4

Fertilization

Beginning a New Organism

FERTILIZATION IS THE PROCESS whereby the sperm and the egg—collectively called the **gametes**—fuse together to begin the creation of a new individual whose genome is derived from both parents. Fertilization accomplishes two separate ends: sex (the combining of genes derived from two parents) and reproduction (the generation of a new organism). Thus, the first function of fertilization is to transmit genes from parent to offspring, and the second is to initiate in the egg cytoplasm those reactions that permit development to proceed.

Although the details of fertilization vary from species to species, conception generally consists of four major events:

1. Contact and recognition between sperm and egg. In most cases, this ensures that the sperm and egg are of the same species.
2. Regulation of sperm entry into the egg. Only one sperm nucleus can ultimately unite with the egg nucleus. This is usually accomplished by allowing only one sperm to enter the egg and actively inhibiting any others from entering.
3. Fusion of the genetic material of sperm and egg.
4. Activation of egg metabolism to start development.

Urge and urge and urge,
Always the procreant urge of the world.
Out of the dimness opposite equals advance,
Always substance and increase, always sex,
Always a knit of identity, always distinction,

WALT WHITMAN (1855)

The final aim of all love intrigues, be they comic or tragic, is really of more importance than all other ends in human life. What it turns upon is nothing less than the composition of the next generation.

A. SCHOPENHAUER
(QUOTED BY C. DARWIN, 1871)

Structure of the Gametes

A complex dialogue exists between egg and sperm. The egg activates the sperm metabolism that is essential for fertilization, and the sperm reciprocates by activating the egg metabolism needed for the onset of development. But before we investigate these aspects of fertilization, we need to consider the structures of the sperm and egg—the two cell types specialized for fertilization.*

Sperm

It is only within the past 135 years that the sperm's role in fertilization has been known. Anton van Leeuwenhoek, the Dutch microscopist who co-discovered sperm in the 1670s, first believed them to be parasitic animals living within the semen (hence the term *spermatozoa*, meaning "seed animals"). Although he originally assumed that they had nothing to do with reproducing the organism in which they were found, he later came to believe that each sperm contained a pre-

*Many courses begin with gametogenesis and meiosis. This author believes that meiosis and gametogenesis are the culminating processes in development, and that they cannot be properly appreciated without first understanding somatic organogenesis and differentiation. Also, having gonad formation and gametogenesis in the last lectures completes a circle.

formed embryo. Leeuwenhoek (1685) wrote that sperm were seeds (both *sperma* and *semen* mean "seed"), and that the female merely provided the nutrient soil in which the seeds were planted. In this, he was returning to a notion of procreation promulgated by Aristotle 2000 years earlier.

Try as he might, Leeuwenhoek was continually disappointed in his attempts to find preformed embryos within spermatozoa. Nicolas Hartsoeker, the other co-discoverer of sperm, drew a picture of what he hoped to find: a miniscule human ("homunculus") within the sperm (Figure 4.1). This belief that the sperm contained the entire embryonic organism never gained much acceptance, as it implied an enormous waste of potential life. Most investigators regarded the sperm as unimportant.*

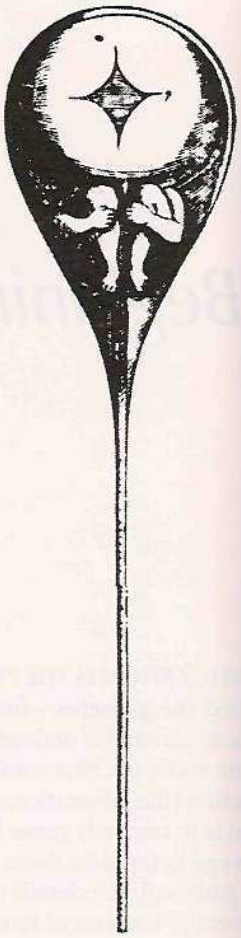


FIGURE 4.1 The human infant preformed in the sperm, as depicted by Nicolas Hartsoeker (1694).

See WEBSITE 4.1
Leeuwenhoek and images of homunculi

The first evidence suggesting the importance of sperm in reproduction came from a series of experiments performed by Lazzaro Spallanzani in the late 1700s. Spallanzani induced male toads to ejaculate into taffeta breeches and found toad semen so filtered to be devoid of sperm; such semen did not fertilize eggs. He even showed that semen had to touch the eggs in order to be functional. However, Spallanzani (like many others) felt that the spermatic "animals" were parasites in the fluid; he thought the embryo was contained within the egg and needed spermatic fluid to activate it (see Pinto-Correia 1997).

The combination of better microscopic lenses and the elucidation of the cell theory (that all life is cellular, and all cells come from preexisting cells) led to a new appreciation of sperm function. In 1824, J. L. Prevost and J. B. Dumas claimed that sperm were not parasites, but rather the active agents of fertilization. They noted the universal existence of sperm in sexually mature males and their absence in immature and aged individuals. These observations, coupled with the known absence of sperm in the sterile mule, convinced them that "there exists an intimate relation between their presence in the organs and the fecundating capacity of the animal." They proposed that the sperm entered the egg and contributed materially to the next generation.

These claims were largely disregarded until the 1840s, when A. von Kolliker described the formation of sperm from cells in the adult testes. He ridiculed the idea that the semen could be normal and yet support such an enormous number of parasites. Even so, von Kolliker denied there was any physical contact between sperm and egg. He believed that the sperm excited the egg to develop in much the same way a magnet communicates its presence to iron. It was not until 1876 that Oscar Hertwig and Herman Fol independently demonstrated sperm entry into the egg and the union of the two cells' nuclei. Hertwig had been seeking an organism suitable for detailed microscopic observations, and he found the Mediterranean sea urchin (*Paracentrotus lividus*) to be perfect for this purpose. Not only was it common throughout the region and sexually mature throughout most of the year, but its eggs were available in large numbers and were transparent even at high magnifications.

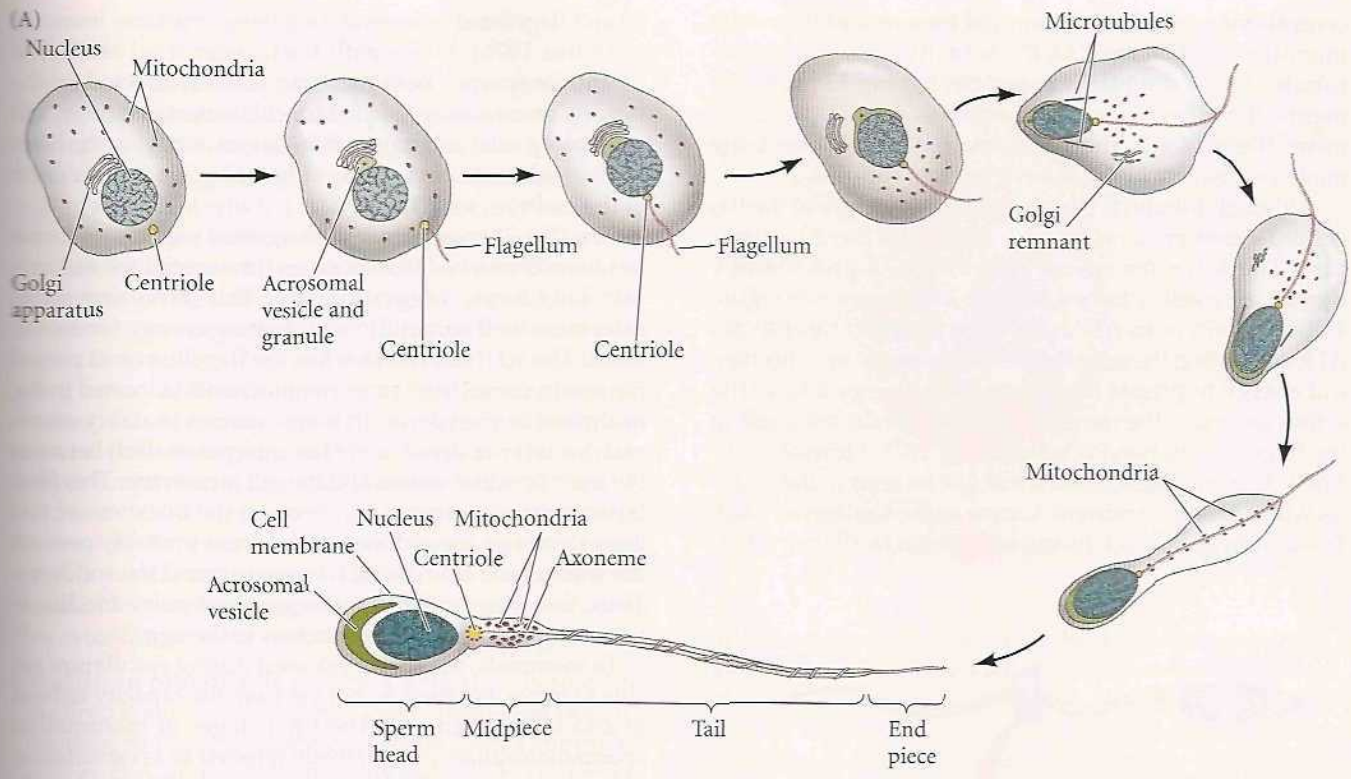
When he mixed suspensions of sperm together with egg suspensions, Hertwig repeatedly observed sperm entering the eggs and saw sperm and egg nuclei unite. He also noted that *only one sperm was seen to enter each egg, and that all the nuclei of the resulting embryo were derived mitotically from the nucleus created at fertilization.* Fol made similar observations and also detailed the mechanism of sperm entry. Fertilization was at last recognized as the union of sperm and egg, and the union of sea urchin gametes remains one of the best-studied examples of fertilization.*

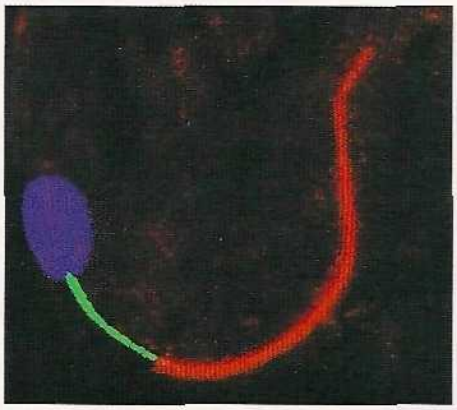
See WEBSITE 4.2
The origins of fertilization research

Each sperm cell consists of a **haploid nucleus**, a propulsion system to move the nucleus, and a sac of enzymes that enable the nucleus to enter the egg. In most species, almost all of the cell's cytoplasm is eliminated during sperm maturation, leaving only certain organelles that are modified for spermatic function (Figure 4.2). During the course of maturation, the sperm's haploid nucleus becomes very streamlined and its DNA becomes tightly compressed. In front of this compressed haploid nucleus lies the **acrosomal vesicle**, or **acrosome**. The acrosome is derived from

*Indeed, sperm was discovered around 1676, while the events of fertilization were not elucidated until 1876. Thus, for nearly 200 years, people had no idea what the sperm actually did. See Pinto-Correia 1997 for details of this remarkable story.

*Hertwig and Fol were actually not the first persons to report fertilization in the sea urchin. At least three other astute observers—Adolphe Dufossé, Karl Ernst von Baer, and Alphonse Derbés—observed sperm-egg contact in 1847. Briggs and Wessel (2006) suggest that the convulsions of Europe during 1848, the low opinion German scientists had of French biology, and the tenuousness of these results (given poor microscopy and the lack of a theory in which to place them) may have confined these papers to obscurity.





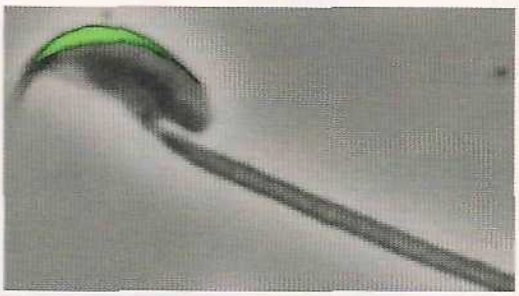


FIGURE 4.2 Modification of a germ cell to form a mammalian sperm. (A) The centriole produces a long flagellum at what will be the posterior end of the sperm, and the Golgi apparatus forms the acrosomal vesicle at the future anterior end. The mitochondria collect around the flagellum near the base of the haploid nucleus and become incorporated into the midpiece ("neck") of the sperm. The remaining cytoplasm is jettisoned, and the nucleus condenses. The size of the mature sperm has been enlarged relative to the other stages. (B) Mature bull sperm. The DNA is stained blue with DAPI, the mitochondria are stained green, and the tubulin of the flagellum is stained red. (C) Acrosome of mouse sperm, stained green by the fusion protein proacrosin-GFP. (A after Clermont and Leblond 1955; B from Sutovsky et al. 1996, courtesy of G. Schatten; C, courtesy of K.-S. Kim and G. L. Gerton.)

the cell's Golgi apparatus and contains enzymes that digest proteins and complex sugars; thus, the acrosome can be considered a modified secretory vesicle. The enzymes stored in the acrosome are used to digest a path through the outer coverings of the egg. In many species, a region of globular actin proteins lies between the sperm nucleus and the acrosomal vesicle. These proteins are used to extend a fingerlike **acrosomal process** from the sperm during the early stages of fertilization. In sea urchins and several other species, recognition between sperm and egg involves molecules on the acrosomal process. Together, the acrosome and nucleus constitute the **sperm head**.

The means by which sperm are propelled vary according to how the species has adapted to environmental conditions. In most species (the major exception is, once again, the nematodes, where the sperm is formed at the sites where fertilization occurs), an individual sperm is able to travel by whipping its **flagellum**. The major motor portion of the flagellum is the **axoneme**, a structure formed by microtubules emanating from the centriole at the base of the sperm nucleus. The core of the axoneme consists of two

central microtubules surrounded by a row of 9 doublet microtubules (Figure 4.3A,B). Actually, only one microtubule of each doublet is complete, having 13 protofilaments; the other is C-shaped and has only 11 protofilaments (Figure 4.3C). The interconnected protofilaments are made exclusively of the dimeric protein **tubulin**.

Although tubulin is the basis for the structure of the flagellum, other proteins are also critical for flagellar function. The force for sperm propulsion is provided by **dynein**, a protein attached to the microtubules (see Figure 4.3B). Dynein is an ATPase, an enzyme that hydrolyzes ATP, converting the released chemical energy into mechanical energy to propel the sperm. This energy allows the active sliding of the outer doublet microtubules, causing the flagellum to bend (Ogawa et al. 1977; Shinyoji et al. 1998). The importance of dynein can be seen in individuals with a genetic syndrome known as the Kartagener triad. These individuals lack functional dynein in all their ciliated and flagellated cells, rendering these structures immotile (Afzelius 1976). Males with Kartagener triad are sterile (immotile sperm). Both men and women affected by the syndrome are susceptible to bronchial infections (immotile respiratory cilia) and have a 50% chance of having the heart on the right side of the body (immotile cilia in the center of the embryo; see Chapter 8).

The "9 + 2" microtubule arrangement with dynein arms has been conserved in axonemes throughout the eukaryotic kingdoms, suggesting that this arrangement is extremely well suited for transmitting energy for movement. The ATP needed to whip the flagellum and propel the sperm comes from rings of mitochondria located in the **midpiece** of the sperm. In many species (notably mammals), a layer of dense fibers has interposed itself between the mitochondrial sheath and the cell membrane. This fiber layer stiffens the sperm tail. Because the thickness of this layer decreases toward the tip, the fibers probably prevent the sperm head from being whipped around too suddenly. Thus, the sperm cell has undergone extensive modification for the transport of its nucleus to the egg.

In mammals, the sperm released during ejaculation are able to move, but they do not yet have the capacity to bind to and fertilize an egg. The final stages of mammalian sperm maturation, cumulatively referred to as *capacitation*, do not occur until the sperm has been inside the female reproductive tract for a certain period of time.

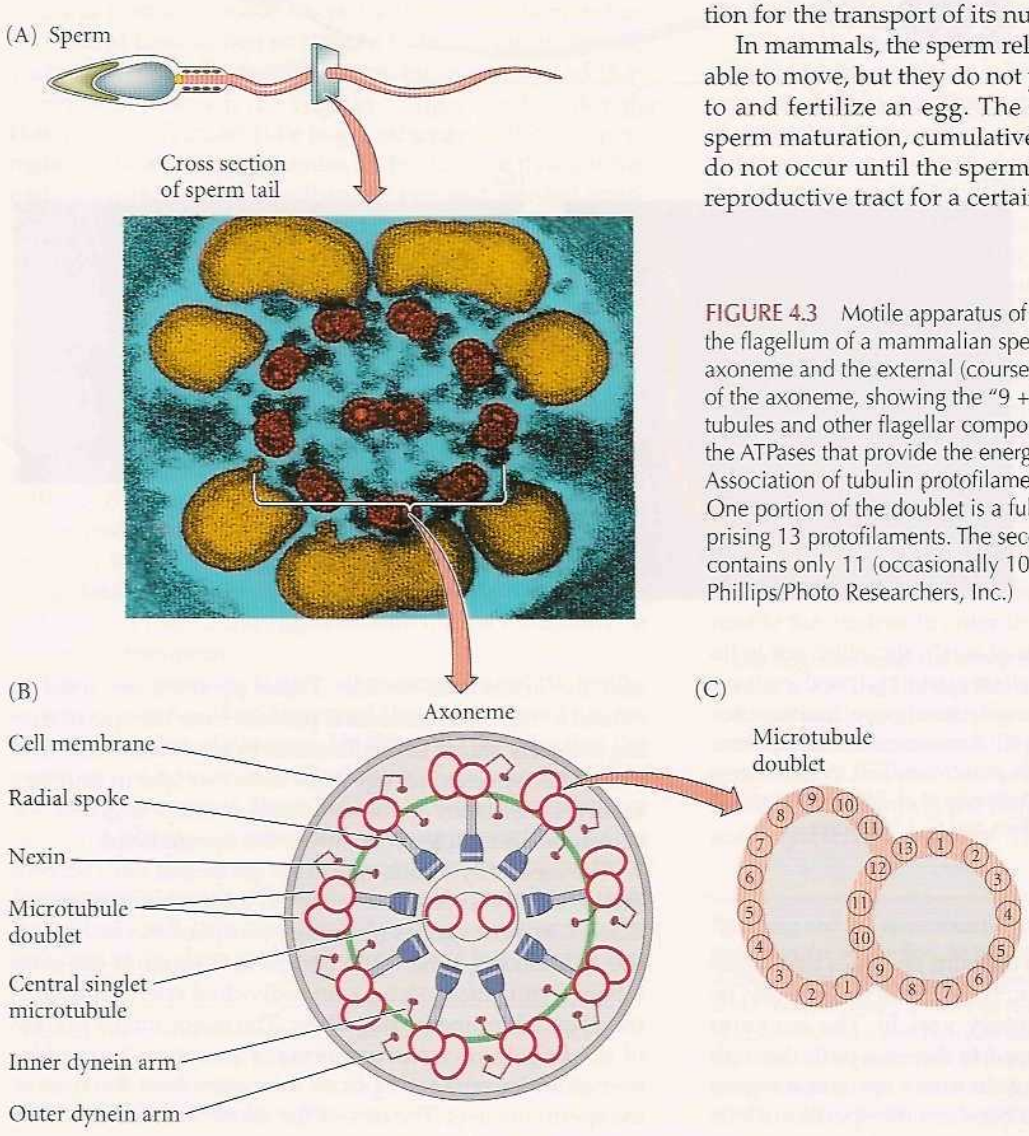


FIGURE 4.3 Motile apparatus of the sperm. (A) Cross section of the flagellum of a mammalian spermatozoon, showing the central axoneme and the external (course) fibers. (B) Interpretive diagram of the axoneme, showing the "9 + 2" arrangement of the microtubules and other flagellar components. The dynein arms contain the ATPases that provide the energy for flagellar movement. (C) Association of tubulin protofilaments into a microtubule doublet. One portion of the doublet is a fully circular microtubule comprising 13 protofilaments. The second portion of the doublet contains only 11 (occasionally 10) protofilaments. (A © D. M. Phillips/Photo Researchers, Inc.)

The egg

All the material necessary for the beginning of growth and development must be stored in the egg, or **ovum**. Whereas the sperm eliminates most of its cytoplasm as it matures, the developing egg (called the **oocyte** before it reaches the stage of meiosis at which it is fertilized*) not only conserves the material it has, but actively accumulates more. The meiotic divisions that form the oocyte conserve its cytoplasm rather than giving half of it away (see Figure 16.30); at the same time, the oocyte either synthesizes or absorbs proteins such as yolk that act as food reservoirs for the developing embryo. Birds' eggs are enormous single cells, swollen with accumulated yolk. Even eggs with relatively sparse yolk are large compared with sperm. The volume of a sea urchin egg is about 200 picoliters (2×10^{-4} mm^3), more than 10,000 times the volume of sea urchin sperm (Figure 4.4). So, even though sperm and egg have equal haploid nuclear components, the egg also accumulates a remarkable cytoplasmic storehouse during its maturation. This cytoplasmic trove includes the following:

- **Nutritive proteins**. It will be a long time before the embryo is able to feed itself or even obtain food from its mother, so the early embryonic cells need a supply of energy and amino acids. In many species, this is accomplished by accumulating yolk proteins in the egg. Many of these yolk proteins are made in other organs (e.g., liver, fat bodies) and travel through the maternal blood to the oocyte.
- **Ribosomes and tRNA**. The early embryo needs to make many of its own structural proteins and enzymes, and in some species there is a burst of protein synthesis soon after fertilization. Protein synthesis is accomplished by ribosomes and tRNA that exist in the egg. The developing egg has special mechanisms for synthesizing ribosomes; certain amphibian oocytes produce as many as 10^{12} ribosomes during their meiotic prophase.
- **Messenger RNAs**. The oocyte not only accumulates proteins, it also accumulates mRNAs that encode proteins for the early stages of development. It is estimated that sea urchin eggs contain thousands of different types of mRNA that remain repressed until after fertilization (see Chapter 2).

*Eggs over easy: The terminology of eggs is confusing. In general, an *egg* is a female gamete capable of binding sperm and being fertilized. An *oocyte* is a developing egg that cannot yet bind sperm or be fertilized (Wessel 2009). The problems in terminology come from the fact that different species of animals have eggs in different stages of meiosis (see Figure 4.5). The human egg, for instance, is in second meiotic metaphase when it binds sperm, whereas the sea urchin egg has finished all of its meiotic divisions when it binds sperm. The contents of the egg also vary greatly from species to species. The synthesis and placement of these materials will be addressed in Chapter 16, when we discuss the differentiation of the germ cells.

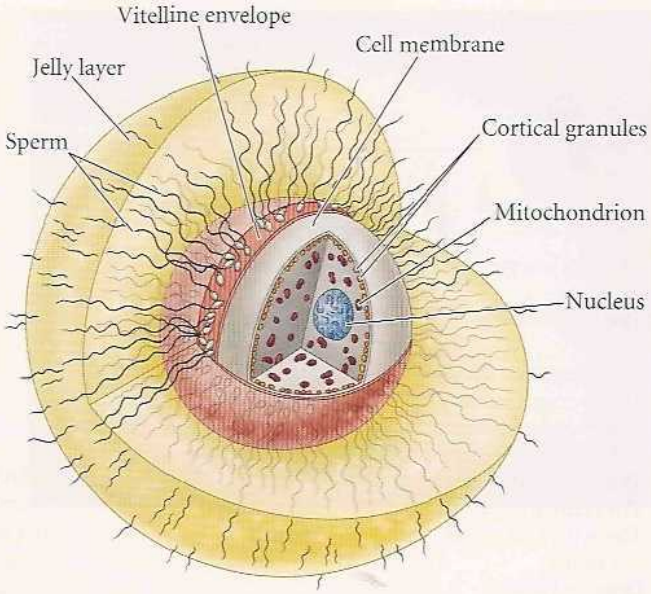


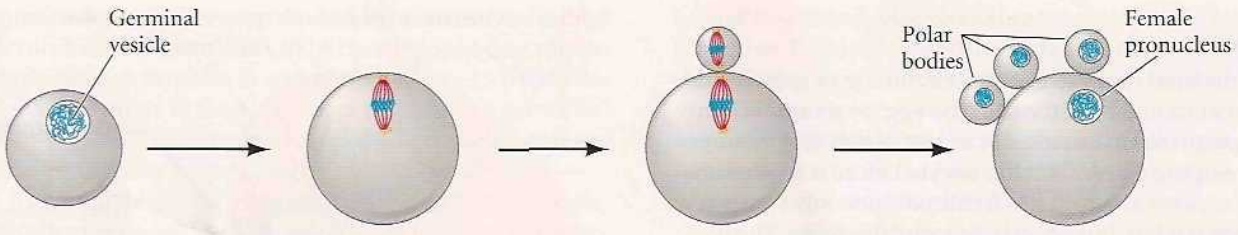
FIGURE 4.4 Structure of the sea urchin egg at fertilization. The drawing shows the relative sizes of egg and sperm. (After Epel 1977.)

- **Morphogenic factors**. Molecules that direct the differentiation of cells into certain cell types are present in the egg. These include transcription factors and paracrine factors. In many species, they are localized in different regions of the egg and become segregated into different cells during cleavage (see Chapter 5).
- **Protective chemicals**. The embryo cannot run away from predators or move to a safer environment, so it must come equipped to deal with threats. Many eggs contain ultraviolet filters and DNA repair enzymes that protect them from sunlight. Some eggs contain molecules that potential predators find distasteful, and the yolk of bird eggs even contains antibodies.

Within this enormous volume of egg cytoplasm resides a large nucleus. In a few species (such as sea urchins), the **female pronucleus** is already haploid at the time of fertilization. In other species (including many worms and most mammals), the egg nucleus is still diploid—the sperm enters before the egg's meiotic divisions are completed (Figure 4.5). In these species, the final stages of egg meiosis will take place after the sperm's nuclear material (the **male pronucleus**) is already inside the egg cytoplasm.

See WEBSITE 4.3 The egg and its environment

Enclosing the cytoplasm is the egg **cell membrane**. This membrane must be capable of fusing with the sperm cell membrane and must regulate the flow of certain ions during fertilization. Outside the cell membrane is an extracellular matrix that forms a fibrous mat around the egg and is often involved in sperm-egg recognition (Correia and Carroll 1997). In invertebrates, this structure is usually



Primary oocyte	First metaphase	Second metaphase	Meiosis complete
The roundworm *Ascaris*	The nemertean worm *Cerebratulus*	The lancelet *Branchiostoma*	Cnidarians (e.g., anemones)
The mesozoan *Dicyema*	The polychaete worm *Chaetopterus*	Amphibians	Sea urchins
The sponge *Grantia*	The mollusc *Dentalium*	Most mammals	
The polychaete worm *Myzostoma*	The core worm *Pectinaria*	Fish	
The clam worm *Nereis*	Many insects		
The clam *Spisula*	Starfish		
The echiuroid worm *Urechis*			
Dogs and foxes			

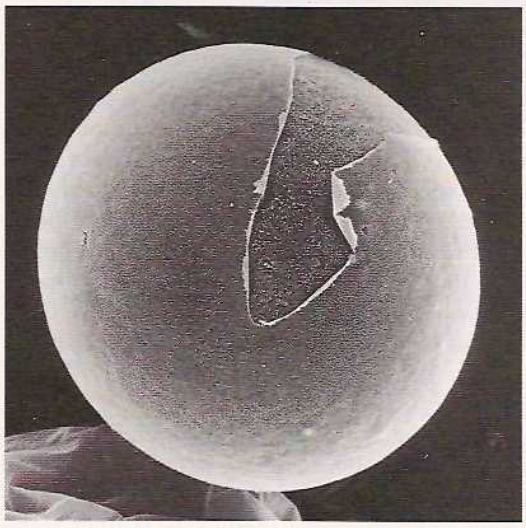
FIGURE 4.5 Stages of egg maturation at the time of sperm entry in different animal species. Note that in most species, sperm entry occurs before the egg nucleus has completed meiosis. The germinal vesicle is the name given to the large diploid nucleus of the primary oocyte. The polar bodies are nonfunctional cells produced by meiosis (see Chapter 16). (After Austin 1965.)

called the **vitelline envelope** (Figure 4.6). The vitelline envelope contains several different glycoproteins. It is supplemented by extensions of membrane glycoproteins from the cell membrane and by proteinaceous "posts" that adhere the vitelline envelope to the membrane (Mozingo and Chandler 1991). The vitelline envelope is essential for the species-specific binding of sperm.

Many types of eggs also have a layer of **egg jelly** outside the vitelline envelope (see Figure 4.4). This glycoprotein meshwork can have numerous functions, but most commonly it is used either to attract or to activate sperm. The egg, then, is a cell specialized for receiving sperm and initiating development.

In mammalian eggs, the extracellular envelope is a separate and thick matrix called the **zona pellucida**. The mammalian egg is also surrounded by a layer of cells called the **cumulus** (Figure 4.7), which is made up of the ovarian follicular cells that were nurturing the egg at the time of its release from the ovary. Mammalian sperm have to get past these cells to fertilize the egg. The innermost layer of cumulus cells, immediately adjacent to the zona pellucida, is called the **corona radiata**.

(A)



(B)

FIGURE 4.6 Sea urchin egg cell surface. (A) Scanning electron micrograph of an egg before fertilization. The cell membrane is exposed where the vitelline envelope has been torn. (B) Transmission electron micrograph of an unfertilized egg, showing microvilli and cell membrane, which are closely covered by the vitelline envelope. A cortical granule lies directly beneath the cell membrane. (From Schroeder 1979, courtesy of T. E. Schroeder.)

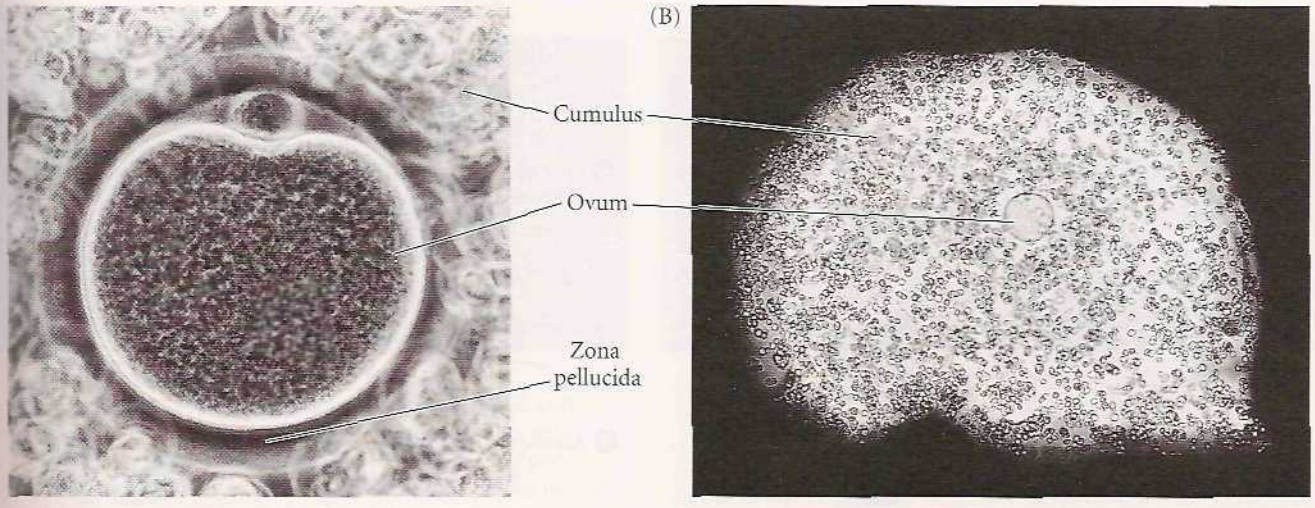


FIGURE 4.7 Mammalian eggs immediately before fertilization. (A) The hamster egg, or ovum, is encased in the zona pellucida. This, in turn, is surrounded by the cells of the cumulus. A polar body cell, produced during meiosis, is also visible within the zona pellucida. (B) At lower magnification, a mouse oocyte is shown surrounded by the cumulus. Colloidal carbon particles (India ink, seen here as the black background) are excluded by the hyaluronidate matrix. (Courtesy of R. Yanagimachi.)

Lying immediately beneath the cell membrane of most eggs is a thin layer (about 5 μm) of gel-like cytoplasm called the **cortex**. The cytoplasm in this region is stiffer than the internal cytoplasm and contains high concentrations of globular actin molecules. During fertilization, these actin molecules polymerize to form long cables of actin known as **microfilaments**. Microfilaments are necessary for cell division. They are also used to extend the egg surface into small projections called **microvilli**, which may aid sperm entry into the cell (see Figure 4.6B; also see Figure 4.16).

Also within the cortex are the **cortical granules** (see Figures 4.4 and 4.6B). These membrane-bound, Golgi-derived structures contain proteolytic enzymes and are thus homologous to the acrosomal vesicle of the sperm. However, whereas a sea urchin sperm contains just one acrosomal vesicle, each sea urchin egg contains approximately 15,000 cortical granules. Moreover, in addition to digestive enzymes, the cortical granules contain mucopolysaccharides, adhesive glycoproteins, and hyalin protein. As we will soon detail, the enzymes and mucopolysaccharides help prevent polyspermy—they prevent additional sperm from entering the egg after the first sperm has entered—and the hyalin and adhesive glycoproteins surround the early embryo and provide support for the cleavage-stage blastomeres.

See VADE MECUM Gametogenesis

Recognition of egg and sperm

The interaction of sperm and egg generally proceeds according to five basic steps (Figure 4.8; Vacquier 1998):

1. The chemoattraction of the sperm to the egg by soluble molecules secreted by the egg
2. The exocytosis of the sperm acrosomal vesicle to release its enzymes
3. The binding of the sperm to the extracellular matrix (vitelline envelope or zona pellucida) of the egg
4. The passage of the sperm through this extracellular matrix
5. Fusion of egg and sperm cell membranes

Sometimes steps 2 and 3 can be reversed (as in mammalian fertilization; see Figure 4.8B), and the sperm binds to the extracellular matrix of the egg before releasing the contents of the acrosome. After these five steps are accomplished, the haploid sperm and egg nuclei can meet and the reactions that initiate development can begin. In this chapter, we will focus on the fertilization events of sea urchins, which undergo external fertilization, and mice, which undergo internal fertilization. In subsequent chapters, the variations of fertilization will be described as we study the development of particular organisms.

External Fertilization in Sea Urchins

In many species, the meeting of sperm and egg is not a simple matter. Many marine organisms release their gametes into the environment. That environment may be as small as a tide pool or as large as an ocean (Mead and Epel 1995). Moreover, this environment is shared with other species that may shed their gametes at the same time. Such organisms are faced with two problems: How can sperm and eggs meet in such a dilute concentration, and how can sperm be prevented from attempting to fertilize

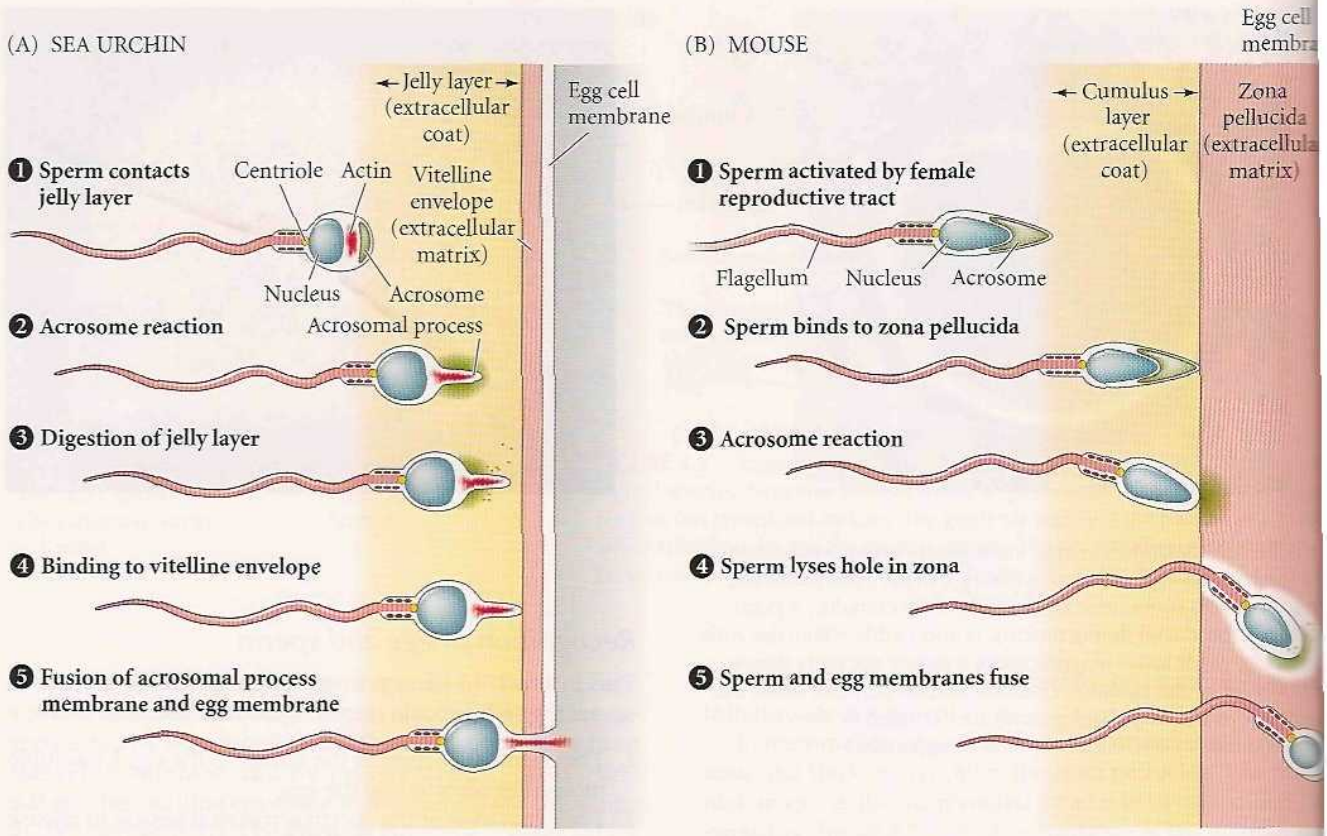


FIGURE 4.8 Summary of events leading to the fusion of egg and sperm cell membranes in (A) the sea urchin and (B) the mouse. (A) Sea urchin fertilization is external. (1) The sperm is chemotactically attracted to and activated by the egg. (2, 3) Contact with the egg jelly triggers the acrosome reaction, allowing the acrosomal process to form and release proteolytic enzymes. (4) The sperm adheres to the vitelline envelope and lyses a hole in it. (5) The sperm adheres to the egg cell membrane and fuses with it. The sperm pronucleus can now enter the egg cytoplasm. (B) Mammalian fertilization is internal. (1) The contents of the female reproductive tract capacitate, attract, and activate the sperm. (2) The acrosome-intact sperm binds to the zona pellucida, which is thicker than the vitelline envelope of sea urchins. (3) The acrosome reaction occurs on the zona pellucida. (4) The sperm digests a hole in the zona pellucida. (5) The sperm adheres to the egg, and their cell membranes fuse.

eggs of another species? In addition to simply producing enormous numbers of gametes, two major mechanisms have evolved to solve these problems: species-specific sperm attraction and species-specific sperm activation. Here we describe these events as they occur in sea urchins.

Sperm attraction: Action at a distance

Species-specific sperm attraction has been documented in numerous species, including cnidarians, molluscs, echinoderms, amphibians, and urochordates (Miller 1985; Yoshida et al. 1993; Burnett et al. 2008). In many species, sperm are attracted toward eggs of their species by **chemotaxis**—that is, by following a gradient of a chemical secreted by the egg. In 1978, Miller demonstrated that the eggs of the cnidarian *Orthopyxis caliculata* not only secrete a chemotactic factor but also regulate the timing of its release. Developing oocytes at various stages in their maturation were fixed on microscope slides, and sperm were released at a certain distance from the eggs. Miller found that when sperm were added to oocytes that had not yet completed their second meiotic division, there was no attraction of sperm to eggs. However, after the second meiotic division was finished and the eggs were ready to be fertilized, the sperm migrated toward them. Thus, these oocytes control not only the type of sperm they attract, but also the time at which they attract them.

The mechanisms of chemotaxis differ among species (see Metz 1978; Eisenbach 2004), and the chemotactic molecules are different even in closely related species. In sea urchins, sperm motility is acquired when the sperm are spawned into seawater. As long as sperm cells are in the testes, they cannot move because their internal pH is kept low (about pH 7.2) by the high concentrations of CO_2 in the gonad. However, once spawned into seawater, sperm pH is elevated to about 7.6, resulting in the activation of the dynein ATPase. The splitting of ATP provides the energy for the flagella to wave, and the sperm begin swimming vigorously (Christen et al. 1982).

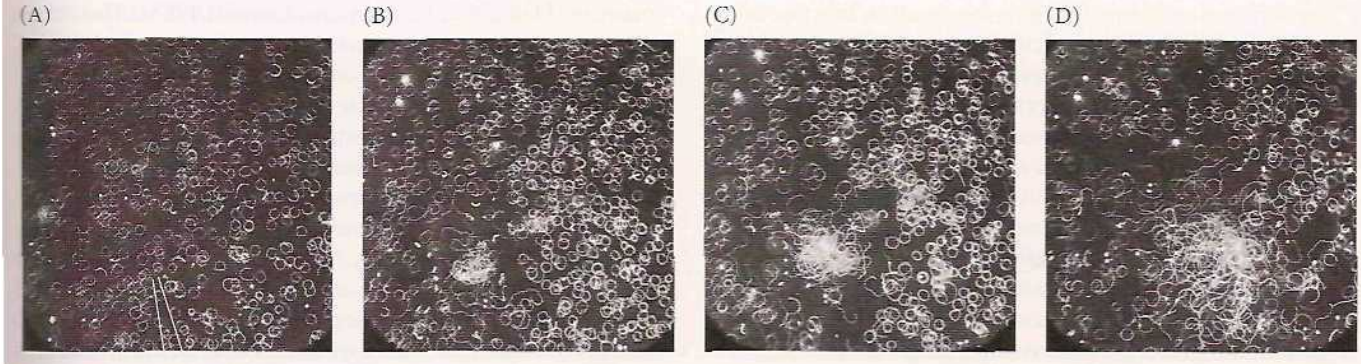
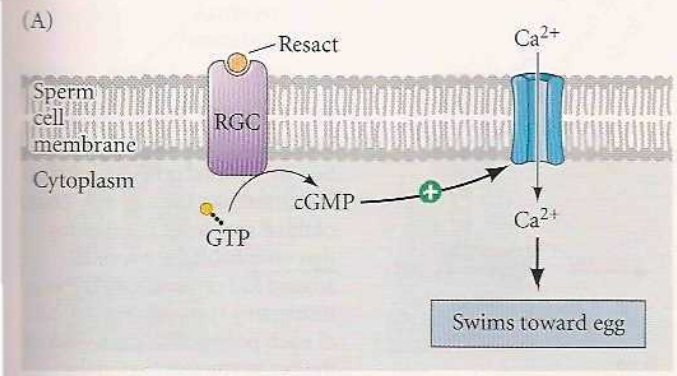


FIGURE 4.9 Sperm chemotaxis in the sea urchin *Arbacia punctulata*. One nanoliter of a 10-n*M* solution of resact is injected into a 20-microliter drop of sperm suspension. (A) A 1-second photographic exposure showing sperm swimming in tight circles before the addition of resact. The position of the injection pipette is shown by the white lines. (B–D) Similar 1-second exposures showing migration of sperm to the center of the resact gradient 20, 40, and 90 seconds after injection. (From Ward et al. 1985, courtesy of V. D. Vacquier.)

But the ability to move does not provide the sperm with direction. In echinoderms, direction is provided by small chemotactic peptides such as resact. **Resact** is a 14-amino acid peptide that has been isolated from the egg jelly of the sea urchin *Arbacia punctulata* (Ward et al. 1985). Resact diffuses readily from the egg jelly into the seawater and has a profound effect at very low concentrations when added to a suspension of *Arbacia* sperm. When a drop of seawater containing *Arbacia* sperm is placed on a microscope slide, the sperm generally swim in circles about 50 μm in diameter. Within seconds after a small amount of resact is injected into the drop, sperm migrate into the region of the injection and congregate there (Figure 4.9). As resact diffuses from the area of injection, more sperm are recruited into the growing cluster. Resact is specific for *A. punctulata* and does not attract sperm of other species. (An analogous compound, speract, has been isolated from the purple sea urchin, *Strongylocentrotus purpuratus*.) *A. punctulata* sperm have receptors in their cell membranes that bind resact (Ramarao and Garbers 1985; Bentley et al. 1986). When the extracellular side of the receptor binds resact, it activates latent guanylyl cyclase activity in the cytoplasmic side of the receptor (Figure 4.10). This causes the sperm cell to make more cyclic GMP (cGMP), a compound that activates a calcium channel, allowing the influx of calcium ions (Ca^{2+}) from the seawater into the sperm, thus providing a directional cue (Nishigaki et al. 2000; Wood et al. 2005). Recent studies have demonstrated that the binding of a single resact molecule is able to provide direction for the sperm, which swim up a concentration gradient of this compound until they reach the egg (Kaupp et al. 2003; Kirkman-Brown et al. 2003).



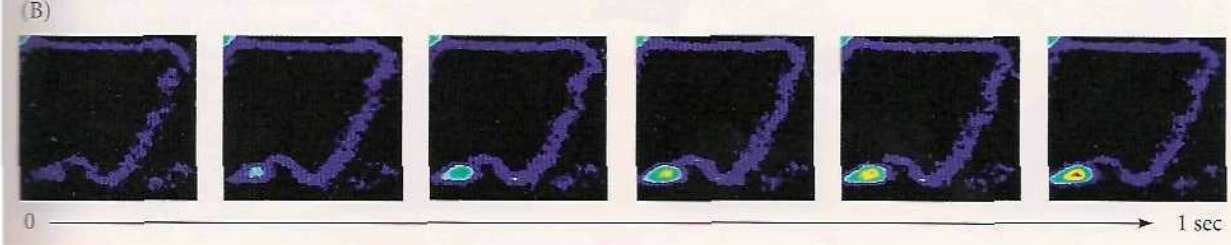


FIGURE 4.10 Model for chemotactic peptides in sea urchin sperm. (A) Resact from *Arbacia* egg jelly binds to its receptor on the sperm. This activates the receptor's guanylyl cyclase (RGC) activity, forming intracellular cGMP in the sperm. The cGMP opens calcium channels in the sperm cell membrane, allowing Ca^{2+} to enter the sperm. The influx of Ca^{2+} activates sperm motility, and the sperm swims up the resact gradient toward the egg. (B) Ca^{2+} levels in different regions of *Strongylocentrotus purpuratus* sperm after exposure to 125 n*M* speract (this species' analog of resact). Red indicates the highest level of Ca^{2+}, blue the lowest. The sperm head reaches its peak Ca^{2+} levels within 1 second. (A after Kirkman-Brown et al. 2003; B from Wood et al. 2003, courtesy of M. Whitaker)

In addition to its function as a sperm-attracting peptide, resact also acts as a **sperm-activating peptide**. One of the major roles of the egg jelly is to increase the motility of sperm, and sperm-activating peptides cause dramatic and immediate increases in mitochondrial respiration and sperm motility (Hardy et al. 1994; Inamdar et al. 2007). The increases in cyclic GMP and Ca^{2+} also activate the mitochondrial ATP-generating apparatus and the dynein ATPase that stimulates flagellar movement in the sperm (Shimomura et al. 1986; Cook and Babcock 1993). Thus, upon meeting resact, *Arbacia* sperm are instructed where to go and are given the motive force to get there.

The acrosome reaction

A second interaction between sperm and egg jelly results in the **acrosome reaction**. In most marine invertebrates, the acrosome reaction has two components: the fusion of the acrosomal vesicle with the sperm cell membrane (an exocytosis that results in the release of the contents of the acrosomal vesicle), and the extension of the acrosomal process (Dan 1952; Colwin and Colwin 1963). The acrosome reaction in sea urchins is initiated by contact of the sperm with the egg jelly. Contact causes the exocytosis of the sperm's acrosomal vesicle and proteolytic enzymes and proteasomes (protein-digesting complexes) that digest a path through the jelly coat to the egg surface (Dan 1967; Franklin 1970). Once the sperm reaches the egg surface, the acrosomal process adheres to the vitelline envelope and tethers the sperm to the egg. It is possible that proteasomes from the acrosome coat the acrosomal process, allowing it to digest the vitelline envelope at the point of attachment and proceed toward the egg (Yokota and Sawada 2007).

In sea urchins, the acrosome reaction is initiated by the interactions of the sperm cell membrane with a specific complex sugar in the egg jelly. These sulfate-containing polysaccharides bind to specific receptors located on the sperm cell membrane directly above the acrosomal vesicle. The egg jelly factors that initiate the acrosome reaction are often highly specific to each species, and egg jelly carbohydrates from one species of sea urchin fail to activate the acrosome reaction even in closely related species (Figure 4.11; Hirohashi

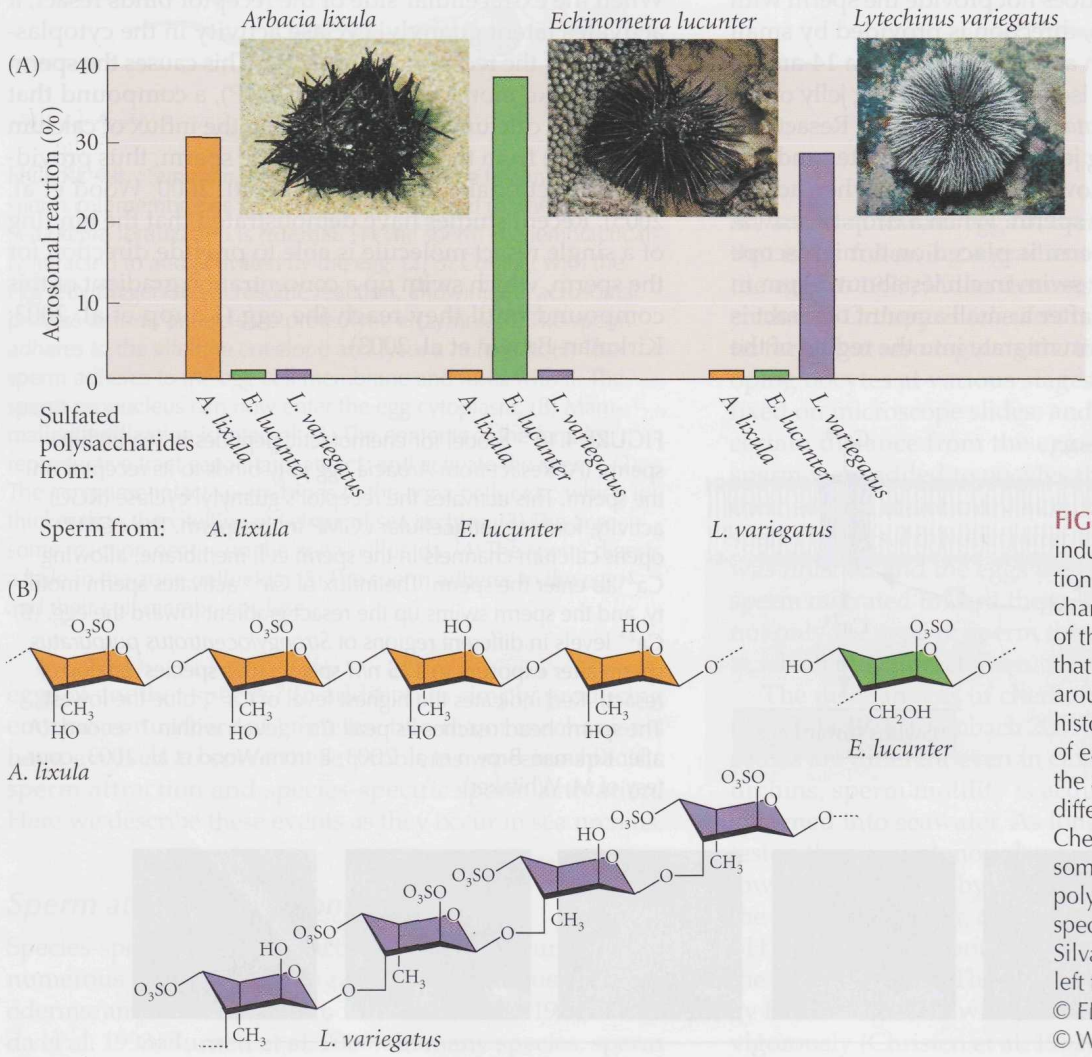


FIGURE 4.11 Species-specific induction of the acrosome reaction by sulfated polysaccharides characterizing the egg jelly coats of three species of sea urchins that co-inhabit the intertidal around Rio de Janeiro. (A) The histograms compare the ability of each polysaccharide to induce the acrosome reaction in the different species of sperm. (B) Chemical structures of the acrosome reaction-inducing sulfated polysaccharides reveal their species-specificity. (After Vilela-Silva et al. 2008; photographs left to right © Interfoto/Alamy; © FLPA/AGE Fotostock; © Water Frame/Alamy.)

and Vacquier 2002; Hirohashi et al. 2002; Vilela-Silva et al. 2008). Thus, the activation of the acrosome reaction constitutes a barrier to interspecies (and thus unviable) fertilizations. This is important when numerous species inhabit the same habitat and when their spawning seasons overlap.

In the sea urchin *Stongylocentrotus purpuratus*, the acrosome reaction is initiated by a repeating polymer of fucose sulfate. When this sulfated carbohydrate binds to its receptor on the sperm, the receptor activates three sperm membrane proteins: (1) a calcium transport channel that allows Ca^{2+} to enter the sperm head; (2) a sodium/hydrogen exchanger that pumps sodium ions (Na^+) into the sperm as it pumps hydrogen ions (H^+) out; and (3) a phospholipase enzyme that makes another second messenger, the phospholipid **inositol trisphosphate** (**IP3**, of which we will hear much more later in the chapter). IP_3 is able to release Ca^{2+} from *inside* the sperm, probably from within the acrosome itself (Domino and Garbers 1988; Domino et al. 1989; Hirohashi and Vacquier 2003). The elevated Ca^{2+} level in a relatively basic cytoplasm triggers the fusion of the acrosomal membrane with the adjacent sperm cell membrane (Figure 4.12A–C), releasing enzymes that can lyse a path through the egg jelly to the vitelline envelope.

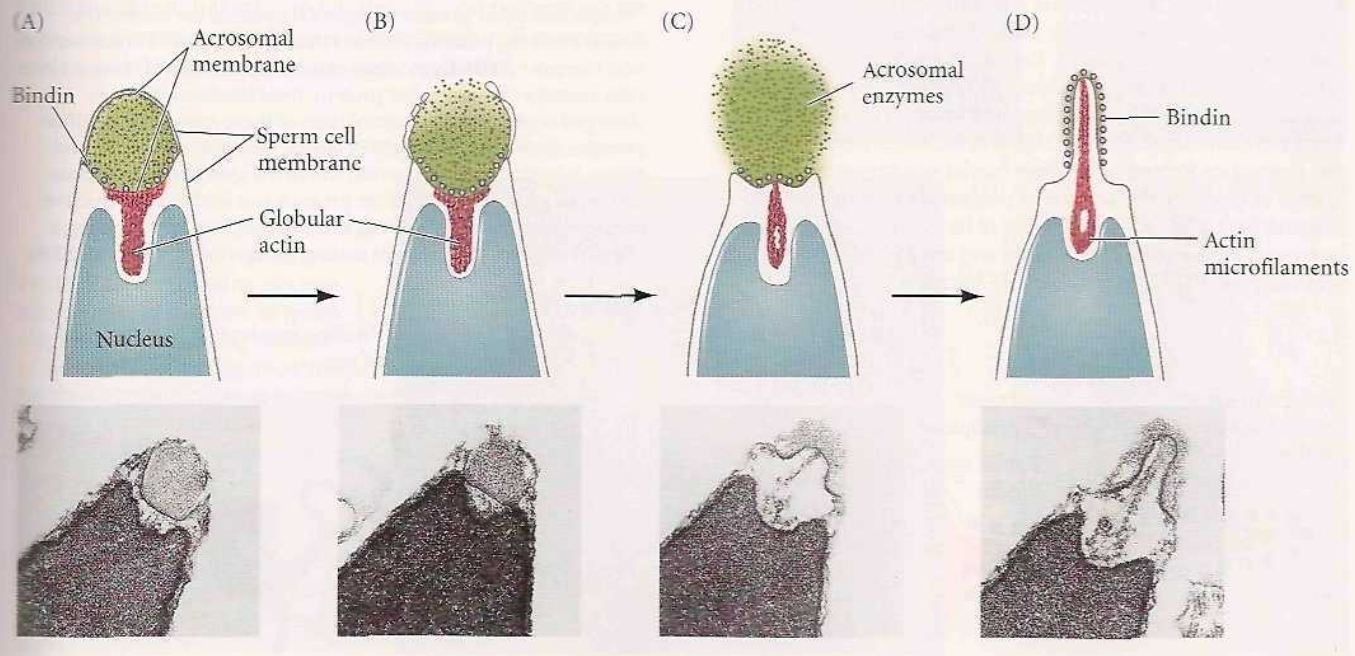
The second part of the acrosome reaction involves the extension of the acrosomal process (Figure 4.12D). This protrusion arises through the polymerization of globular actin molecules into actin filaments (Tilney et al. 1978). The influx of Ca^{2+} is thought to activate the protein RhoB in the acrosomal region and midpiece of sea urchin sperm (Castellano et al. 1997; de la Sancha 2007). This GTP-binding protein helps organize the actin cytoskeleton in many types of cells, and it is thought to be active in polymerizing actin to make the acrosomal process.

Recognition of the egg's extracellular coat

The sperm's contact with an egg's jelly coat provides the first set of species-specific recognition events (i.e., sperm attraction, activation, and acrosome reaction). Another critical species-specific binding event must occur once the sea urchin sperm has penetrated the jelly and the acrosomal process of the sperm contacts the surface of the egg (Figure 4.13A). The acrosomal protein mediating this recognition in sea urchins is called **bindin**. In 1977, Vacquier and co-workers isolated this insoluble, 30,500-Da protein from the acrosome of *Strongylocentrotus purpuratus* and found it to be capable of binding to dejellied eggs of the same species. Further, its interaction with eggs is often species-specific: bindin isolated from the acrosomes of *S. purpuratus* binds to its own dejellied eggs but not to those of *S. franciscanus* (Figure 4.13B; Glabe and Vacquier 1977; Glabe and Lennarz 1979). Using immunological techniques, Moy and Vacquier (1979) demonstrated that bindin is located specifically on the acrosomal process—exactly where it should be for sperm-egg recognition (Figure 4.14).

See WEBSITE 4.4
The Lillie-Loeb dispute over sperm-egg binding

FIGURE 4.12 Acrosome reaction in sea urchin sperm. (A–C) The portion of the acrosomal membrane lying directly beneath the sperm cell membrane fuses with the cell membrane to release the contents of the acrosomal vesicle. (D) The actin molecules assemble to produce microfilaments, extending the acrosomal process outward. Actual photographs of the acrosome reaction in sea urchin sperm are shown below the diagrams. (After Summers and Hylander 1974; photographs courtesy of G. L. Decker and W. J. Lennarz.)



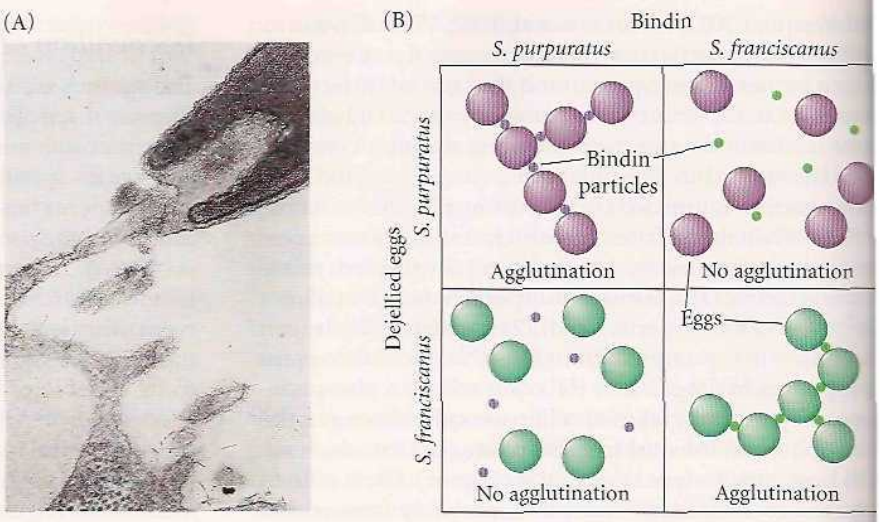
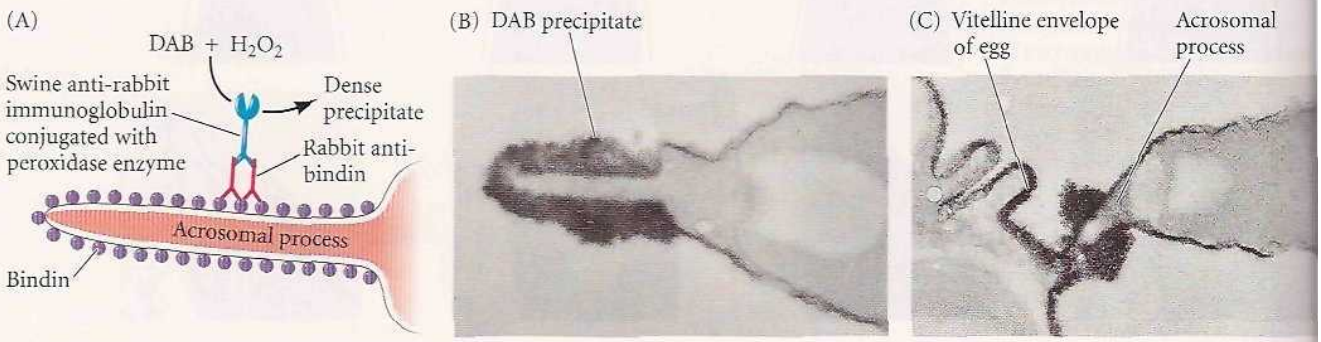


FIGURE 4.13 Species-specific binding of acrosomal process to egg surface in sea urchins. (A) Actual contact of a sea urchin sperm acrosomal process with an egg microvillus. (B) In vitro model of species-specific binding. The agglutination of dejellied eggs by bindin was measured by adding bindin particles to a plastic well containing a suspension of eggs. After 2–5 minutes of gentle shaking, the wells were photographed. Each bindin bound to and agglutinated only eggs from its own species. (A from Epel 1977, courtesy of F. D. Collins and D. Epel; B based on photographs in Glabe and Vacquier 1977.)

Biochemical studies have shown that the bindins of closely related sea urchin species indeed have different protein sequences. This finding implies the existence of species-specific bindin *receptors* on the egg vitelline envelope. Such receptors were suggested by the experiments of Vacquier and Payne (1973), who saturated sea urchin eggs with sperm. As seen in Figure 4.15A, sperm binding does not occur over the entire egg surface. Even at saturating numbers of sperm (approximately 1500), there appears to be room on the ovum for more sperm heads, implying a limiting number of sperm-binding sites. **EBR1**, a 350-kD glycoprotein that displays the properties expected of a bindin receptor, has been isolated from sea urchin eggs (Figure 4.15B; Kamei and Glabe 2003). These bindin receptors are thought to be aggregated into complexes on the vitelline envelope, and hundreds of such complexes may be needed to tether the sperm to the egg. The receptor for sperm bindin on the egg vitelline envelope appears to recognize the protein portion of bindin in a species-specific manner. Closely related species of sea urchins (different species in the same genus) have divergent bindin receptors, and eggs will adhere only to the bindin of their own species* (Figure 4.15C). Thus, species-specific recognition of sea urchin gametes can occur at the levels of sperm attrac

FIGURE 4.14 Localization of bindin on the acrosomal process. (A) Immunochemical technique used to localize bindin. Rabbit antibody was made to the bindin protein, and this antibody was incubated with sperm that had undergone the acrosome reaction. If bindin were present, the rabbit antibody would remain bound to the sperm. After any unbound antibody was washed off, the sperm were treated with swine antibody that had been covalently linked to peroxidase enzymes. The swine antibody bound to the rabbit antibody, placing peroxidase molecules wherever bindin was present. Peroxidase catalyzes the formation of a dark precipitate from diaminobenzidine (DAB) and hydrogen peroxide. Thus, this precipitate formed only where bindin was present. (B) Localization of bindin to the acrosomal process after the acrosome reaction (×33,200). (C) Localization of bindin to the acrosomal process at the junction of the sperm and the egg. (B,C from Moy and Vacquier 1979, courtesy of V. D. Vacquier.)

*Bindin and other gamete recognition proteins are among the fastest evolving proteins known (Metz and Palumbi 1996; Swanson and Vacquier 2002). Even when closely related urchin species have near-identity of every other protein, their bindins may have diverged significantly. The evolution of these gamete recognition proteins shows the hallmarks of sexual selection and coevolved genes. It is thought that coevolution of the genes encoding male and female gamete recognition proteins can lead to reproductive barriers that have the potential to drive speciation by dividing a population into two different mating groups (see Clark et al. 2009).



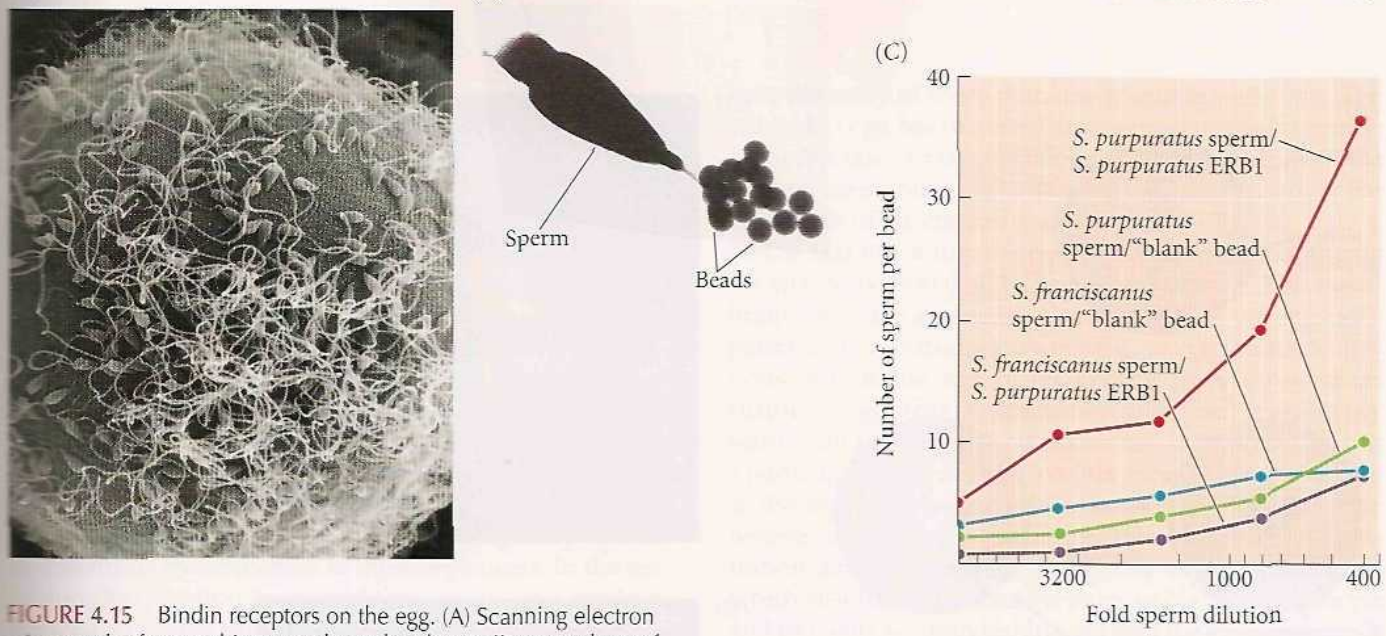
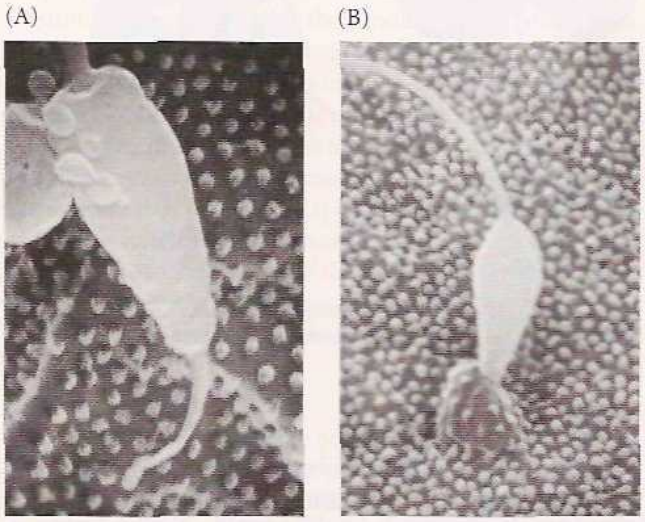


FIGURE 4.15 Bindin receptors on the egg. (A) Scanning electron micrograph of sea urchin sperm bound to the vitelline envelope of an egg. Although this egg is saturated with sperm, there appears to be room on the surface for more sperm, implying the existence of a limited number of bindin receptors. (B) *Strongylocentrotus purpuratus* sperm bind to polystyrene beads that have been coated with purified bindin receptor protein. (C) Species-specific binding of sea urchin sperm to ERB1. *S. purpuratus* sperm bound to beads coated with EBR1 bindin receptor purified from *S. purpuratus* eggs, but *S. franciscanus* sperm did not. Neither sperm bound to uncoated "blank" beads. (A © Mia Tegner/SPL/Photo Researchers, Inc.; B from Foltz et al. 1993; C after Kamei and Glabe 2003.)

tion, sperm activation, the acrosome reaction, and sperm adhesion to the egg surface.

Fusion of the egg and sperm cell membranes

Once the sperm has traveled to the egg and undergone the acrosome reaction, the fusion of the sperm cell membrane with the cell membrane of the egg can begin.



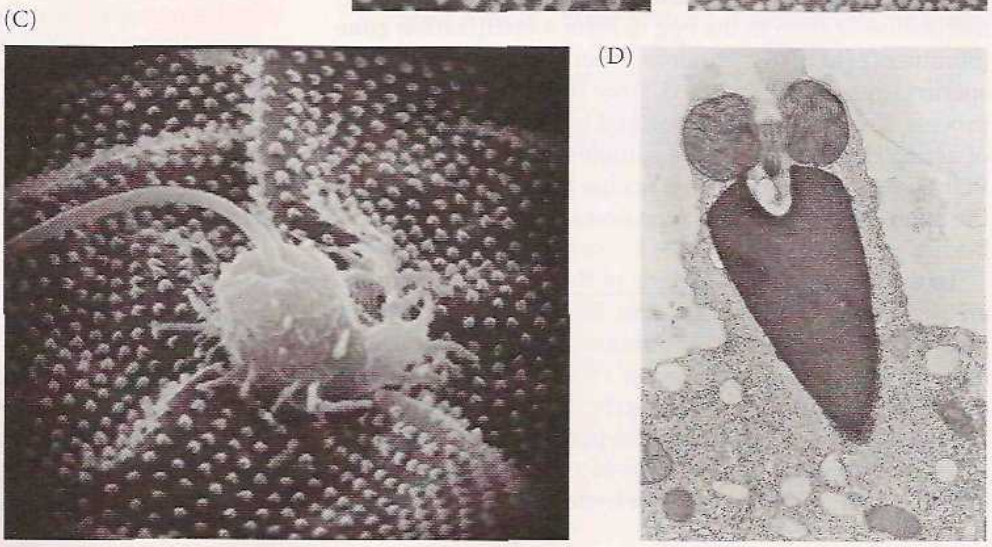
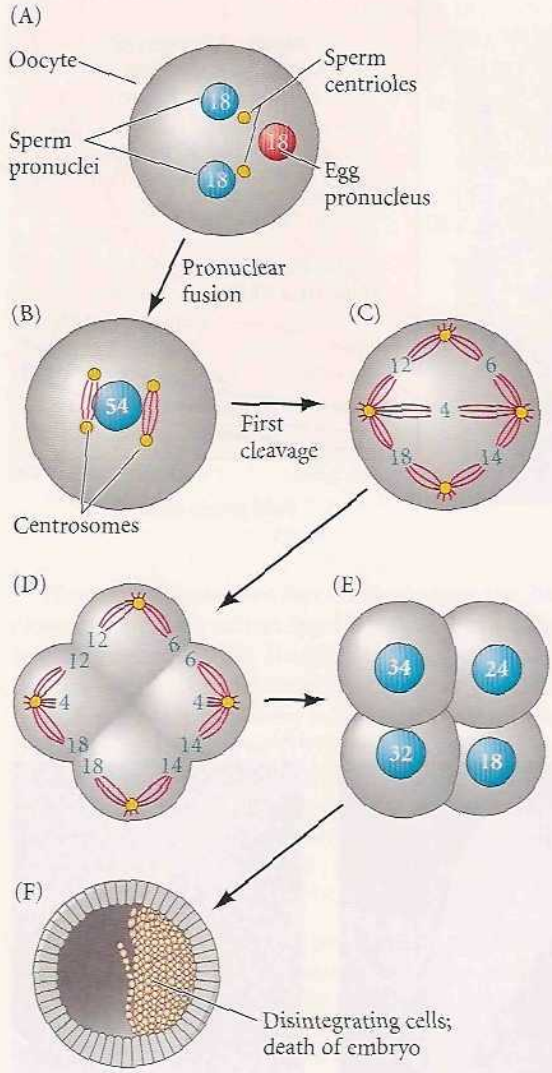


FIGURE 4.16 Scanning electron micrographs of the entry of sperm into sea urchin eggs. (A) Contact of sperm head with egg microvillus through the acrosomal process. (B) Formation of fertilization cone. (C) Internalization of sperm within the egg. (D) Transmission electron micrograph of sperm internalization through the fertilization cone. (A–C from Schatten and Mazia 1976, courtesy of G. Schatten; D courtesy of F. J. Longo.)



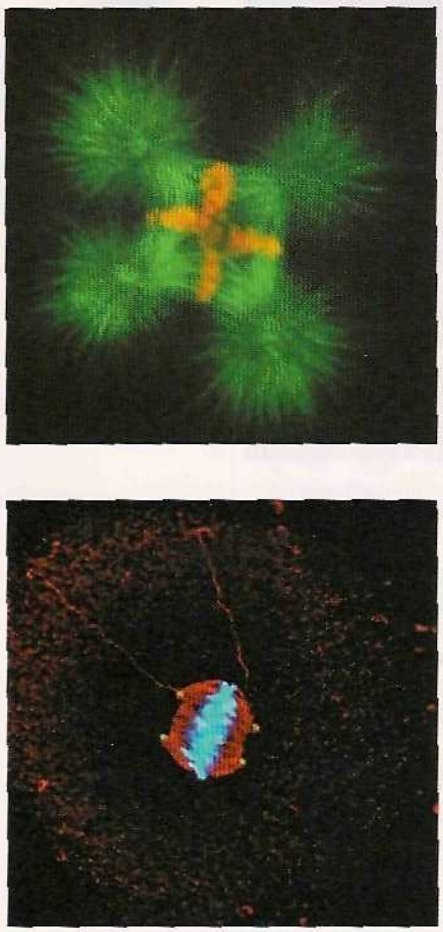


FIGURE 4.17 Aberrant development in a dispermic sea urchin egg. (A) Fusion of three haploid nuclei, each containing 18 chromosomes, and the division of the two sperm centrioles to form four centrosomes (mitotic poles). (B,C) The 54 chromosomes randomly assort on the four spindles. (D) At anaphase of the first division, the duplicated chromosomes are pulled to the four poles. (E) Four cells containing different numbers and types of chromosomes are formed, thereby causing (F) the early death of the embryo. (G) First metaphase of a dispermic sea urchin egg akin to (D). The microtubules are stained green; the DNA stain appears orange. The triploid DNA is being split into four chromosomally unbalanced cells instead of the normal two cells with equal chromosome complements. (H) Human dispermic egg at first mitosis. The four centrioles are stained yellow, while the microtubules of the spindle apparatus (and of the two sperm tails) are stained red. The three sets of chromosomes divided by these four poles are stained blue. (A–F after Boveri 1907; G courtesy of J. Holy; H from Simerly et al. 1999, courtesy of G. Schatten.)

The entry of a sperm into a sea urchin egg is illustrated in Figure 4.16. Sperm-egg fusion appears to cause the polymerization of actin in the egg to form a **fertilization cone** (Summers et al. 1975). Homology between the egg and the sperm is again demonstrated, since the sperm's acrosomal process also appears to be formed by the polymerization of actin. The actin from the gametes forms a connection that widens the cytoplasmic bridge between the egg and the sperm. The sperm nucleus and tail pass through this bridge.

In the sea urchin, all regions of the egg cell membrane are capable of fusing with sperm. In several other species, certain regions of the membrane are specialized for sperm recognition and fusion (Vacquier 1979). Fusion is an active process, often mediated by specific "fusogenic" proteins. Indeed, sea urchin sperm bindin plays a second role as a fusogenic protein. In addition to recognizing the egg, bindin contains a long stretch of hydrophobic amino acids near its amino terminus, and this region is able to fuse phospholipid vesicles in vitro (Ulrich et al. 1999; Gage et al. 2004). Under the proper ionic conditions (those in the mature unfertilized egg), bindin can cause the sperm and egg membranes to fuse.

The fast block to polyspermy

As soon as one sperm has entered the egg, the fusibility of the egg membrane—which was so necessary to get the sperm inside the egg—becomes a dangerous liability. In most animals, any sperm that enters the egg can provide a haploid nucleus and a centriole to the egg. In normal **monospermy**, only one sperm enters the egg, and a haploid sperm nucleus and a haploid egg nucleus combine to form the diploid nucleus of the fertilized egg (zygote), thus restoring the chromosome number appropriate for the species. The centriole provided by the sperm divides to form the two poles of the mitotic spindle during cleavage, while the egg-derived centriole is degraded.

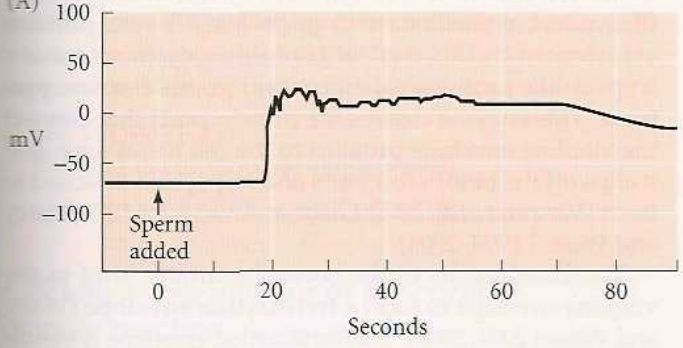
The entrance of multiple sperm—**polyspermy**—leads to disastrous consequences in most organisms. In the sea urchin, fertilization by two sperm results in a triploid nucleus, in which each chromosome is represented three times rather than twice. Worse, each sperm's centriole divides to form the two poles of a mitotic apparatus; so instead of a bipolar mitotic spindle separating the chromosomes into two cells, the triploid chromosomes may be divided into as many as four cells. Because there is no mechanism to ensure that each of the four cells receives the proper number and type of chromosomes, the chromosomes are apportioned unequally: some cells receive extra copies of certain chromosomes, while other cells lack them (Figure 4.17). Theodor Boveri demonstrated in 1902 that such cells either die or develop abnormally.

Species have evolved ways to prevent the union of more than two haploid nuclei. The most common way is to prevent the entry of more than one sperm into the egg. The sea urchin egg has two mechanisms to avoid polyspermy: a fast reaction, accomplished by an electric change in the egg cell membrane, and a slower reaction, caused by the exocytosis of the cortical granules (Just 1919).

The **fast block to polyspermy** is achieved by changing the electric potential of the egg cell membrane. This membrane provides a selective barrier between the egg cytoplasm and the outside environment, so that ion concentrations within the egg differ greatly from those of its surroundings. This concentration difference is especially significant for sodium and potassium ions. Seawater has a particularly high sodium ion (Na^+) concentration, whereas the egg cytoplasm contains relatively little Na^+. The reverse is the case with potassium ions (K^+). These concentration differences are maintained by the cell membrane, which steadfastly inhibits the entry of Na^+ into the oocyte and prevents K^+ from leaking out into the environment. If we insert an electrode into an egg and place a second electrode outside it, we can measure the constant difference in charge across the egg cell membrane. This **resting membrane potential** is generally about 70 mV, usually expressed as –70 mV because the inside of the cell is negatively charged with respect to the exterior.

Within 1–3 seconds after the binding of the first sperm, the membrane potential shifts to a positive level, about +20 mV (Longo et al. 1986). This change is caused by a small influx of Na^+ into the egg (Figure 4.18A). Although sperm can fuse with membranes having a resting potential of –70 mV, they cannot fuse with membranes having a positive resting potential, so no more sperm can fuse to the egg. It is not known whether the increased sodium permeability of the egg is due to the *binding* of the first sperm, or to the *fusion* of the first sperm with the egg (Gould and Stephano 1987, 1991; McCulloh and Chambers 1992). However, recent data

(A)



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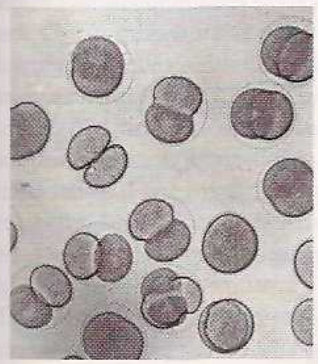

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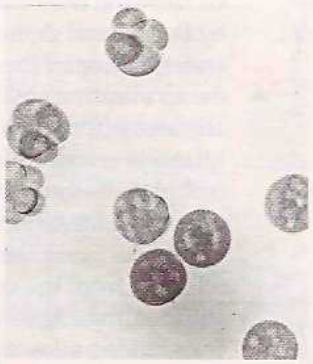


FIGURE 4.18 Membrane potential of sea urchin eggs before and after fertilization. (A) Before the addition of sperm, the potential difference across the egg cell membrane is about –70 mV. Within 1–3 seconds after the fertilizing sperm contacts the egg, the potential shifts in a positive direction. (B,C) *Lytechinus* eggs photographed during first cleavage. (B) Control eggs developing in 490 m*M* Na^+. (C) Polyspermy in eggs fertilized in similarly high concentrations of sperm in 120 m*M* Na^+ (choline was substituted for sodium). (D) Table showing the rise of polyspermy with decreasing Na^+ concentration. Salt water is about 600 m*M* NaCl. (After Jaffe 1980; B,C courtesy of L. A. Jaffe.)

(D)

Na^+ (m*M*)	Polyspermic eggs (%)
490	22
360	26
120	97
50	100

suggest that the fusogenic region of bindin will not function on a positively charged surface (Rocha et al. 2007).

The importance of Na^+ and the change in resting potential was demonstrated by Laurinda Jaffe and colleagues. They found that polyspermy can be induced if sea urchin eggs are artificially supplied with an electric current that keeps their membrane potential negative. Conversely, fertilization can be prevented entirely by artificially keeping the membrane potential of eggs positive (Jaffe 1976). The fast block to polyspermy can also be circumvented by lowering the concentration of Na^+ in the surrounding water (Figure 4.18B–D). If the supply of sodium ions is not sufficient to cause the positive shift in membrane potential, polyspermy occurs (Gould-Somero et al. 1979; Jaffe 1980).

It is not known how the change in membrane potential acts on the sperm to block secondary fertilization. Most likely, the sperm carry a voltage-sensitive component (possibly a positively charged fusogenic protein), and the insertion of this component into the egg cell membrane could be regulated by the electric charge across the membrane (Iwao and Jaffe 1989). An electric block to polyspermy also occurs in frogs* (Cross and Elinson 1980), but probably not in most mammals (Jaffe and Cross 1983).

See WEBSITE 4.5 Blocks to polyspermy

See VADE MECUM E. E. Just

The slow block to polyspermy

The fast block to polyspermy is transient, since the membrane potential of the sea urchin egg remains positive for only about a minute. This brief potential shift is not sufficient to prevent polyspermy permanently, and polyspermy can still occur if the sperm bound to the vitelline envelope are not somehow removed (Carroll and Epel 1975). This sperm removal is accomplished by the **cortical granule reaction**, a slower, mechanical block to polyspermy that becomes active about a minute after the first successful sperm-egg fusion (Just 1919). This reaction—also known as the **slow block to polyspermy**—is found in many animal species, including sea urchins and most mammals.

Directly beneath the sea urchin egg cell membrane are about 15,000 cortical granules, each about 1 μm in diameter (see Figure 4.6B). Upon sperm entry, these cortical granules fuse with the egg cell membrane and release their contents into the space between the cell membrane and the fibrous mat of vitelline envelope proteins. Several proteins are released by this cortical granule exocytosis. One is a trypsin-like protease called **cortical granule serine protease**. This enzyme cleaves the protein posts that connect the vitelline envelope proteins to the cell membrane, and it clips off the bindin receptors and any sperm attached to them (Vacquier et al. 1973; Glabe and Vacquier 1978; Haley and Wessel 1999, 2004).

The components of the cortical granules bind to the vitelline envelope to form a **fertilization envelope** (Wong and Wessel 2004, 2008). This fertilization envelope is elevated from the cell membrane by *glycosaminoglycans* released by the cortical granules. These viscous compounds absorb water to expand the space between the cell membrane and the fertilization envelope, so that the envelope moves radially away from the egg (see Figures 4.19 and 4.20). The fertilization envelope is then stabilized by crosslinking adjacent proteins through egg-specific *peroxidase enzymes* (soluble ovoperoxidase from the cortical granules and Udx1 in the former cortical granule membrane) and a *transglutaminase* released from the cortical granules (Foerder and Shapiro 1977; Wong et al. 2004; Wong and Wessel 2009). This crosslinking allows the egg and early embryo to resist the shear forces of the intertidal waves. As shown in Figure 4.19,

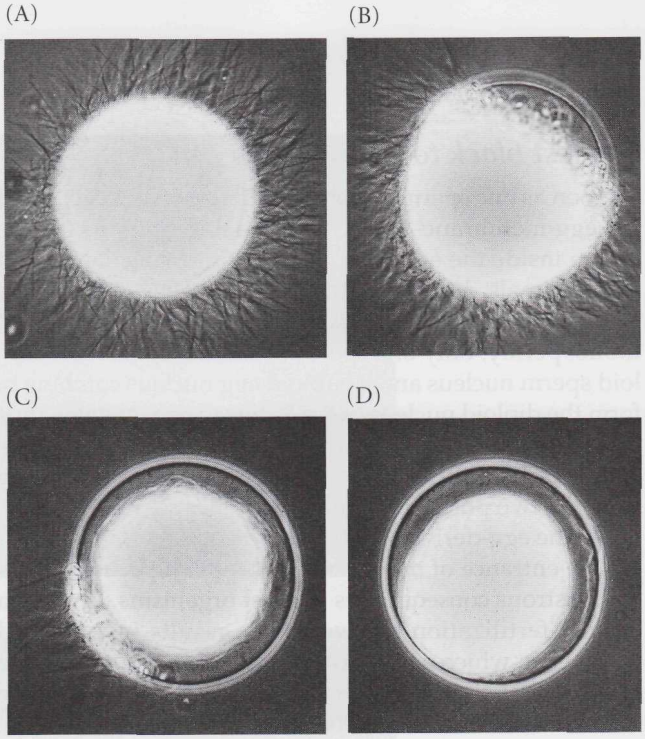


FIGURE 4.19 Formation of the fertilization envelope and removal of excess sperm. To create these photographs, sperm were added to sea urchin eggs, and the suspension was then fixed in formaldehyde to prevent further reactions. (A) At 10 seconds after sperm addition, sperm surround the egg. (B,C) At 25 and 35 seconds after insemination, respectively, a fertilization envelope is forming around the egg, starting at the point of sperm entry. (D) The fertilization envelope is complete, and excess sperm have been removed. (From Vacquier and Payne 1973, courtesy of V. D. Vacquier.)

*One might ask, as did a recent student, how amphibians could have a fast block to polyspermy, since their eggs are fertilized in pond water, which lacks high amounts of sodium ions. It turns out that the ion channels that open in frog egg membranes at fertilization are chloride channels instead of sodium channels as in sea urchin eggs. The concentration of Cl^- inside the frog egg is much higher than that of pond water. Thus, when chloride channels open at fertilization, the negatively charged chloride ions flow *out* of the cytoplasm, leaving the inside of the egg at a positive potential (see Jaffe and Schlicter 1985; Glahn and Nuccitelli 2003).

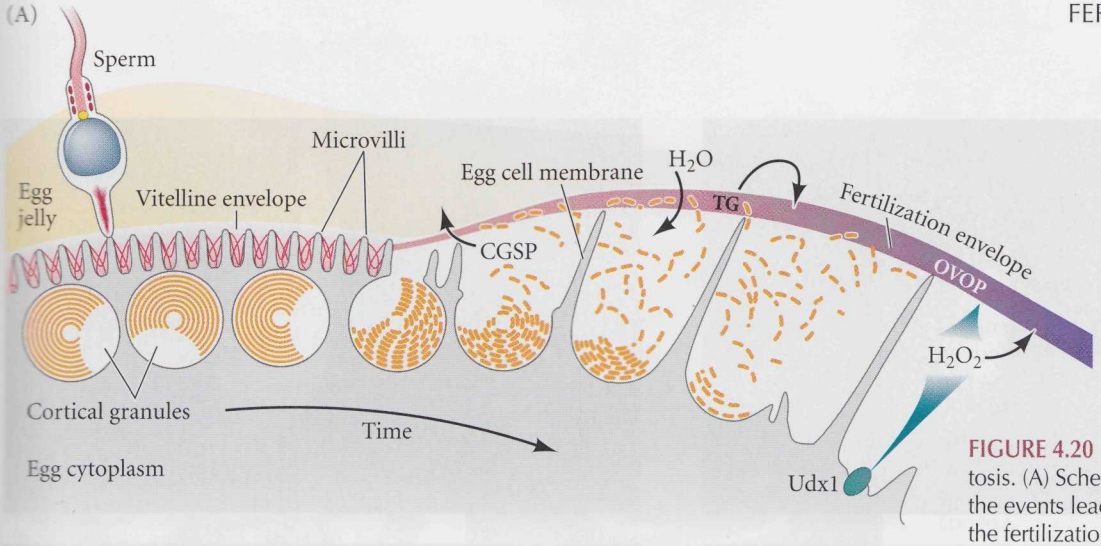


FIGURE 4.20 Cortical granule exocytosis. (A) Schematic diagram showing the events leading to the formation of the fertilization envelope and hyaline layer. As cortical granules undergo exocytosis, they release cortical granule serine protease (CGSP), which cleaves the proteins linking the vitelline envelope to the cell membrane. Mucopolysaccharides released by the cortical granules form an osmotic gradient, thereby causing water to enter and swell the space between the vitelline envelope and the cell membrane. Peroxidases (OVOP and Udx1) and transglutaminases (TG) then harden the vitelline envelope, now called the fertilization envelope. Transmission electron micrographs of the cortex of an unfertilized sea urchin egg (B) and the same region of a recently fertilized egg (C). The raised fertilization envelope and the points at which the cortical granules have fused with the egg cell membrane of the egg (arrows) are visible in (C). (A after Wong et al. 2008; B,C from Chandler and Heuser 1979, courtesy of D. E. Chandler.)

the fertilization envelope starts to form at the site of sperm entry and continues its expansion around the egg. This process starts about 20 seconds after sperm attachment and is complete by the end of the first minute of fertilization. As this is happening, a fourth set of cortical granule proteins, including *hyalin*, forms a coating around the egg (Hylander and Summers 1982). The egg extends elongated microvilli whose tips attach to this **hyaline layer**. This layer provides support for the blastomeres during cleavage.

See WEBSITE 4.6
Building the egg's extracellular matrix

See VADE MECUM Sea urchin fertilization

Calcium as the initiator of the cortical granule reaction

The mechanism of cortical granule exocytosis is similar to that of the exocytosis of the acrosome, and it may involve many of the same molecules.* Upon fertilization, the concentration of free Ca^{2+} in the egg cytoplasm increases greatly. In this high-calcium environment, the cortical granule membranes fuse with the egg cell membrane, releasing their contents (Figure 4.20). Once the fusion of the cortical granules begins near the point of sperm entry, a wave of cortical granule exocytosis propagates around the cortex to the opposite side of the egg.

In sea urchins and mammals, the rise in Ca^{2+} concentration responsible for the cortical granule reaction is not due to an influx of calcium into the egg, but rather comes from within the egg itself. The release of calcium from intracellular storage can be monitored visually using calcium-activated luminescent dyes such as aequorin (like GFP, a protein isolated from luminescent jellyfish) or fluorescent dyes such as fura-2. These dyes emit light when they bind free Ca^{2+}. When a sea urchin egg is injected with dye and then fertilized, a striking wave of calcium release

*Exocytotic reactions like the cortical granule reaction and the acrosome reaction are also seen in the release of insulin from pancreatic cells and in the release of neurotransmitters from synaptic terminals. In all cases, there is Ca^{2+}-mediated fusion of the secretory vesicle and the cell membrane. Indeed, the similarity of acrosomal vesicle exocytosis and synaptic vesicle exocytosis may be quite deep. Studies of acrosome reactions in sea urchins and mammals suggest that when the receptors for the sperm-activating ligands bind these molecules, the resulting depolarization of the membrane opens voltage-dependent Ca^{2+} channels in a manner reminiscent of synaptic transmission (González-Martínez et al. 1992; Tulsani and Abou-Haila 2004). The proteins that dock the cortical granules of the egg to the cell membrane also appear to be homologous to those used in the axon terminal. The synaptic granules of the neurons, the acrosomal vesicle of the sperm, and the cortical granules of the egg all appear to use synaptotagmin to bind calcium and initiate fusion of the vesicle with the cell membrane (Bi et al. 1995; Leguia et al. 2006; Roggero et al. 2007).

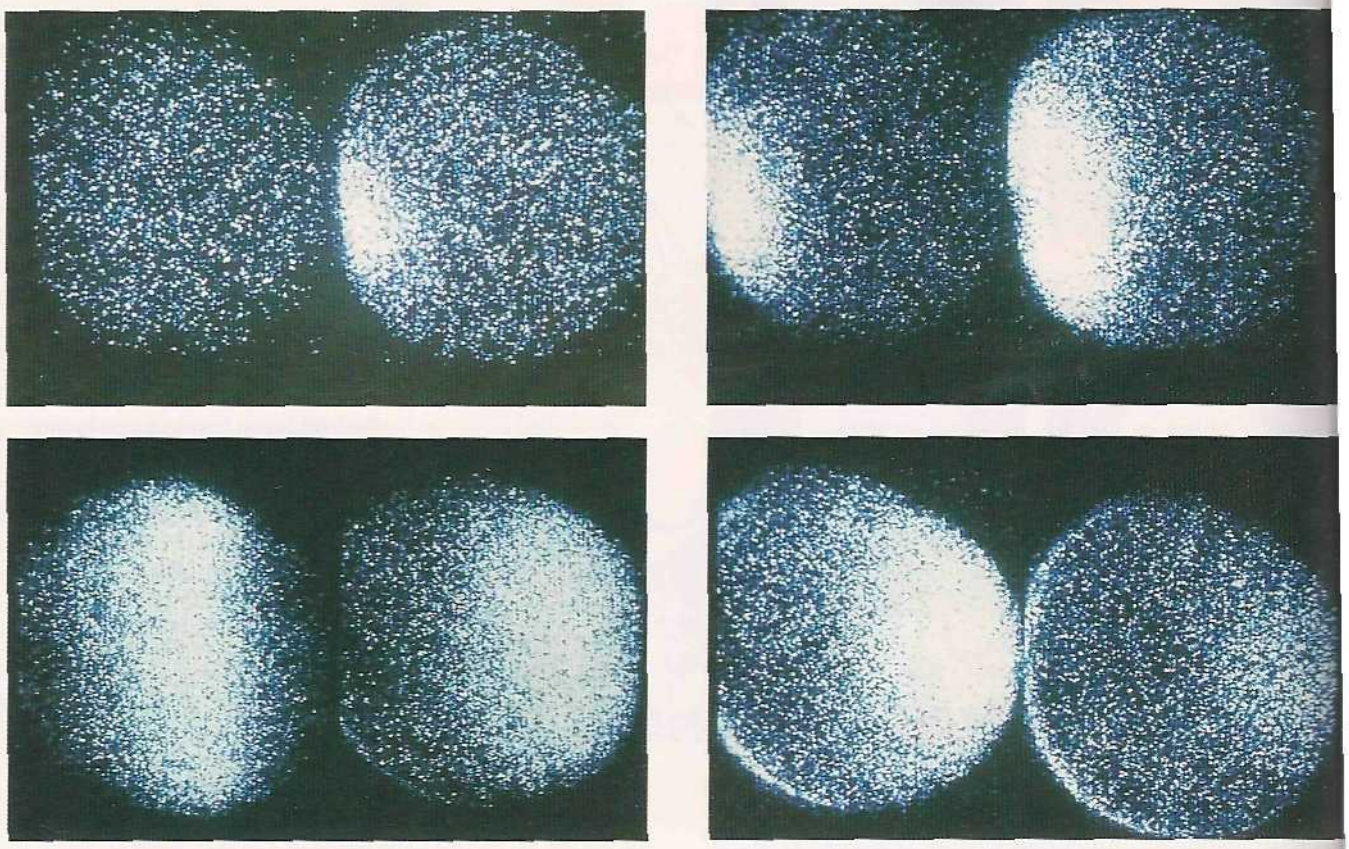


FIGURE 4.21 Wave of Ca^{2+} release across a sea urchin egg during fertilization. The egg is pre-loaded with a dye that fluoresces when it binds Ca^{2+}. When a sperm fuses with the egg, a wave of calcium release is seen, beginning at the site of sperm entry and propagating across the egg. The wave takes 30 seconds to traverse the egg. (Courtesy of G. Schatten.)

propagates across the egg (Figure 4.21). Starting at the point of sperm entry, a band of light traverses the cell (Steinhardt et al. 1977; Hafner et al. 1988). The calcium ions do not merely diffuse across the egg from the point of sperm entry. Rather, the release of Ca^{2+} starts at one end of the cell and proceeds actively to the other end, creating a wave of calcium across the egg. The entire release of Ca^{2+} is complete within roughly 30 seconds, and free Ca^{2+} is re-sequestered shortly after being released. If two sperm enter the egg cytoplasm, Ca^{2+} release can be seen starting at the two separate points of entry on the cell surface (Hafner et al. 1988).

Several experiments have demonstrated that Ca^{2+} ions are directly responsible for propagating the cortical granule reaction, and that these ions are stored within the egg itself. The drug A23187 is a calcium *ionophore* (a compound that allows the diffusion of ions such as Ca^{2+} across lipid membranes, permitting them to traverse otherwise impermeable barriers). Placing unfertilized sea urchin eggs into seawater containing A23187 causes the cortical granule reaction and the elevation of the fertilization envelope. Moreover, this reaction occurs in the absence of any Ca^{2+} in the surrounding water. Therefore, the A23187 must be stimulating the release of Ca^{2+} already sequestered in organelles within the egg (Chambers et al. 1974; Steinhardt and Epel 1974).

In sea urchins and vertebrates (but not snails and worms), the calcium ions responsible for the cortical granule reaction are stored in the endoplasmic reticulum of the egg (Eisen and Reynolds 1985; Terasaki and Sardet 1991). In sea urchins and frogs, this reticulum is pronounced in the cortex and surrounds the cortical granules (Figure 4.22; Gardiner and Grey 1983; Luttmer and Longo 1985). The cortical granules are themselves tethered to the cell membrane by a series of integral membrane proteins that facilitate calcium-mediated exocytosis (Conner et al. 1997; Conner and Wessel 1998). Thus, as soon as Ca^{2+} is released from the endoplasmic reticulum, the cortical granules fuse with the cell membrane above them. Once initiated, the release of calcium is self-propagating. Free calcium is able to release sequestered calcium from its storage sites, thus causing a wave of Ca^{2+} release and cortical granule exocytosis.

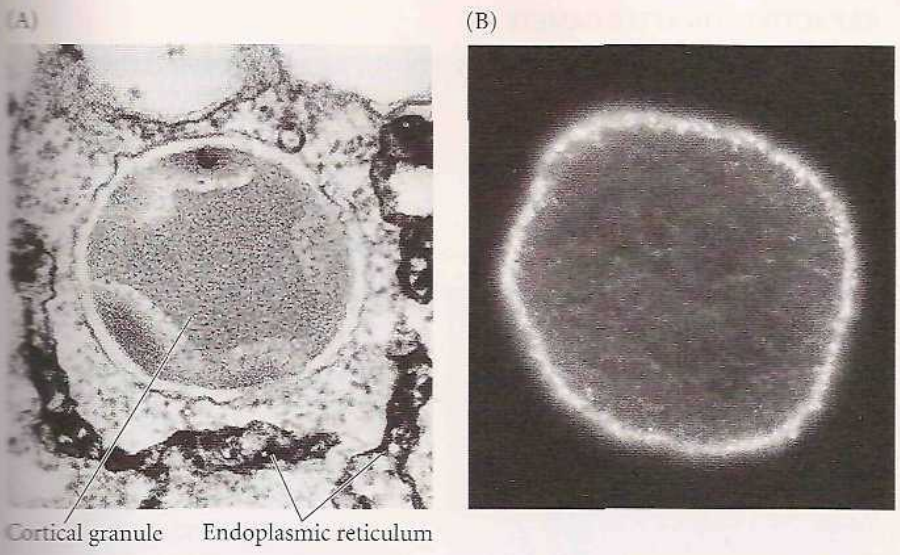


FIGURE 4.22 Endoplasmic reticulum surrounding cortical granules in sea urchin eggs. (A) The endoplasmic reticulum has been stained to allow visualization by transmission electron microscopy. The cortical granule is seen to be surrounded by dark-stained endoplasmic reticulum. (B) An entire egg stained with fluorescent antibodies to calcium-dependent calcium release channels. The antibodies show these channels in the cortical endoplasmic reticulum. (A from Luttmer and Longo 1985, courtesy of S. Luttmer; B from McPherson et al. 1992, courtesy of F. J. Longo.)

Activation of Egg Metabolism in Sea Urchins

Release of intracellular calcium ions

Although fertilization is often depicted as merely the means to merge two haploid nuclei, it has an equally important role in initiating the processes that begin development. These events happen in the cytoplasm and occur without the involvement of the parental nuclei.* In addition to initiating the slow block to polyspermy (through cortical granule exocytosis), the release of Ca^{2+} that occurs when the sperm enters the egg is critical for activating the egg's metabolism and initiating development. Calcium ions release the inhibitors from maternally stored messages, allowing these mRNAs to be translated; they also release the inhibition of nuclear division, thereby allowing cleavage to occur. Indeed, throughout the animal kingdom, calcium ions are used to activate development during fertilization.

However, the way calcium ions are released varies between species (see Parrington et al. 2007). One way, first proposed by Jacques Loeb (1899, 1902), is that a soluble factor from the sperm is introduced into the egg at the time of cell fusion, and this substance activates the egg by changing the ionic composition of the cytoplasm (Figure 4.23A). This mechanism, as we will see later, probably works in mammals. The other mechanism, proposed by Loeb's rival Frank Lillie (1913), is that the sperm acts like a big hormone, binding to receptors on the egg cell surface and changing their conformation, thus initiating reactions within the cytoplasm that activate the egg (Figure 4.23B). This is probably what happens in sea urchins.

IP_3: THE RELEASER OF CALCIUM IONS If Ca^{2+} from the egg's endoplasmic reticulum is responsible for the cortical granule reaction and the reactivation of development, what releases Ca^{2+}? Throughout the animal kingdom, it has been found that inositol 1,4,5-trisphosphate (IP_3) is the primary mechanism for releasing Ca^{2+} from intracellular storage.

The IP_3 pathway is shown in Figure 4.24. The membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is split by the enzyme **phospholipase C** (**PLC**) to yield two active compounds: IP_3 and **diacylglycerol** (**DAG**). IP_3 is able to release Ca^{2+} into the cytoplasm by opening the calcium channels of the endoplasmic reticulum. DAG activates protein kinase C, which in turn activates a protein that exchanges sodium ions for hydrogen ions, raising the pH of the egg (Nishizuka 1986; Swann and Whitaker 1986). This Na^+/H^+ exchange pump also requires Ca^{2+} for its activity. The result of PLC activation, therefore, is the liberation of Ca^{2+} and the alkalinization of the egg, and both of the compounds this activation creates—IP_3 and DAG—are involved in the initiation of development.

In sea urchin eggs, IP_3 is formed initially at the site of sperm entry and can be detected within seconds of the eggs being fertilized. The inhibition of IP_3 synthesis prevents Ca^{2+} release (Lee and Shen 1998; Carroll et al. 2000), while injected IP_3 can release sequestered Ca^{2+}, leading to cortical granule exocytosis (Whitaker and Irvine 1984; Busa et al. 1985). Moreover, these IP_3-mediated effects can be

*In certain salamanders, this developmental function of fertilization has been totally divorced from the genetic function. The silver salamander (*Ambystoma platineum*) is a hybrid subspecies consisting solely of females. Each female produces an egg with an unreduced chromosome number. This egg, however, cannot develop on its own, so the silver salamander mates with a male Jefferson salamander (*A. jeffersonianum*). The sperm from the male Jefferson salamander only stimulates the egg's development; it does not contribute genetic material (Uzzell 1964). For details of this complex mechanism of procreation, see Bogart et al. 1989.

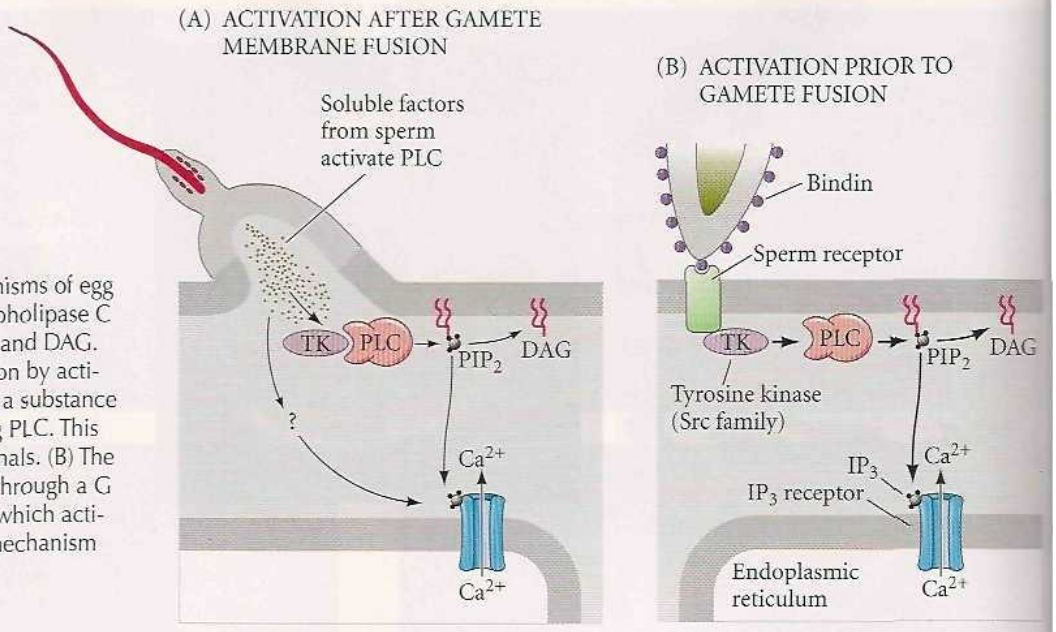


FIGURE 4.23 Probable mechanisms of egg activation. In both cases, a phospholipase C (PLC) is activated and makes IP_3 and DAG. (A) Ca^{2+} release and egg activation by activated PLC from the sperm, or by a substance from the sperm that activates egg PLC. This may be the mechanism in mammals. (B) The bindin receptor (perhaps acting through a G protein) activates an Src kinase, which activates PLC. This is probably the mechanism used by sea urchins.

thwarted by preinjecting the egg with calcium-chelating agents (Turner et al. 1986).

IP_3-responsive calcium channels have been found in the egg endoplasmic reticulum. The IP_3 formed at the site of sperm entry is thought to bind to the IP_3 receptors of these channels, effecting a local release of calcium (Ferris et al. 1989; Furuichi et al. 1989). Once released, Ca^{2+} can diffuse directly, or it can facilitate the release of more Ca^{2+} by binding to calcium-release receptors, also located in the cortical endoplasmic reticulum (McPherson et al. 1992). The binding of Ca^{2+} to these receptors releases more Ca^{2+}, which binds to more receptors, and so on. The resulting wave of calcium release is propagated throughout the cell, starting at the point of sperm entry. The cortical granules, which fuse with the cell membrane in the presence of high calcium concentrations, respond with a wave of exocytosis that follows the calcium wave. Mohri and colleagues (1995) have shown that IP_3-released Ca^{2+} is both necessary and sufficient for initiating the wave of calcium release.

PHOSPHOLIPASE C, THE GENERATOR OF IP_3 The question then becomes, What activates phospholipase C enzymes?

This question has not been easy to address, since (1) there are numerous types of PLC that (2) can be activated through different pathways, and (3) different species use different mechanisms to activate PLC. Results from studies of sea urchin eggs suggest that the active PLC in echinoderms is a member of the γ (gamma) family of PLCs (Carroll et al. 1997, 1999; Shearer et al. 1999). Inhibitors that specifically block PLCγ inhibit IP_3 production as well as Ca^{2+} release. Moreover, these inhibitors can be circumvented by microinjecting IP_3 into the egg.

KINASES: A LINK BETWEEN SPERM AND PLCγ The finding that the γ class of PLCs was responsible for generating IP_3 during echinoderm fertilization spurred investigators to look at exactly which proteins activated this particular class of phospholipases. Their work soon came to focus on the Src family of protein kinases. Src proteins are found in the cortical cytoplasm of sea urchin and starfish eggs, where they can form a complex with PLCγ. Inhibition of Src protein kinases lowered and delayed the amount of Ca^{2+} released (Kinsey and Shen 2000; Giusti et al. 2003; Townley et al. 2009).

So what activates Src kinase activity? One possibility is heterotrimeric G proteins in the cortex of the egg (Figure 4.25). Such G proteins are known to activate Src kinases in mammalian somatic cells, so the cortical G proteins of sea urchin eggs seem like good candidates; blocking these G proteins prevented Ca^{2+} release (Voronina and Wessel 2003, 2004). It is also possible that these G proteins activate PLC directly. Indeed, there may be more than one pathway and more than one way to activate Ca^{2+} release.

Thus, in sea urchins, it is thought that the binding of sperm to the egg (or possibly the fusion of sperm and egg) activates PLCγ through G proteins and Src kinases. The IP_3 thus generated opens calcium channels in the nearby cortical endoplasmic reticulum, allowing the initial and local outflow of Ca^{2+}. This first efflux of ions opens calcium-gated calcium release channels, causing a wave of Ca^{2+} that flows across the egg from the point of sperm entry to the opposite side of the egg. In so doing, some of the Ca^{2+} initiates cortical granule exocytosis, fusing the cortical granule with the egg cell membrane. Other Ca^{2+} would be bound by proteins such as calmodulin, which is activated by Ca^{2+} and can regulate numerous functions.

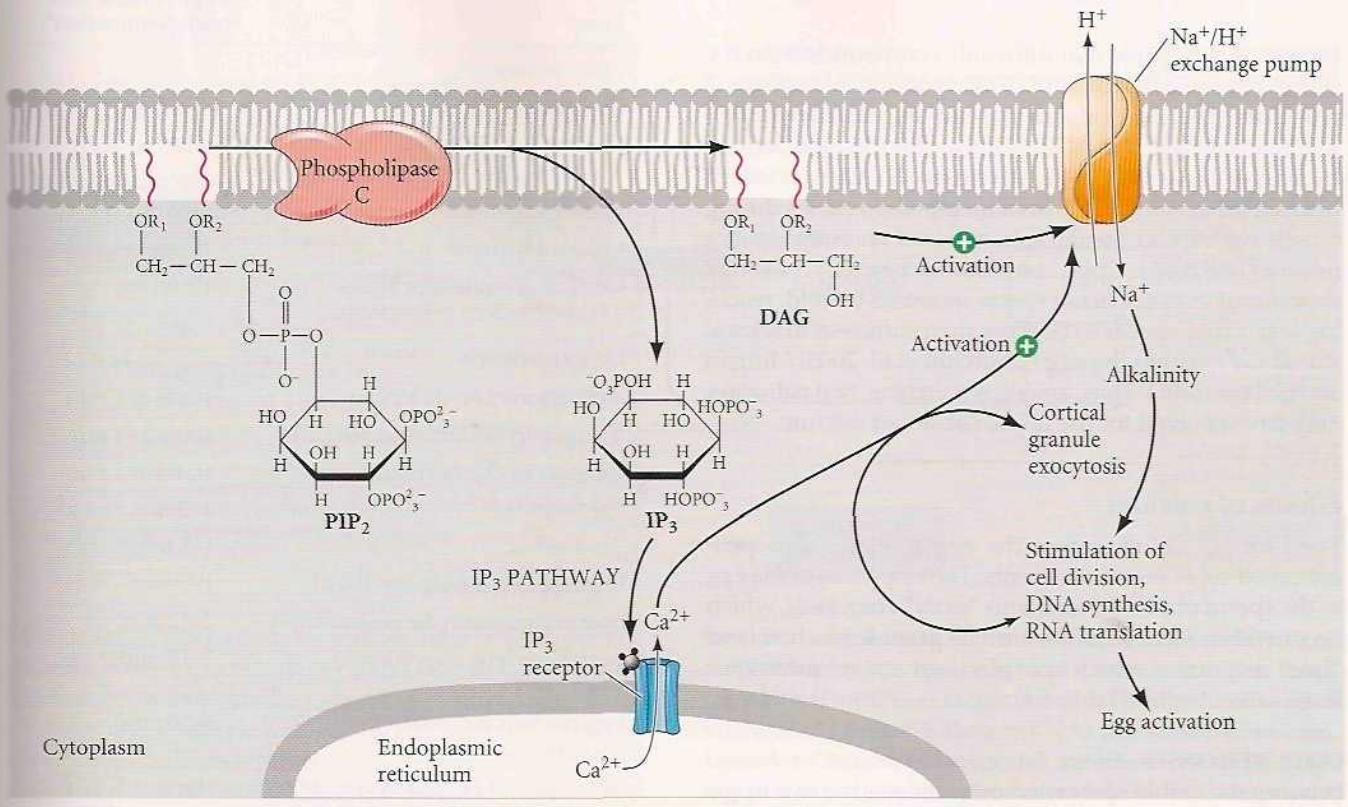


FIGURE 4.24 Roles of inositol phosphates in releasing calcium from the endoplasmic reticulum and the initiation of development. Phospholipase C splits PIP_2 into IP_3 and DAG. IP_3 releases calcium from the endoplasmic reticulum, and DAG, with assistance from the released Ca^{2+}, activates the sodium-hydrogen exchange pump in the membrane.

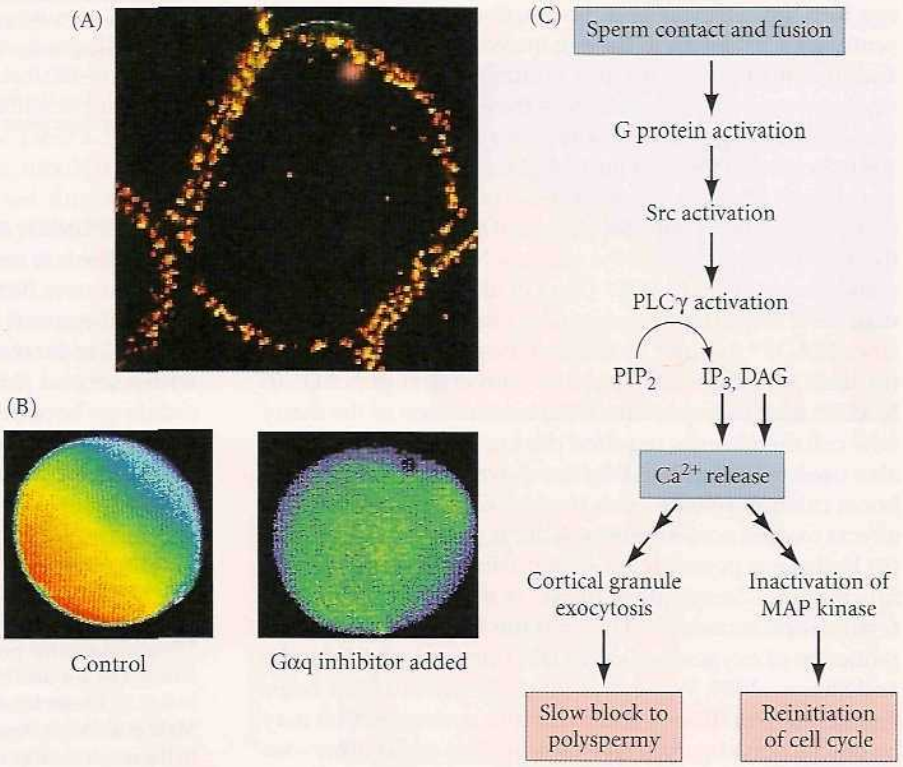


FIGURE 4.25 G protein involvement in Ca^{2+} entry into sea urchin eggs. (A) Mature sea urchin egg immunologically labeled for the cortical granule protein hyaline (red) and the G protein Gαq (green). (The overlap of signals produces the yellow color.) Gαq is localized to the cortex. (B) A wave of Ca^{2+} appears in the control egg (computer-enhanced to show relative intensities, with red being the highest), but not in the egg injected with an inhibitor of the Gαq protein. (C) Possible model for egg activation by the influx of Ca^{2+}. (After Voronina and Wessel 2003; photographs courtesy of G. M. Wessel.)

NAADP It is possible that diffusible compounds from the sperm also release calcium directly to the endoplasmic reticulum. There is evidence that *nicotinic acid adenine dinucleotide phosphate* (NAADP), a linear dinucleotide derived from NADP, serves as a sperm-borne Ca^{2+} releaser. NAADP frees stored Ca^{2+} from membrane vesicles during muscle contraction, insulin secretion, and neurotransmitter release (Lee 2001). Upon contact with egg jelly, NAADP concentration in sea uchin sperm increases tenfold, reaching levels that appear to be more than sufficient to release stored Ca^{2+} within the egg (Churchill et al. 2003; Morgan and Galione 2007). Thus, among sea urchins, two pathways may have evolved for the release of stored calcium.

Effects of calcium

The flux of calcium across the egg activates a preprogrammed set of metabolic events. The responses of the egg to the sperm can be divided into "early" responses, which occur within seconds of the cortical granule reaction, and "late" responses, which take place several minutes after fertilization begins (Table 4.1).

EARLY RESPONSES As we have seen, contact or fusion between sea urchin sperm and egg activates the two major blocks to polyspermy: the fast block, initiated by sodium influx into the cell; and the slow block, initiated by the intracellular release of Ca^{2+}.

The same release of Ca^{2+} responsible for the cortical granule reaction is also responsible for the re-entry of the egg into the cell cycle and the reactivation of egg protein synthesis. Ca^{2+} levels in the egg increase from 0.1 to 1 m*M*, and in almost all species, this occurs as a wave or succession of waves that sweep across the egg beginning at the site of sperm-egg fusion (Jaffe 1983; Terasaki and Sardet 1991; Stricker 1999; see Figure 4.21).

Calcium release activates a series of metabolic reactions that initiate embryonic development (Figure 4.26). One of these is the activation of the enzyme NAD^+ kinase, which converts NAD^+ to $NADP^+$ (Epel et al. 1981). This change may have important consequences for lipid metabolism, since $NADP^+$ (but not NAD^+) can be used as a coenzyme for lipid biosynthesis. Thus the conversion of NAD^+ to $NADP^+$ may be important in the construction of the many new cell membranes required during cleavage. $NADP^+$ is also used to make NAADP (see above), which appears to boost calcium release even further. Calcium release also affects oxygen consumption. A burst of oxygen reduction (to hydrogen peroxide) is seen during fertilization, and much of this "respiratory burst" is used to crosslink the fertilization envelope. The enzyme responsible for this reduction of oxygen is also NADPH-dependent (Heinecke and Shapiro 1989; Wong et al. 2004). Lastly, NADPH helps regenerate glutathione and ovothiols, molecules that may be crucial scavengers of free radicals that could otherwise damage the DNA of the egg and early embryo (Mead and Epel 1995).

TABLE 4.1 Events of sea urchin fertilization

Event	Approximate time postinsemination[a]
EARLY RESPONSES	
Sperm-egg binding	0 seconds
Fertilization potential rise (fast block to polyspermy)	within 1 sec
Sperm-egg membrane fusion	within 1 sec
Calcium increase first detected	10 sec
Cortical granule exocytosis (slow block to polyspermy)	15–60 sec
LATE RESPONSES	
Activation of NAD kinase	starts at 1 min
Increase in $NADP^+$ and NADPH	starts at 1 min
Increase in O_2 consumption	starts at 1 min
Sperm entry	1–2 min
Acid efflux	1–5 min
Increase in pH (remains high)	1–5 min
Sperm chromatin decondensation	2–12 min
Sperm nucleus migration to egg center	2–12 min
Egg nucleus migration to sperm nucleus	5–10 min
Activation of protein synthesis	starts at 5–10 min
Activation of amino acid transport	starts at 5–10 min
Initiation of DNA synthesis	20–40 min
Mitosis	60–80 min
First cleavage	85–95 min

Main sources: Whitaker and Steinhardt 1985; Mohri et al. 1995.

[a]Approximate times based on data from *S. purpuratus* (15–17°C), *L. pictus* (16–18°C), *A. punctulata* (18–20°C), and *L. variegatus* (22–24°C). The timing of events within the first minute is best known for *Lytechinus variegatus*, so times are listed for that species.

LATE RESPONSES: RESUMPTION OF PROTEIN AND DNA SYNTHESIS The late responses of fertilization include the activation of a new burst of DNA and protein synthesis. The fusion of egg and sperm in sea urchins causes the intracellular pH to increase.* This rise in intracellular pH begins with a second influx of sodium ions, which causes a 1:1 exchange between sodium ions from the seawater and hydrogen ions from the egg. The loss of hydrogen ions causes the pH of the egg to rise (Shen and Steinhardt 1978; Michael and Walt 1999).

It is thought that pH increase and Ca^{2+} elevation act together to stimulate new DNA and protein synthesis

*This is due to the production of diacylglycerol, as mentioned above. The sea urchin egg has a Na^+/H^+ antiport protein that is regulated by the ionic and cytoskeletal changes at fertilization (Rangel-Mata et al. 2007). Again, variation among species may be prevalent. In the much smaller egg of the mouse, there is no elevation of pH after fertilization, and there is no dramatic increase in protein synthesis immediately following fertilization (Ben-Yosef et al. 1996).

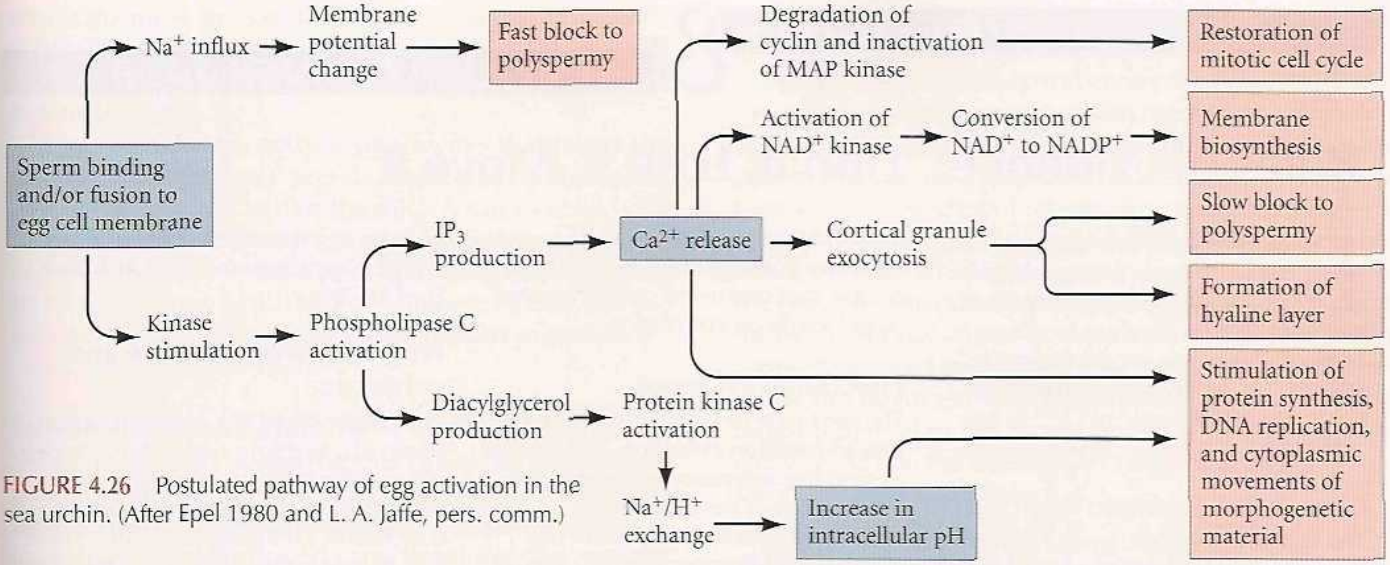


FIGURE 4.26 Postulated pathway of egg activation in the sea urchin. (After Epel 1980 and L. A. Jaffe, pers. comm.)

(Winkler et al. 1980; Whitaker and Steinhardt 1982; Rees et al. 1995). If one experimentally elevates the pH of an unfertilized egg to a level similar to that of a fertilized egg, DNA synthesis and nuclear envelope breakdown ensue, just as if the egg were fertilized (Miller and Epel 1999). Calcium ions are also critical to new DNA synthesis. The wave of free Ca^{2+} inactivates the enzyme MAP kinase, converting it from a phosphorylated (active) to an unphosphorylated (inactive) form, thus removing an inhibition on DNA synthesis (Carroll et al. 2000). DNA and protein synthesis can then resume.

In sea urchins, a burst of protein synthesis usually occurs within several minutes after sperm entry. This protein synthesis does not depend on the synthesis of new messenger RNA; rather, it uses mRNAs already present in the oocyte cytoplasm (Figure 4.27; also see Table 2.2). These mRNAs encode proteins such as histones, tubulins, actins, and morphogenetic factors that are used during early development. Such a burst of protein synthesis can be induced by artificially raising the pH of the cytoplasm using ammonium ions (Winkler et al. 1980).

One mechanism for this global rise in the translation of messages stored in the oocyte appears to be the release of inhibitors from the mRNA. In Chapter 2, we discussed maskin, an inhibitor of translation in the unfertilized amphibian oocyte. In sea urchins, a similar inhibitor binds translation initiation factor eIF4E at the 5′ end of several mRNAs and prevents these mRNAs from being translated. Upon fertilization, however, this inhibitor—the eIF4E-binding protein—becomes phosphorylated and is degraded, thus allowing eIF4E to complex with other translation factors and permit protein synthesis from the stored sea urchin mRNAs (Cormier et al. 2001; Oulhen et al. 2007). One of the mRNAs "freed" by the degradation of eIF4E-binding pro-

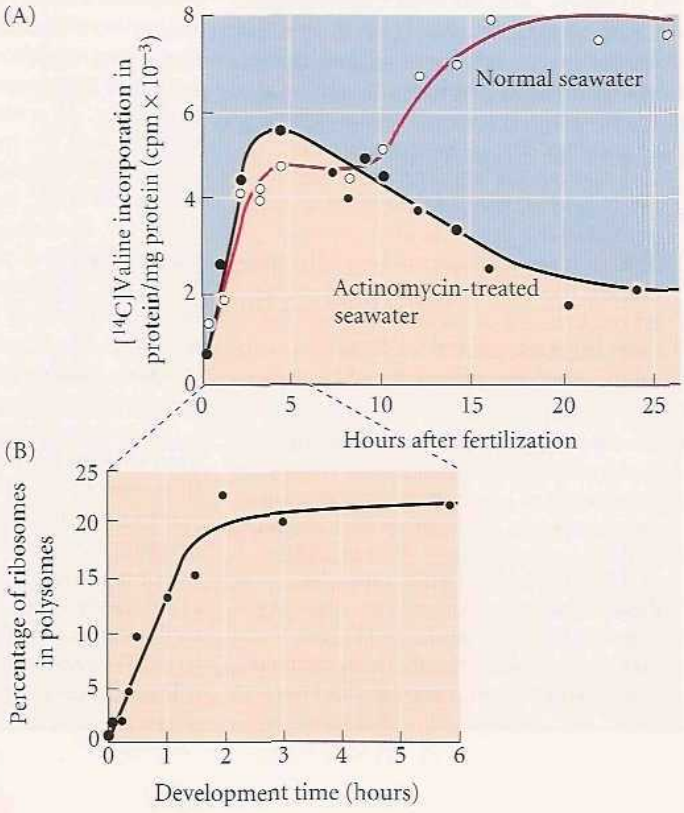


FIGURE 4.27 A burst of protein synthesis at fertilization uses mRNAs stored in the oocyte cytoplasm. (A) Protein synthesis in embryos of the sea urchin *Arbacia punctulata* fertilized in the presence or absence of actinomycin D, an inhibitor of transcription. For the first few hours, protein synthesis occurs with very little new transcription from the zygote or embryo nuclei. A second burst of protein synthesis occurs during the mid-blastula stage, at about 12 hours after fertilization. This burst represents translation of newly transcribed messages and therefore is not seen in embryos growing in actinomycin. (B) The percentage of ribosomes recruited into polysomes increases during the first hours of sea urchin development, especially during the first cell cycle. (A after Gross et al. 1964; B after Humphreys 1971.)

SIDELIGHTS & SPECULATIONS

Rules of Evidence: "Find It, Lose It, Move It"

Biology, like all science, does not deal with Facts; rather, it deals with evidence. Several types of evidence will be presented in this book, and they are not equivalent in strength. As an example, we will use the analysis of the role of Ca^{2+} in egg activation.

Correlative evidence

The first, and weakest, type of evidence is **correlative evidence**. Here, we find correlations between two or more events and then make the inference that one event causes the other. For example, upon the meeting of sea urchin sperm and egg, a wave of free Ca^{2+} spreads across the egg (see Figure 4.21), and this wave of Ca^{2+} is thought to activate the egg. This chain of events has been shown in several ways, most convincingly by aequorin fluorescence (Steinhardt et al. 1977; Shimomura 1995; Steinhardt 2006).

Although one might infer that the meeting of egg and sperm caused the Ca^{2+} wave, and that this Ca^{2+} wave caused egg activation, such a correlation of events with one another does not necessarily demonstrate a causal relationship. It is possible the meeting of gametes first caused the flow of Ca^{2+} across the egg and then, separately and by some other mechanism, activated the egg. It is also conceivable that some aspect of egg activation caused the Ca^{2+} release. The correlated occurrence of these events could even be coincidental and have no relationship to one another.* Correlative evidence provides a starting point for many investigations, but one cannot say that one event causes another based solely on correlation.

*In a tongue-in-cheek letter spoofing such correlative evidence, Sies (1988) demonstrated a remarkably good correlation between the number of storks seen in West Germany from 1965 to 1980 and the number of babies born during those same years. Any cause-and-effect scenario between storks and babies, however, would certainly fly in the face of the evidence presented in this chapter.

Functional evidence

The next type of evidence is called **loss-of-function evidence**, also known as **negative inference evidence**. Here, the absence of the postulated cause is associated with the absence of the effect. While stronger than correlative evidence, loss-of-function evidence still does not exclude other explanations. For instance, when calcium chelators such as EDTA were injected into the egg prior to fertilization, released Ca^{2+} failed to activate the egg. This would imply that Ca^{2+} is necessary for egg activation. However, data from such inhibitory studies (including studies from loss-of-function mutations) always leave open the possibility that the inhibitor suppresses more than just the process being studied. For instance, when protease inhibitors caused the failure of mammalian fertilization, it was assumed that these inhibitors were blocking the action of proteases released from the acrosome. As a result, biologists thought that the mammalian acrosome releases soluble proteases that digest the zona. Later experiments, however, demonstrated that the protease inhibitors inhibited the acrosome reaction itself so that the proteases were never released (Llanos et al. 1993). Thus, one couldn't tell whether soluble proteases played any role in mammalian fertilization.

The strongest type of evidence is **gain-of-function evidence**. Here the initiation of the first event causes the second event to happen even in instances where or when neither event usually occurs. Thus, when calcium ionophores (which can shuttle Ca^{2+} across membranes) were added to *unfertilized* eggs, Ca^{2+} was released from intracellular storage and the egg became activated even without fertilization (Steinhardt and Epel 1974).

Progression of evidence and coherence

Correlative ("find it"), loss-of-function ("lose it"), and gain-of-function ("move it") evidence must consistently support each other to establish and solidify a conclusion. This progression of "find it; lose it; move it" evidence is at the core of nearly all studies of developmental mechanism (Adams 2000). Sometimes it can be found in a single paper, and sometimes, as the case above illustrates, the evidence comes from many laboratories. "Every scientist," writes Fleck (1979), "knows just how little a single experiment can prove or convince." Rather, "an entire system of experiments and controls is needed." Science is a communal endeavor, and it is doubtful that any great discovery is the achievement of a single experiment, or of any individual.

Science also accepts evidence better when it fits into a system of other findings. This is often called **coherence**. For instance, the ability of calcium to activate the egg became a standard part of fertilization physiology when Ca^{2+} was shown to cause both the resumption of cell division and the initiation of translation—two separate components of egg activation. Also, once the sperm was found to activate phopholipase C—the enzyme that synthesizes IP_3—and IP_3 was found to activate intracellular calcium release in numerous cells, the release of Ca^{2+} became understood as being the central element of sea urchin egg activation. It fit into a much wider picture of physiological calcium release, and the mechanisms for its synthesis and its effects all fit together.

tein is the message encoding cyclin B (Salaun et al. 2003, 2004). The cyclin B protein combines with Cdk1 cyclin to create **mitosis-promoting factor** (**MPF**), which is required to initiate cell division.

Thus fertilization activates pathways that target the translational inhibitory protein for degradation, and the newly accessible 5′ end of the mRNA can interact with those proteins that allow the message to be translated. One of these mRNAs encodes a protein critical for cell division. In such a manner, fertilization can initiate mitosis and the sea urchin can begin to form a multicellular organism.

Fusion of genetic material

In sea urchins, the sperm nucleus enters the egg perpendicular to the egg surface. After the sperm and egg cell membranes fuse, the sperm nucleus and its centriole separate from the mitochondria and flagellum. The sperm's mitochondria and the flagellum disintegrate inside the egg, so very few, if any, sperm-derived mitochondria are found in developing or adult organisms. Thus, although each gamete contributes a haploid genome to the zygote, the *mitochondrial* genome is transmitted primarily by the maternal parent. Conversely, in almost all animals studied (the mouse being the major exception), the centrosome needed to produce the mitotic spindle of the subsequent divisions is derived from the sperm centriole (see Figure 4.17; Sluder et al. 1989, 1993).

Fertilization in sea urchin eggs occurs after the second meiotic division, so there is a haploid female pronucleus in the cytoplasm of the egg when the sperm enters. Once inside the egg, the sperm nucleus undergoes a dramatic transformation as it decondenses to form the haploid male pronucleus. First, the nuclear envelope vesiculates into small packets, exposing the compact sperm chromatin to the egg cytoplasm (Longo and Kunkle 1978; Poccia and Collas 1997). Then proteins holding the sperm chromatin in its condensed, inactive state are exchanged for other proteins derived from the egg cytoplasm. This exchange permits the decondensation of the sperm chromatin. Once decondensed, the DNA adheres to the nuclear envelope, where DNA polymerase can initiate replication (Infante et al. 1973).

In sea urchins, sperm chromosome decondensation appears to be initiated by the phosphorylation of the nuclear envelope lamin protein and the phosphorylation of two sperm-specific histones that bind tightly to the DNA. The process begins when sperm comes into contact with a certain glycoprotein in the egg jelly that elevates the level of cAMP-dependent protein kinase activity. These protein kinases phosphorylate several of the basic residues of the sperm-specific histones and thereby interfere with their binding to DNA (Garbers et al. 1980; Porter and Vacquier 1986; Stephens et al. 2002). This loosening is thought to facilitate the replacement of the sperm-specific histones with other histones that have been stored in the oocyte cytoplasm (Green and Poccia 1985).

After the sea urchin sperm enters the egg cytoplasm, the male pronucleus separates from the tail and rotates 180 degrees so that the sperm centriole is between the sperm pronucleus and the egg pronucleus. The sperm centriole then acts as a microtubule organizing center, extending its own microtubules and integrating them with egg microtubules to form an aster.* Microtubules extend throughout the egg and contact the female pronucleus, at which point the two pronuclei migrate toward each other. Their fusion forms the diploid **zygote nucleus** (Figure 4.28). DNA synthesis can begin either in the pronuclear stage (during migration) or after the formation of the zygote nucleus, and depends on the level of Ca^{2+} released earlier in fertilization (Jaffe et al. 2001).

Internal Fertilization in Mammals

It is very difficult to study any interactions between the mammalian sperm and egg that might take place prior to these gametes making contact. One obvious reason for this is that mammalian fertilization occurs inside the oviducts of the female: while it is relatively easy to mimic the conditions surrounding sea urchin fertilization using either natural or artificial seawater, we do not yet know the components of the various natural environments that mammalian sperm encounter as they travel to the egg.

A second reason why it is difficult to study mammalian fertilization is that the sperm population ejaculated into the female is probably very heterogeneous, containing spermatozoa at different stages of maturation. Out of the 280×10^6 human sperm normally ejaculated during coitus, only about 200 reach the vicinity of the egg (Ralt et al. 1991). Thus, since fewer than 1 in 10,000 sperm even gets close to the egg, it is difficult to assay those molecules that might enable the sperm to swim toward the egg and become activated.

Getting the gametes into the oviduct: Translocation and capacitation

The female reproductive tract is not a passive conduit through which sperm race, but a highly specialized set of tissues that actively regulates the transport and maturity of both gametes. Both the male and female gametes use a

*When Oscar Hertwig observed this radial array of sperm asters forming in his newly fertilized sea urchin eggs, he called it "the sun in the egg" and thought it was the happy indication of a successful fertilization (Hertwig 1877). More recently, Simerly and co-workers (1999) found that certain types of human male infertility are due to defects in the centriole's ability to form these microtubular asters. This deficiency results in the failure of pronuclear migration and the cessation of further development.

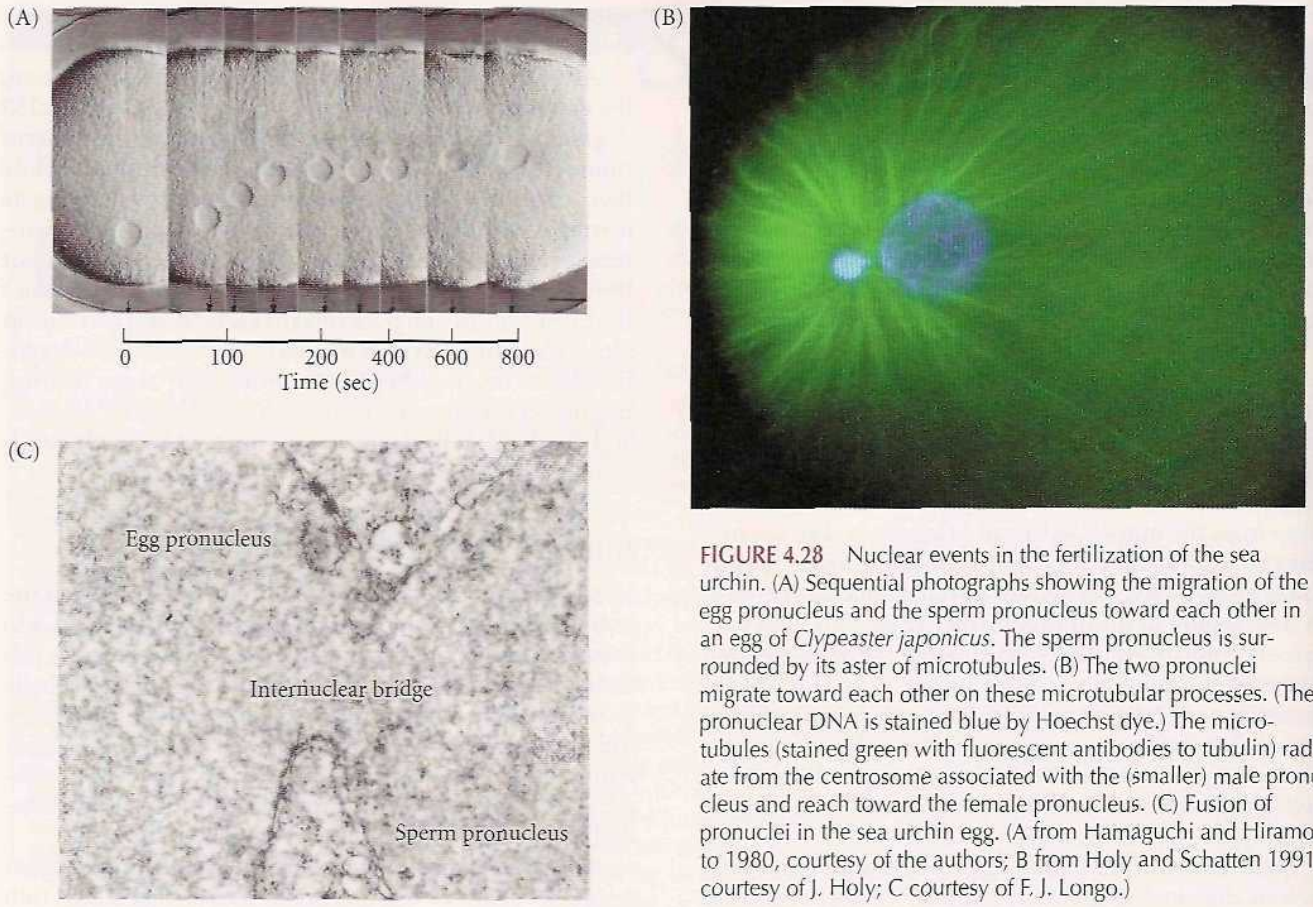


FIGURE 4.28 Nuclear events in the fertilization of the sea urchin. (A) Sequential photographs showing the migration of the egg pronucleus and the sperm pronucleus toward each other in an egg of *Clypeaster japonicus*. The sperm pronucleus is surrounded by its aster of microtubules. (B) The two pronuclei migrate toward each other on these microtubular processes. (The pronuclear DNA is stained blue by Hoechst dye.) The microtubules (stained green with fluorescent antibodies to tubulin) radiate from the centrosome associated with the (smaller) male pronucleus and reach toward the female pronucleus. (C) Fusion of pronuclei in the sea urchin egg. (A from Hamaguchi and Hiramoto 1980, courtesy of the authors; B from Holy and Schatten 1991, courtesy of J. Holy; C courtesy of F. J. Longo.)

combination of small-scale biochemical interactions and large-scale physical propulsion to get to the **ampulla**, the region of the oviduct where fertilization takes place.

TRANSLOCATION A mammalian oocyte just released from the ovary is surrounded by a matrix containing cumulus cells. (Cumulus cells are the cells of the ovarian follicle to which the developing oocyte was attached; see Figures 4.7 and 16.31). If this matrix is experimentally removed or significantly altered, the fimbriae of the oviduct will not "pick up" the oocyte-cumulus complex (see Figure 8.15), nor will the complex be able to enter the oviduct (Talbot et al. 1999). Once it is picked up, a combination of ciliary beating and muscle contractions transport the oocyte-cumulus complex to the appropriate position for its fertilization in the oviduct.

The translocation of sperm from the vagina to the oviduct involves many processes that work at different times and places. Sperm motility (i.e., flagellar action) is probably a minor factor in getting the sperm into the oviduct, although motility is required for mouse sperm to travel through the cervical mucus, and for sperm to encounter the egg once they are in the oviduct. Sperm are found in the oviducts of mice, hamsters, guinea pigs, cows, and humans within 30 minutes of sperm deposition in the vagina—a time "too short to have been attained by even the most Olympian sperm relying on their own flagellar power" (Storey 1995). Rather, sperm appear to be transported to the oviduct by the muscular activity of the uterus. Recent studies of sperm motility have led to several conclusions, including the following:

1. Uterine muscle contractions are critical in getting the sperm into the oviduct.
2. The region of the oviduct before the ampulla may slow down sperm and release them slowly.
3. Sperm (flagellar) motility is important once sperm arrive within the oviduct; sperm become hyperactive in the vicinity of the oocyte.
4. Sperm may receive directional cues from temperature gradients between the regions of the oviduct and from chemical cues derived from the oocyte or cumulus.
5. During this trek from the vagina to the ampullary region of the oviduct, the sperm matures such that it has the capacity to fertilize the egg when the two finally meet.

CAPACITATION Newly ejaculated mammalian sperm are unable to undergo the acrosome reaction or fertilize an egg until they have resided for some time in the female reproductive tract (Chang 1951; Austin 1952). The set of physiological changes by which sperm become competent to fertilize the egg is called **capacitation**. Sperm that are not capacitated are "held up" in the cumulus matrix and are unable to reach the egg (Austin 1960; Corselli and Talbot 1987). Capacitation can be accomplished in vitro by incubating sperm in a tissue culture medium (such media contain calcium ions, bicarbonate, and serum albumin) or in fluid taken from the oviducts.

Contrary to the opening scenes of the *Look Who's Talking* movies, "the race is not always to the swiftest." Wilcox and colleagues (1995) found that nearly all human pregnancies result from sexual intercourse during a 6-day period ending on the day of ovulation. This means that the fertilizing sperm could have taken as long as 6 days to make the journey. Although some human sperm reach the ampulla of the oviduct within a half-hour after intercourse, "speedy" sperm may have little chance of fertilizing the egg, since they have not undergone capacitation. Eisenbach (1995) has proposed a hypothesis wherein capacitation is a transient event, and sperm are given a relatively brief window of competence during which they can successfully fertilize the egg. As the sperm reach the ampulla, they acquire competence—but they lose it if they stay around too long. By binding and capacitating sperm, the oviduct releases "packets" of capacitated sperm at various intervals, thereby prolonging the time that fertilization can be successful.

The molecular events that take place during capacitation (Figure 4.29) have not yet been fully accounted for, but five sets of molecular changes are considered to be important:

1. The sperm cell membrane is altered by the removal of cholesterol by albumin proteins in the female reproductive tract (Cross 1998). The cholesterol efflux from the sperm cell membrane changes the location of "lipid rafts," isolated regions of the cell membrane that often contain receptor proteins. Originally located throughout the sperm cell membrane, lipid rafts now cluster over the anterior sperm head. These lipid microdomains contain proteins that can bind the zona pellucida and participate in the acrosome reaction (Bou Khalil et al. 2006; Gadella et al. 2008).
2. Particular proteins or carbohydrates on the sperm surface are lost during capacitation (Lopez et al. 1985; Wilson and Oliphant 1987). It is possible that these compounds block the recognition sites for the sperm proteins that bind to the zona pellucida. It has been suggested that the unmasking of these sites might be one of the effects of cholesterol depletion (Benoff 1993).

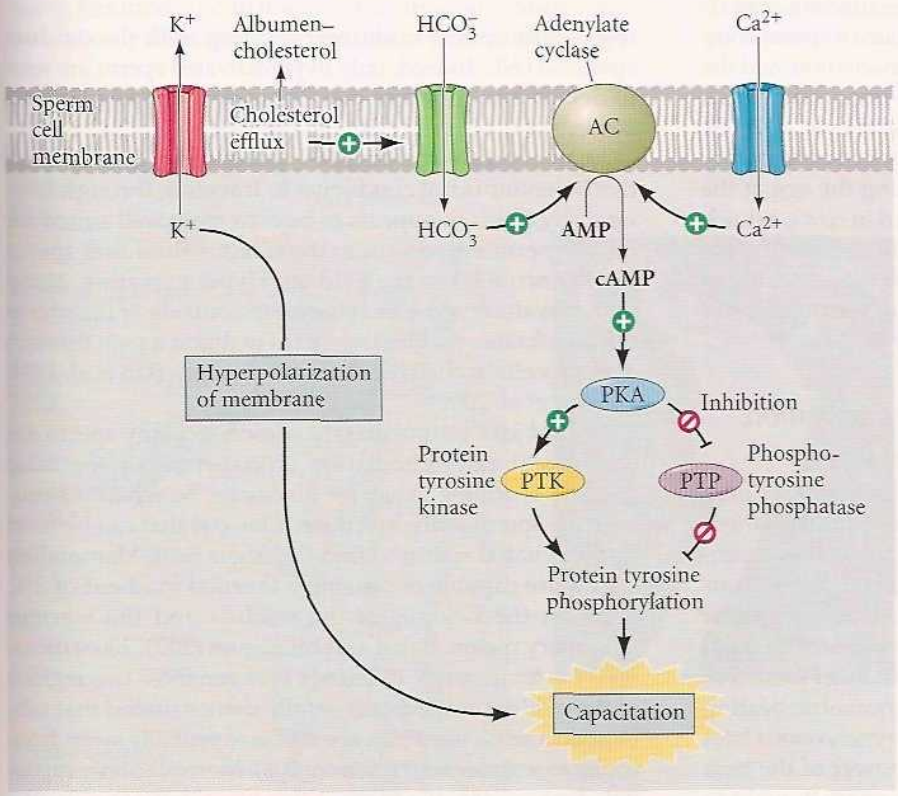


FIGURE 4.29 Hypothetical model for mammalian sperm capacitation. The efflux of potassium (the mechanism of which still remains unknown) results in a change in the resting potential of the sperm cell membrane. The removal of cholesterol by albumin stimulates ion channels that enable calcium and bicarbonate ions to enter the sperm. These ions promote the activity of a sperm-specific soluble adenylate cyclase, which makes cAMP from AMP. The rise in cAMP activates protein kinase A, causing it to activate the protein tyrosine kinases (while inactivating the protein phosphatases). The kinases phosphorylate proteins that are essential for capacitation. (After Visconti and Kopf 1998; Hess et al. 2005.)

3. The membrane potential of the sperm cell membrane becomes more negative as potassium ions leave the sperm. This change in membrane potential may allow calcium channels to be opened and permit calcium to enter the sperm. Calcium and bicarbonate ions may be critical in activating cAMP production and in facilitating the membrane fusion events of the acrosome reaction (Visconti et al. 1995; Arnoult et al. 1999).
4. Protein phosphorylation occurs (Galantino-Homer et al. 1997; Arcelay et al. 2008). In particular, two chaperone (heat-shock) proteins migrate to the surface of the sperm head when they are phosphorylated. Here, they may play an essential role in forming the receptor that binds to the zona pellucida (Asquith et al. 2004, 2005).
5. The outer acrosomal membrane changes and comes into contact with the sperm cell membrane in a way that prepares it for fusion (Tulsiani and Abou-Haila 2004).

It is uncertain whether these events are independent of one another and to what extent each of them contributes to sperm capacitation.

There may be an important connection between sperm translocation and capacitation. Smith (1998) and Suarez (1998) have documented that before entering the ampulla of the oviduct, the uncapacitated sperm bind actively to the membranes of the oviduct cells in the narrow passage (isthmus) preceding it (Figure 4.30; see also Figure 8.15). This binding is temporary and appears to be broken when the sperm become capacitated. Moreover, the life span of the sperm is significantly lengthened by this binding, and its capacitation is slowed down. This restriction of sperm entry into the ampulla, the slowing down of capacitation, and the expansion of sperm life span may have important consequences (Töpfer-Petersen et al. 2002; Gwathmey et al. 2003). The binding action may function as a block to polyspermy by preventing many sperm from reaching the egg at the same time; if the oviduct isthmus is excised in cows, a much higher rate of polyspermy results. In addition, slowing the rate of sperm capacitation and extending the active life of sperm may maximize the probability that sperm will still be available to meet the egg in the ampulla.

FIGURE 4.30 Scanning electron micrograph (artificially colored) showing bull sperm as it adheres to the membranes of epithelial cells in the oviduct of a cow prior to entering the ampulla. (From Lefebvre et al. 1995, courtesy of S. Suarez.)

In the vicinity of the oocyte: Hyperactivation, thermotaxis, and chemotaxis

Different regions of the female reproductive tract may secrete different, regionally specific molecules, and these molecules may influence sperm motility as well as capacitation. During capacitation, sperm become hyperactivated—they swim at higher velocities and generate greater force. This hyperactivation appears to be mediated through the opening of a sperm-specific calcium channel located in the sperm tail (Quill et al. 2003). The asymmetric beating of the flagellum is changed into a rapid synchronous beat with a higher degree of bending. The power of the beat and the direction of sperm head movement are thought to release the sperm from their binding with the oviduct epithelial cells. Indeed, only hyperactivated sperm are seen to detach and continue their journey to the egg (Suarez 2008a,b).

Suarez and co-workers (1991) have shown that although this behavior is not conducive to traveling through low-viscosity fluids, it appears to be extremely well suited for linear sperm movement in the viscous fluid that sperm might encounter in the oviduct. Hyperactivation, along with a hyaluronidase enzyme on the outside of the sperm cell membrane, enables the sperm to digest a path through the extracellular matrix of the cumulus cells (Lin et al. 1994; Kimura et al. 2009).

An old joke claims that the reason so many sperm are released at each ejaculation is that none of the male gametes is willing to ask for directions. So what *does* provide the sperm with directions? One cue that can be used by capacitated sperm to find the egg is heat. Mammalian sperm are capable of sensing a thermal gradient of 2°C between the isthmus of the oviduct and the warmer ampullary region. Bahat and colleagues (2003, 2006) measured the temperature difference between these two regions of the oviduct and experimentally demonstrated that rabbit sperm sense the difference and preferentially swim from cooler to warmer sites (thermotaxis). Moreover, only capacitated sperm are able to sense this temperature gradient.

Once in the ampullary region, a second sensing mechanism, chemotaxis, may come into play. It appears that the oocyte and its accompanying cumulus cells secrete molecules that attract the sperm toward the egg during the last stages of sperm migration. Ralt and colleagues (1991) tested this hypothesis using follicular fluid from human follicles whose eggs were being used for in vitro fertilization. When the researchers microinjected a drop of follicular fluid into a larger drop of sperm suspension, some of the sperm changed direction and migrated toward the source of follicular fluid. Microinjection of other solutions did not have this effect. Moreover, these investigations uncovered a fascinating correlation: the fluid from only about half the follicles tested showed a chemotactic effect; in nearly every case, the egg was fertilizable if, and only if, the fluid showed chemotactic ability ($P < 0.0001$; see Eisenbach 1999; Sun et al. 2005). Further research has shown that the ability of human follicular fluid to attract human sperm only occurs if the sperm has been capacitated (Cohen-Dayag et al. 1995; Eisenbach and Tur-Kaspa 1999; Wang et al. 2001).

The identity of these chemotactic compounds is being investigated, but one of them appears to be the hormone **progesterone**. Guidobaldi and colleagues (2008) have shown that progesterone secreted from the cumulus cells surrounding the rabbit oocyte is bound by capacitated sperm and used as a directional cue by the sperm. It is possible that, like certain invertebrate eggs, the human egg secretes a chemotactic factor only when it is capable of being fertilized, and that sperm are attracted to such a compound only when they are capable of fertilizing the egg.

Recognition at the zona pellucida

Before the mammalian sperm can bind to the oocyte, it must first bind to and penetrate the egg's zona pellucida. The zona pellucida in mammals plays a role analogous to that of the vitelline envelope in invertebrates; the zona, however, is a far thicker and denser structure than the vitelline envelope. The binding of sperm to the zona is relatively, but not absolutely, species-specific.

There appear to be several steps in the binding of a hyperactivated, wiggling mouse sperm to the zona pellucida. The mouse zona pellucida is made of three major glycoproteins—**ZP1**, **ZP2**, and **ZP3** (**zona proteins 1, 2,** and **3**)—along with accessory proteins that bind to the zona's integral structure. This glycoprotein matrix, which is synthesized and secreted by the growing oocyte, binds the sperm and, once the sperm is bound, initiates the acrosome reaction (Saling et al. 1979; Florman and Storey 1982; Cherr et al. 1986).

In recent years, a new model for mammalian sperm-zona binding has emerged, emphasizing sequential interactions between several sperm proteins and the components of the zona (Figure 4.31). The first step appears to be a relatively weak binding accomplished by the recognition of a sperm protein by a peripheral protein that coats the zona pellucida. This is followed by a somewhat stronger association between the zona and the sperm's SED1 protein. Last, a protein on the sperm (and possibly several other factors) forms strong links with the ZP3 of the zona. This last binding will cause the mouse sperm to undergo its acrosome reaction directly on the zona pellucida.

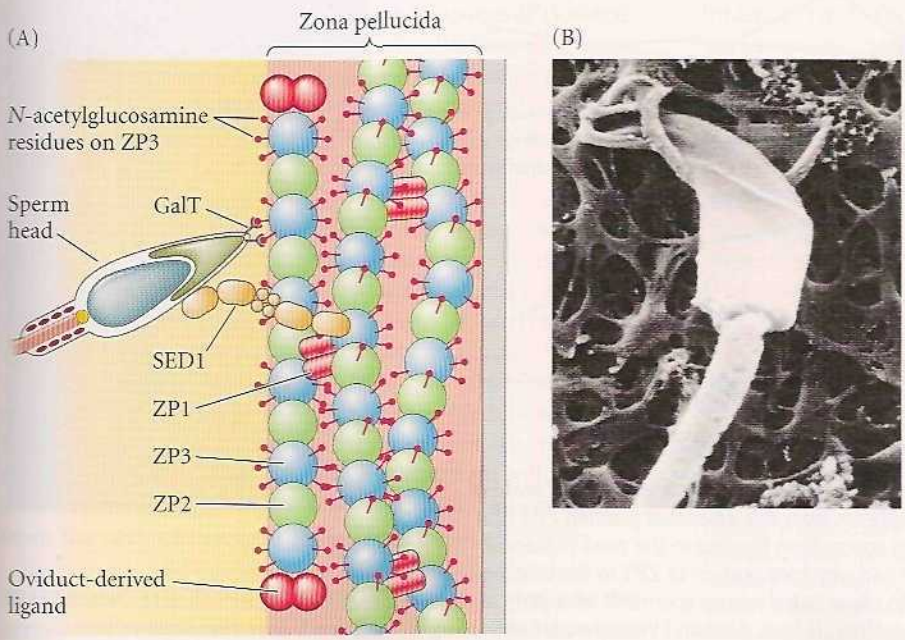


FIGURE 4.31 Sperm-zona binding. (A) Possible model of proteins involved in mouse sperm-egg adhesion. First the sperm binds weakly but specifically to a ligand protein secreted by the oviduct and coating the zona pellucida. The sperm surface protein SED1 (which is localized in the correct area of the sperm head for lateral sperm adhesion) then binds to the ZP complex on the zona. Sperm galactosyltransferase (GalT) crosslinks tightly and specifically to *N*-acetylglucosamine sugar residues on zona protein 3 (ZP3). The clustering of GalT proteins in the sperm cell membrane activates G proteins that open calcium channels and initiate the acrosome reaction. (The diagram is not drawn to scale.) (B) Electron micrograph showing sperm-zona binding in the golden hamster. (A based on data of B. Shur, courtesy of B. Shur; B courtesy of R. Yanagimachi.)

EARLY STAGES OF GAMETE ADHESION Before strong and specific binding of the sperm and the egg's zona pellucida, an initial tethering is accomplished. It appears that sperm initially bind to a 250-kDa protein that is associated with, but not integrally part of, the zona pellucida (Rodeheffer and Shur 2004). This protein can be washed away by preparative techniques (which is probably why it wasn't discovered earlier). Sperm binding is also facilitated by the sperm adhesion protein SED1, which binds to the zona protein complex (Ensslin and Shur 2003). SED1 is found in a discrete domain of the sperm cell membrane, directly overlying the acrosome, and it only binds to the zona of unfertilized oocytes (and not to those of fertilized oocytes). Antibodies against SED1 or solubilized SED1 proteins will inhibit sperm-zona binding. Indeed, the sperm of males whose *SED1* gene has been knocked out are unable to bind to the zona pellucida.

THE FINAL STAGE OF SPERM-ZONA RECOGNITION: BINDING TO ZP3 There are several pieces of evidence demonstrating that ZP3 is the major sperm-binding glycoprotein in the mouse zona pellucida. The binding of mouse sperm to the mouse zona pellucida can be inhibited by first incubating the sperm with solubilized zona glycoproteins. Using this inhibition assay, Bleil and Wassarman (1980, 1986, 1988) found that ZP3 was the active competitor for sperm binding sites (Figure 4.32A). This conclusion that sperm bound soluble ZP3 was further supported by the finding that radiolabeled ZP3 (but not ZP1 or ZP2) bound to the heads of mouse sperm with intact acrosomes (Figure 4.32B).

The cell membrane overlying the sperm head can bind to thousands of ZP3 glycoproteins in the zona pellucida. Moreover, there appear to be several different proteins on sperm that are capable of binding ZP3 (Wassarman et al. 2001; Buffone et al. 2008). Some of these sperm proteins bind to the serine- and threonine-linked carbohydrate chains of ZP3, and one of the zona-binding proteins, a sperm-surface galactosyltransferase, recognizes carbohydrate residues on ZP3 (Miller et al. 1992; Gong et al. 1995; Lu and Shur 1997). Another set of proteins that bind to the zona are ADAM3 and ADAM2 (also called cyritestin and fertilin β, respectively). These proteins form a complex on the tip of the sperm head, and sperm deficient in either one cannot bind to the zona (Kim et al. 2004; Nishimura et al. 2007).

Although the precise carbohydrate groups on ZP3 to which the proteins of the sperm membrane bind are still undefined, the conclusion that the carbohydrate moieties of ZP3 are critical for sperm attachment to the zona has been confirmed by the finding that if these carbohydrate groups are removed from ZP3, it will not bind sperm as well as intact ZP3 (see Figure 4.32A; Florman and Wassarman 1985; Kopf 1998).

INDUCTION OF THE MOUSE ACROSOME REACTION BY ZP3 ZP3 is the specific glycoprotein in the mouse zona pellucida to which sperm bind. ZP3 also initiates the acrosome reaction after sperm have bound to it. The mouse zona pellucida, unlike the sea urchin vitelline envelope, is a thick structure. By undergoing the acrosome reaction on the zona

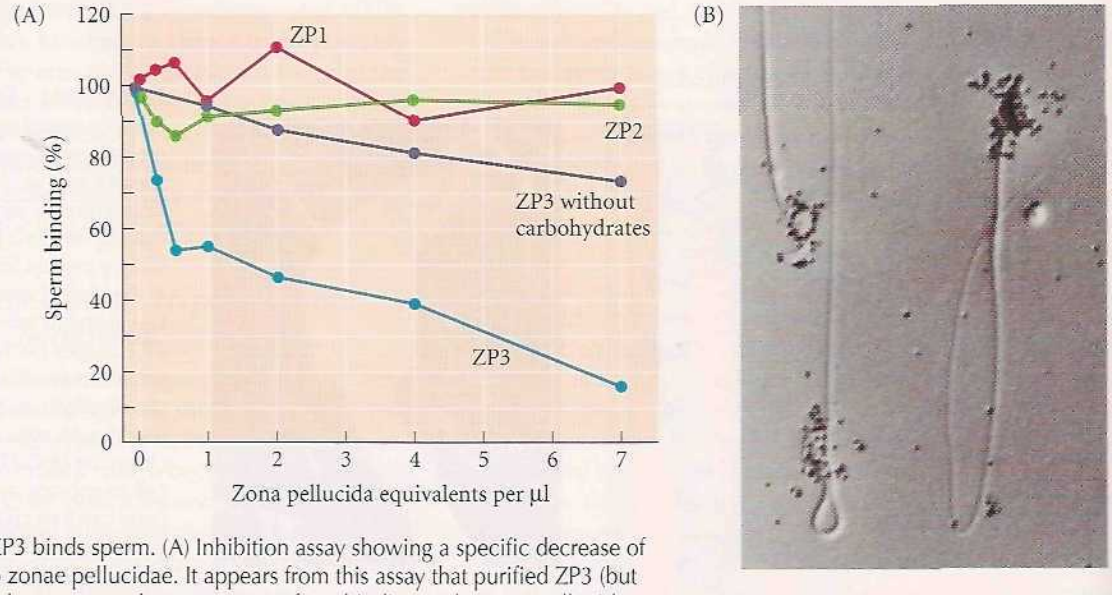


FIGURE 4.32 Mouse ZP3 binds sperm. (A) Inhibition assay showing a specific decrease of mouse sperm binding to zonae pellucidae. It appears from this assay that purified ZP3 (but not ZP1 or ZP2) can bind to sperm and prevent sperm from binding to the zona pellucida. The assay also illustrates the importance of the carbohydrate portion of ZP3 to the binding reaction. (B) Radioactively labeled ZP3 binds to capacitated mouse sperm. (A after Bleil and Wassarman 1980 and Florman and Wassarman 1985; B from Bleil and Wassarman 1986, courtesy of the authors.)

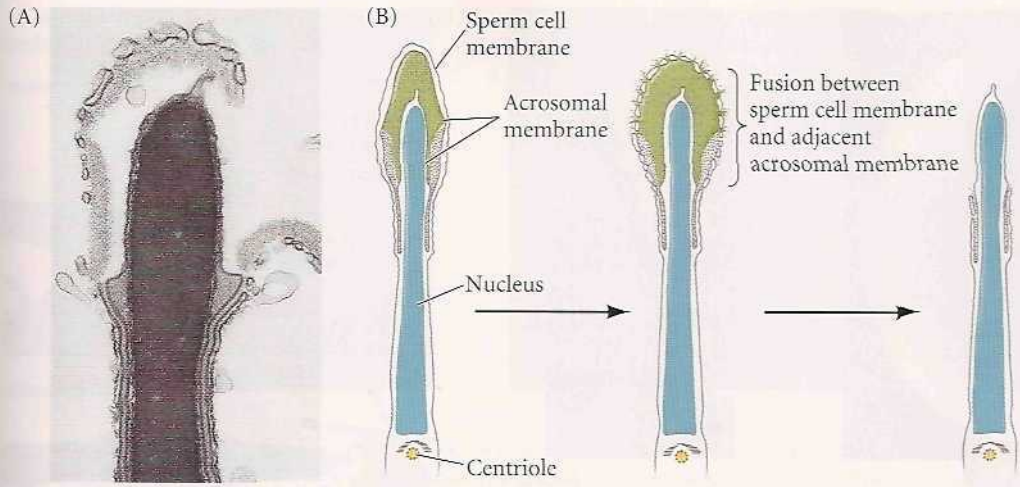


FIGURE 4.33 Acrosome reaction in hamster sperm. (A) Transmission electron micrograph of hamster sperm undergoing the acrosome reaction. The acrosomal membrane can be seen to form vesicles. (B) Interpretive diagram of electron micrographs showing the fusion of the acrosomal and cell membranes in the sperm head. (A from Meizel 1984, courtesy of S. Meizel; B after Yanagimachi and Noda 1970.)

pellucida, the mouse sperm can concentrate its proteolytic enzymes directly at the point of attachment and digest a hole through this extracellular layer (see Figure 4.8B). Indeed, mouse sperm that undergo the acrosome reaction before they reach the zona pellucida are unable to penetrate it (Florman et al. 1998).

ZP3 is able to cause the Ca^{2+}-mediated exocytosis of the acrosomal vesicle. How it does this is still under intensive investigation, although it is known that following ZP3 binding, phospholipase C (which synthesizes IP_3) is activated, the sperm head becomes more alkaline, and membrane Ca^{2+} channels are opened (see Florman et al. 2008). The exact sequence of these events remains undiscovered, but one possibility is that ZP3 initiates these events by crosslinking the sperm cell surface galactosyltransferases, whose active site faces outward and binds to the carbohydrate residues of ZP3 (see Figure 4.31A). This crosslinking activates specific G proteins in the sperm cell membrane, initiating a cascade that opens the membrane's Ca^{2+} channels and results in the calcium-mediated exocytosis of the acrosomal vesicle (Figure 4.33; Leyton and Saling 1989; Leyton et al. 1992; Florman et al. 1998; Shi et al. 2001). The source of the Ca^{2+} is also still unknown (Publicover et al. 2007). It is possible that there is a transient IP_3-mediated calcium influx from the outside environment followed by a longer-acting opening of Ca^{2+} channels, releasing Ca^{2+} from the neck of the sperm (see Florman et al. 2008).

TRAVERSING THE ZONA PELLUCIDA The exocytosis of the acrosomal vesicle releases a variety of proteases that lyse the zona pellucida. These enzymes, which may include proteosomes (Yi et al. 2007a,b), create a hole through which the sperm can travel toward the egg. However, during the acrosome reaction, the anterior portion of the sperm cell membrane (i.e., the region containing the ZP3 binding sites) is shed from the sperm. But if sperm are going to penetrate the zona pellucida, they must somehow retain some adhesion to it. In mammals, there appear to be several adhesion systems that keep the sperm in its channel. First, **zonadhesin** protein from the acrosome may fix the sperm to the point of attachment and provide a pivot point for the sperm to enter into the zona (Hardy and Garbers 1995). Second, certain proteins in the inner acrosomal membrane bind specifically to the ZP2 glycoprotein (Bleil et al. 1988). Whereas acrosome-intact sperm will not bind to ZP2, acrosome-reacted sperm will. Moreover, antibodies against the ZP2 glycoprotein will not prevent the binding of acrosome-intact sperm to the zona but will inhibit the attachment of acrosome-reacted sperm.* Third, in some species, proacrosin, a protein that adheres to the inner acrosomal membrane, binds to sulfated carbohydrate groups on the zona pellucida glycoproteins (Gaboriau et al. 2007).

*In guinea pigs, secondary binding to the zona is thought to be mediated by the protein PH-20. Moreover, when this inner acrosomal membrane protein was injected into adult male or female guinea pigs, 100% of them became sterile for several months (Primakoff et al. 1988). The blood sera of these sterile guinea pigs had extremely high concentrations of antibodies to PH-20. The antiserum from guinea pigs sterilized in this manner not only bound specifically to PH-20, but also blocked sperm-zona adhesion in vitro. The contraceptive effect lasted several months, after which fertility was restored. More recently, O'Rand and colleagues (2004) provided reversible immunological contraception by injecting male monkeys with eppin, a sperm-surface protein that interacts with semen components. The antibodies block these interactions, probably slowing down the sperm. These experiments show that the principle of immunological contraception is well founded.

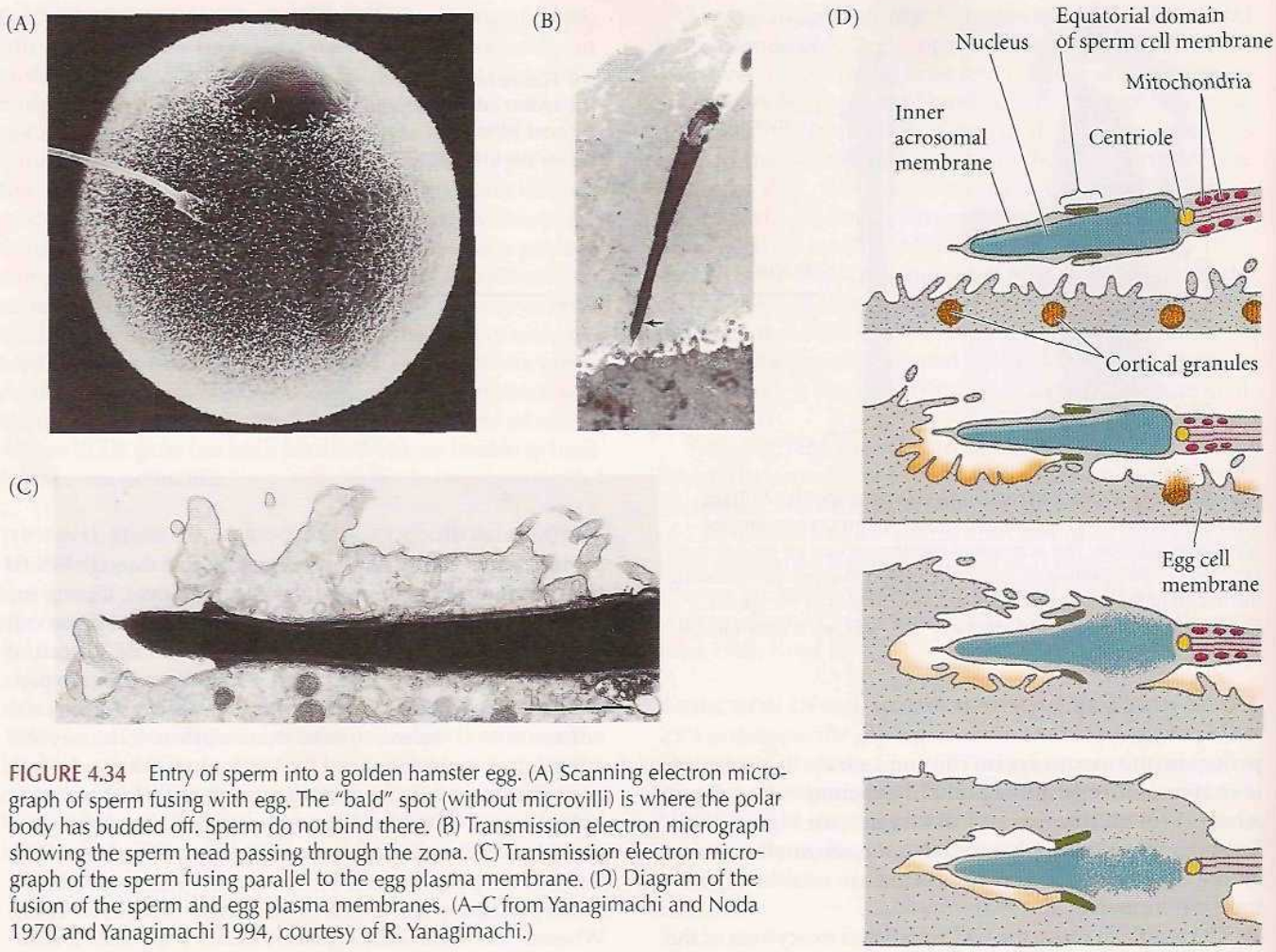


FIGURE 4.34 Entry of sperm into a golden hamster egg. (A) Scanning electron micrograph of sperm fusing with egg. The "bald" spot (without microvilli) is where the polar body has budded off. Sperm do not bind there. (B) Transmission electron micrograph showing the sperm head passing through the zona. (C) Transmission electron micrograph of the sperm fusing parallel to the egg plasma membrane. (D) Diagram of the fusion of the sperm and egg plasma membranes. (A–C from Yanagimachi and Noda 1970 and Yanagimachi 1994, courtesy of R. Yanagimachi.)

Gamete fusion and the prevention of polyspermy

In mammals, the sperm contacts the egg not at its tip (as in the case of sea urchins), but on the side of the sperm head . The acrosome reaction, in addition to expelling the enzymatic contents of the acrosome, also exposes the inner acrosomal membrane to the outside. The junction between this inner acrosomal membrane and the sperm cell membrane is called the **equatorial region**, and this is where membrane fusion between sperm and egg begins (Figure 4.34). As in sea urchin gamete fusion, the sperm is bound to regions of the egg where actin polymerizes to extend microvilli to the sperm (Yanagimachi and Noda 1970).

The mechanism of mammalian gamete fusion is still controversial (see Primakoff and Myles 2002; Ikawa et al. 2008). Gene knockout experiments suggest that mammalian gamete fusion may depend on interaction between a sperm protein and integrin-associated CD9 protein on the egg (Le Naour et al. 2000; Miyado et al. 2000; Evans 2001). CD9 protein has been localized to the membranes of the egg microvilli, and female mice with the *CD9* gene knocked out are infertile because their eggs fail to fuse with sperm (Kaji et al. 2002; Runge et al. 2006). This infertility can be reversed by the microinjection of mRNA encoding either mouse or human CD9 protein. It is not known exactly how these proteins facilitate membrane fusion, but CD9 is also known to be critical for the fusion of myocytes (muscle cell precursors) to form striated muscle (Tachibana and Hemler 1999).

On the sperm side of the mammalian fusion process, Inoue and colleagues (2005) have implicated the immunoglobulin-like protein Izumo (named after a Japanese shrine dedicated to marriage). Sperm from mice carrying loss-of-function mutations in the *Izumo* gene are able to bind and penetrate the zona pellucida, but are not able to fuse with the egg cell membrane. Human sperm also contain Izumo protein, and antibodies directed against Izumo prevent sperm-egg fusion in humans as well. There are other candidates for sperm fusion proteins; indeed, there may be several sperm-egg binding systems operating, and each of them may be necessary but not sufficient to insure proper gamete binding and fusion. It is not yet known whether the Izumo on the sperm and the CD9 on the egg bind one another.

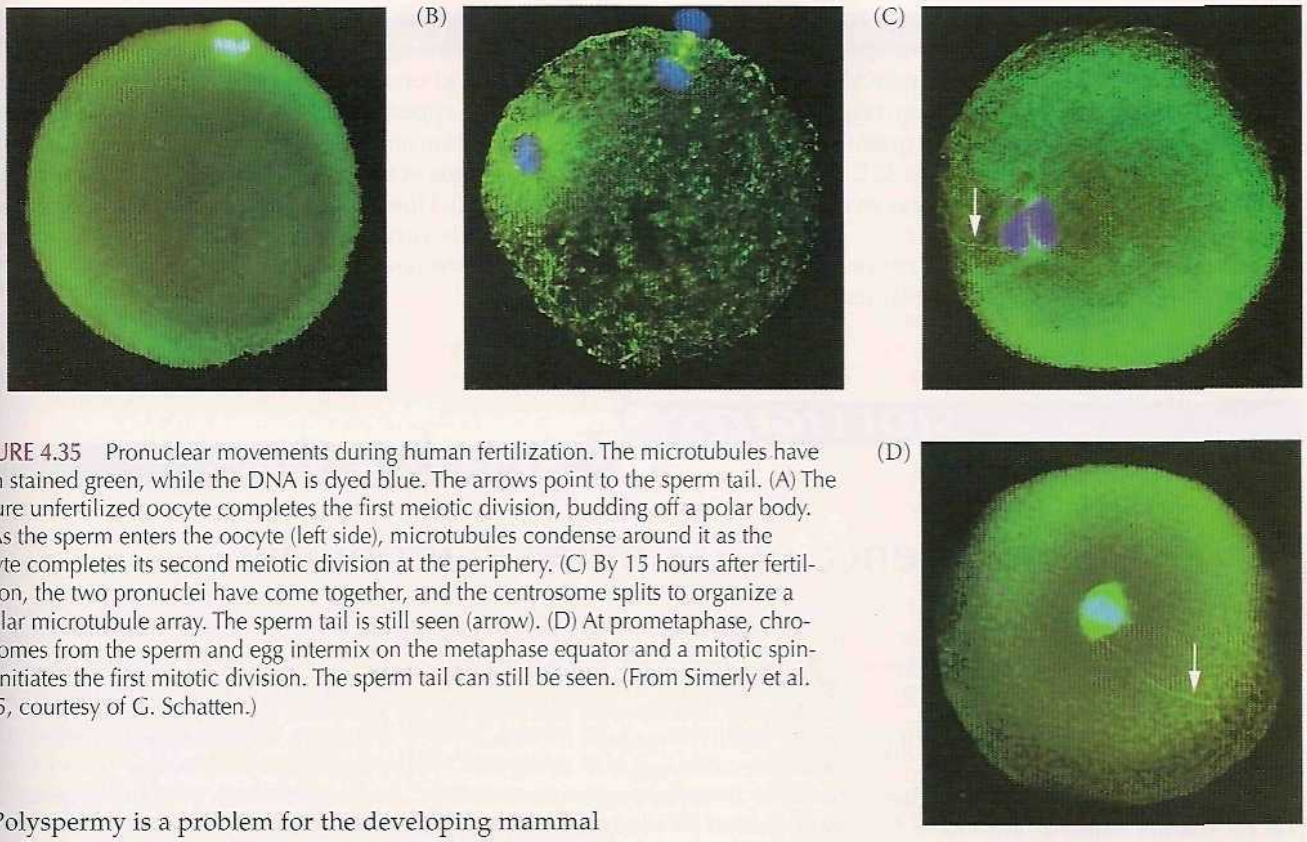


FIGURE 4.35 Pronuclear movements during human fertilization. The microtubules have been stained green, while the DNA is dyed blue. The arrows point to the sperm tail. (A) The mature unfertilized oocyte completes the first meiotic division, budding off a polar body. (B) As the sperm enters the oocyte (left side), microtubules condense around it as the oocyte completes its second meiotic division at the periphery. (C) By 15 hours after fertilization, the two pronuclei have come together, and the centrosome splits to organize a bipolar microtubule array. The sperm tail is still seen (arrow). (D) At prometaphase, chromosomes from the sperm and egg intermix on the metaphase equator and a mitotic spindle initiates the first mitotic division. The sperm tail can still be seen. (From Simerly et al. 1995, courtesy of G. Schatten.)

Polyspermy is a problem for the developing mammal as well as for the sea urchin (see Figure 4.17). In mammals, an electrical "fast" block to polyspermy has not yet been detected; and it may not even be needed, given the limited number of sperm that reach the ovulated egg (Gardner and Evans 2006). However, a *slow* block to polyspermy in mammals occurs by enzymes from the cortical granules modifying the zona pellucida proteins. Released enzymes modify the zona pellucida sperm receptors such that they can no longer bind sperm (Bleil and Wassarman 1980). Cortical granules of mouse eggs have been found to contain *N*-acetylglucosaminidase enzymes capable of cleaving *N*-acetylglucosamine from ZP3 carbohydrate chains. *N*-acetylglucosamine is one of the carbohydrate groups to which sperm can bind. Miller and co-workers (1992, 1993) have demonstrated that when the *N*-acetylglucosamine residues are removed at fertilization, ZP3 will no longer serve as a substrate for the binding of other sperm. ZP2 is clipped by another cortical granule protease and loses its ability to bind sperm as well (Moller and Wassarman 1989). Thus, once one sperm has entered the egg, other sperm can no longer initiate or maintain their binding to the zona pellucida and are rapidly shed.

Fusion of genetic material

As in sea urchins, the mammalian sperm that finally enters the egg carries its genetic contribution in a haploid pronucleus. In mammals, the process of pronuclear migration takes about 12 hours, compared with less than 1 hour in the sea urchin. The mammalian sperm enters almost tangentially to the surface of the egg rather than approaching it perpendicularly, and it fuses with numerous microvilli (see Figure 4.34A). The DNA of the sperm nucleus is bound by basic proteins called *protamines*, which are tightly compacted through disulfide bonds. Glutathione in the egg cytoplasm reduces these disulfide bonds and allows the uncoiling of the sperm chromatin (Calvin and Bedford 1971; Kvist et al. 1980; Perreault et al. 1988).

The mammalian sperm enters the oocyte while the oocyte nucleus is "arrested" in metaphase of its second meiotic division (Figure 4.35A,B; see also Figure 4.5). The calcium oscillations brought about by sperm entry inactivate MAP kinase and allow DNA synthesis. But unlike the sea urchin egg, which is already in a haploid state, the mammalian oocyte still has chromosomes in the middle of meiotic metaphase. Oscillations in the level of Ca^{2+} activate another kinase that leads to the proteolysis of cyclin (thus allowing the cell cycle to continue, eventually resulting in a haploid female pronucleus) and securin (the protein holding the metaphase chromosomes together) (Watanabe et al. 1991; Johnson et al. 1998). Mammals appear to undergo several waves of Ca^{2+} release; it is possible that events initiated by the first wave might not go to completion without additional calcium waves (Ducibella et al. 2002).

DNA synthesis occurs separately in the male and female pronuclei. The centrosome (new centriole) accompanying the male pronucleus produces its asters (largely from proteins stored in the oocyte). The microtubules join the two

pronuclei and enable them to migrate toward one another other. Upon meeting, the two nuclear envelopes break down (Figure 4.35C). However, instead of producing a common zygote nucleus (as in sea urchins), the chromatin condenses into chromosomes that orient themselves on a common mitotic spindle (Figure 4.35C,D). Thus, a true diploid nucleus in mammals is first seen not in the zygote, but at the 2-cell stage.

Each sperm brings into the egg not only its pronucleus but also its mitochondria, its centriole, and a small amount of cytoplasm. The sperm mitochondria and their DNA are degraded in the egg cytoplasm, so that all of the new individual's mitochondria are derived from its mother. The egg and embryo appear to get rid of the paternal mitochondria both by dilution and by actively targeting them for destruction (Cummins et al. 1998; Shitara et al. 1998; Schwartz and Vissing 2002). However, in most mammals, the sperm centriole not only survives but serves as the organizing agent for making the new mitotic spindle.

SIDELIGHTS & SPECULATIONS

The Nonequivalence of Mammalian Pronuclei

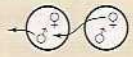
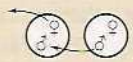
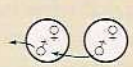
It is generally assumed that males and females carry equivalent haploid genomes. Indeed, one of the fundamental tenets of Mendelian genetics is that genes derived from the sperm are functionally equivalent to those derived from the egg. However, as we saw in Chapter 2, genomic imprinting can occur in mammals such that the sperm-derived genome and the egg-derived genome may be functionally different and play complementary roles during certain stages of development. This *imprinting* is thought to be caused by the different patterns of cytosine methylation on the genome.

The first evidence for nonequivalence came from studies of a human tumor called a **hydatidiform mole**, which resembles placental tissue. A majority of such moles have been shown to arise when a haploid sperm fertilizes an egg in which the female pronucleus is absent. After entering the egg, the sperm chromosomes duplicate themselves, thereby restoring the diploid chromosome number. However, the entire genome is derived from the sperm (Jacobs et al. 1980; Ohama et al. 1981). The cells survive, divide, and have a normal chromosome number, but development is abnormal. Instead of forming an embryo, the egg becomes a mass of placenta-like cells. Normal development does not occur when the entire genome comes from the male parent.

Normal development also does not occur when the genome is derived totally from the egg. The ability to develop an embryo without spermatic contribution is called **parthenogenesis** (Greek, "virgin birth"). The eggs of many invertebrates and some vertebrates are capable of developing normally in the absence of sperm (see Chapter 16). Mammals, however, do not exhibit parthenogenesis. Placing mouse oocytes in a culture medium that artificially activates the oocyte while suppressing the formation of the second polar body produces diploid mouse eggs whose genes are derived exclusively from the oocyte (Kaufman et al. 1977). These eggs divide to form embryos with spinal cords, muscles, skeletons, and organs, including beating hearts. However, development does not continue, and by day 10 or 11 (halfway through the mouse's gestation), these parthenogenetic embryos deteriorate. Neither human nor mouse development can be completed solely with egg-derived chromosomes.

That male and female pronuclei are both needed for normal development was also shown by pronuclear transplantation experiments (Surani and Barton 1983; McGrath and Solter 1984; Surani et al. 1986). Either male or female pronuclei can be removed from recently fertilized mouse eggs

TABLE 4.2 Pronuclear transplantation experiments

Class of reconstructed zygotes	Operation	Number of successful transplants	Number of progeny surviving
Bimaternal		339	0
Bipaternal		328	0
Control		348	18

Source: McGrath and Solter 1984.

SIDELIGHTS & SPECULATIONS (Continued)

and added to other recently fertilized eggs. (The two pronuclei can be distinguished at this stage because the female pronucleus is the one beneath the polar bodies.) Thus, zygotes with two male or two female pronuclei can be constructed. Although these eggs will form diploid cells that undergo normal cleavage, eggs whose genes are derived solely from sperm nuclei or solely from oocyte nuclei do not develop to birth. Control eggs undergoing such transplantation (i.e., eggs containing one male and one female pronucleus taken from different zygotes) can develop normally (Table 4.2). Thus, for mammalian development to occur, both the sperm-derived and the egg-derived pronuclei are critical.

The importance of DNA methylation in this block to parthenogenesis was demonstrated when Kono and colleagues (2004) generated a female mouse whose genes came exclusively from two oocytes. To accomplish this feat, they had to mutate the DNA methylation system in one of the oocyte genomes to make it more like that of a male mouse, and then they had to perform two rounds of nuclear transfer. "Men," as one reviewer remarked, "do not need to fear becoming redundant any time soon" (Vogel 2004).

Activation of the mammalian egg

As in every other animal studied, a transient rise in cytoplasmic Ca^{2+} is necessary for egg activation in mammals. The sperm induces a series of Ca^{2+} waves that can last for hours, terminating in egg activation (i.e., the resumption of meiosis, cortical granule exocytosis, and the release of the inhibition on maternal mRNAs) and the formation of the male and female pronuclei. And, again as in sea urchins, fertilization triggers intracellular Ca^{2+} release through the production of IP_3 by the enzyme phospholipase C (Swann et al. 2006; Igarashi et al. 2007).

However, the mammalian PLC responsible for egg activation and pronucleus formation may in fact come from the sperm rather than from the egg. Some of the first observations for a sperm-derived PLC came from studies of intracytoplasmic sperm injection (ICSI), an experimental treatment for curing infertility. Here, sperm are directly injected into oocyte cytoplasm, bypassing any interaction with the egg plasma membrane. To the surprise of many biologists (who had assumed that sperm *binding* to some egg receptor protein was critical for egg activation), this treatment worked. The human egg was activated and pronuclei formed. Injecting mouse sperm into mouse eggs will also induce fertilization-like Ca^{2+} oscillations in the egg and lead to complete development (Kimura and Yanagimachi 1995).

It appeared that an activator of Ca^{2+} release was stored in the sperm head. This activator turned out to be a soluble sperm PLC enzyme, **PLCξ** (zeta), that is delivered to the egg by gamete fusion. In mice, expression of PLCζ mRNA in the egg produces Ca^{2+} oscillations, and removing PLCζ from mouse sperm (by antibodies or RNAi) abolishes the sperm's calcium-inducing activity (Saunders et al. 2002; Yoda et al. 2004; Knott et al. 2005). Human sperm that are unsuccessful in ICSI have been shown to have little or no functional PLCζ. In fact, normal human sperm can activate Ca^{2+} oscillations when injected into mouse eggs, but sperm lacking PLCζ do not (Yoon et al. 2008).

Whereas sea urchin eggs usually are activated as a single wave of Ca^{2+} crosses from the point of sperm entry, the mammalian egg is traversed by numerous waves of Ca^{2+} (Miyazaki et al. 1992; Ajduk et al. 2008; Ducibella and Fissore 2008). In mammals, the calcium ions released by IP_3 bind to a series of proteins including calmodulin-activated protein kinase (which will be important in eliminating the inhibitors of mRNA translation), MAP kinase (which allows the resumption of meiosis), and synaptotagmin (which helps initiate cortical granule fusion). Calcium ions that are not used are pumped back into the endoplasmic reticulum, and additional calcium ions are acquired from outside the cell. If mammalian oocytes are cultured in media without Ca^{2+}, the number and amplitude of oscillations decrease (Igusa and Miyazake 1983; Kline and Kline 1992). The extent (i.e., amplitude, duration, and number) of Ca^{2+} oscillations appears to regulate the timing of egg activation (Ozil et al. 2005; Toth et al. 2006). Thus, cortical granule exocytosis occurs before the resumption of meiosis and the translation of maternal mRNAs.

Coda

Fertilization is not a moment or an event, but a process of carefully orchestrated and coordinated events including the contact and fusion of gametes, the fusion of nuclei, and the activation of development. It is a process whereby two cells, each at the verge of death, unite to create a new organism that will have numerous cell types and organs. It is just the beginning of a series of cell-cell interactions that characterize animal development.

Snapshot Summary: *Fertilization*

1. Fertilization accomplishes two separate activities: sex (the combining of genes derived from two parents), and reproduction (the creation of a new organism).
2. The events of fertilization usually include (1) contact and recognition between sperm and egg; (2) regulation of sperm entry into the egg; (3) fusion of genetic material from the two gametes; and (4) activation of egg metabolism to start development.
3. The sperm head consists of a haploid nucleus and an acrosome. The acrosome is derived from the Golgi apparatus and contains enzymes needed to digest extracellular coats surrounding the egg. The midpiece and neck of the sperm contain mitochondria and the centriole that generates the microtubules of the flagellum. Energy for flagellar motion comes from mitochondrial ATP and a dynein ATPase in the flagellum.
4. The female gamete can be an egg (with a haploid nucleus, as in sea urchins) or an oocyte (in an earlier stage of development, as in mammals). The egg (or oocyte) has a large mass of cytoplasm storing ribosomes and nutritive proteins. Some mRNAs and proteins that will be used as morphogenetic factors are also stored in the egg. Many eggs also contain protective agents needed for survival in their particular environment.
5. Surrounding the egg cell membrane is an extracellular layer often used in sperm recognition. In most animals, this extracellular layer is the vitelline envelope. In mammals, it is the much thicker zona pellucida. Cortical granules lie beneath the egg's cell membrane.
6. Neither the egg nor the sperm is the "active" or "passive" partner. The sperm is activated by the egg, and the egg is activated by the sperm. Both activations involve calcium ions and membrane fusions.
7. In many organisms, eggs secrete diffusible molecules that attract and activate the sperm.
8. Species-specific chemotactic molecules secreted by the egg can attract sperm that are capable of fertilizing it. In sea urchins, the chemotactic peptides resact and speract have been shown to increase sperm motility and provide direction toward an egg of the correct species.
9. The acrosome reaction releases enzymes exocytotically. These proteolytic enzymes digest the egg's protective coating, allowing the sperm to reach and fuse with the egg cell membrane. In sea urchins, this reaction in the sperm is initiated by compounds in the egg jelly. Globular actin polymerizes to extend the acrosomal process. Bindin on the acrosomal process is recognized by a protein complex on the sea urchin egg surface.
10. Fusion between sperm and egg is probably mediated by protein molecules whose hydrophobic groups can merge the sperm and egg cell membranes. In sea urchins, bindin may mediate gamete fusion.
11. Polyspermy results when two or more sperm fertilize an egg. It is usually lethal, since it results in blastomeres with different numbers and types of chromosomes.
12. Many species have two blocks to polyspermy. The fast block is immediate and causes the egg membrane resting potential to rise. Sperm can no longer fuse with the egg. In sea urchins this is mediated by the influx of sodium ions. The slow block, or cortical granule reaction, is physical and is mediated by calcium ions. A wave of Ca^{2+} propagates from the point of sperm entry, causing the cortical granules to fuse with the egg cell membrane. The released contents of these granules cause the vitelline envelope to rise and harden into the fertilization envelope.
13. The fusion of sperm and egg results in the activation of crucial metabolic reactions in the egg. These reactions include reinitiation of the egg's cell cycle and subsequent mitotic division, and the resumption of DNA and protein synthesis.
14. Genetic material is carried in a male and a female pronucleus, which migrate toward each other. In sea urchins, the male and female pronuclei merge and a diploid zygote nucleus is formed. DNA replication occurs after pronuclear fusion.
15. In all species studied, free Ca^{2+}, supported by the alkalization of the egg, activates egg metabolism, protein synthesis, and DNA synthesis. Inositol trisphosphate (IP_3) is responsible for releasing Ca^{2+} from storage in the endoplasmic reticulum. DAG (diacylglycerol) is thought to initiate the rise in egg pH.
16. IP_3 is generated by phospholipases. Different species may use different mechanisms to activate the phospholipases.
17. Mammalian fertilization takes place internally, within the female reproductive tract. The cells and tissues of the female reproductive tract actively regulate the transport and maturity of both the male and female gametes.
18. The translocation of sperm from the vagina to the egg is regulated by the muscular activity of the uterus, by the binding of sperm in the isthmus of the oviduct, and by directional cues from the oocyte (immature egg) and/or the cumulus cells surrounding it.

19. Mammalian sperm must be capacitated in the female reproductive tract before they are capable of fertilizing the egg. Capacitation is the result of biochemical changes in the sperm cell membrane.
20. Capacitated mammalian sperm must penetrate the cumulus and bind to the zona pellucida before undergoing the acrosome reaction. In the mouse, this binding is mediated by ZP3 (zona protein 3) and several sperm proteins that recognize it.
21. ZP3 initiates the mammalian acrosome reaction on the zona pellucida, and the acrosomal enzymes are concentrated there.
22. In mammals, blocks to polyspermy include the modification of the zona proteins by the contents of the cortical granules so that sperm can no longer bind to the zona.
23. The rise in intracellular free Ca^{2+} at fertilization in amphibians and mammals causes the degradation of cyclin and the inactivation of MAP kinase, allowing the second meiotic metaphase to be completed and the formation of the haploid female pronucleus.
24. In mammals, DNA replication takes place as the pronuclei are traveling toward each other. The pronuclear membranes disintegrate as the pronuclei approach each other, and their chromosomes gather around a common metaphase plate.
25. The male and female pronuclei of mammals are not equivalent. If the zygote's genetic material is derived solely from one parent or the other, normal development will not take place. This difference in the male and female genomes is thought to be the result of different methylation patterns on the genes.

For Further Reading

Complete bibliographical citations for all literature cited in this chapter can be found at the free-access website **www.devbio.com**

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Go Online

WEBSITE 4.1 Leeuwenhoek and images of homunculi. Scholars in the 1600s thought that either the sperm or the egg carried the rudiments of the adult body. Moreover, these views became distorted by contemporary commentators and later historians.

WEBSITE 4.2 The origins of fertilization research. Studies by Hertwig, Fol, Boveri, and Auerbach investigated fertilization by integrating cytology with genetics. The debates over meiosis and nuclear structure were critical in these investigations of fertilization.

WEBSITE 4.3 The egg and its environment. The laboratory is not where most eggs are found. Eggs have evolved remarkable ways to protect themselves in particular environments.

WEBSITE 4.4 The Lillie-Loeb dispute over sperm-egg binding. In the early 1900s, fertilization research was framed by a dispute between F. R. Lillie and Jacques Loeb, who disagreed over whether the sperm recognized the egg through soluble factors or through cell-cell interactions.

WEBSITE 4.5 Blocks to polyspermy. Theodore Boveri's analysis of polyspermy is a classic of experimental and descriptive biology. E. E. Just's delineation of the fast and slow blocks was a critical paper in embryology. Both papers are reprinted here, along with commentaries.

Vade Mecum

Gametogenesis. Stained sections of testis and ovary illustrate the process of gametogenesis, the streamlining of developing sperm, and the remarkable growth of the egg as it stores nutrients for its long journey. You can see this in movies and labeled photographs that take you at each step deeper into the mammalian gonad.

E. E. Just. Blocks to polyspermy were discovered in the early 1900s by the African American embryologist Ernest Just, who became one of the few embryologists ever to be honored on a postage stamp. The Sea Urchin segment contains videos of Just's work on sea urchin fertilization.

WEBSITE 4.6 Building the egg's extracellular matrix. In sea urchins, the cortical granules secrete not only hyalin, but a number of proteins that construct the extracellular matrix of the embryo. This highly coordinated process results in sequential layers.

Sea urchin fertilization. The remarkable reactions that prevent polyspermy in a fertilized sea urchin egg can be seen in the raising of the fertilization envelope. The Sea Urchin segment contains movies of this event shown in real time.