverse reaction, lipoamide was added to the Tris buffer and the buffer contribution was nullified by setting to auto zero at 340 nm. Subsequently NADH, the sperm extract or purified enzyme was added to the cuvette, mixed well and the decrease in OD340 was recorded continuously for 100 seconds. The forward reaction was performed by adding NAD+ to the Tris buffer and then nullifying the buffer contribution by setting to auto zero at 340 nm. Then the sperm extract was added followed by the reduced lipoamide from the air tight container with the help of a Hamilton syringe. The in-
crease OD$_{340}$ was then monitored continuously for 100 seconds. The unit of forward or reverse activity was expressed as change in OD$_{340}$ for 100 seconds. The values obtained were normalized with factor 10$^{-3}$.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Lipoamide</th>
<th>Dihydrolipoamide</th>
<th>NADH</th>
<th>NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward reaction</td>
<td>Not detected</td>
<td>8 mM</td>
<td>Increase in OD$_{340}$ 0.32 mM</td>
<td></td>
</tr>
<tr>
<td>Reverse reaction</td>
<td>4 mM</td>
<td>Not detected</td>
<td>0.16 mM</td>
<td>Decrease in OD$_{340}$</td>
</tr>
</tbody>
</table>

*Table 2.6 Reagents for *in vitro* dehydrogenase assay for hamster sperm E3*

2.2.6.3 Diaphorase zymogram

The diaphorase activity of dihydrolipoamide dehydrogenase was studied qualitatively in a zymogram performed essentially by the method described by Caldwell (Caldwell *et al*., 1976) using the soluble sperm lysate as the enzyme source (as used in the dehydrogenase assay). The extract (30 µl) was mixed with 5X loading buffer (same as SDS-PAGE loading buffer without SDS and β-ME) and immediately loaded onto a native PAGE. The gel for PAGE (stacking 5% and resolving 8%) was prepared in the same manner as mentioned in the SDS-PAGE section. The gel was then run at 4°C using native buffer (which was essentially same as SDS-PAGE running buffer without SDS) at 20 mA till the bromophenol blue dye front reached the bottom edge of the gel. After the run was over the gel was incubated in Tris buffer (0.25 mM, pH 8.4) for 30 minutes at room temperature. Further MTT and NADH were added at the working concentration of 0.25% and 0.05%, respectively. The gel was incubated in dark till the violet diaphorase bands appeared.

2.2.7 *Sperm physiology studies*

2.2.7.1 Assessment of Acrosome reaction

Acrosome reaction of sperm samples was monitored essentially by the method described in (Kulanand and Shivaji, 2001). Sperm suspension (15 µl) was put on a pre-warmed slide and mixed with equal volume of Eosin Y (0.25% made in TALP-PVA medium). A coverslip was now carefully put on the sperm suspension on the slide, which
was then scored under a phase contrast microscope (Leitz) with 40X objective. A minimum of 100 spermatozoa was counted for each sample. The viable spermatozoa undergoing or having undergone acrosome reaction were counted as positive. The results were further expressed as percentage of acrosome reacted spermatozoa.

2.2.7.2 Assessment of Hyperactivation

The hyperactivation status of spermatozoa was assessed essentially as described by Uma et al., (2000). Non-hyperactivated and hyperactivated hamster spermatozoa exhibit different types of motility; non-hyperactivated sperm exhibit planar type while hyperactivated sperm exhibit circular or helical type of motility. Based on these criteria, motile spermatozoa could be visually categorized on the CASA monitor as hyperactivated or non-hyperactivated. Sperm suspensions maintained in the required media were dispersed on pre-warmed slide chambers (made with Parafilm on glass slides), covered with cover slips and 3-4 fields of each slide were recorded using HTM-CEROS. The hyperactivation status of the sperm population was assessed by manually counting the number of motile spermatozoa in the recorded fields. The percentage of hyperactivated sperm was thus counted and results show the average of 3-4 fields covering a minimum of hundred spermatozoa. Hyperactivation was assessed in detail by studying the kinematics of the motile spermatozoa.

2.2.7.3 Sperm Kinematics

Sperm kinematics (study of velocity parameters) was studied using a system called Computer Aided Sperm Analyzer (CASA) in an HTM-CEROS set up (Hamilton Thorne, Maryland, USA). This set up consists of a trinocular microscope attached to a close circuit television through a CCD camera, a software with a frame grabber capturing the image from the television, and analysis software analyzing the image. The set up includes a video recorder using which the movement of the sperm can be captured and replayed later for offline analysis. The set up also includes a slide warmer set at 37°C, which can be put on the stage of the microscope for studying the sperm movement at the physiological temperature. Sperm suspensions maintained in different media were dispersed on pre-warmed slide chambers (made with Parafilm on glass slides), covered with cover slips (Uma Devi et al., 2000) and 3-4 fields of each slide were recorded using HTM-CEROS. The percentage motility status of the sperm population was assessed by manually counting the number of motile spermatozoa in the recorded fields and subse-
quenty expressed as percentage of total number of spermatozoa. The movement of the spermatozoa can be quantitatively characterized by looking at individual motility parameters like VAP (average path velocity), VCL (curvilinear velocity) and LIN (linearity). The same recorded samples were further analyzed for these velocity parameters using the same software with the following set of parameters: frames acquired: 50; frame rate (Hz): 60; minimum contrast: 25; minimum cell size (pixels): 10; straightness threshold (%): 80; low VAP cut off (μm/sec): 7.5; medium VAP cut off (μm/sec): 12.5; low VSL cut off (μm/sec): 5; static head intensity limits: 0.2-1.47; static head size limits: 0.12-7.37; static elongation limits: 1-98; magnification: 1.14 (4X); video frequency (Hz): 60; bright field: no; slide warmer temperature (°C): 37; field selection mode: manual. About 100 spermatozoa from each recorded slide were analyzed for the velocity parameters.

2.2.8 N-terminal sequencing

Sperm proteins were resolved by 2D-PAGE (as mentioned in the section 2.2.2.2), transferred to PVDF membranes (soaked in methanol), stained with Ponceau S and visualized, the way it has been described in the section 2.2.3.1. The protein spot of interest was then excised with a sterile blade and washed with 50% methanol (in deionized water to remove glycine in the transfer procedure). The spots were again washed with deionised water, dried between fresh tissue paper and subjected to N-terminal sequencing using an automated protein sequencer (Applied Biosystems, California, USA).

2.2.9 Generation of antiserum for dihydrolipoamide dehydrogenase

Antibody against dihydrolipoamide dehydrogenase (E3) was raised in both rabbit and mouse. For raising antibody in mouse 30-40 μg of purified pig heart E3 was used for each subcutaneous injection. The purified antigen was diluted to the required concentration in sterile PBS (pH 7.5), which was then diluted with an equal volume of Freund’s complete adjuvant. An emulsion was prepared by vortexing the mixture as long as no oil separated out on standing. The formation of a perfect emulsion was ensured by checking if a drop of it could float when put on water. First, second and third boosters were
given at intervals of 7-8 days in Freund's incomplete adjuvant. The titre of antibody was checked after the third booster and total blood was collected after the fourth booster by sacrificing the animals. The same protocol was used to raise antibodies in rabbit by using 200 μg of purified pig heart dihydrolipoamide dehydrogenase for each injection given at intervals of 20-21 days. The animal was bled at the end of the third booster. Premune sera were collected from each animal before the first injection. The blood collected was let to clot at room temperature for 3 hours and kept at 4°C overnight for clot retraction. Next day the clotted blood was centrifuged to collect the serum from top. The antisera were aliquoted in small volumes and stored at -20°C.

2.2.10 Total RNA isolation

Total RNA was isolated from the adult testis following the manufacturer's protocol (Sigma, St. Louis, Missouri, USA) by a single step liquid phase separation method using Tri-Reagent. The glassware and the homogenizer probe were baked in hot air oven (200°C) overnight. Immediately after sacrificing the animal, approximately 200-300mg of the tissue was taken in 2-3 ml of the Tri-reagent, homogenized and centrifuged at 4°C at 13,000 rpm, for 2 minutes. The supernatant was then transferred to fresh microfuge tubes to which 0.2 ml of chloroform per ml of Tri-reagent was added. The tubes were then vortexed, set at room temperature, for 5-15 minutes, and centrifuged at 13,000 rpm, for 10 minutes, at 4°C. The aqueous phase was recovered in a fresh tube to which 0.5 ml of iso-propanol per ml of Tri-reagent was added. The contents were then mixed and set at room temperature, for 10-15 minutes, for precipitation, centrifuged at 13,000 rpm, for 10 minutes, at 4°C, supernatant discarded and the pellet washed with 70% ethanol. The pellet was air dried, re-suspended in autoclaved deionized water and stored at -70°C. The integrity of the RNA was checked by the criteria that a good RNA preparation has OD\textsubscript{260} : OD\textsubscript{280} equivalent to approximately 1.8 and in a formaldehyde-agarose gel the band intensity of the 28S ribosomal RNA was approximately double that of the 18S rRNA.
2.2.11 RT-PCR

Reverse transcription was done using 3 μg of total RNA. The total RNA sample was first denatured at 65°C (10 minutes) and immediately kept on ice. Denatured RNA was then subjected to reverse transcription to synthesize the first strand of cDNAs by using 50 units of expand RT (Roche, Mannheim, Germany) with oligo dT (250ng/20μl) (Roche, Mannheim, Germany) as the primer at 42°C for 1 hour. PCR was further performed on 1 μl aliquots of cDNA samples using gene specific forward and reverse primers, which were synthesized in-house. Each PCR was done in a 10 μl reaction volume using 1 pmole/μl of each primer (made by the in house facility) along with 1 mM dNTPs and 1 unit of AmpliTaq gold (Roche, Mannheim, Germany). The combination of overlapping primers used were BLF (5'ATGCAGAGCTGGAGTCGTGTACC3'-forward) / KAS2R (5'CTCTAAAAGCCTCTGATAAGGTCGGATG3'reverse) and KASC1F (5'TTGGTGTAGAATGGGTTTCAGTTTGCAAGAC3'-forward) / BLR (5'TTAAAAGTTGATTGGTTTTCAAAAGCTGCAG3'-reverse). The amplification was performed for 36 cycles (in MJ research DNA Engine thermal cycler) with each of the primer sets as follows: denaturation at 94°C (1 minute); annealing at 56°C (1 minute), extension at 72°C (2 minutes). A final extension was also carried out at 72°C (5 minutes). These primer sets amplified two overlapping fragments separately. The PCR products were run on 1% agarose gels. The bands were cut and eluted from the gel with gel elution kit (Qiagen, Hilden, Germany) and sequenced in an automated sequencer (ABI prism 3700, Applied Biosystem, California, USA) with the respective primers.

2.2.12 Software

Sequences were analyzed and compared using BLAST programme (Altschul et al., 1990) available from the National Centre for Biotechnological Information (NCBI, http://www.ncbi.nlm.nih.gov/blast). The accessory help for the sequence analysis was taken from SeWeR programme (Basu, 2001) from http://www.bioinformatics.org/ sewer. The sequence alignments have been done by the ClustalW programme from the European Bioinformatics Institute (EBI) server.
2.2.13 Histochemistry

2.2.13.1 Preparations of tissue sections

Testes were dissected out immediately after sacrifice of the animal and rinsed in TBS to remove blood. The tissue was then immediately immersed in freshly prepared Bouin's fixative (70% water saturated picric acid, 20% formaldehyde, 5% acetic acid) and left for 16 hours at room temperature. After 4-5 hours (when the tissue had hardened) it was cut carefully transversely so that the fixative reached the inside of the tissue. After 16 hours of fixation the tissue pieces were rinsed in large volumes of 70% alcohol with several changes. The fixed tissue was then submitted to the tissue sectioning facility of CCMB. Moulds were prepared with paraffin wax and 5 μm thick sections were obtained on slides coated with 0.5% gelatin. Prior to any staining the sections were deparaffinized in xylene for 20 minutes. They were further shifted to absolute alcohol for 10 minutes and then through a series of graded alcohol (95%, 80%, 70% and 50%) after every 5 minutes. Ultimately, the sections on the slides were hydrated in two changes of distilled water each for 10 minutes and then processed for staining.

2.2.13.2 PAS-Hematoxylin staining

Deparaffinized hydrated sections were incubated in freshly prepared periodic acid (2% in water) for 30 minutes. After rinsing the sections in distilled water they were then stained with Schiff's reagent (Sigma, St Louis, Missouri) overnight. After rinsing them in water, sections were dipped in Harris' Hematoxylin solution for 1 minute. The dye was cleared by adding acid-alcohol (0.5% acetic acid: 95.5% ethanol), for 10 seconds. The sections were then thoroughly rinsed with water and semidried before mounting in 30% glycerol.

2.2.13.3 Immunohistochemistry

Deparaffinized sections were rinsed in TBS and then blocked with 5% BSA (in TBS) for 1 hour. Primary antibody (1:100 dilution made in 1% BSA in TBS) incubations were carried out overnight at 4°C. Incubations with secondary antibody (1:50 dilution made in 1% BSA in TBS) conjugated with alkaline phosphatase was for 30 minutes at room temperature. Each incubation was interspersed with gentle washes in running
TBS. Finally, colour was developed by incubating the section with NBT/BCIP, a substrate for alkaline phosphatase, in presence of 0.1 mM levamisole to inhibit any endogenous alkaline phosphatase activity. The sections were rinsed in water, semidried and mounted in 30% glycerol.

2.2.14 Dissolution of acrosomal matrix

The acrosomal matrices were dislodged from the sperm following the protocol of Olson et al. (Olson et al., 1988) with little modifications. The sperm were arrested at different stages of capacitation by putting them on ice at individual time points during capacitation. Finally, sperm were washed twice with cold TBS at 500g for 5 minutes each. The sperm pellet were then resuspended in HEPES buffer (10 mM HEPES with 140 mM NaCl) containing 0.1% TritonX-100 and 0.25 mM PMSF and kept on ice for 1 hour. After vortexing, the sperm were then subjected to homogenization in a Dounce homogenizer (5 up and down strokes at power 10) which dislodges the acrosomal matrices from the rest of the sperm. They were further kept on ice for 30 minutes after which sperm were centrifuged at 350g for 5 minutes. TBS washes were given twice to the sperm pellet at the same speed and finally the spermatozoa were centrifuged at 10,000 rpm to form a tight pellet. SDS-PAGE extracts were prepared as mentioned in the previous section. Protein estimation was done with the extracts according to the method of Karlsson et al., (1994). Briefly, 100 µl 60% TCA was added to sample adjusted to 50 µl and volume made up to 250 µl in microtitre plates. The samples were shaken in the microtitre plate for 30 minutes and OD was taken at 575 nm in an ELISA reader (Moleculardevices, California, USA).

Acrosomal matrices were partially dislodged from the sperm head using the method described for guinea pig spermatozoa (Foster et al., 1997). Hamster spermatozoa were collected from the cauda epididymides in a buffer having 20 mM sodium acetate (pH 5.2) and 0.15 M NaCl (along with protease inhibitor cocktail as mentioned before) and were washed at 500g, for 5 minutes, at 4°C. The sperm pellet was suspended in the same buffer with 0.625% TritonX-100 and the suspension was passed through a 26 gauge needle 20 times. This, although did not dislodge all the acrosomal matrices
completely, loosened them and sperms could be identified at different stages of acro-
sonal matrix loss.

2.3 References

All references are listed at the end of the thesis.