A FINE STRUCTURAL STUDY OF CYTODIFFERENTIATION DURING CLEAVAGE, BLASTULA, AND GASTRULA STAGES OF *FUNDULUS HETEROCLITUS*

THOMAS L. LENTZ and J. P. TRINKAUS

From the Departments of Anatomy and Biology, Yale University, New Haven, Connecticut, and The Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT

The fine structure of cleavage, blastula, and gastrula stages of *Fundulus heteroclitus* was investigated. Cleavage blastomeres are relatively unspecialized, containing few or poorly developed organelles. Beginning in blastula stages, signs of differentiation were noted, including development of the endoplasmic reticulum and Golgi apparatus and appearance of a primary nucleolus and polyribosomes. More extensive structural specializations occur in gastrula stages, including further development of the endoplasmic reticulum and appearance of a granular component in the nucleolus. These changes are associated with cell differentiation and an increased capacity for protein synthesis, and may be preparatory to subsequent histogenesis. The periblast is a continuous syncytial cytoplasmic layer located between the blastodisc and yolk and is formed during late cleavage by incomplete division of the cytoplasm of the blastodisc. Cytoplasmic projections extend from the periblast (and from the basal region of cleavage blastomeres prior to formation of the periblast) into the yolk and function in uptake of yolk material in the absence of pinocytosis. Yolk material appears to be digested by the periblast and transferred into the segmentation cavity where it is available to the blastomeres. Protein granules, lipid droplets, glycogen, crystalline arrays, and multivesicular bodies are related to food storage and utilization by blastomeres. The yolk gel layer enclosing the yolk sphere was found to be a thin layer of cytoplasm continuous with the margin of the periblast and is renamed the yolk cytoplasmic layer.

INTRODUCTION

The egg of *Fundulus heteroclitus* has often served as material for the analysis of development (9, 28, 37, 39). Recurrent interest in this egg has stemmed in part from its favorable properties as experimental material (40) and from certain special features of its development, in which phenomena of general developmental interest are represented in exaggerated form. Like all teleost eggs, the *Fundulus* egg develops meroblastically. The embryo forms from a superficial blastoderm, which at first surmounts and later surrounds a relatively large fluid yolk mass. Interposed between the blastoderm and the yolk is a syncytial protoplasmic layer, the periblast, through which nutrients must pass from the yolk to the blastoderm. The manner in which nutrient materials are transferred from the yolk to the periblast and thence to the blastomeres of the overlying blastoderm is an important but as yet poorly understood process. Epiboly, or the spreading of the blasto-

derm over the uncleaved yolk, is extensive in *Fundulus*. The blastoderm, including the periblast, is confined to the animal pole of the egg during cleavage and blastula stages and then, during gastrulation, it spreads over the large yolk sphere eventually to encompass it (37). The present studies were performed in order to investigate these phenomena in greater detail.

It also was of interest to investigate the fine structural changes occurring during the differentiation of blastomeres of cleavage, blastula, and gastrula stages. These stages precede histogenesis and are probably involved in preparations for these later events. Furthermore, changes in cell surface and locomotory activities begin in the blastula stages (38) and are presumably accompanied by structural modifications. Whether changes as yet undetected occur still earlier, during cleavage stages, is also a pressing question. This paper is concerned with the fine structure of the blastomeres and periblast, as it relates to the movement of nutrient materials from the yolk into the blastoderm and to the cellular organelles involved in differentiation. The following paper will deal with the contact relations of the blastomeres, with each other and with the periblast, as they relate to cell surface activity and migratory behavior during gastrulation (42).

MATERIALS AND METHODS

Eggs were collected at Woods Hole, Massachusetts, during June and July and fertilized following procedures outlined by Armstrong and Child (2) and Trinkaus (40). Embryos were staged according to Oppenheimer's series (27), even though the more recent stages of Armstrong and Child (2) are more detailed. Oppenheimer stages have been the standard for 30 years among workers studying *F. heteroclitus* and have the advantage of familiarity. Eggs were prepared for fixation by mechanical removal of the chorion with watchmaker's forceps (37). Blastoderms were isolated in the following manner: the bulk of the fluid, viscous yolk was removed by cutting into the yolk surface layer just vegetal to the margin of the blastoderm in early cleavage stages and just vegetal to the margin of the periblast in blastula and gastrula stages. In this manner the entire blastoderm, including the periblast when present and a thin layer of yolk just beneath, was isolated for fixation. Since blastoderms curl immediately after severance of the yolk surface layer, they were isolated in the fixative to reduce curling.

Because of the difficulties generally encountered in the preservation of embryonic tissues, several combinations of fixatives (glutaraldehyde and osmium tetroxide) and buffers (cacodylate, phosphate, Veronal-acetate) were tried. Only the most successful procedure is described, because all the micrographs illustrated are of tissues prepared in this manner. Whole eggs were placed in a solution of cold 5% glutaraldehyde (33) containing 0.4 M sucrose, with the pH adjusted to 7.4 with 0.05 M cacodylate buffer, and the blastoderm was isolated immediately. As soon as it was removed, the blastoderm was transferred to fresh cold fixative where it was left for 1 hr. It was then placed overnight in cold 0.05 M cacodylate buffer (pH 7.4) containing 0.4 M sucrose, followed by fixation for 1 hr in cold 1% osmium tetroxide buffered with Veronal-acetate (pH 7.4) containing 0.4 M sucrose. Even though fixation was carried out in a solution of high osmolality (900 milliosmols/kg), the cells did not shrink appreciably. The use of fixative solution of lower osmolality resulted in variable swelling and disruption of mitochondria and intracellular membrane-bounded spaces and an apparent distortion of cell relationships.

The fixed tissues were dehydrated rapidly in graded concentrations of ethanol and embedded in Maraglas (14). Thin sections were cut on a Porter-Blum microtome and stained with lead hydroxide (12). A few sections were stained with uranyl acetate. The blocks were oriented so that the blastoderms were sectioned transversely. In this manner, it was possible to observe the relationships of yolk, periblast, blastomeres, and enveloping layer in each section. Sections 1–2 μ thick were cut from the same blocks and stained with 0.2% toluidine blue for light microscopy. All thin sections were examined with an RCA EMU 3F electron microscope. Stages 2 (fertilized one cell) through 12⅛ (mid-gastrula) were examined.

OBSERVATIONS

Light Microscope Observations

Considerably more morphological detail was apparent in the 1-μ plastic sections than has been obtained previously in paraffin sections. Prior to fertilization, the egg cytoplasm is distributed in a thin surface layer that constitutes the cortex of the fluid yolk sphere (stage 1). With sperm penetration and egg activation the cortical cytoplasm streams to the animal pole, where the zygote nucleus is located, to form a thickened cap of cytoplasm called a blastodisc (stage 2). This leaves the yolk sphere covered by a thin surface layer (previously termed the yolk gel layer). Cleavage begins within an hour or two of fertilization and involves only the blastodisc (Fig. 1). The following stages comprise cleavage: 2, one cell; 3, two cells; 4, four cells; 5, eight cells; 6, 16 cells; 7, 32 cells.

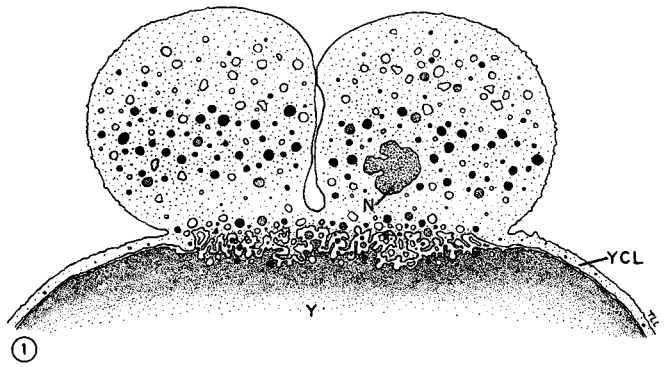


FIGURE 1 Diagram of a blastoderm at the four-cell stage (stage 4, early cleavage). The large round blastomeres contain a basal nucleus (*N*), vacuoles, and granules. Vacuoles are most abundant in the apical region of the cells, whereas granules are more common in a band in the lower half. The cleavage furrow separating the two blastomeres does not extend the entire distance to the yolk (*Y*), leaving the cells joined by a basal protoplasmic bridge. Cytoplasmic projections extend from the blastomeres into the yolk. Granules and vacuoles are abundant in this region. A space separates the membrane of the blastomeres from the moderately dense yolk. The yolk cytoplasmic layer (*YCL*) (formerly yolk gel layer) encloses the yolk sphere and is continuous with the margin of the blastoderm. × 85.

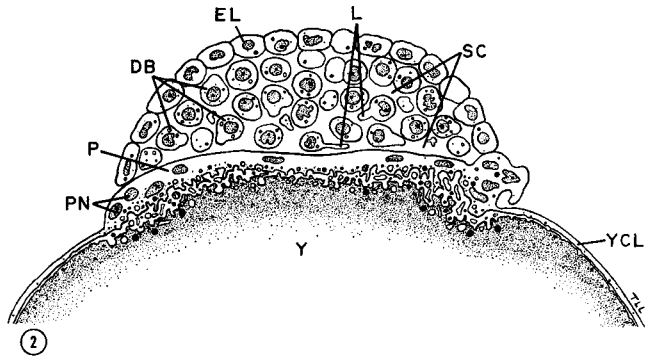


FIGURE 2 Diagram of an early blastula blastoderm (stage 8). The enveloping layer (*EL*), or outermost layer of the blastoderm, consists of a single layered sheet of closely united cells. The enveloping layer terminates at the margin of the periblast (*P*) and encloses the deep blastomeres (*DB*) and the segmentation cavity (*SC*). Most of the deep blastomeres are rounded and contain granules and a central nucleus with a nucleolus. Lobopodia (*L*) project from some of the deep blastomeres. The periblast (*P*) is a syncytial protoplasmic sheet separating the blastomeres and yolk (*Y*). Nuclei (*PN*) are most numerous in its widened lateral margins. Cytoplasmic projections, most extensive in the marginal region, extend from the periblast into the yolk. Granules and vacuoles are present in the basal portion of the periblast, especially within the projections. The yolk cytoplasmic layer (*YCL*) is continuous with the margin of the periblast. × 85.

The blastomeres of the cleavage stages are large and in direct contact with the yolk (Fig. 1). The cells contain a basal lobulated nucleus and granules and vacuoles of different sizes. Whereas the outer surface of the blastomeres is relatively smooth, the region adjacent to the yolk is highly irregular. Numerous slender branching cytoplasmic processes extend into the yolk. Granules are abundant within and immediately above these extensions. A thin layer of cytoplasm extends outward from the periphery of the blastoderm to enclose the yolk sphere.

The blastoderm gradually becomes transformed into the three-layered blastula (stages 8–10) (Fig. 2). The outermost layer is a tightly coherent surface layer, one cell thick, which covers the entire blastoderm from late cleavage on through blastula and gastrula stages into embryogenesis. Because of

its morphological position, relative to the deep cells, it has been called the enveloping layer (*die Deckschicht* or *la couche enveloppante*). Beneath the enveloping layer are the deep blastomeres. They are rounded or lobular and separated from each other and the enveloping layer by spaces of different dimensions. These spaces represent the remnants of cleavage furrows which have merged and are known collectively as the segmentation cavity. During the blastula stages and at the beginning of gastrulation, the deep blastomeres are rather loosely arranged in a segmentation cavity which is confined on the outside by the enveloping layer and below by the periblast. As gastrulation advances, the deep cells become more tightly packed. The deep blastomeres of the blastula are considerably smaller than the blastomeres of the cleavage stages. A centrally situated nucleus is present and a nucleolus can be discerned in blastomeres of early blastulae (stage 8). Small granules are present in the cytoplasm.

Beneath the deep cells, located between them and the yolk, is a continuous syncytial layer formed during cleavage by incomplete division of the cytoplasm of the blastodisc. The vertical cleavage furrows do not extend all the way to the yolk (Fig. 1), leaving an uncleaved basal region in contact with the yolk. As horizontal cleavages occur, this uncleaved basal cytoplasm is cut off to form a continuous layer. It is soon invaded by nuclei from blastomeres, mostly at the periphery, which are still continuous with it until stage 7, and becomes a syncytium (1, 47). It has been called either the yolk syncytium or the periblast (1). Since the marginal cells of the enveloping layer adhere tightly to the peripheral region of the periblast (Figs. 2 and 3), the two layers form a sac completely enclosing the segmentation cavity and the deep cells. The cytoplasmic layer enclosing the yolk is now continuous with the periphery of the periblast (Figs. 2 and 3).

The periblast is about 5–15 μ thick in blastulae, being widest at its lateral margin. It is thinner during gastrula stages. Nuclei are numerous at its margin (Fig. 2). The cytoplasm of the periblast appears relatively empty, except in the basal portion adjacent to the yolk where granules are numerous. Projections of cytoplasm extend from the periblast into the yolk. During early blastula (Fig. 2), these project from all portions of the periblast. In gastrulae, however, the extensions are smaller and concentrated at the edge of the periblast (Fig. 3). The central region is relatively thin and smooth. Since the periblast always lies between the yolk and the cells of the blastoderm, all nutrients that pass from the yolk into the blastoderm must pass through it.

After the blastula (stages 8–10) has divided into a few thousand cells, it flattens and spreads over the yolk in an extensive movement of epiboly. The blastomeres of gastrulae are smaller than those of blastulae (Fig. 3). Cells comprising the enveloping layer are flattened and tightly joined. Lobopodia project from the deep blastomeres, which are now

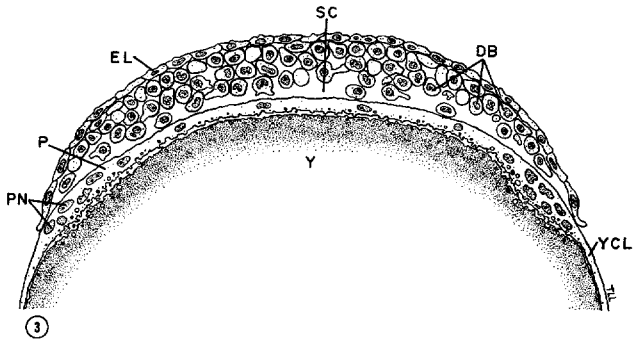


FIGURE 3 Diagram of an early gastrula (stage 12). The blastoderm is flattened and extends over one-third of the yolk sphere as a result of epiboly. The cells of the enveloping layer (*EL*) are flattened and closely united. The deep blastomeres (*DB*) show a further reduction in size and are more closely packed. Lobopodia extend from some of the blastomeres. The basal membrane of the periblast (*P*) does not contain so many projections into the yolk, and granules and vacuoles are not so numerous. *SC*, segmentation cavity; *PN*, periblast nucleus; *Y*, yolk; *YCL*, yolk cytoplasmic layer. × 85.

more closely packed. The segmentation cavity is reduced in size and most prominent just above the periblast. The lower surface of the periblast does not appear as active as in blastulae because extensions into the yolk are not so numerous and there are fewer granules and vacuoles. The basal membrane of the central portion of the periblast is relatively smooth. Our observations extend to the mid-gastrula (stage 12⅓), when the blastoderm has spread halfway over the yolk toward the vegetal pole.

Cleavage Stages

Blastomeres of the cleavage stages are characterized by a lack of cytoplasmic specializations, as evidenced by the paucity or poor development of organelles (Fig. 4). The hyaloplasm is of low density and contains few ribosomes. Mitochondria are small, round or oval in shape and contain loosely packed cristae and a few opaque granules. Smooth-surfaced vesicles and irregular cisternae are numerous, as well as larger membrane-bounded electron-transparent spaces probably corresponding to the clear vacuoles observed with the light microscope. Several Golgi complexes are located in a single cell, but these are small and consist of a few membranous lamellae and small vesicles. The most conspicuous feature of the cells are large (0.5–3 μ) membrane-bounded granules (Fig. 4) corresponding to the granules observed in the light micrographs. The contents of these structures are usually either homogeneously dense or finely granular. However, some are heterogeneous with granular, homogeneous, and lucent regions (Fig. 4). In some cases, small vesicles or dense rods are embedded in the finely granular material (Fig. 4). A few lipid droplets are situated in the same zone as the granules.

The apical surface of the blastomeres is irregular, containing large rounded processes, blunt villus-like extensions or slender microvilli projecting into the perivitelline space (Figs. 4 and 5). Invaginations of the plasma membrane are present at the bases of some microvilli (Fig. 5). The density of the extracellular space (perivitelline space) continuous with these invaginations is identical with that of the contents of membrane-bounded spaces in the apical cytoplasm. Small vesicles are also situated in the apical cytoplasm and often occur in close proximity to the plasma membrane. Vesicles in the latter position are often connected to the surface membrane by a slender membranous stalk or tube about 250 A in diameter (Fig. 5). Both surface projections and vesicles are more common in the uncleaved blastodisc and blastomeres through the eight-cell stage (stages 2–5). By the 16- to 32-cell stage (stages 6 and 7), the surface is less irregular and there are fewer associated vesicles.

The basally situated nucleus of the cleavage stages is lobulated and small in relation to the entire cell. The nucleoplasm is occupied by tiny filaments and finely granular material which is evenly distributed, forming few clumps. Nucleoli were not noted. The nuclear envelope contains pores that in cross-section, often appear to be bridged by a diaphragm.

In contrast to the upper portion of the cell, the region adjacent to the yolk is highly specialized. Long, branching, and anastomosing cytoplasmic processes extend for varying distances into the yolk material (Fig. 6). Secondary branches often extend into adjacent cytoplasmic projections or are enclosed by another arm of cytoplasm (Fig. 6). Most of the basal extensions emanate from the central regions of the blastoderm; few are present laterally. The plasma membrane of the projections is smooth with few invaginations or associated pinocytotic vesicles (Fig. 6). Phagocytosis in the form of cytoplasmic arms extending outward to enclose extracellular material was not observed, although yolk is present at the bases of the villi and in the interstices between anastomosing arms.

The contents of the extensions are in marked contrast to both the cytoplasm of the overlying blastomeres and the surrounding yolk. Most conspicuous are small dense granules, about 250 A in diameter (Figs. 6 and 7). These particles are irregular in shape and their density is increased with lead hydroxide staining. Because these granules are similar to the particles in glycogenic areas of other vertebrate tissues (11, 31), they are presumed to be glycogen. The particles are situated in areas of the cytoplasm devoid of other organelles. Other particles of unknown nature are about 150 A in diameter and usually occur in short chains (Fig. 8). These particles, resembling ribosomes, are also confined to discrete areas of cytoplasm and are common in dilated terminal portions of many projections. There is little if any density to the ground substance of these extensions (Fig. 8). Large granules[1] are composed of finely

[1] Membrane-bounded granules of *Fundulus* blastomeres and periblast differ greatly in internal structure. There is a continuous spectrum in density from

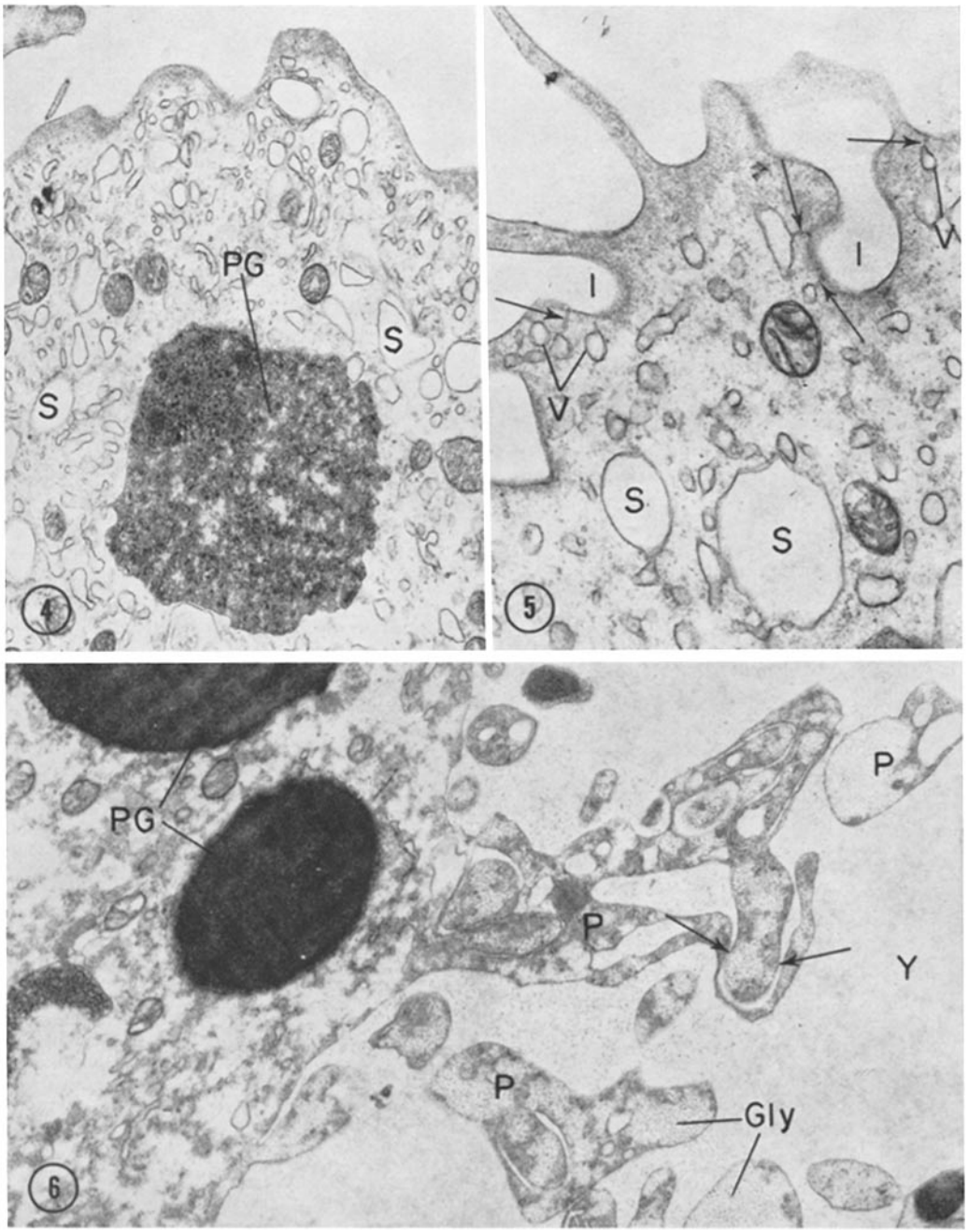
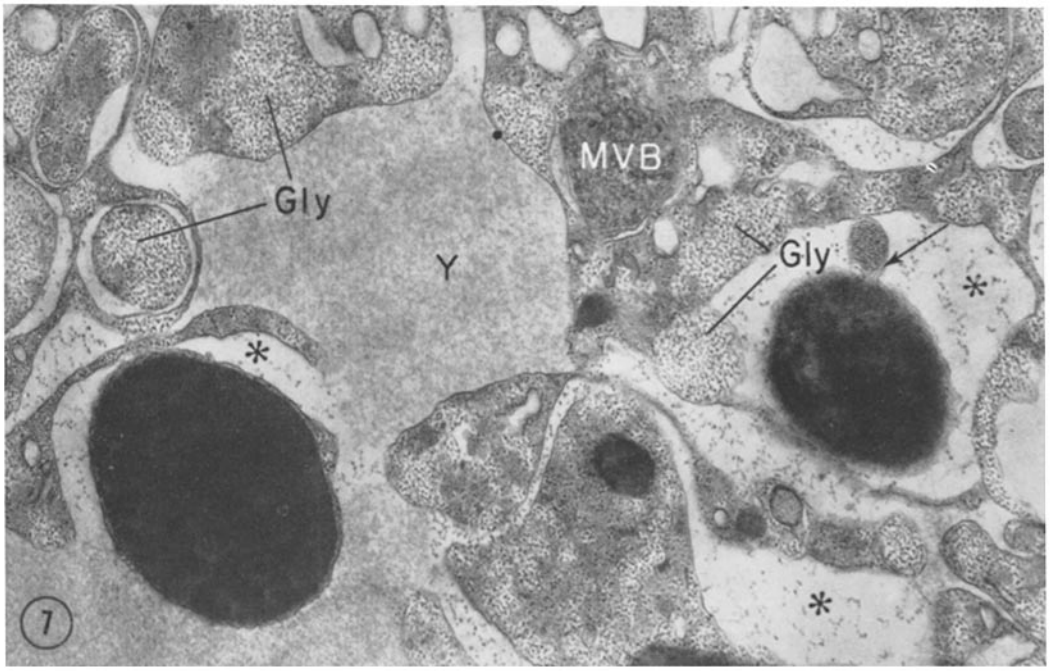


FIGURE 4 Apical region of a blastomere of the four-cell stage (stage 4). Short blunt projections occur on the surface. The cell contains membrane-bounded clear spaces (*S*), membranous profiles, and mitochondria. A large protein granule (*PG*) is filled with moderately dense granular material within which are embedded small vesicles and dense rods. Regions of lower density occur within the granule. × 13,500.

FIGURE 5 Apical surface of a blastomere of the four-cell stage. Slender microvilli project from the surface. The cytoplasm is condensed beneath the plasma membrane. A few vesicles (*V*) are situated in this zone and some are connected to the surface by a short neck (arrows). Large, membrane-bounded spaces (*S*) are deeper in the cytoplasm. *I*, invaginations at bases of villi. × 24,000.

FIGURE 6 Basal portion of a blastomere of the four-cell stage, adjacent to the yolk (*Y*). Blunt, villus-like projections (*P*) extend into the yolk. The projections branch and sometimes protrude into adjacent arms (arrows). Glycogenic areas (*Gly*) are present in the terminal portions of some of the villi. Large, dense protein, proteid, or yolk granules (*PG*) occur in the cytoplasm of the blastomere. × 14,000.



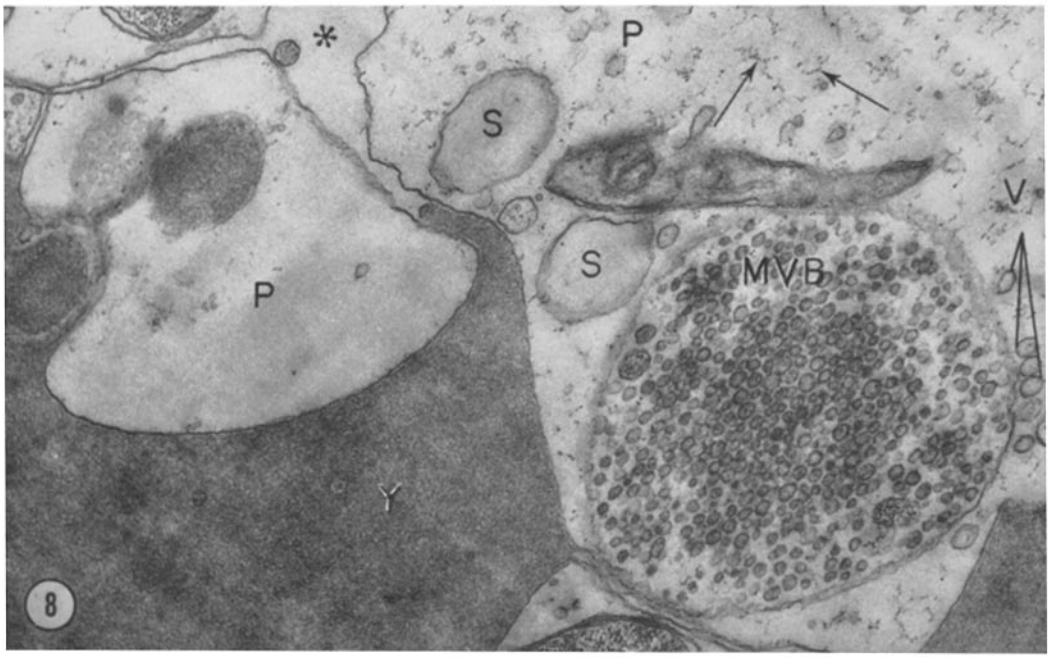


FIGURE 7 Interface between cytoplasm and yolk (*Y*) of a one-cell blastodisc prior to cleavage (stage 2). The numerous cytoplasmic projections contain glycogen (*Gly*), dense granules, and a multivesicular body (*MVB*). A small vacuole containing material of medium density is fusing with a large dense granule (arrow). Note the absence of pinocytotic vesicles in association with the plasma membrane. The yolk is composed of finely granular, moderately dense material. Adjacent to some of the processes, and particularly in between processes, the yolk space is electron-transparent (*) and contains granules similar in size and density to the cytoplasmic glycogen. × 24,000.

FIGURE 8 Dilated terminal portions of cytoplasmic projections (*P*) or extensions into the dense yolk material (*Y*) of a one-cell stage (stage 2). The hyaloplasm is of low density and contains a few small vesicles and dense granules. The granules are 150 A in diameter and occur in clusters or chains (arrows). A large multivesicular body (*MVB*) and membrane-bounded spaces (*S*) are located in one of the extensions. Vesicles (*V*) are situated at the periphery of the multivesicular body. Yolk material is lacking in an electron-transparent space (*) continuous with the yolk space. × 24,000.

granular or homogeneous and moderately or extremely dense materials (Figs. 6 and 7). The denser granules resemble the proteid granules of the mosquito oocyte (32). Another type of vacuole is filled with smooth-surfaced vesicles, 300–500 A in diameter, forming a large multivesicular body (Figs. 7 and 8). Other vesicles, usually slightly larger than those within the multivesicular body, are present in the surrounding cytoplasm, sometimes in close proximity to the membrane of the vacuole (Fig. 8). The cytoplasmic vesicles contain electron-transparent material or a few tiny dense particles. The contents of the vesicles within the multivesicular body are usually moderately dense (Fig. 8). A number of small or large membrane-bounded profiles are also located in the projections (Fig. 8).

The yolk adjacent to the cells is composed of moderately dense, finely granular material (Figs. 7 and 8). This material extends into the spaces between the irregular cytoplasmic projections (Figs. 7 and 8). In the immediate vicinity of the plasma membranes, however, this moderately dense yolk material is often lacking. Instead, the region adjacent to the membrane is electron-transparent and sometimes contains granules identical to the intracellular presumed glycogen granules (Figs. 7 and 8). Clusters of dense vesicles, granules, and membranous elements have also been observed in the yolk (Fig. 13). These accumulations resemble the fatty yolk center of the oocyte of the frog (44). In some sections, the yolk appears similar to the protein granules of the blastomeres (compare yolk in Fig. 8 with granules in Fig. 7).

The periblast was first observed at the 16-cell stage (stage 6) (Fig. 9). At this time, it forms as a distinct cytoplasmic layer, about 5 μ in thickness, between the blastomeres and the yolk, separated from the blastomeres by a narrow cleft of about 0.5 μ. Peripherally, it is still continuous with the cytoplasm of the lateral blastomeres. This cleft represents the beginning of the segmentation cavity. Microvilli extend into this space from both periblast and blastomeres. The portion of the periblast adjacent to the yolk is identical with the cell-yolk interfaces of earlier stages, with numerous villus-like projections extending into the yolk (Fig. 9). The cytoplasm of the periblast is of low density like that of the early blastomeres and contains a few irregular membranous cisternae and large spaces with clear contents. Protein or proteid granules of different sizes and densities, lipid droplets, and multivesicular bodies are abundant (Fig. 9). Glycogen particles are confined to the basal villi.

Blastula

Cells of the blastula stages show additional cytoplasmic specializations, especially by late blastula (Fig. 10). Membranous cisternae are more numerous and sometimes form elongated profiles (Fig. 10). Some of the membranes have associated ribosomes, but most remain smooth-surfaced at this time. Finely granular material of medium density is present within the cisternae of the endoplasmic reticulum (Fig. 10). Cytoplasmic ribosomes are more numerous. They usually occur singly in the hyaloplasm, but in some cells clusters of four to six ribosomes occupy regions relatively free of other organelles (Fig. 10, inset). Some of the mitochondria are more elongated and more complex, containing numerous cristae. Membranous sacs or spaces with electron-transparent contents are present, but not nearly so numerous as in cleavage stages. The Golgi apparatus, usually adjacent to the nucleus, is composed of parallel lamellae and a larger number of small vesicles. Protein granules and multivesicular bodies are scattered throughout the cytoplasm of the blastomeres (Fig. 10). Lipid droplets are smaller and less numerous than in the cleavage stages. Glycogen particles are occasionally encountered.

Another cytoplasmic structure frequently observed in blastomeres of early blastulae are crystalline arrays composed of aggregates of dense granules, 100 A in diameter (Fig. 11). The granules are arranged in parallel linear bands to produce a crystalline appearance. The chains of granules are separated by a space of 50 A. Within a band, granules are 50 A apart. Three to ten chains of granules are arranged side by side to form a cluster. Several clusters compose the entire structure, which is free in the cytoplasm and is not enclosed by a membrane. These crystalline arrays were observed most frequently in blastomeres of early blastulae (stage

medium to nearly opaque. The denser granules closely resemble lipid droplets. Because most of these structures are derived from the yolk material and probably differ only in the relative proportion of phosphoprotein, ribonucleic acid, and lipid (29), they are referred to as protein granules. Presumably, the dense granules contain a large amount of lipid. Completely opaque structures are referred to as lipid droplets, and it is possible that these are derived from some of the slightly less dense granules.

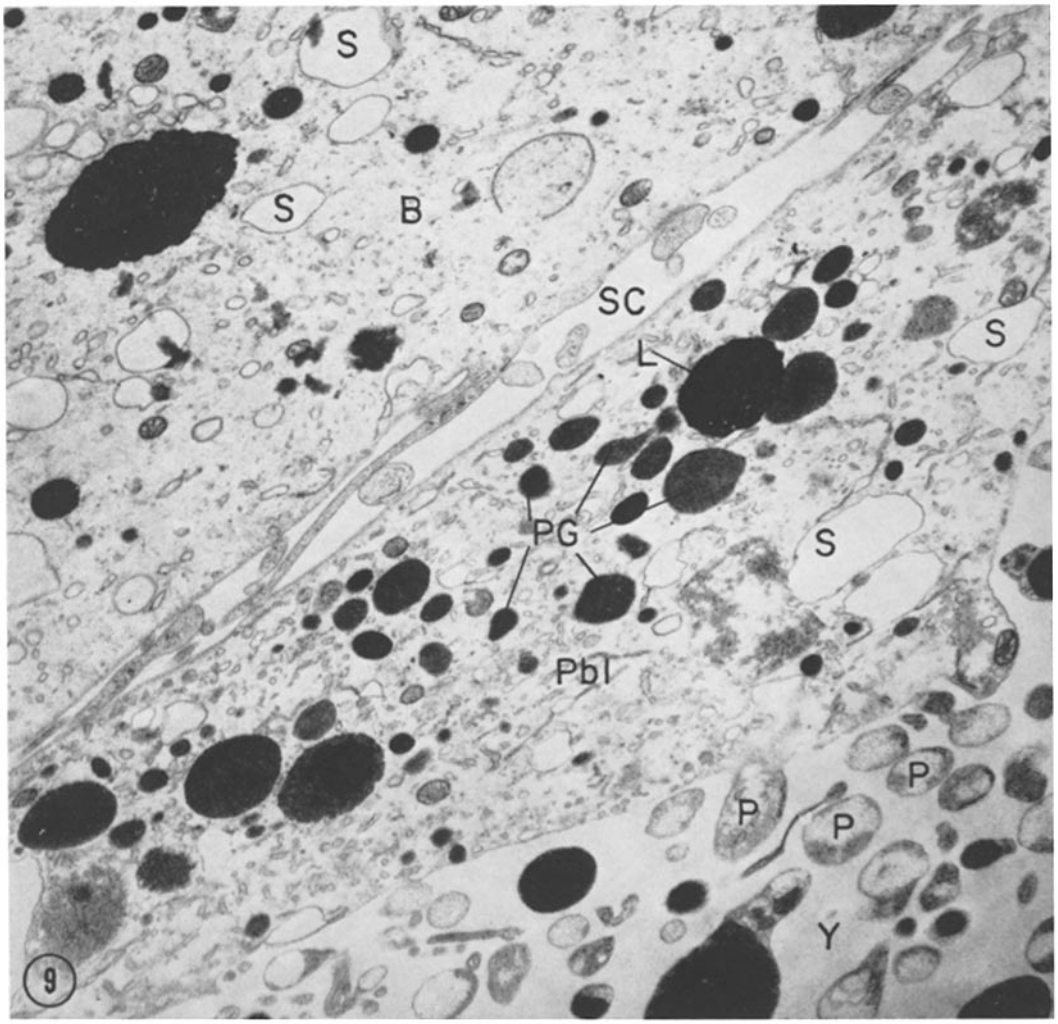


FIGURE 9 Eight-cell blastoderm (stage 6). The periblast (*Pbl*) extends as a sheet of cytoplasm between the yolk (*Y*) and blastomeres (*B*). Villous projections (*P*) extend from the periblast into the yolk. The cytoplasm of the periblast and blastomere contains electron-transparent spaces (*S*), membranous profiles, a few small mitochondria, opaque lipid droplets (*L*), and dense protein or proteid granules (*PG*) of different densities. Villi extend from both periblast and blastomere into the segmentation cavity (*SC*). × 27,000.

8). Although the composition of these structures is unknown, they closely resemble the crystalline structure of amphibian yolk platelets (18).

The surfaces of both the enveloping layer and deep blastomeres are usually smooth without pinocytotic vesicles (Fig. 10). The hyaloplasm adjacent to the plasma membrane of enveloping layer cells is dense and rarely contains vesicles, in contrast to the apex of cleavage blastomeres. Large pseudopodium-like protrusions of cytoplasm called lobopodia are common in deep cells of the blastula and gastrula stages (Fig. 10). They contain a paucity of organelles, usually only a few membranous vesicles or cisternae (Fig. 10). Polyribosomes are present in some lobopodia. Mitochondria and protein granules or lipid droplets are absent. A thin band of finely granular material of medium density separates the lobopodium from the cytoplasm proper

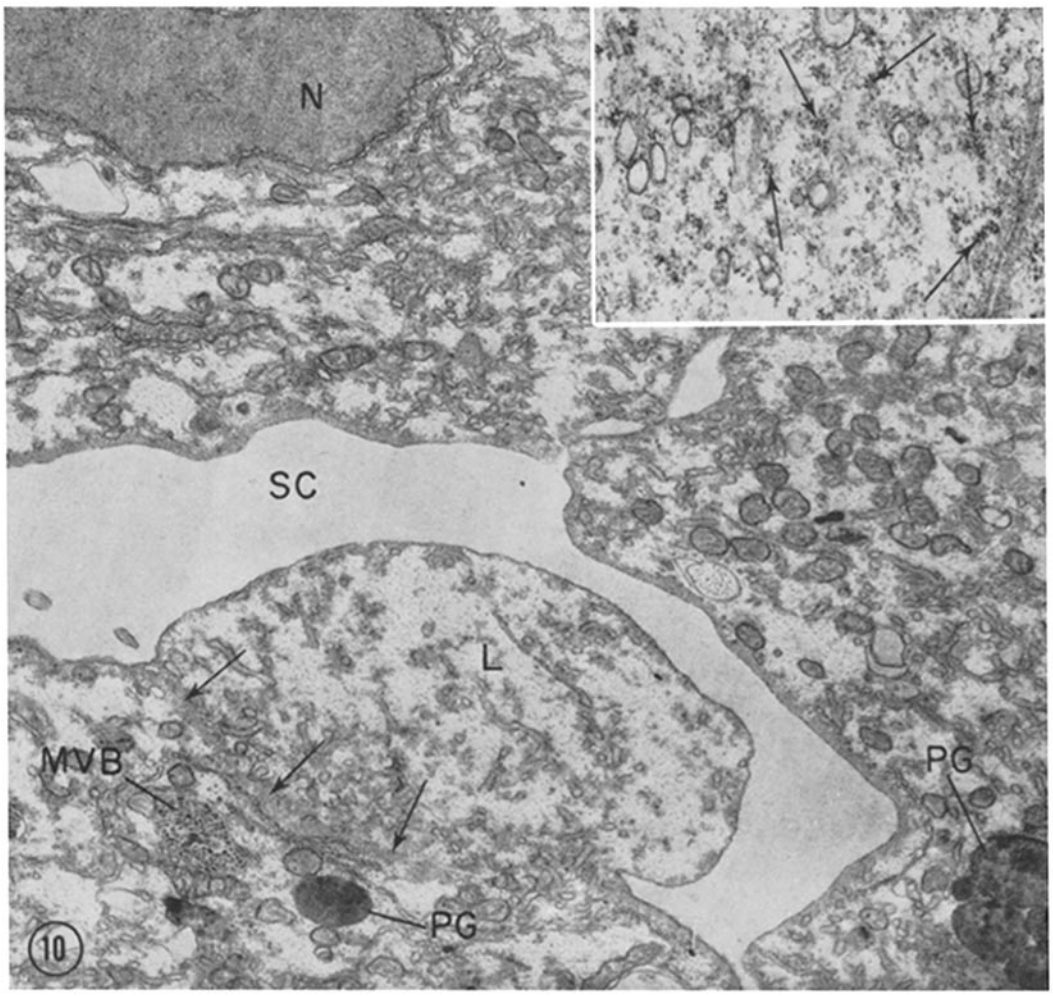


FIGURE 10 Blastomeres of a late blastula (stage 10). Membranous cisternae are more numerous at this time. A lobopodium (*L*) extends from one of the blastomeres into the segmentation cavity (*SC*). The cytoplasm of the lobopodium contains fewer organelles than the cell proper. A thin band of condensed material (arrows) separates the lobopodium from the remainder of the cell. *N*, nucleus; *PG*, protein granule; *MVB*, multivesicular body. Inset. Ribosomes occurring in clusters (arrows). The area occupied by the polyribosomes is sparse in other organelles. Fig. 10, × 13,500; inset, × 32,000.

(Fig. 10). Fibrillar or tubular elements were not observed in relation to the lobopodium.

Nuclei of blastula stages are smaller than those of cleavage stages, but still irregular in outline with many lobes and indentations. Although the nuclei are smaller at this time, the nucleocytoplasmic ratio is greater, because of a considerable decrease in the size of the blastomeres as a result of cleavage. The nucleoplasm contains fibrillar and finely granular material. A nucleolus was observed first in blastomeres of early blastulae (stage 8) (Fig. 12). It is spherical, usually less than 2 μ in diameter, eccentrically situated, and is composed of densely packed, very finely textured material (Fig. 12). To distinguish this type of nucleolus from those observed in later developmental stages, Karasaki (21) has termed it a primary nucleolus.

At the beginning of the blastula stages, the periblast is present as a discrete cytoplasmic layer

beneath the blastomeres and overlying the yolk. It is continuous with the yolk cytoplasmic layer and in contact laterally with the enveloping layer. The syncytial nature of this layer was confirmed in the present fine structural study: no vertical plasma membranes were observed. Irregularly shaped nuclei with nucleoli are unevenly distributed and are most numerous in the wide margin. The hyaloplasm of the periblast is of low density, resembling that of blastomeres of early cleavage stages (Fig. 13). Within it, mitochondria, smooth-surfaced vesicles and membranous cisternae, and larger vacuoles with clear contents are evenly distributed. Golgi complexes are numerous and are composed of a few lamellae and many small vesicles. Lipid droplets and protein granules are largest and most numerous in the regions adjacent to the yolk (Fig. 13). As one proceeds apically, they are smaller and fewer in number. Many of the larger vacucles are surrounded by smaller vesicles (Fig. 13). The density of the latter varies greatly, from electron-transparent to electron-opaque. Some of the small vesicles appear to fuse with the membranes of the larger droplets (Fig. 13). Large multivesicular bodies are also present. The region of the periblast immediately adjacent to the yolk is similar to the cell-yolk interface of the cleavage stages.

Gastrula

Blastomeres of the gastrula stages are more differentiated than those of previous stages. The hyaloplasm is of greater density. Elongated membranous cisternae of the endoplasmic reticulum are more numerous (Fig. 14 *a*). Some of the membranes contain a few ribosomes on their outer surfaces, although the majority remain smooth-surfaced. Mitochondria are similar to those of blastula. The Golgi apparatus is of greater complexity and has a large number of small vesicles associated with it (Fig. 14 *b*). Protein granules are less numerous, whereas the membrane-bounded structures containing small vesicles and granules in addition to moderately dense material (multivesicular bodies) are more abundant, especially in beginning gastrulae (stage 11) (Figs. 14 *a* and 15). Few lip-

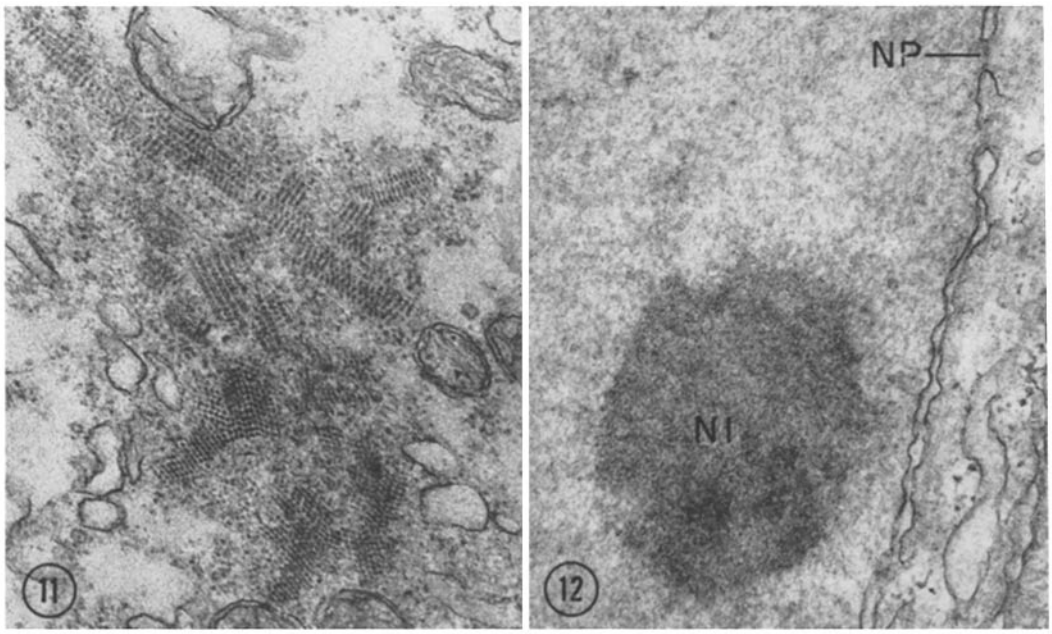


FIGURE 11 Crystalline lattice in a blastomere of the early blastula stage (stage 8). Parallel bands of dense granules comprise the configuration. × 46,500.

FIGURE 12 Nucleolus (*Nl*) of a blastomere of the early blastula stage (stage 8). The nucleolus is composed of a dense aggregate of very fine-textured material. Evenly distributed chromatin fibrils occur in the nucleoplasm. *NP*, nuclear pore. × 50,000.

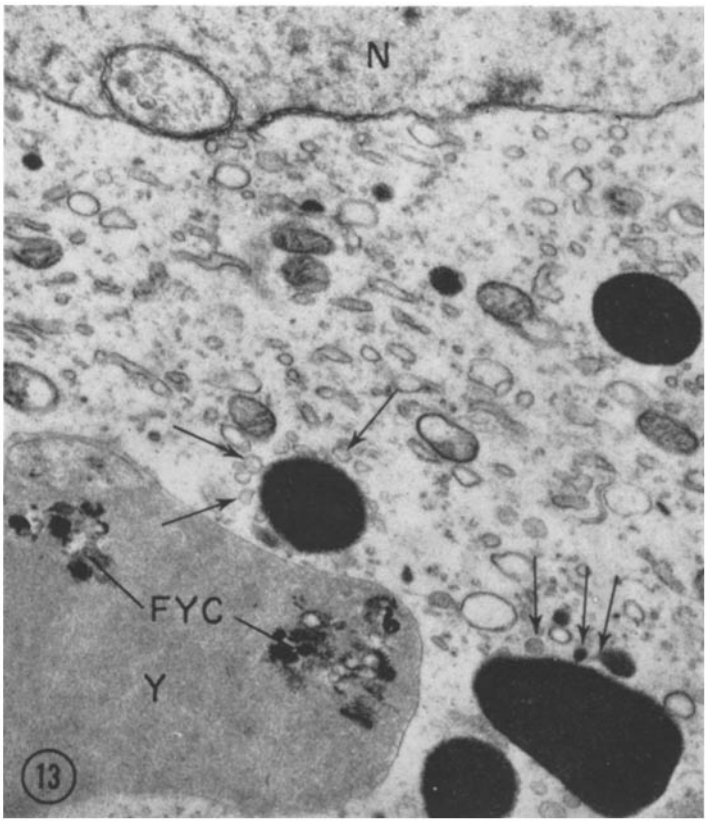


FIGURE 13 Region of the periblast adjacent to the yolk (*Y*) of a late blastula (stage 10). The periblast contains large dense granules and small vesicles with clear or dense contents. Some of the vesicles are clustered around and appear to be fusing with the large granules (arrows). Fatty yolk centers (*FYC*) are composed of dense globules and membranous elements. *N*, nucleus. × 17,000.

id droplets are present, but glycogen particles are more numerous and are scattered throughout the hyaloplasm (Fig. 14 *c*). The surface blastomeres that comprise the enveloping layer are flattened and elongated, but do not differ in cytoplasmic structure from the deep blastomeres.

The nucleoplasm contains larger granules, about 200 A in diameter, in addition to the fibrillar and finely granular material (Fig. 15). These granules have been referred to as interchromatin granules (3, 21, 35). Nucleoli are larger and sometimes multiple. In addition to the very fine material comprising the primary nucleolus, granules are now present (Fig. 15, inset). The latter are about 150 A in diameter and resemble cytoplasmic ribosomes. These dense granules often are situated on the periphery of the fine-textured component. Nuclear pores are more numerous than in previous stages.

The periblast of the gastrula stages is similar to that of the blastula, except that there appear to be fewer lipid droplets, protein granules, and multivesicular bodies. There is little density to the hyaloplasm, and ribosomes are scarce or absent (Fig. 16). The Golgi apparatus of the periblast remains complex (Fig. 16). Basal projections of cytoplasm into the yolk are not numerous, except in the marginal region. Apically, slender microvilli project into the segmentation cavity (Fig. 16). The structure of the nuclei and the nucleoli of the periblast at all stages generally parallels the changes observed in the blastomeres. Thus, the nucleoli of gastrula periblast are composed of a fine-textured component and a granular component. The nucleoplasm, however, is not so dense and does not

contain as many interchromatin granules as blastomere nuclei do.

The Surface Layer of the Yolk

The yolk gel layer is continuous with the margins of the peripheral blastomeres during cleavage (stages 2–6) and subsequently with the margin of the periblast. Its structure is that of a layer of cytoplasm, similar to the periblast (Fig. 17). Indeed, the yolk gel layer differs from the periblast only in that it is thinner (2–5 μ), its external membrane is smooth, and immediately under the membrane the cytoplasm is condensed, as beneath all membranes directly exposed to the perivitelline fluid (surface cells of the blastoderm and the marginal periblast). Otherwise, the cytoplasm is of low density and contains small and large membrane-bounded spaces with electron-transparent contents, a few mitochondria, and an occasional uncomplicated Golgi apparatus (Fig. 17). A few protein granules, lipid droplets, and glycogen granules are situated in the basal portion of the layer near its connection to the periblast. The cytoplasm is separated from the yolk by a membrane that, unlike that of the periblast, is relatively smooth in contour with only an occasional blunt villus extending into the yolk (Fig. 17).

DISCUSSION

The cells of *Fundulus* blastoderms, although relatively undifferentiated in comparison with cells of adult tissues, contain some specializations related to the performance of specific functions. Of particular significance are the cytoplasmic specializations concerned with uptake and utilization of yolk. Furthermore, the blastomeres undergo changes, especially during blastula and gastrula stages, related to the acquisition of the nuclear and cytoplasmic machinery necessary for subsequent differentiation.

The periblast subjects the yolk material to enzymatic hydrolysis (43) and transfers materials to the overlying blastoderm (8). Isolated blastoderms cultured in yolk medium show limited differentia-

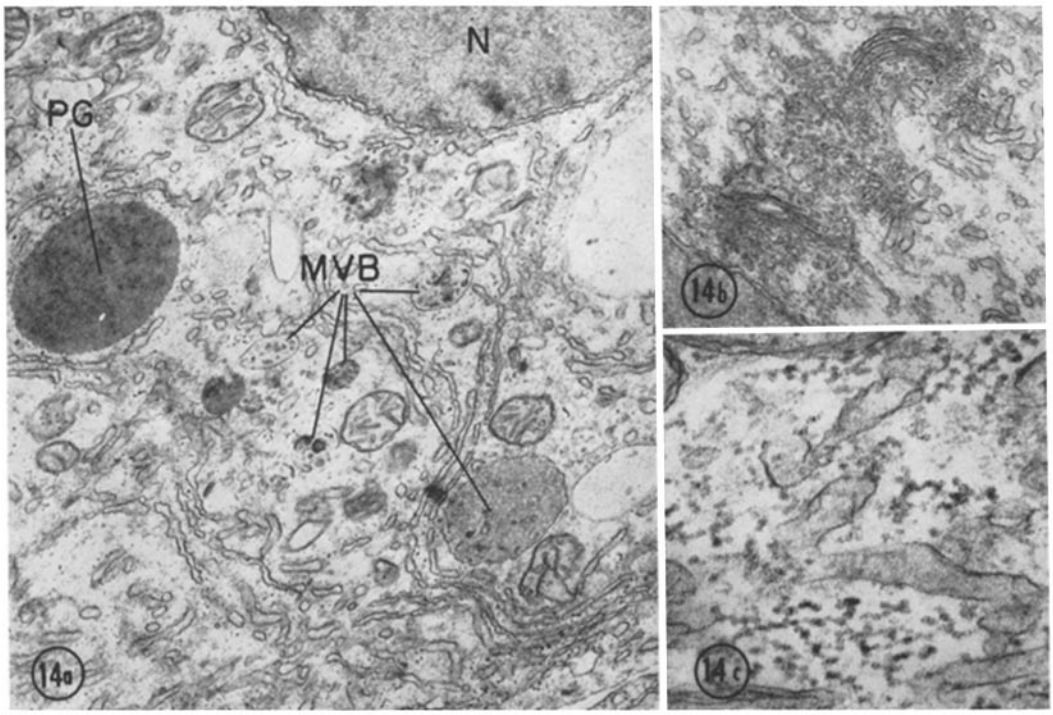


FIGURE 14 Cytoplasm of gastrula blastomeres. Fig. 14 *a* Note the elongated cisternae of endoplasmic reticulum. *N*, nucleus; *PG*, protein granule; *MVB*, multivesicular body. Stage 11. Fig. 14 *b* Golgi apparatus. The contents of some of the vesicles are moderately dense. Stage 11. Fig. 14 *c* Dense cytoplasmic granules, presumably glycogen. Stage 12⅓. Fig. 14 *a*, × 17,000; *b*, 14,000; *c*, 77,000.

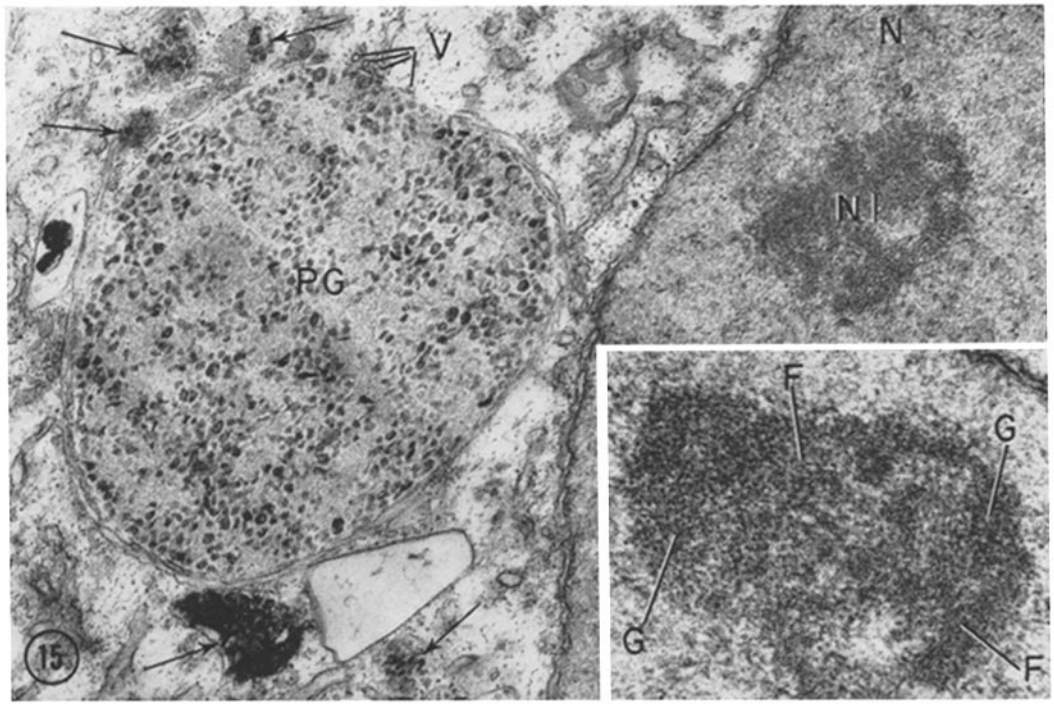


FIGURE 15 Blastomere from an early gastrula (stage 11). A large protein granule (*PG*) is composed of granular material of medium density and small vesicles with dense contents. Smaller granules of similar structure (arrows) as well as a few small vesicles (*V*) are situated outside the limiting membrane of the large granule. Dense interchromatin granules are dispersed in the nucleoplasm. *N*, nucleus; *Nl*, nucleolus. Inset. Nucleolus of an early gastrula blastomere (stage 11). The nucleolus contains finely textured fibrous regions (*F*) and granular areas (*G*) composed of 150 A particles. Fig. 15, × 28,500; inset, × 42,000.

tion, but if the periblast is included with the blastoderm, development of the embryo proceeds to a much more advanced degree (41). These findings indicate that the periblast is essential for the nutrition of the blastoderm. The elongated, branching projections of cytoplasm extending into the yolk from the blastomeres of the early cleavage stages, and the periblast of later stages appear to be of primary importance in the uptake of material from the yolk. The plasma membranes of these projections are smooth with no associated pinocytotic vesicles. Although phagocytosis in the usual sense was not observed, some yolk material could be trapped and incorporated in the interstices at the bases of the branching and anastomosing projections. Because of the apparent absence of pinocytosis and phagocytosis in *Fundulus*, we infer that the bulk of the food material enters the cytoplasmic projections directly, by passing through the plasma membrane, presumably as small molecules (i.e., glucose, fatty acids, triglycerides, amino acids, peptides). This implies that yolk material must be solubilized or partially digested extracellularly. This suggestion is supported by the presence of an electron-transparent zone, perhaps representing the area of solubilization, between the plasma membrane and moderately dense yolk.

Protein or proteid granules, lipid droplets, and presumed glycogen particles are abundant within the villous projections of the periblast and are probably formed from the precursor materials absorbed from the yolk. Some may represent yolk material trapped at the bases of the projections. In many cases, small vesicles containing either electron-opaque or electron-transparent material fuse with large vacuoles, contributing to the growth of the vacuole or forming large multivesicular bodies similar to those in the rat ovum (34). Some of the multivesicular bodies also contain moderately dense material and closely re-

semble protein granules in the fat body of insects (25). According to Locke and Collins (25), the latter are formed by the fusion of Golgi vesicles. Some yolk globules in ova and embryos of amphibians also appear to originate from multivesicular bodies (16, 23, 46). The Golgi apparatus also plays a role in the formation of yolk protein granules in the teleost *Lebistes* (10). The periblast of *Fundulus* contains many Golgi complexes composed of a few lamellae and many small vesicles. It is possible that some of these vesicles are involved in the growth of protein granules, at least in the immediate vicinity of the yolk.

Nutrient materials originating from the yolk must pass through the periblast to reach the cells of the blastoderm. The apical cytoplasm of the periblast is of low density and relatively free of protein granules, lipid droplets, or glycogen particles. It appears, therefore, that the materials present in the basal regions of the periblast in the form of lipid droplets and protein granules are again broken down to their precursors, which then diffuse out of the periblast into the segmentation cavity. However, some of the materials absorbed from the yolk may pass directly through the periblast without a storage stage. Small vesicles containing hydrolytic enzymes may originate from the numerous Golgi complexes and fuse with the protein or yolk granules. Thus, some of the multivesicular bodies probably represent stages in the destruction of protein granules (although these could not be distinguished from those in the process of formation).

Nutrient materials which have passed through the periblast into the segmentation cavity continually bathe the deep blastomeres and the inner surfaces of cells of the enveloping layer. Materials in the segmentation cavity are absorbed by the blastomeres in the apparent absence of pinocytosis. The blastomeres of all stages contain lipid droplets and protein granules whose contents vary in density and texture. Some small cytoplasmic particles

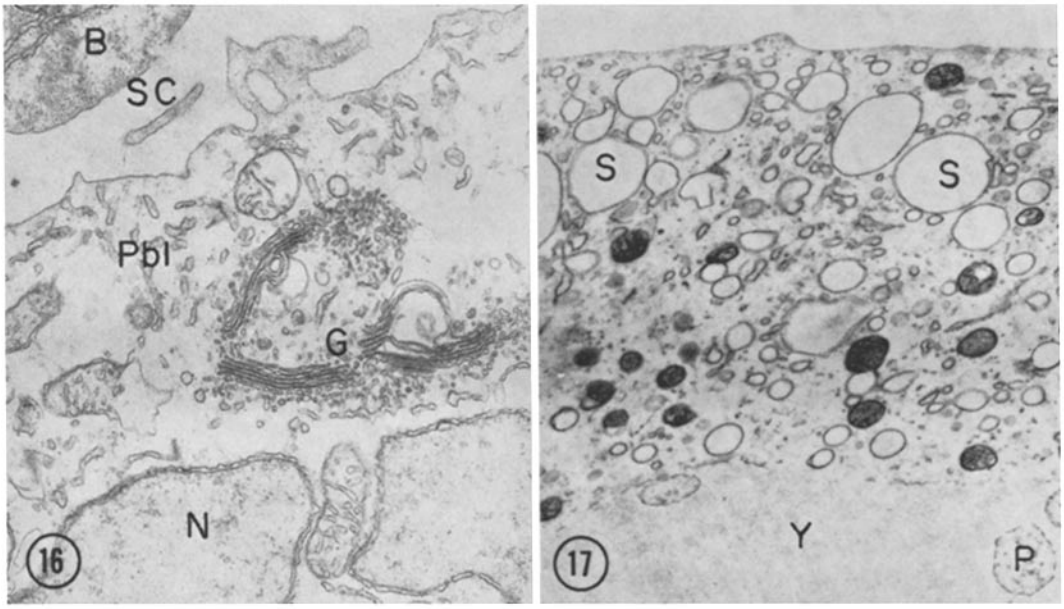


FIGURE 16 Periblast (*Pbl*) and blastomere (*B*) of a mid-gastrula (stage 12⅓). The hyaloplasm of the periblast is of low density. Note the elaborate Golgi apparatus (*G*). Microvilli extend from the periblast into the segmentation cavity (*SC*). *N*, nucleus. × 19,000.

FIGURE 17 Yolk cytoplasmic layer from a four-cell stage in a region close to the blastoderm. This layer is a cytoplasmic sheet containing small mitochondria, a few irregular membranous profiles, and large clear spaces (*S*). Both the surface membrane and the basal membrane adjacent to the yolk (*Y*) are relatively smooth in contour with few projections (*P*). The cortical cytoplasm beneath the surface membrane is slightly condensed, but not to the degree observed in most areas of the yolk cytoplasmic layer. × 10,000.

are aggregated in crystalline formations in blastomeres of the blastula, but we have not observed an internal crystalline structure in membrane-bounded granules like that found in the yolk platelets of amphibians (18).

The protein granules, lipid droplets, and glycogen observed in the blastomeres could have been originally present in the cytoplasm of the oocyte and not yet utilized. However, these structures, especially protein granules, appear more numerous in the blastomeres of blastula stages than of earlier cleavage stages (although large granules may decompose into numerous smaller ones), indicating the uptake of materials from the segmentation cavity and their storage in granules in the blastomeres prior to utilization. As emphasized by Worley and Moriber (48), protein material exists in a dynamic state during embryonic development, with constant formation, breakdown, and reformation of protein granules.

Large and small multivesicular bodies, identical with those observed in the periblast, are also present in the blastomeres. As in the periblast, some of these structures may be involved in the formation and others in the destruction of protein granules. It may be significant that multivesicular bodies are most abundant in early gastrula cells (stage 11). Protein granules not containing vesicles, on the other hand, are more numerous in the blastula stages (8–10), but much less numerous by mid-gastrula (stage 12⅛). In amphibians, decomposition of yolk begins in the gastrula stage (19), under the influence of phosphoprotein phosphatase (13). At the same time, in *Fundulus* the Golgi apparatus is highly developed, containing a large number of small vesicles. Small vesicles, originating in the Golgi zone and carrying hydrolytic enzymes, may fuse with the protein granule and transform it into a multivesicular body. Thus, the multivesicular bodies at stage 11 may represent stages in the breakdown and digestion of protein granules. This system resembles one in which the Golgi apparatus supplies hydrolytic enzymes to a phagocytotic vacuole, transforming it into a phagolysosome, digestive vacuole, or multivesicular body (15).

The present study has revealed the yolk gel layer to be a layer of cytoplasm. It is continuous with and an extension of the periblast, and, like it, has a membrane separating the cytoplasm from the yolk beneath. This structure possesses remarkable contractile properties and heals wounds readily (36, 37). For these reasons, it has been thought to play an important role in epiboly (9, 24). Although its precise role in epiboly remains undefined, its importance in containing the yolk and maintaining the spherical shape of the egg is undisputed. Now that we possess a clear notion of the structure of this layer, a noncommittal term, such as yolk gel layer, is no longer adequate. We propose that it now be called the yolk cytoplasmic layer.

Important structural changes occur in the nucleus and nucleolus during early development of the *Fundulus* egg. In early blastulae, the nucleolus appears as a mass of fine fibrillar material. Then, near the onset of gastrulation, dense granules resembling cytoplasmic ribosomes appear. In this respect, the nucleolus of *Fundulus* gastrulae approximates the nucleolus of most other plant, invertebrate, and vertebrate cells (3, 17, 30, 35). At the same time, nuclear chromatin material shows a tendency to aggregate into small clumps, and interchromatin granules become numerous. These changes are identical with the sequence of changes described by Karasaki (21) during early development of the newt *Triturus*.

The appearance of the nucleolus has been associated, in other forms, with an increase in the number of cytoplasmic ribosomes and subsequent cytoplasmic specialization and increased capacity for protein synthesis (6, 7, 20–22). The present studies reveal a similar situation in *Fundulus*; cytoplasmic ribosomes, especially polyribosomes, become more numerous at the same time the nucleolus is first noted. In gastrulae, the endoplasmic reticulum becomes more extensive and acquires material within its cisternae, indicating synthetic activity.

A different situation occurs in the periblast. A nucleolus composed of a fibrous region and dense granules appears, as in the blastomeres. However, no ribosomes become evident in the cytoplasm, indicating that transfer of ribosomal RNA or its precursor to the cytoplasm is prevented in the periblast. Transfer of this material to the cytoplasm, therefore, appears to be independent of the accumulation of ribosome-like particles in the nucleolus. Differentiation of the periblast should be affected, as a result, because protein synthesis cannot occur in the absence of ribosomes. It is, therefore, of interest that the cytoplasm of the periblast remains unspecialized, like the cytoplasm of cleavage blastomeres from which the periblast is derived. This absence of differentiation of periblast cytoplasm correlates also with the future history of the periblast; it remains a cytoplasmic layer

during embryogenesis, not engaging in histogenesis (47).

During cleavage, the blastomeres undergo cell division and show DNA replication in the absence of growth. The blastomeres are undifferentiated: the hyaloplasm is of low density, ribosomes and membranous elements comprising the endoplasmic reticulum are sparse, and mitochondria are relatively simple in structure. Beginning during the blastula stage, when the blastoderm consists of a few hundred cells, the blastomeres show an increase in cytoplasmic specializations. In addition to the nuclear changes, cytoplasmic ribosomes, including polyribosomes associated with protein synthesis (26, 45), and membranous elements become more numerous. The Golgi apparatus and mitochondria are more complex. These signs of differentiation in blastula stages occur earlier than expected. This phase of development is generally assumed to be characterized by extensive cell division and formation of a segmentation cavity, but during which there is neither growth nor any overt sign of differentiation. The changes occurring during the blastula stage may represent the beginnings of more extensive specialization during gastrula stages. At the same time these changes occur, the surface activity of the deep blastomeres increases (42).

The differentiation of the cytoplasm during gastrulation is not unexpected. In other embryos, it has been shown that ribosomal RNA synthesis increases rapidly at gastrulation (4, 5, 6), and this occurrence is correlated with an increase in protein synthesis and growth (5, 22). An immediate consequence of gastrulation is organogenesis, with accompanying cellular differentiation into the many histological types. The increased elaboration of synthetic machinery which occurs during gastrulation, especially the endoplasmic reticulum and Golgi apparatus, is necessary for the production of a variety of different proteins during histogenesis. In addition, cellular locomotor activity and adhesiveness increase in relation to the mass cell movements of gastrulation (38). These activities also represent a form of differentiation and presumably have their basis in the increased specializations of the cytoplasm.

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