DEVELOPMENTAL BIOLOGY 5
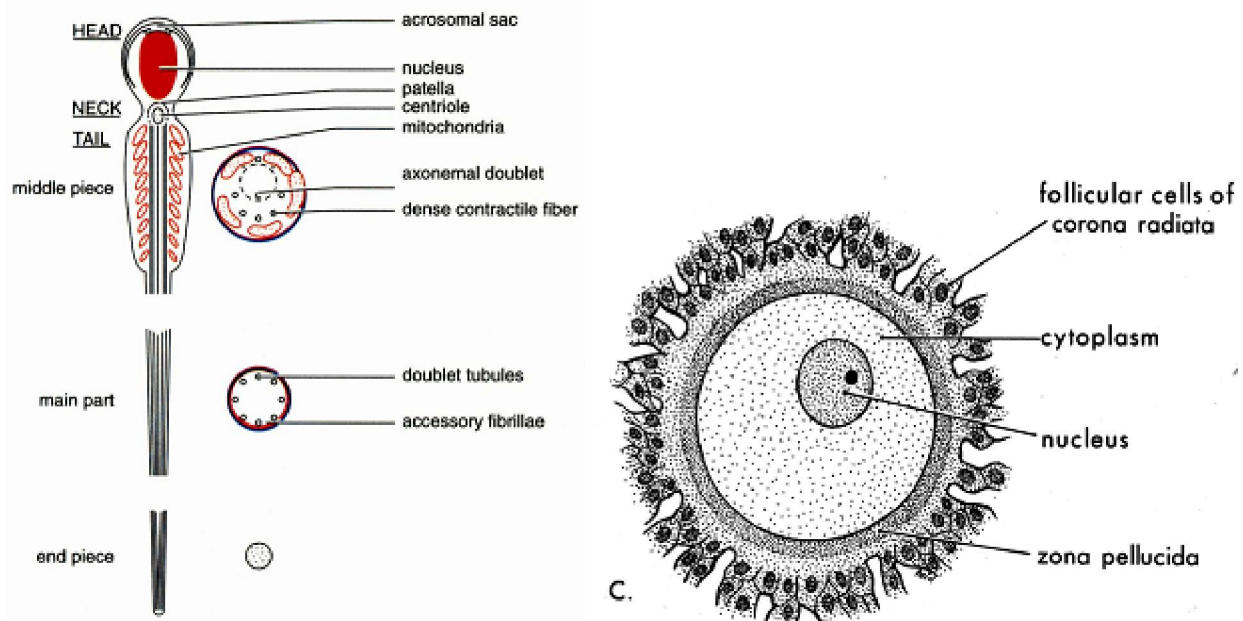
FERTILIZATION

Introduction:

Special features of the gametes for fertilization: Both egg and sperm acquire structural specializations for fertilization.

Eggs are non-motile, surrounded by protective egg coverings. These serve to recognize the sperm specifically and prevent fertilization by more than one sperm (polyspermy). The mammalian egg has zona pellucida layer around the plasma membrane beneath which cortical granules are present. The zona pellucida layer makes the egg impenetrable to more than one sperm.

Sperms are highly motile cells consisting of nucleus, mitochondria to provide energy source and a flagellum for movement. The anterior end of the sperm is highly specialized which aids in penetration of the egg. Sperms are typically designed to activate the egg and to deliver their nuclei into the egg cytoplasm.

Basic requirements of fertilization: Fertilization requires a fluid medium in most animals. It may be seawater in marine forms, fresh water in fresh water forms and body fluid in viviparous animals. To increase the probability of fertilization, the number of sperms must exceed the number of eggs. Moreover the lifespan of gametes is limited; therefore fertilization must take place within a short duration of time. Eggs that are shed in water like that of most invertebrates, fishes and amphibians, have shorter life span while those fertilized within the body of female, generally have longer life span.

Site of fertilization: Fertilization may be either external or internal. In external fertilization the gametes are discharged in the aquatic medium and the fertilization occurs outside the body of both male and female parents, as in most invertebrates and some vertebrates (fish and frog). The aquatic medium for external fertilization may be either seawater or fresh water. When fertilization occurs inside the body of female parent, it is internal fertilization, as in Drosophila, birds and mammals.

Mechanism of fertilization: The process of fertilization has been mostly studied in invertebrates such as sea urchins and in vertebrates like amphibians and mammals. Fertilization begins with the approach of the sperm to the egg and ends up with the formation of diploid zygote. The process of fertilization requires five general events:

1. Recognition of egg and sperm (approach of spermatozoan to the egg, attachment and binding)
2. Acrosome reaction and penetration
3. Fusion of plasma membranes of egg and spermatozoa.
4. Activation of egg
5. Fusion of egg and sperm pronuclei

1. Recognition of Egg and Sperm:

Encounter of spermatozoa and ova: The meeting of sperm and egg is not a simple task. Most animals accomplish close approximation of gametes through special devices or a particular behaviour.

Among fresh water animals the timing of spawning of eggs by females and shedding of sperms by male parent are very specific. The sperms are delivered directly to the eggs immediately after laying.

In marine forms the time interval between shedding of gametes may be longer by weeks or months. The task of meeting sperm and egg is further intensified as they release their gametes into the open sea, where they are readily dispersed. For this reason, a large number of gametes may be produced to maintain a sufficient gamete concentration in the water. Moreover, the aquatic medium is shared by other species that may shed their gametes at the same time. In aquatic medium the movement of spermatozoa may either be entirely at random or the consequence of directed movements caused by some attractive force associated with the egg.

During internal fertilization, such as in mammals, the gametes of both sexes are deposited in the female reproductive tract. The fluid movements within the reproductive tract, assist in transporting the gametes to the site of fertilization.

Sperm attraction: In many animals, sperms are attracted towards eggs of their species by "chemotaxis" i.e. following a gradient of a chemical secreted by the egg. Chemotaxis has been demonstrated in cnidarians, molluscs, echinoderm and urochordates (Miller, 1985; Yoshida et al, 1993). Similarly in the egg jelly of the sea urchin, chemotactic factors are present for sperm attraction. A chemotactic factor called **resace**t, a 14 – amino acid peptide, has been isolated from the egg jelly of sea urchin *Arbacia punctulata* (Ward et al 1985). A peptide of 10 – amino acid in the

jelly of *Strongylocentrotus purpuratus* and *Hemicentrotus pulcherimus* has been named as **speract** (Garbers et al 1982). They diffuse readily into seawater and are species specific.

Fertilizin and Anti fertilizin interactions: Factors that mediate sperm– egg interactions even before they make contact were identified by F.R. Lillie (1912). He proposed the first theory of physiology of fertilization called fertilization theory. He observed that the egg water (seawater surrounding unfertilized sea urchin eggs), agglutinated the sperm and activated their motility. The reaction was species specific. This factor called **fertilizin** came from the egg jelly coat. It slowly dissolved as in sea water. Fertilizin was later shown to be the constituent of both jelly coat and egg membrane such as vitelline membrane and plasma membrane. Fertilizinis a proteoglycan. Both the amino acids and monosaccharides of fertilizin vary from one species to another so that each species possesses its specific type of fertilizin. Each molecule of fertilizin has more than one 'active group' so that one fertilizin particle may attach to two or more sperms and bind them together.

The receptor sites for fertilizing are present on the sperm plasma membrane called **anti fertilizin**. These are acid proteins. Adhesion of spermatozoa to the surface of the egg is brought about by linking of fertilizin molecule with antifertilizin molecules. The reaction between fertilizin and anti-fertilizin is similar to antigen – antibody reaction. In both cases, a chemical lock is formed between two complimentary substances. It has been suggested that the main function of fertilizin – antifertilizin is to thin out the number of spermatozoa around the egg, so that the chances of two or more spermatozoa fusing with the egg at the same time are diminished.
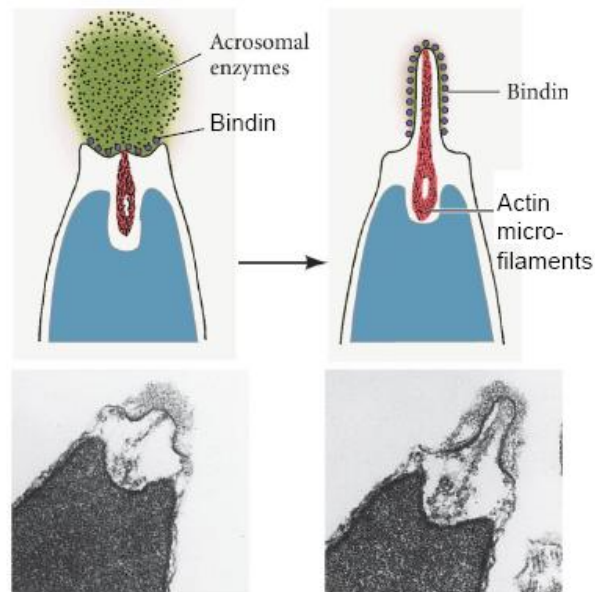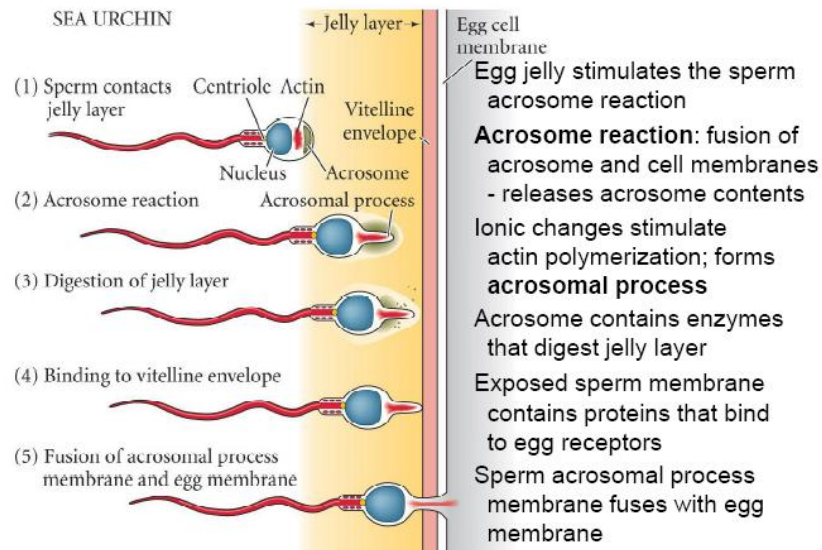
2. **Acrosome reaction and penetration:**

The acrosomal reaction in sea urchin

Once the sperm makes contact with the egg, then it has to penetrate surface coats that surround the egg. The penetration is facilitated by the acrosome reaction in which the membrane enclosing the acrosome is shed, releasing the contents of acrosome. The acrosomal reaction involves two processes:
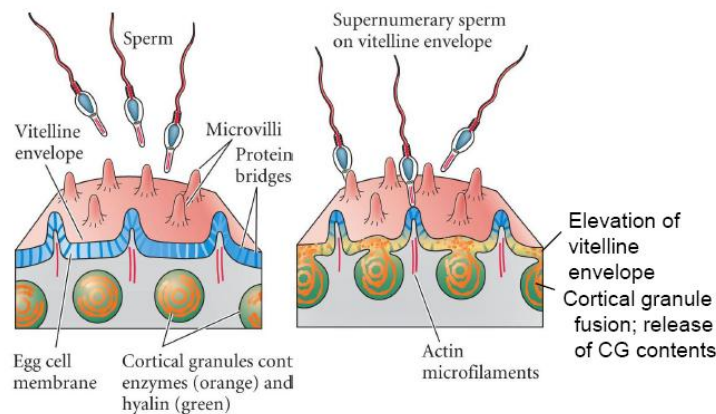
a) exocytosis of acrosomal vesicle and
b) extension of acrosomal process.

a) Exocytosis of acrosomal vesicle: Contact of the sperm with the egg jelly coat component, a fucose containing polysaccharide triggers the acrosome reaction and causes influx of calcium into the sperm head. This initiates fusion of the outer acrosomal membrane with sperm plasma membrane and ultimate breakdown of acrosomal vesicle. Hydrolytic enzymes called lysins present in the acrosomal vesicle, are released. Lysins digest the egg envelope locally and clear the path for spermatozoa to reach the egg surface (vitelline membrane).The exocytosis of acrosomal vesicle is thus caused by calcium – mediated fusion of acrosomal membrane with the adjacent sperm plasma membrane. The egg jelly factors that stimulate the acrosome reaction are highly species specific.

Sea Urchin Acrosome Reaction

SEA URCHIN

(1) Sperm contacts jelly layer — Centriole, Actin, Vitelline envelope, Nucleus, Acrosome

(2) Acrosome reaction — Acrosomal process

(3) Digestion of jelly layer

(4) Binding to vitelline envelope

(5) Fusion of acrosomal process membrane and egg membrane

← Jelly layer →

Egg cell membrane

- Egg jelly stimulates the sperm acrosome reaction
- **Acrosome reaction**: fusion of acrosome and cell membranes - releases acrosome contents
- Ionic changes stimulate actin polymerization; forms **acrosomal process**
- Acrosome contains enzymes that digest jelly layer
- Exposed sperm membrane contains proteins that bind to egg receptors
- Sperm acrosomal process membrane fuses with egg membrane

Acrosomal enzymes
Bindin
Bindin
Actin micro-filaments

Cortical Granule Exocytosis

b) Extension of acrosomal process: The second part of the acrosomal reaction involves the extension of the acrosomal process / tube / filament. It is formed by polymerization of globular active molecules into actin filaments.

Gamete binding and species specific recognition in sea urchins:

Once the sea urchin sperm has penetrated the egg jelly, the acrosomal process of the sperm contacts the vitelline envelope of the egg. The attachment between the acrosomal process and the vitelline envelope is species - specific. The specificity is due to interactions between sperm **bindin** present on the acrosomal process and a specific sperm receptor on the vitelline envelope. Bindin is located specifically on the acrosomal process and it binds to species - specific bindin receptors present on the egg vitelline membrane.

Gamete binding and recognition in mammals:

a) Capacitation

In mammals fertilization is internal. The reproductive tract plays a very active role in fertilization. The differentiated sperms are unable to undergo the acrosome reaction without residing for some time in the female reproductive tract where they undergo physiological changes. The change in the mammalian spermatozoan, which makes it capable of fertilizing the egg, is called **capacitation**. There are four sets of molecular changes, which take place during capacitation:

• Albumin proteins, present in the female reproductive tract, remove the cholesterol thereby altering the fluidity of sperm plasma membrane.

• Certain proteins or carbohydrates on the sperm surface are lost during capacitation.

• Membrane potential of the sperm becomes more negative, as potassium ions leave the sperm. This change in membrane potential opens up the calcium channels and allows calcium to enter the sperm facilitating the process of membrane fusion during acrosomal reaction.

• Protein phosphorylation occurs. However it not known whether these events are independent of one another and to what extent each one of them causes sperm capacitation.

b) Gamete binding

The mammalian egg is surrounded by extracellular envelope called **zona pellucida**. Around zona pellucida is a layer of cumulus cells (corona radiate) embedded in a cementing substance, hyaluronic acid. Hyaluronidase activity on the surface of the sperm head helps it to penetrate this layer. Next, sperm must bind to zona pellucida before they make contact with the surface of egg itself. The zona pellucida in mammals plays a role analogous to that of viteline envelope in lower vertebrates and invertebrates. The zona pellucida is a glycoprotein matrix synthesized and secreted by the growing oocyte. It plays two important roles in fertilization .It binds the sperm and initiates acrosome reaction.
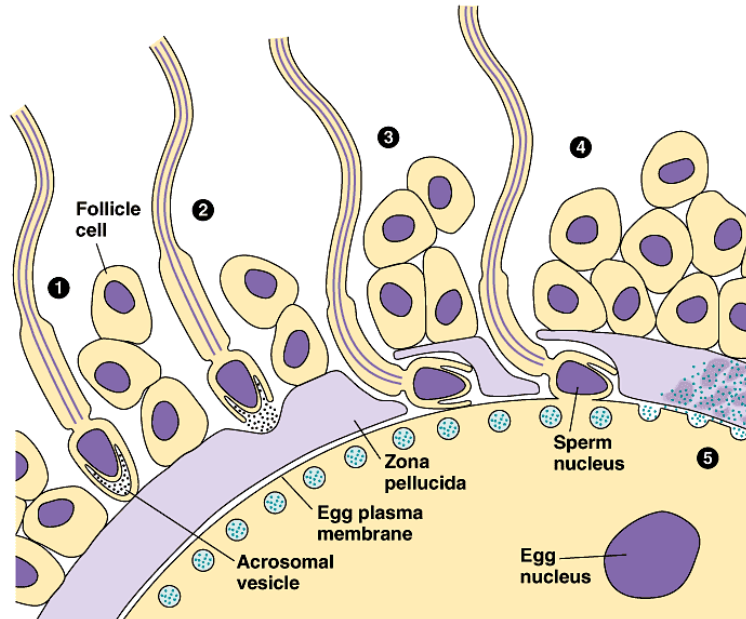
c) Acrosome reaction in mammals

Binding of the spermtozona triggers the acrosome reaction, which allows the sperm to penetrate the zona. Acrosome reaction in mammals involves the fusion of the outer membrane of the acrosome with the sperm plasma membrane. After the fusion, the acrosomal membrane vesiculates which results in the release of acrosomal contents. Subsequently, the outer portion of the acrosomal membrane disappears and only the inner portion adjacent to the nucleus remains intact. When the acrosomal contents are exocytosed, several enzymes are released. These enzymes allow the spermto approach the egg plasma membrane. The mammalian acrosome reaction differs from that of sea urchin in that no acrosomal process is formed.
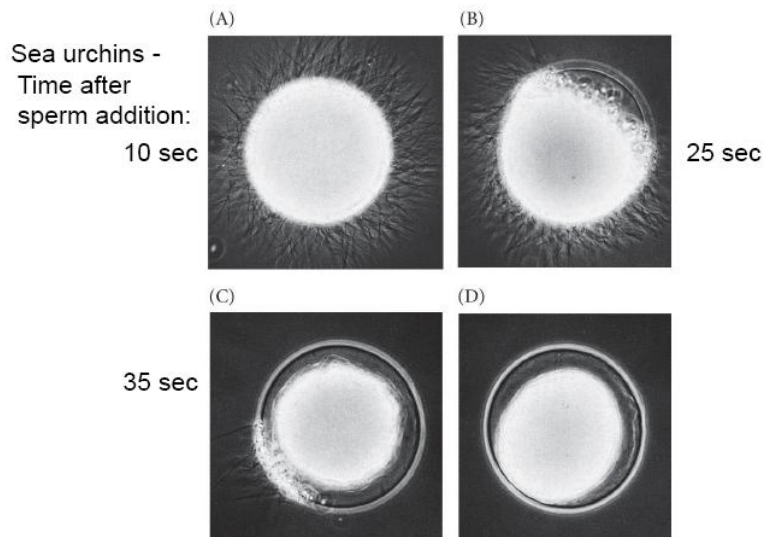
3. Gamete fusion & Prevention of polyspermy

Sperm & egg plasma membrane fusion

After penetration of the extracellular layers by sperm, there occurs the fusion of sperm plasma membrane with that of the egg.

In sea urchin, all regions of the egg plasma membrane are capable of fusing with sperm The cytoplasm of the egg bulges forward at the point of contact producing a process of hyaline cytoplasm called the **fertilization cone**. Sperm egg fusion appears to cause the polymerization of actin into microfilaments and extension of several microvilli to form the fertilization cone. Fertilization cone and microfilaments facilitate sperm entry. The sperm and egg membrane join together forming cytoplasmic bridge. The sperm nucleus and tail pass through the cytoplasmic bridge, which is widened by the actin polymerization.

Follicle cell

Zona pellucida

Egg plasma membrane

Acrosomal vesicle

Sperm nucleus

Egg nucleus

Fertilization Envelope

Sea urchins - Time after sperm addition:

(A) 10 sec

(B) 25 sec

(C) 35 sec

(D)

In mammals, after penetrating the zona, the sperm enters the perivitelline space surrounding the egg and lands on the egg plasma membrane, where fusion begins at the equatorial region of the sperm head. The plasma membrane of egg and sperm become continuous forming a cytoplasmic bridge through which the sperm nucleus enters the egg cytoplasm. Usually the entire sperm including the nucleus, centriole, mitochondria and flagellum enters the egg cytoplasm. Once the sperm enters the egg, fertilization cone is formed as in sea urchin. Fertilization cone is extension of

the egg cytoplasm around the spermhead. A sperm protein **fertilin**, is thought to be involved in mediating fusion of sperm egg membrane.

The prevention of polyspermy

The entry of the sperm into the egg activates the egg. Although many sperm attach to the egg surface, it is important that only one sperm enters the egg(monospermy). Entry of more than one sperm(polyspermy) may result in several abnormalities such as polyploidy, abnormal mechanism of chromosomal separation during cell division and ultimate death of the embryo. Organisms have evolved ways to prevent the union of more than two haploid nuclei.

In fishes the sperm can enter into the egg only through the narrow opening, the **micropyle**, the rest of the egg being covered by impermeable chorion.

In sea urchin and mammals, there is restriction on the number of sperm that are able to penetrate the extra cellular coats and fuse with the egg. In mammals, the sperm has to migrate the long female reproductive tract to reach the egg and further, structural changes in zona pellucida block polyspermy.

The most common way to prevent polyspermy is to prevent the entry of more than one sperm into the egg. The polyspermy is blocked in many animals as soon as the first sperm fuses with the egg plasma membrane. The sea urchin egg has evolved two mechanisms to avoid polyspermy, a) fast reaction that is accomplished b yan electric change in the egg plasma membrane and b) a slower reaction caused by exocytosis of the cortical granules.

a) The fast and temporary block to polyspermy

The fast block is a temporary measure, which is mediated by a transient depolarization of the egg plasma membrane, caused by sperm-egg fusion. Within 1-3 seconds after entry of the first sperm the electrical membrane potential across the egg plasmamembrane shifts from–70 mvto +20 mv. This change is caused by a small influx of sodium ions into the egg & lasts for about 60 seconds after which the membrane potential returns to its original level. Some acrosomal proteins of sperm open the sodium channel in the egg that causes influx of sodium ions into the egg & depolarizes the egg membrane. This resultsin the fast block to polyspermy.

b) The slow & permanent block to polyspermy.

The fast block to polyspermy is for a very short duration (about a minute only). This brief period is not sufficient to prevent polyspermy. Therefore, the fast block to polyspermy is supplemented by a second mechanism, known as **cortical reaction**. It is a slower mechanical block to polyspermy.

Sperm entry into the sea urchin egg results in the release of intracellular calcium ions that are stored in the endoplasmic reticulum in egg cortex. The calciumions are first released at the site of spermentry and with in a minute, a wave of calcium ions traverses the entire egg. This wave of released calcium ions initiates cortical reaction. The cortical reaction consists of a wave of exocytosis of cortical granules, which are present just beneath the plasma membrane in the mature

egg. The cortical granules fuse with the egg plasma membrane and release their contents into the perivitelline space. This space lies between the plasma membrane and the vitelline envelope. Several proteins are released by this cortical granule exocytosis, which are as follows :

• Proteolytic enzymes (proteases) released, break the bonds that bind the vitelline envelope to the egg plasma membrane. This creates a perivitelline space. These enzymes also clip off the bindin receptors and any sperm attached to it.

• Mucopolysacchharides (glycosamineglycans) released, produce an osmotic gradient that causes water to rush into the perivitelline space. As a result, vitelline envelope expands and is elevated. It now becomes fertilization envelope.

• A third protein, a peroxidase enzyme released during cortical reaction, hardens the fertilization envelope by cross-linking tyrosin residues on adjacent proteins of fertilization envelope.

• Finally, cortical granule protein, **hyaline**, forms a coating around the egg. The egg plasma membrane adheres to this protein and the hyaline provides a support for blastomeres during cleavage.

Both fertilization envelope and hyaline layer prevent further sperm from binding to the egg plasma membrane.

In mammals, the cortical reaction is same as ins ea urchin except that a fertilization envelope is not formed. Exocytosis of cortical granules causes release of hydrolytic enzymes into peri-vitelline space. These enzymes modify the zona pellucida sperm receptors so that sperm can no longer bind to zona pellucida. The changes in the zona pellucida are called the zona reaction. A block to polyspermy thus allows only one sperm to fuse with the egg and deliver its nucleus into the egg cytoplasm.

4. The activation of egg metabolism:

After the sperm penetrates the egg a series of diverse cytoplasmic reactions are initiated. The response of the egg to the sperm can be divided into "early" responses, which occur within seconds of the cortical reaction and "late" responses which take place several minutes after fertilization begins.

Early responses:

The early responses to the activation are the prevention of polyspermy, consisting of two major mechanisms. The fast block, which is initiated by sodium influx into the cell, and the slow block initiated by the intracellular release of calcium ions. Within one second, the membrane potential of egg rises and sperm – egg fusion takes place within 6 seconds followed by cortical vesicle exocytosis within 15 – 60 seconds.

Late responses:

Late responses include many metabolic changes, which are as follows:

• Increased rate of respiration due to utilization of glycogen and other food stuffs for getting energy ATP molecules.

• Activation of NAD kinase and increase in NADH and NADPH: NAD kinase converts NAD to NADP, a co enzyme for lipid biosynthesis, which is essential in formation of new cell membrane during cleavage.

• Ionic changes: certain intracellular changes occur in the concentration of cations such as sodium, potassium and calcium. There is increase in pH (remains high). The change in calcium ion concentration has great significance in the metabolic activation of the egg.

• Activation of protein and DNA synthesis: there is increase in the rate of protein synthesis by utilizing mRNA already present in the oocyte cytoplasm. After 5 minutes of fertilization, the rate of protein synthesis increases three to twelve folds. About 20 minutes after fertilization DNA synthesis is initiated.

• Resumption of meiosis: in most animals meiosis is arrested at a particular stage and resumes only after fertilization. The time of fertilization varies from species to species. It has been found that spermatozoan may enter the egg at different stages of maturation in different animals (fig 2.12)

• Initiation of mitosis: the initiation of mitosis occurs because (a) the rate of DNA synthesis increases after fertilization and (b) by the contribution of centriole by sperm to the egg, which is needed for proper mitosis.

To summarize, late responses include many metabolic changes such as the activation of potassium ions and amino acid transport, an increase in the rate of protein synthesis, initiation of DNA replication and several major regulatory events. These events include production of inositol triphosphate, diacylglycerol, release of cytoplasmic free calcium ions and rise in hydrogen ions concentration .

5. Fusion of genetic material in sea urchins and mammals:

In sea urchins, the sperm nucleus enters the egg perpendicular to the egg surface. After entry, the sperm nucleus and its centriole separate from the mitochondria and flagellum. The mitochondria and the flagellum disintegrate inside the egg. Second meiotic division is completed after the entry of the sperm and the resulting haploid egg nucleus is known as female pronucleus. The sperm nucleus once inside the egg, undergoes several changes and becomes male pronucleus. Its nuclear envelope becomes vesicular. De-condensation of chromatin and the formation of pronuclear envelope take place. The male pronucleus inside the egg rotates at 180 degrees so that the sperm centriole comes to lie between male pronucleus and the female pronucleus. The female pronucleus is located in the center of the egg and male pronucleus is in the cortical region of egg at the site of sperm entry. For fusion the male pronucleus has to travel a considerable distance through the egg cytoplasm to reach

female pronucleus. The sperm asters mediate this movement. The sperm aster is a complex of long microtubules that radiate from the sperm centriole. The sperm centriole acts as a microtubule-organizing center for sperm aster. The microtubules of the sperm aster push the male pronucleus towards the center of the egg. The astral microtubules also make contact with female pronucleus and pull it towards the male pronucleus. Thus the two pronuclei migrate towards each other and fuse to form the diploid zygote nucleus. The fusion of male and female pronuclei is called **amphimixis**.

In mammals the entry of sperm is tangential to the eggs surface. Once inside the egg, the sperm nucleus becomes male pronucleus. The sperm centrosome produces asters and contacts the female pronucleus. Male & female pronuclei migrate towards each other, become apposed but do not fuse. They remain adjacent to each other; their nuclear envelopes break down but instead of forming a zygote nucleus the chromatin condenses into chromosomes orienting them on a common mitotic spindle. Thus, only after completion of the first division of fertilized egg, the paternal and maternal chromosomes become enclosed by a common nuclear membrane to form the nuclei of two blastomeres.

Rearrangement of egg cytoplasm:

One of the consequences of egg activation during fertilization is reorganization of egg cytoplasm. In mammals or sea urchin eggs, the cytoplasmic movements are not obvious but in several other animals these cytoplasmic displacements are crucial for the determining cell fate during development. Most spectacular cytoplasmic movements have been observed in ascidian, Styela partita and in frog. In both these animals, a bilateral symmetry is established in the cytoplasm of fertilized egg. In *Styela partita*, the different regions of the cytoplasm have distinct pigmentation. In the mature egg, a layer of cortical cytoplasm containing yellow lipid granules surrounds a central gray cytoplasm. After sperm entry in vegetal hemisphere of the oocyte, the yellow cortical cytoplasm and the clear cytoplasm derived from the breakdown of the oocyte nucleus starts moving vegetally towards the sperm. As the male pronucleus penetrates deeper into the cytoplasm and moves towards a female pronucleus, the clear and the yellow cytoplasm move with it. Just before the first cleavage, the yellow cytoplasm forms a crescentric area (mesodermal crescent) just below the equator of the egg.

Simultaneously a crescent of light grey cytoplasm (notochordal crescent) appears subequatorially on the opposite side of the egg. As a result of cytoplasmic displacements four different kinds of cytoplasmic regions are seen:

i)   the yellow cytoplasm on one side,

ii) the light cytoplasm on the other side. These two together forma belt surrounding the egg just below the equator. Below the zone towards the vegetal pole

iii). The cytoplasm containing abundant yolk granules and

iv). the clear cytoplasm in the animal hemisphere. This state of cytoplasmic localization before the first division displays asymmetrical distribution of cytoplasm that will be irreversibly fixed after the first division with each cell receiving different cytoplasmic constituents.

Unfertilized egg of frog is radially symmetrical about animal – vegetal axis. A single sperm can enter anywhere on the animal hemisphere of the egg. After sperm entry, the cortical cytoplasm rotates 30 degrees towards the sperm entry point, relative to the inner cytoplasm. As a result of this rotation, the underlying cytoplasm located near the equator on the opposite side of sperm entry point contains diffuse pigment granules and therefore appears gray. This region has been referred to as **gray crescent**.

The rotation of the cortical cytoplasm is important as the grey crescent area, which is exposed, marks the region where gastrulation is initiated in amphibian embryos. The rotation of cortical cytoplasm with respect to inner cytoplasm also causes marked changes within the inner cytoplasm. The cytoplasm of the future dorsal side becomes different from the future ventral side where sperm enters. Thus radially symmetrical embryo is transformed into bilaterally symmetrical embryo. These cytoplasmic movements are also responsible for laying down dorsal – ventral axis of the future embryo.

Preparation for cleavage:

Before fertilization, the egg, which has been under metabolic arrest, is released from this arrest on the entry of the sperm. This initiates the process of development by active protein and DNA synthesis in the egg leading to the beginning of cleavage. The first cleavage is not random but tends to be specified by the point of sperm entry and the subsequent rotation of the egg cytoplasm.