

DIFFERENTIATION OF THE JUNCTIONAL COMPLEX OF SURFACE CELLS IN THE DEVELOPING *FUNDULUS* BLASTODERM

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ABSTRACT

The structure of the junctional complex between surface cells was investigated in blastula, mid gastrula, late gastrula, and early embryo of the teleost fish *Fundulus heteroclitus*. In blastulae, the intercellular complex is simple and consists of an apical region where the adjacent membranes are closely apposed (40–60 Å) and in places touch, an intermediate zone with a wider intercellular space (>100 Å), and incipient desmosomes. In gastrulae, there are frequent points of fusion of membranes along the apical zone of the complex. Dilatations and an increased number of desmosomes in different stages of development are found along the intermediate zone. In mid gastrula, a close or gap junction with an intercellular space of 20 Å occurs below the level of the desmosomes. In late gastrula, the gap junction is reduced in extent and desmosomes are better developed. In the early embryo, the basic organization of the complex is the same, although the deeply situated close junctions are no longer apparent and desmosomes and their associated system of filaments are well developed. At this time, the junctional complex is comparable to that of many epithelia and consists of an apical zonula occludens, a short zonula adherens, and deeply situated maculae adherentes.

INTRODUCTION

The surface cells of the blastoderm of the teleost fish *Fundulus heteroclitus* possess apical contact specializations (Trinkaus and Lentz, 1967). A major function of the junctional complex of *Fundulus* has been thought to be cell adhesion, because the junctions are more highly developed during the process of epiboly, when greater stress is placed on cell-to-cell attachments (Trinkaus and Lentz, 1967). This adhesion function seemed all the more likely because cells of this surface layer, the enveloping layer, appear to show contact inhibition of movement (Abercrombie and Heaysman, 1953). They do not crawl over each other during epiboly, in spite of much sur-

face activity, but instead remain in contact along their lateral borders to form a cohesive monolayer (Trinkaus and Lentz, 1967).

More recently, cells of the enveloping layer have been found to be electrically coupled, both by way of intercellular junctions and extracellular space (Bennett and Trinkaus, 1970). Along with this, the resistance between the segmentation cavity and the exterior of the egg is very high, indicating that the cavity is sealed off from the exterior, presumably by the apical circumferential junctions connecting enveloping layer cells (Trinkaus and Lentz, 1967). In view of both the contact-inhibiting behavior of these

cells and their electrical properties, structural details of the junctions between them have taken on added significance. These intercellular contacts may somehow mediate contact inhibition of cell movement (see Trinkaus, 1966 and 1969) and may play a role in the intercellular transmission of ionic and other chemical signals (Bennett and Trinkaus, 1970).

The present communication presents in further detail the structure of the junctional complex of cells of the enveloping layer of *Fundulus*, with particular attention to regions of close apposition and fusion. In addition, the structure of the junctional complex, including desmosomes, is compared in blastula, gastrula, and early embryo stages to determine the sequence of events in the development of its various components.

MATERIALS AND METHODS

Eggs of *Fundulus heteroclitus* were obtained at Woods Hole, Massachusetts, and generally handled according to the procedures we have already outlined (Lentz and Trinkaus, 1967). The following Oppenheimer (1937) stages were examined: 8 (blastula); $12\frac{1}{4}$ and $12\frac{1}{2}$ (mid gastrula); $13\frac{1}{2}$ and 14 (late gastrula); and 18 (early embryo, trunk region).

Blastoderms were obtained and fixed in the following manner. The whole dechorionated egg was placed in cold 3% glutaraldehyde in 0.05 M Na cacodylate buffer (pH 7.4) in the depression of a paramecium slide and allowed to rest for 1 min. Then the animal half of the egg was dissected off by cutting into the yolk below the blastoderm and the margin of the periblast with watchmaker's forceps. When possible, the blastoderm was shaken off from the yolk. Otherwise, the yolk was trimmed away from beneath the blastoderm. Approximately 2 min were required for this operation. The isolated blastoderm was then transferred to another depression containing fresh, cold glutaraldehyde and allowed to fix for 1 hr in the refrigerator. It was then rinsed and stored in cold buffer prior to fixation for 1 hr in cold 1% osmium tetroxide buffered with Na cacodylate (pH 7.4).

After osmium tetroxide fixation, we stained the blastoderm en bloc with aqueous uranyl acetate to enhance membranes and fibrillar structures.¹ The

¹ The morphology of tight and gap junctions has been shown to be affected by the choice of fixation, staining, and dehydration procedures (Brightman and Reese, 1969; Goodenough and Revel, 1970). Uranyl block treatment after aldehyde or osmium tetroxide fixation stabilizes the outer leaflets of the plasma membranes which become visible, delimiting gaps when these are present, following lead staining of the plastic sections (Brightman and Reese, 1969).

method of Farquhar and Palade (1965) was used; in accordance with this method, the tissues were placed in 0.5% uranyl acetate in Michaelis buffer (pH 5.0) for 2 hr at room temperature. The tissues were then dehydrated and embedded in Maraglas (The Marlette Co., Div. of Allied Products Corp., Long Island City, N. Y.). Thin sections cut on a Porter-Blum microtome were stained with uranyl acetate and lead citrate (Reynolds, 1963) or lead hydroxide (Feldman, 1962) and examined with an RCA EMU 3F electron microscope.

RESULTS

The plasma membranes of the enveloping layer cells of *Fundulus* are 70–80 Å in diameter. They form contact specializations along the lateral borders of adjacent cells at all stages of development. Even at the earliest stage examined (stage 8, blastula), regions comparable to the zonula occludens, zonula adherens, and macula adherens of the classical junctional complex (Farquhar and Palade, 1963) could be identified. The components of the junctional complex are relatively unspecialized at this time and show an increased complexity as development proceeds to the early embryo stage. In addition, a fourth component of the junctional complex, a close or gap junction, is present in gastrulae. The cellular relationships and surface features in the enveloping layer at different developmental stages are summarized diagrammatically in Fig. 1. The detailed structure of the junctional complex during development is illustrated in Fig. 14.

Blastula

At the blastula stage (stage 8), the surface of the large, enveloping layer cells is rounded and relatively smooth in contour with a few shallow indentations (Fig. 1). Contact specializations are present along the lateral borders of the enveloping layer cells (Figs. 2, 3). Three components form the junctional complex at this stage: an apical region where the plasma membranes are closely apposed, an intermediate region, and, at deeper levels, small or incipient desmosomes.

The surface plasma membranes of adjacent cells turn inward and appear to touch (Figs. 3 a, 3 b). The distance between the inner leaflets of the plasma membranes is 130–140 Å, indicating that the membranes are fused. The membranes immediately diverge to a distance of 40–60 Å which is maintained over most of the length of this initial portion of the junctional

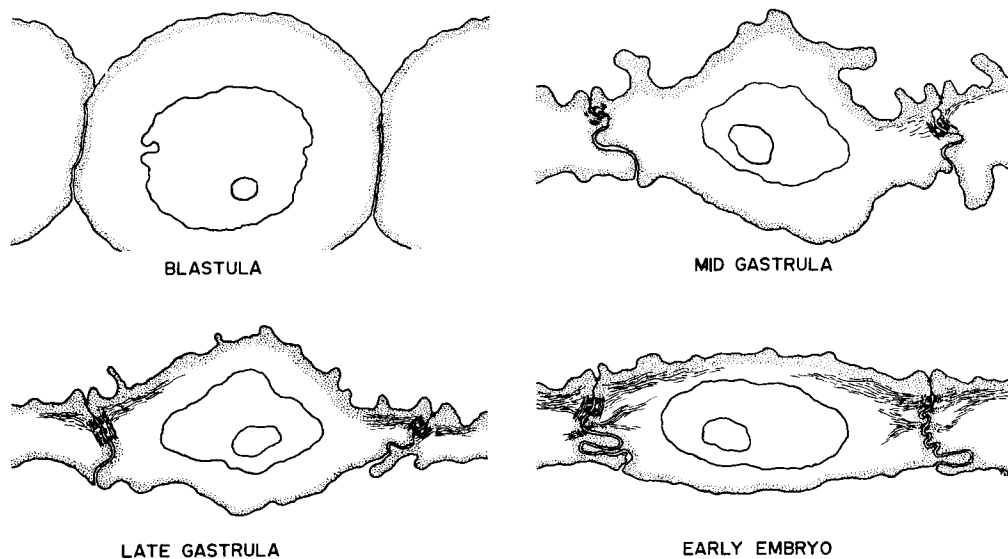


FIGURE 1 Diagram illustrating the surface contours and intercellular relationships of enveloping layer cells of *Fundulus* during development. In blastulae, the cells are large and rounded and the cell surface is relatively smooth. Early junctional complexes occur on the lateral surfaces which are straight. Enveloping layer cells of mid gastrulae are more flattened and show extensive surface activity in the form of surface projections and undulations. (The cells may undergo some shrinkage or change in shape during fixation, because living cells appear even more flattened and attenuated than those in $1\ \mu$ thick plastic sections). Low folds occur on the surface at the lateral margins. The junctional complex, which is more highly developed at this time, begins at the bottom of these lateral folds. The lateral borders are more irregular. In late gastrulae, the surface still shows irregularities. Desmosomes and associated filamentous systems are better developed. In the early embryo, the cells are more flattened and show less surface activity. The lateral surfaces of adjacent cells show more folds and interdigitations. An extensive system of cytoplasmic filaments is associated with the junctional complex. There is a zone of ectoplasmic density largely devoid of organelles beneath all the cell surfaces and thickest apically.

complex which corresponds to the zonula occludens. The intercellular space contains material with some density (Fig. 3 *b*). At irregular intervals along this region the membranes converge and touch again at focal points (Fig. 3 *b*). In some of these contacts, the width of the junction between inner leaflets of the membranes is greater than $140\ \text{A}$, indicating that the membranes are not fused but are only in apposition. In the cytoplasm, there appears to be a slight accumulation of fine material adjacent to and along the inner aspect of the membranes.

Below the apical junction, the membranes diverge to produce an intercellular space of $100\text{--}200\ \text{A}$ (zonula adherens or intermediate junction) (Fig. 3 *a*). The membranes are roughly parallel and do not fuse to obliterate the space. The lateral cell surfaces are generally straight, without folds or complex interdigitations. There

are no marked accumulations of dense or fibrillar material in the cytoplasm alongside the junction.

The third portion of the complex was variably seen at this stage, being absent between many cells. It consists of localized accumulations of fine-textured material of moderate density in the cytoplasm, against the plasma membranes of adjacent cells and opposite each other (Fig. 3 *a*). Fibrils were not associated with the dense material. The intercellular cleft is at least $100\ \text{A}$ wide in this region and contains amorphous material of low to medium density. The length of this junction, interpreted as an early desmosome, does not exceed $0.15\ \mu$.

Mid Gastrula

In the mid gastrula (stage $12\frac{1}{3}\text{--}12\frac{1}{2}$), the enveloping layer cells are smaller and flatter (Fig. 1). Their surface contour is more irregular, with numerous cytoplasmic extensions and surface

undulations. Surface projections usually occur at the lateral edge of each cell so that the junctional complexes begin at the bottom of pairs of folds along the surface contour of the enveloping layer (Figs. 1, 4, 7, 13).

At low magnification, four distinct regions can be seen to comprise the junctional complex (Figs. 4, 8 *a*). In the apical region, the plasma membranes are closely apposed but a narrow intercellular space is apparent over most of their extent. Below this region and extending for a greater distance is a zone in which the intercellular space is wider. Small desmosomes in different stages of formation and intercellular lakes occur along this region. Deep to the desmosomes is a short region which at low magnifications appears as a dense line with no apparent intercellular space (Figs. 4, 8 *a*).

The components of the junctional complex at this stage show additional specializations in comparison to blastulae. The apical region of the complex (zonula occludens) extends about 0.3 μ deep (Fig. 5) and forms a continuous band around the cells (Fig. 6). Over most of the depth of the junction, the plasma membranes are parallel and separated by a space of 40–60 A, and occasionally by as much as 100 A (Figs. 5, 7). The intercellular cleft in places seems to contain amorphous material of low to medium density. At the surface and at irregular intervals along

the junction, however, the membranes come together to form focal tight junctions (Fig. 7). The outer leaflets of the membranes seem to touch at these points, obliterating the intercellular space. The distance between the inner leaflets of the plasma membranes at the points of fusion is 130–140 A.

In the cytoplasm and symmetrically apposed to the regions of focal contact are small accumulations of dense material (Figs. 5, 7). In sections transverse to the junction, these button-like masses are round and about 200 A in diameter. Two pairs of densities were usually seen at this stage. The first pair is invariably present in the most apical region of the junction where the plasma membranes of adjacent cells first make contact.

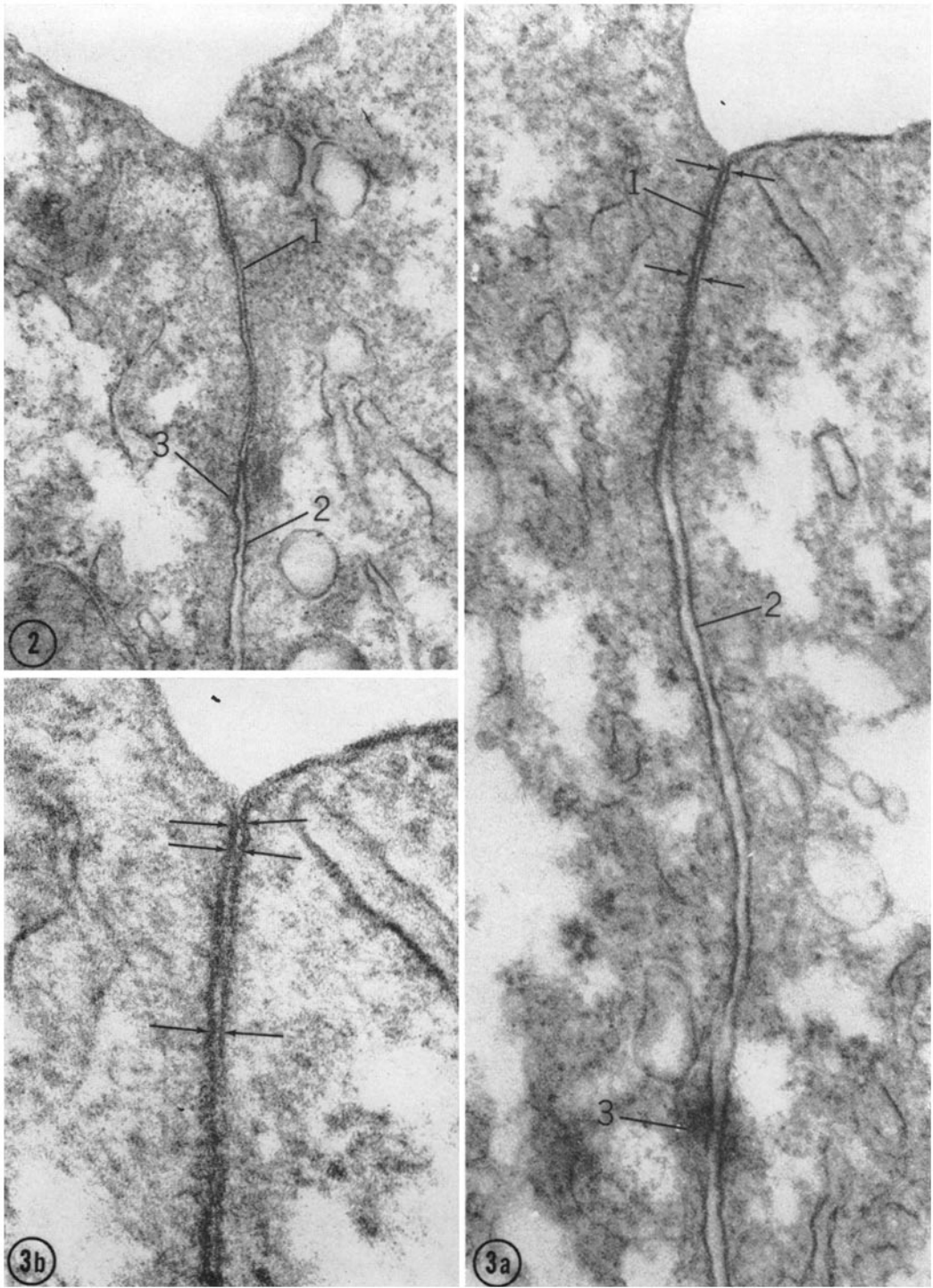
In a few instances in the apical junctions of gastrulae and early embryos, the outer leaflets of the plasma membranes are fused over a considerable distance (Fig. 10). The width of the junction between inner leaflets of the plasma membranes is 130–140 A. Bands of dense material about 200 A wide are applied to the cytoplasmic surfaces of the fused membranes. The dense material extends the same distance as the length of membrane fusion. Where the membranes separate and reflect onto the surface, they are viewed obliquely, indicating that the section is not in a plane at right angles to the surface, but

Abbreviations: 1, apical junction (zonula occludens); 2, intermediate junction (zonula adherens); 3, desmosome (macula adherens); 4, deep gap junction; *DM*, dense material; *F*, cytoplasmic filaments; *L*, intercellular lake.

FIGURE 2 Low magnification view of adjacent surface blastomeres of a stage 8 blastula. Where the cells contact each other apically, the membranes are closely apposed (zonula occludens) (1). Below this, they are separated by a large intercellular space (zonula adherens) (2). A slight, localized increase in cytoplasmic density along the latter zone may represent an early desmosome (macula adherens) (3). $\times 69,000$.

FIGURE 3 a Higher magnification of a junction between surface blastomeres of the blastula (stage 8). In the apical portion of the junction (1), the membranes are closely apposed but generally separated by a narrow intercellular space. Below this, the membranes are parallel and separated by a larger space of about 100–200 A (2). At a deeper level, localized masses of moderately dense cytoplasmic material are symmetrically apposed to the plasma membranes (3). The intercellular space shows an increase in density in this region. These densities appear to represent the first stage in desmosome development. $\times 89,000$.

FIGURE 3 b Enlargement of the apical region of the junction illustrated in Fig. 3 a. Note that where the membranes first turn inward, and in a few places along this part of the junction, the membranes appear to touch or come into very close apposition (*arrows*). Elsewhere, the membranes are separated by a space of 40–60 A. $\times 163,000$.



instead passes through the intercellular region at an angle to the surface of the enveloping layer.

Below the apical junction, the plasma membranes diverge to produce an irregular cleft 100–200 Å across (zonula adherens). In places, the membranes diverge to form large intercellular lakes (Fig. 4). The contents of the cleft appear electron lucent, although there is a suggestion of small amounts of material adhering to the plasma membranes.

Small desmosomes (maculae adherentes) of various complexities are found in this portion of the junctional complex where the membranes are separated by 200 Å. The simplest are the same as those in blastulae and consist of a small amount of dense material adjacent to the inner aspect of the plasma membrane (Fig. 4). In more advanced desmosomes, there is a further accumulation of dense, fine-textured material, increasing the overall dimensions of the desmosomes (Fig. 4). The dense cytoplasmic material merges with a band of filaments that parallels the plasma membranes in the region of the developing desmosomes. The plasma membranes are separated by a space of 100 Å or more, and material of medium density occupies the intercellular cleft in the region of the junction.

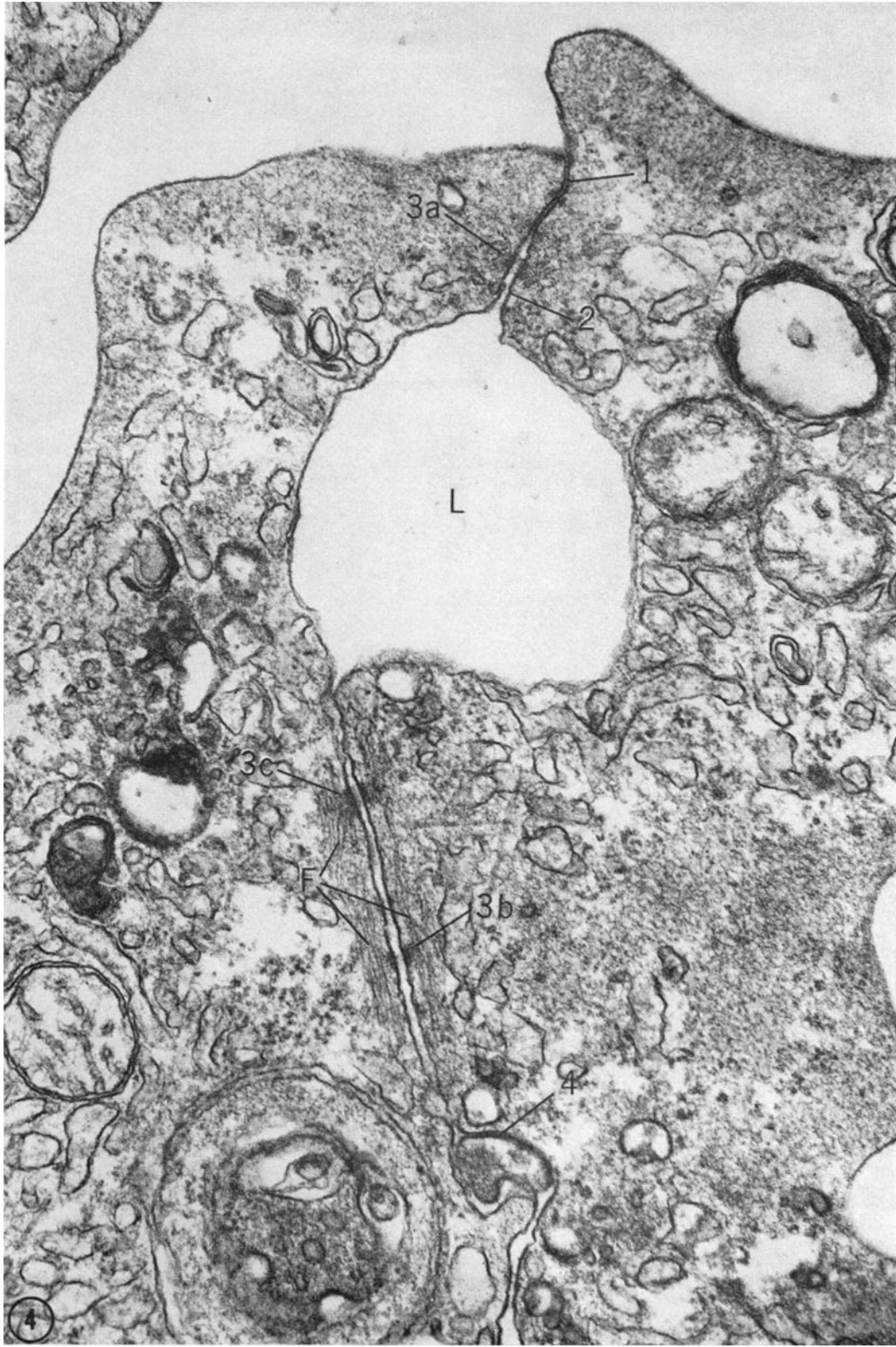
The last element of the junctional complex occurs below the other regions. It appears to be a plaquelike (fascia) structure 0.2–0.4 μ in depth, and is usually straight with no sharp bends or curves along its length (Figs. 4, 5, 8). The membranes of adjacent cells converge and approach each other to within a distance of 20 Å but do not fuse (Fig. 8*b*). This junction, then, corresponds to a close or gap junction. The extremely narrow gap in this junction is occupied

by moderately dense material, making visualization of the membrane leaflets more difficult because of lower contrast. The width of the entire junction between inner leaflets of the membrane is 160–180 Å, accounting for the narrow gap, since the plasma membranes are 70–80 Å in diameter.

Late Gastrula

At the late gastrula stage (stage 13½–14), surface processes are still abundant but not quite as large or elongated as in mid gastrulae (Fig. 1). An elevation or fold is usually seen on each side of the junctional complex, so that, as in mid gastrulae, the complex occurs at the base of a surface indentation. The basic organization of the junctional complex is the same as in mid gastrulae. It consists of an apical region of close membrane apposition with points of fusion (zonula occludens), a region in which the intercellular spaces are wider (zonula adherens), and desmosomes (maculae adherentes) (Fig. 9). Several differences are apparent, however. In the apical junction, only one pair of dense, button-like masses is usually seen, situated where the converging membranes first touch most apically. The intermediate region of the complex is shorter, because desmosomes occur at higher levels. Sometimes the intercellular space of the intermediate junction, as well as of the apical junction, contains moderately dense material (Fig. 10). A diffuse accumulation of material also occurs along the inner surfaces of the membranes in these regions (Figs. 10, 12). The desmosomes are generally more highly developed, and there are fewer early stages compared with the mid gas-

FIGURE 4 Survey view of the junctional complex of a stage 12 ½ gastrula. The surface contour of the enveloping layer cells is irregular with broad undulations. In the most apical portion of the junction (zonula occludens) (1), which extends for about 0.3 μ, the membranes are in close apposition, although an intercellular space can be discerned. Below this, in the intermediate region (zonula adherens) (2), the membranes are separated generally by an intercellular space of over 100 Å. A large, dilated, intercellular lake (L) occurs along this region. In addition, desmosomes (3) in different stages of formation are found. The earliest (3*a*), consists of paired densities in the cytoplasm with a minimum of associated filaments. In more advanced desmosomes (3*b*, 3*c*), both the dense plaques and cytoplasmic filaments (F) are more prominent. The latter run parallel to, and a short distance from, the plasma membranes, but contact the dense material. Dense material also occurs in the intercellular space in the desmosome region. Finally, below these components of the junction is a short zone where the plasma membranes are again closely apposed; no intercellular space is apparent at this magnification (4). × 51,000.



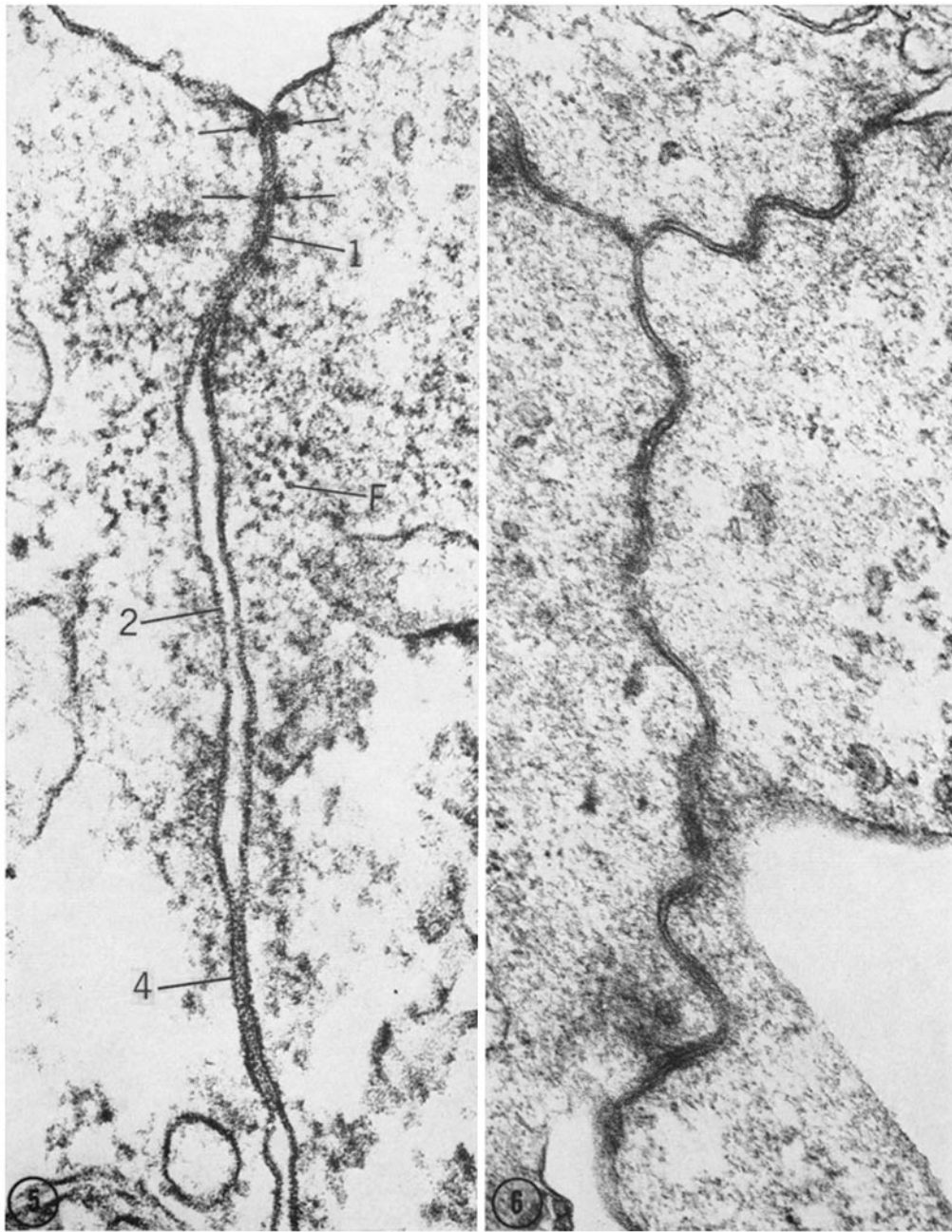


FIGURE 5 Higher magnification of the junctional complex at the mid gastrula stage ($12\frac{1}{3}$) illustrating the apical occluding region (1), intermediate region (2), and a short zone of very close membrane apposition (4). Note that paired, button-like masses of extremely dense material occur in the cytoplasm where the membranes first touch and along the apical region (arrows). Below this, some filaments (F) are seen in transverse section and there are some cytoplasmic densities adjacent to the membranes elsewhere, but a definitive early desmosome is not apparent. The deep zone of membrane apposition is ~ 180 A wide, greater than twice the width of the membrane (~ 80 A). It is presumably a gap junction. $\times 143,000$.

FIGURE 6 Mid gastrula (stage $12\frac{1}{3}$). The section passes through the zonula occludens of the junctional complex and parallel to the surface of the enveloping layer, demonstrating the continuous or zonular nature of this contact. In most places, a narrow intercellular space is apparent. $\times 67,000$.

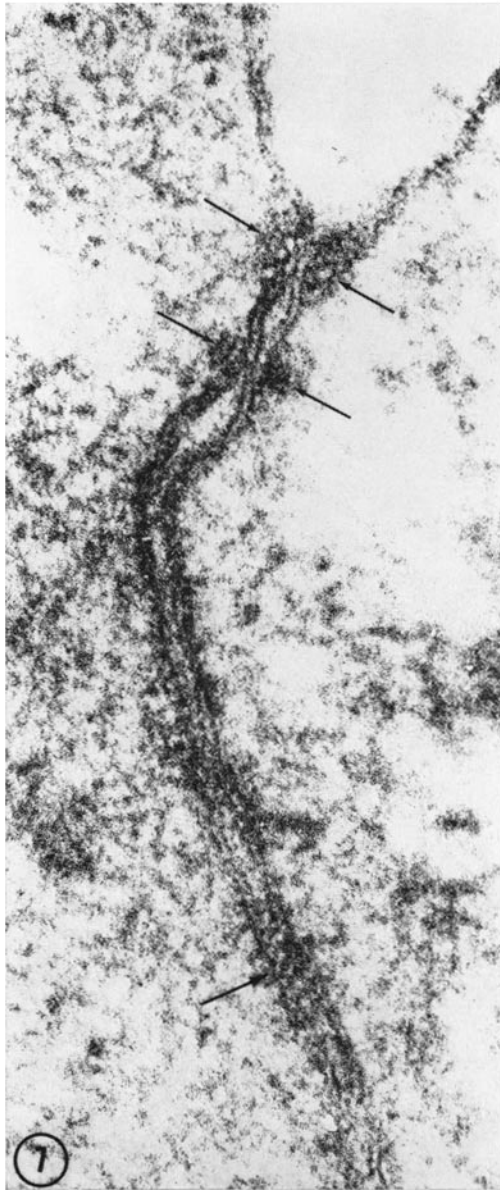


FIGURE 7 High magnification of the apical region of the junctional complex at the mid gastrula stage ($12\frac{1}{3}$). The cells make contact at the base of surface folds so that the junction begins at the bottom of an indentation on the surface. The membranes first come together, obliterating the intercellular space, diverge to a distance of 100 A, and then converge again at another focal point of fusion (small *arrows*). The width of the junction between the inner leaflets of the plasma membranes at the focal points of fusion is 130–140 A. There is another local dilation below the second point of fusion, but along the rest of the apical junction the intercellular space is most often about 40–60 A across. Note that the first two pairs of dense cytoplasmic buttons occur at the points of membrane contact. A more diffuse accumulation of material occurs in the cytoplasm along the rest of this region. Toward the bottom of the junction, some densities occur at intervals in the intercellular space (large *arrow*). $\times 250,000$.

the intercellular space of the desmosome, and is sometimes bisected by an intermediate dense line (Figs. 9, 11). The dense cytoplasmic plaques are more distinct at this time and may be separated from the plasma membrane by a narrow space (Figs. 9, 11). A prominent bundle of filaments parallels the lateral plasma membrane a short distance from the plaque of dense material (Figs. 9–12). Short filaments extend from the dense plaques to enter the large bundles of filaments at right angles (Fig. 12). In the apical cytoplasm, filaments are also more abundant. These run parallel to the apical cell surface and connect with the lateral bundles (Fig. 1). The filaments associated with the desmosomes are 60–80 A in diameter (Figs. 10, 12).

Early Embryo

In the trunk region of the early embryo (stage 18), the enveloping layer cells are spindle shaped and the surface undulations and projections are reduced further in number and size (Fig. 1) in comparison with the late gastrula. The junctional complex at this stage consists of a zonula occludens, zonula adherens, and macula adherens (Fig. 13). In the zonula occludens, only the most apical pair of button-like cytoplasmic densities was seen (Fig. 13). The intercellular space of the intermediate junction contains moderately dense material. Desmosomes are numerous and their associated fibrillar systems are prominent. The longitudinal system of filaments in the apical

trula. Below the desmosomes, the plasma membranes pursue a more wavy course and do not form large intercellular lakes. Gap junctions are not seen as frequently, and those observed are usually shorter than those in mid gastrulae (Fig. 9).

In sections parallel to the surface, the desmosomes are observed as button-like structures occurring at close intervals along the membranes (Fig. 11). Moderately dense material occurs in

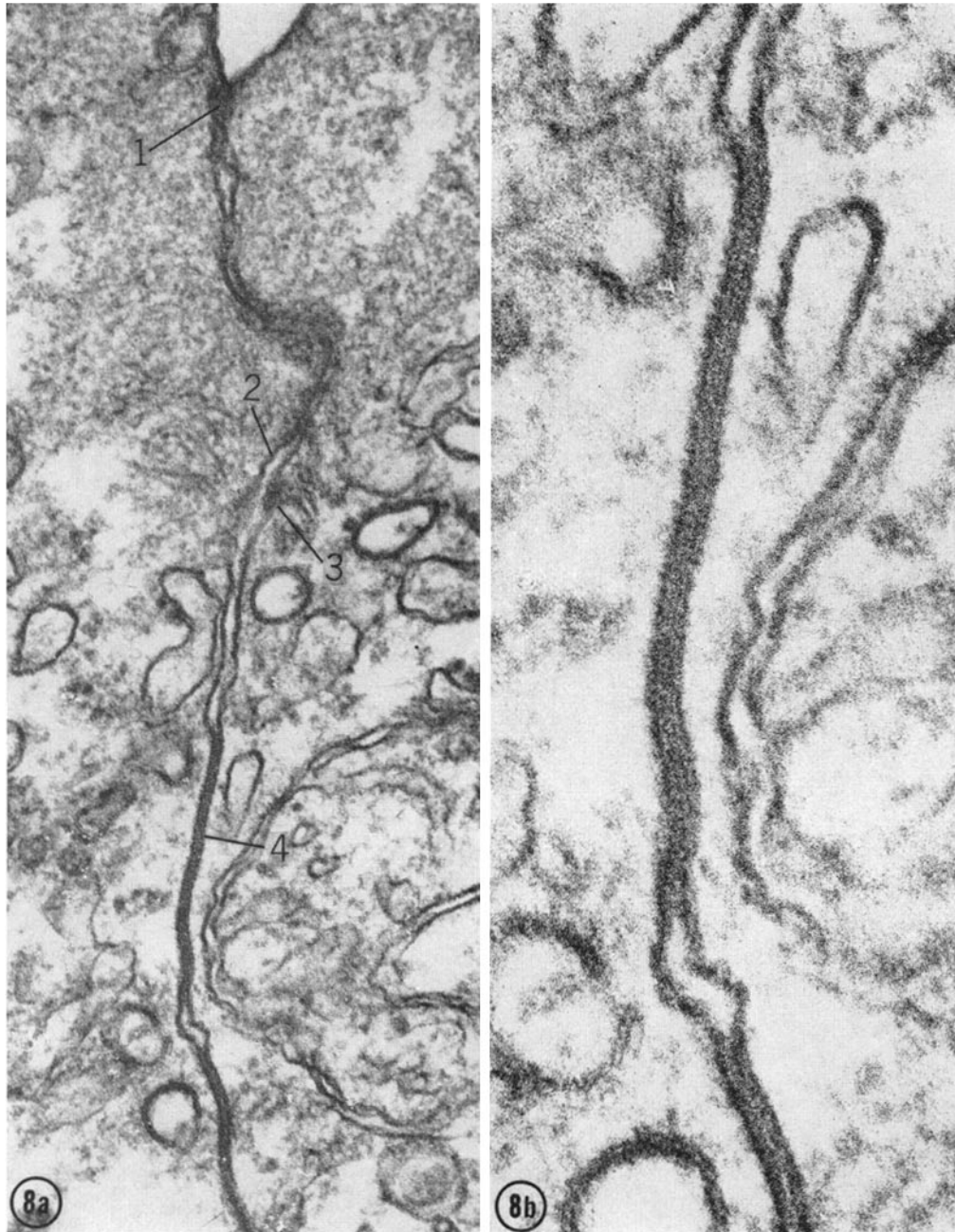


FIGURE 8 *a* Junctional complex (stage 12 $\frac{1}{3}$, mid gastrula). The four components of the complex are an apical occluding zonule (1), an intermediate zone (2), a desmosome (3), and a deeply situated gap junction (4). The latter is a relatively straight region in which the membranes are parallel and very closely apposed. $\times 89,000$.

FIGURE 8 *b* High magnification of the zone of close membrane apposition in Fig. 8 *a*. Note that a very narrow space of 20 A separates the adjacent membranes, indicating that this structure is a close or gap junction. The thin gap is filled with material of moderate density. The over-all dimension of the junction is 160–180 A, whereas a single membrane is 70–80 A. $\times 250,000$.

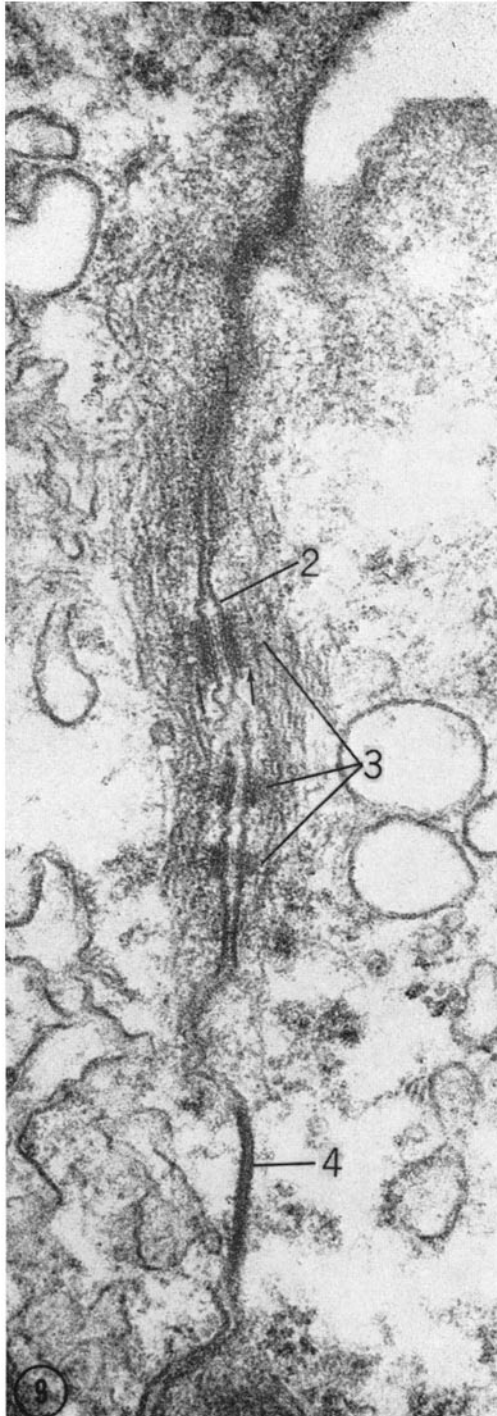


FIGURE 9 Junctional complex (late gastrula, stage 14). The four elements of the complex are an apical zonula occludens (sectioned obliquely for most of its length) (1), an intermediate zone (2), maculae adherentes (3), and a gap junction (4). The gap junction is 180 A in over-all width and is shorter than in mid gastrulae. The first desmosome is well developed. The intercellular material is bisected by a dense line. The cytoplasmic plaques of dense material are separated from the membranes by a thin line of low density. Some short filaments (arrows) extend out from the dense plaques into the prominent bundle of filaments running parallel to the lateral borders. $\times 83,000$.

vously (Fig. 13). Below the desmosomes, the membranes are roughly parallel and separated by an intercellular space of 100–200 A. Sometimes adjacent cells form complex interdigitations. The deeply situated region below the desmosomes, where the membranes came together to form a 20 A gap in gastrulae, is very rarely seen between surface cells of the trunk at this later stage. In other respects, the structural details of the components of the junctional complex are the same as those of the previous stage (late gastrula).

DISCUSSION

Development of the Junctional Complex

Although the contact specializations of adjacent enveloping layer cells of *Fundulus* embryos differ in some details from the characteristic tripartite junctional complex found in a number of more highly developed epithelial tissues (Farquhar and Palade, 1963), they are similar in basic organization (see Fig. 14). In the initial apical region of the complex, the membranes touch in places to obliterate the intercellular space. Although over a larger extent of this apical region the membranes are separated by a space of 60 A or less, this region as a whole corresponds to the zonula occludens.² Below the zonula occludens,

² In our earlier study (Trinkaus and Lentz, 1967), we found dense bars extending across the intercellular space of some apical junctions and noted their similarity to invertebrate septate desmosomes. With the present methods of fixation, nothing clearly comparable to a septate desmosome was seen, although in some places poorly defined periodic densities occurred in the 40–60 A intercellular space of apical junctions (Fig. 7). We now believe that these periodic structures as well as those described earlier could represent the polygonally packed subunits on

cytoplasm is extensive and joins the filaments paralleling the lateral surfaces (Fig. 13). A few desmosomes are found at deeper levels than pre-

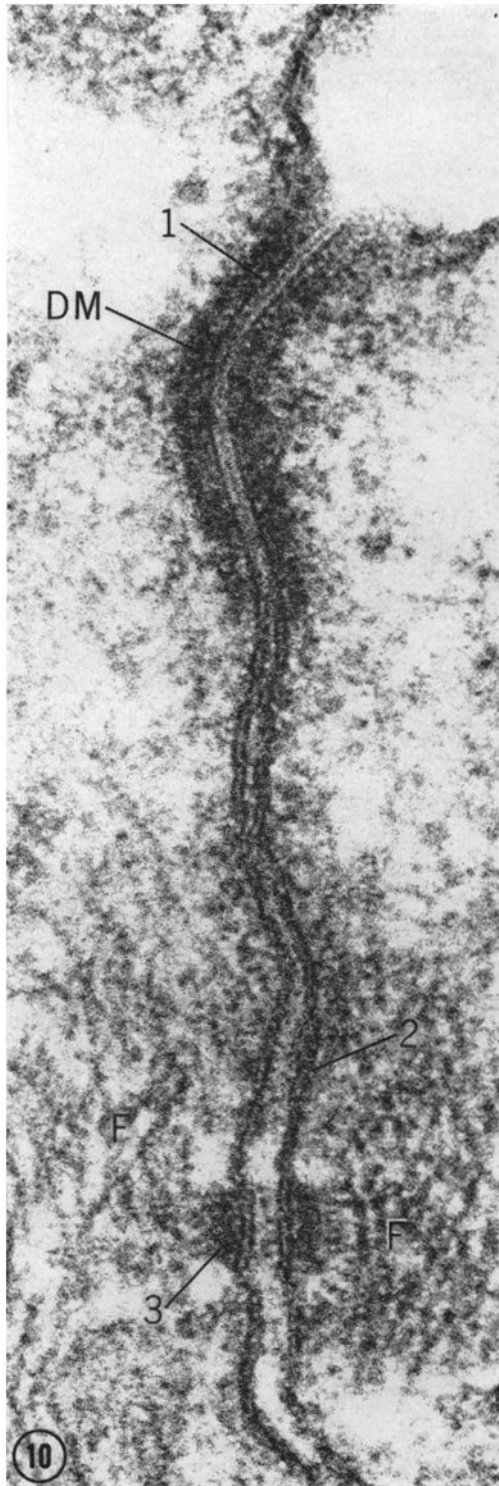


FIGURE 10. Junctional complex in the late gastrula (stage 14). The section is interpreted as passing along the length of a region of membrane fusion seen as a point contact in transverse sections (Fig. 7) (see text). In the apical region (1), the outer leaflets of the plasma membranes are fused, obliterating the intercellular space and forming an intermediate line (zonula occludens). The distance between inner leaflets is 130–140 Å, less than twice the width of a single membrane. A band of extremely dense material (DM) is applied to the inner leaflets for the length of membrane fusion. Below this, the membranes are separated by a space of 40–60 Å that contains some moderately dense material. The intercellular space is wider in the short zonula adherens (2) and also contains dense material. There is a diffuse accumulation of cytoplasmic material against the inner surface of the membranes. A well-developed macula adherens (3) is present deep to the other elements. A dense intermediate line bisects the intercellular material. A few short filaments run from the dense cytoplasmic plaques into prominent bundles of filaments (F) running parallel to the lateral surface. $\times 220,000$.

the membranes are over 100 Å apart and roughly parallel, except for focal dilatations of the intercellular space. This intermediate portion of the complex, then, can be identified as a zonula adherens. Below this region, the more deeply located desmosomes or maculae adherentes are found.

Proximal to the desmosomes, and often extending for a considerable distance, an additional specialization is seen in mid and advanced gastrulae. In these regions, the plasma membranes approach to within 20 Å of each other but do not fuse. This region corresponds to a close or gap junction (Revel and Karnovsky, 1967). By early embryogenesis, though, the deeply situated close junctions have apparently disappeared in these surface cells so that the junctional complex resembles that of most epithelia.

The changes in the junctional complex at different developmental stages are summarized in Fig. 14. In the earliest stage studied here (stage 8, blastula), the junctional complex is the least specialized. An apical occluding zonule,

the surfaces of the plasma membranes (Robertson, 1963; Revel and Karnovsky, 1967; Brightman and Reese, 1969; see also Furshpan and Potter, 1968). The striated lattice appearance such as seen here is produced by oblique views along the polygonally packed subunits (Revel and Karnovsky, 1967).

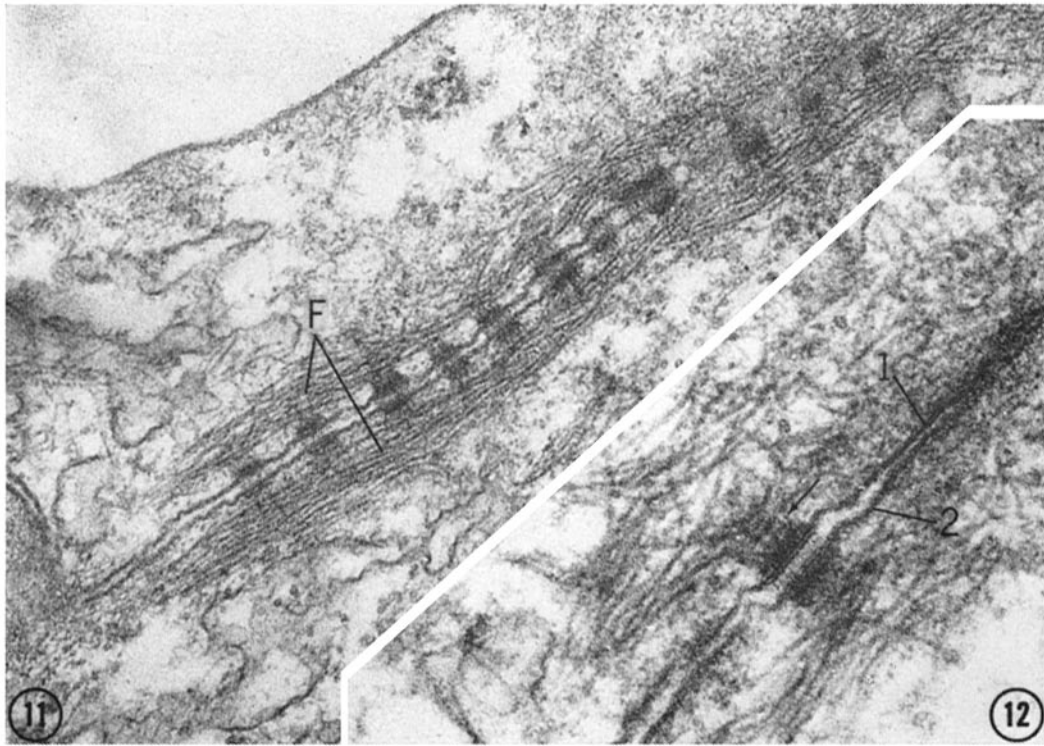


FIGURE 11 Stage 14, late gastrula. The section passes in a plane roughly parallel to the surface at the level of the desmosomes. Desmosomes are closely spaced along the membranes. The bundles of cytoplasmic filaments (*F*) paralleling the membranes bind the individual desmosomes into a continuous system. $\times 72,000$.

FIGURE 12 Desmosome, late gastrula, stage 14. The desmosome is moderately well developed. A more advanced stage is illustrated in Figs. 9 and 10. The intercellular space contains material of moderate density. Short filaments (*arrow*) leave the plaques to enter the large bundles of filaments at right angles. Note the accumulation of moderately dense material against the inside of the membranes in the intermediate (*2*) and apical regions (*1*) of the junctional complex above the desmosome. $\times 96,000$.

however, is a constant feature of enveloping layer cells. Desmosomes are variably present between cells and rudimentary in structure, indicating their first appearance at this stage. Desmosomes have not been observed in earlier stages in *Fundulus* (Trinkaus and Lentz, 1967).

The most extensive structural changes occur between the blastula and gastrula stages. The major difference in the apical region (zonula occludens) is an increase in number of focal points of membrane fusion and the appearance of small masses of extremely dense material in the cytoplasm adjacent to the areas of fusion. These masses differ from the more diffuse accumulations of material along the apical and intermediate

regions and the plaques adjacent to the desmosomes in being narrower, denser, and more discretely localized. In the late gastrula and early embryo, only the apical pair of densities is usually seen. Rarely, more extensive zones of membrane fusion and elongated bands of dense cytoplasmic material are present in the apical junction. The cytoplasmic densities applied to the inner aspect of the plasma membrane in the zonula occludens have also been observed in adult *Fundulus* (E. D. Hay and J.-P. Revel, personal communication). In the adult, the material is more extensively distributed along the junction and is densest at the surface.

In freeze-etch preparations, the regions of

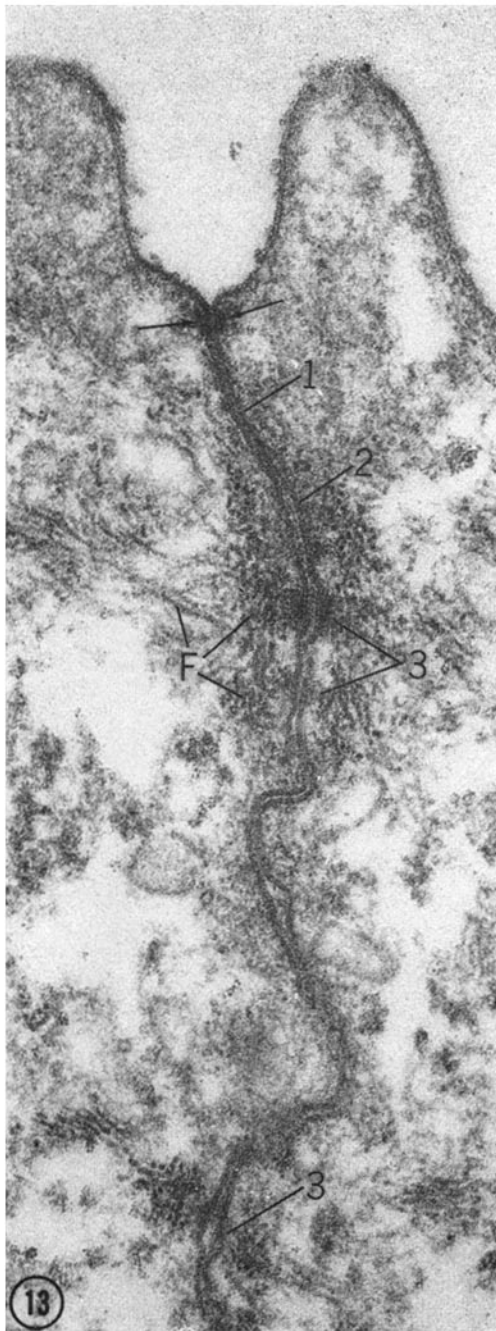


FIGURE 13 Junctional complex in the trunk region of the early embryo (stage 18). The complex consists of an apical zonula occludens (1), a zonula adherens (2), and maculae adherentes (3). Only the most apical pair of button-like densities (arrows) is present. Moderately dense material occupies the intercellular space in the intermediate region and also occurs along the cytoplasmic surfaces of the membranes. Note that one desmosome occurs at a deep level. The bundles of filaments (F) lateral to the desmosomes are sectioned transversely. Other filaments (F) extend into the cytoplasm at right angles from the lateral bundles. The gap junction was not seen at this stage. $\times 95,000$.

regions of fusion are seen as focal or point contacts. However, the images in which there is an extensive length of membrane fusion are most likely due to sections passing at an angle or parallel to the cell surface and directly along one of the grooves of membrane fusion. In both cases, the extent of the dense cytoplasmic material in *Fundulus* coincides with the region of membrane fusion, raising the possibility that they determine the zones of membrane fusion. Staehelin et al. (1969) have suggested that sets of particles along the grooves on the cytoplasmic faces of the apposed membranes could be linked, explaining the adhesive properties of the tight junction.

The intermediate junction or zonula adherens shows an increased complexity in the late gastrula and early embryo, although it is reduced in extent by the occurrence of desmosomes at higher levels. Changes in this region are the appearance of moderately dense material in the intercellular space and in the cytoplasm against the plasma membranes.

The desmosomes are generally more complex beyond the blastula stage and several details are apparent in this material which have not been seen in a previous study of desmosome development (Overton, 1962). The following sequence of events in the formation of desmosomes was observed: accumulation of dense cytoplasmic material applied to and near the plasma membrane; appearance of intercellular dense material and, at the same time, cytoplasmic fibrils running parallel to and contacting the electron-opaque plaque; widening of the intercellular space; separation of the dense cytoplasmic plaque material from the plasma membrane; increase in compactness of the cytoplasmic plaques; and appearance of an intermediate line in the inter-

membrane fusion in the zonula occludens have been shown to be elongated grooves or strands that branch and anastomose (Staehelin et al., 1969; Goodenough and Revel, 1970). When the section passes at right angles to the grooves, the

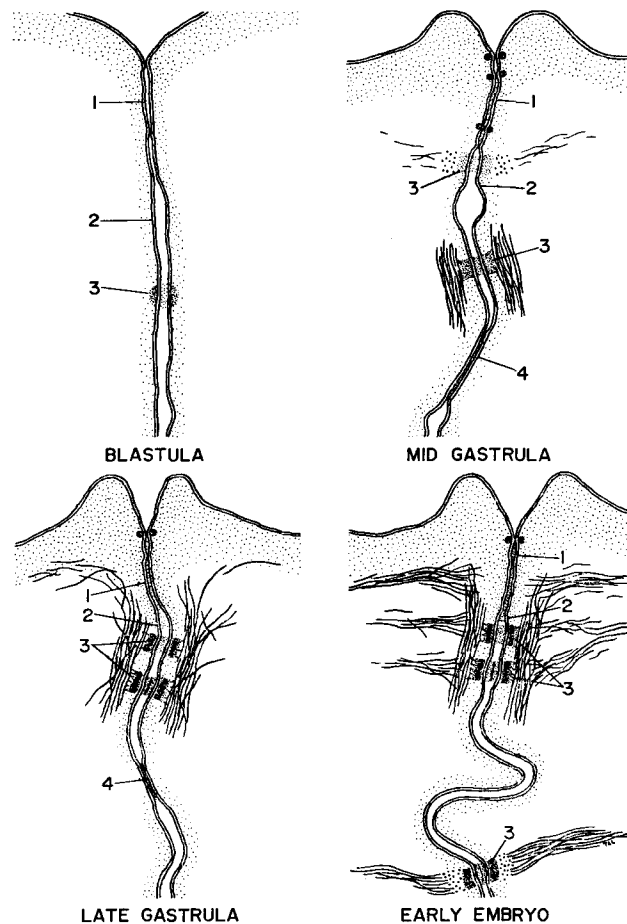


FIGURE 14 Diagram summarizing the stages of development of the junctional complex in developing *Fundulus* embryos. In blastulae (stage 8), the intercellular junction consists of an apical region (zonula occludens) where the membranes are closely apposed (40–60 Å) and in places touch (1), an intermediate region (zonula adherens) in which the membranes are separated by an intercellular space of >100 Å (2), and incipient desmosomes (3). The latter consist of a pair of cytoplasmic densities opposite one another along the intermediate zone. In mid gastrulae (stage $12\frac{1}{3}$), there are frequent points of focal fusion of membranes along the apical zone (1) of the complex. Dense, button-like masses of material occur in the cytoplasm adjacent to the point contacts. Dilations as well as an increased number of desmosomes are found along the intermediate zone (2). The desmosomes or maculae adherentes (3) exhibit stages in development which include increased density of the cytoplasmic plaques, association of filaments with the plaques, and appearance of a plug of moderately dense intercellular material. Below the desmosomes, the membranes approach to within 20 Å of each other and form a close or gap junction with an over-all width of 160–180 Å (4). In late gastrulae (stage 14), the desmosomes (3) show further differentiation by the separation of the cytoplasmic plaques from the membrane by a narrow space and the appearance of an intermediate dense line in the intercellular material. The intermediate zone (2) is reduced in length by the appearance of more apically situated desmosomes, but the intercellular space may contain some moderately dense material. The diffuse cytoplasmic material against the inner surfaces of the membranes is also increased in density. The gap junction, when present, is short. By the early embryo stage (stage 18), the gap junction is no longer apparent. Only the most apical pair of extremely dense, button-like masses persists. Desmosomes (3) are increased in number, and occur at deeper levels, and their associated system of cytoplasmic filaments is more extensive. See text for further details.

cellular material. In comparison with the chick (Overton, 1962), desmosomes first appear and differentiate at correspondingly earlier stages of development in *Fundulus*. The cytoplasmic filaments associated with desmosomes also appear to be more prominent in *Fundulus*; but this may be due to differences in fixation and staining. Half or hemi-desmosomes were not seen in *Fundulus*.

Functions of Contact Specializations

CELL ADHESION: Desmosomes (macula adherens) show the greatest development between blastula and gastrula stages. Since these structures are generally thought to play a role in cell-to-cell attachment (Farquhar and Palade, 1965), we have suggested that their appearance in gastrulae could be in response to the greater stress placed on enveloping layer cells during the extensive stretching caused by epiboly (Trinkaus and Lentz, 1967). At the same time, the cytoplasmic system of filaments associated with the developing desmosomes becomes more prominent and could serve to transmit uniformly the force within this spreading sheet of cells. Adjacent to the desmosomes, the filaments run along the lateral margins, but elsewhere they are oriented parallel to the apical cell surface. The latter are arranged along the lines of stress produced by stretching of the enveloping layer, and, through their connection with the filaments next to the desmosomes, could serve to transmit the force to the desmosomes. Thus, it is not surprising that the desmosomes and filaments develop simultaneously and at the time when greater force is exerted on the cells. An increased incidence of desmosomes has also been observed during the spreading of the chick epiblast (Balinsky and Walther, 1961; Overton, 1962; Bellairs, 1963; Trelstad et al., 1967).

The apical junction (zonula occludens) is present at all stages studied and probably subserves more than one function. Along with the desmosomes, these apical junctions no doubt also function in cell attachment. In other material, such junctions have been shown to be the last junctional element to be disrupted under stretching or tension (Sedar and Forte, 1964; Farquhar and Palade, 1965; Hays et al., 1965; Muir, 1965).

The 100–200 Å junction (zonula adherens) is also probably an area of cell-to-cell adhesion

(Farquhar and Palade, 1965; Trinkaus and Lentz, 1967), but it very likely binds cells less firmly than do desmosomes and zonulae occludentes. Gap junctions and interdigitations of cytoplasmic flanges also may aid cell-to-cell adhesion, as evidenced by their appearance during epiboly. It is possible that these junctions, like desmosomes, also appear in response to the increased stress placed on cells of the enveloping layer during epiboly, when there is undoubtedly an increased need for cell-to-cell attachments.

ELECTRICAL PROPERTIES: In addition to its adhesive function, the apical junction of an epithelium also serves to seal off the intercellular space from the exterior (Farquhar and Palade, 1965). In *Fundulus*, the electrical resistance between the segmentation cavity underlying the enveloping layer and the exterior is exceptionally high (Bennett and Trinkaus, 1970); thus it can be inferred both that the outer cell membrane is a high resistance membrane, and that the clefts between the surface cells are occluded by circumferential junctions. The apical junctional complexes observed in the electron microscope are zonular and have regions of membrane fusion, and thus are the only apparent candidates for this function. It seems reasonable to conclude, therefore, that they are functionally occluding junctions (zonula occludens), even though the membranes are not fused over the entire depth of the junction. The presence of these occluding junctions and the highly resistant outer cell membrane probably account for the high degree of impermeability of the enveloping layer of *Fundulus* (Brown, 1905). Dechorionated *Fundulus* eggs develop equally well in sea water and distilled water and have very low permeability to water, as shown by their limited uptake of tritiated water (Dunham et al., 1970).

Another possible function of the apical junction is electrical coupling of adjacent cells. The possibility that cells of the enveloping layer of *Fundulus* are electrically coupled has been explored recently with microelectrodes (Bennett and Trinkaus, 1970). Cells of all stages studied (cleavage, blastula, gastrula) were found to be coupled electrically. Coupling has been found to occur by way of both intercellular junctions and extracellular space of the segmentation cavity. The latter pathway is possible when the resistance of the membrane that faces on the cavity is low compared to that of the surface membrane, and

the cavity is sealed off from the exterior by circumferential junctions. Junctional coupling was demonstrated in gastrulae when it was found that cells near a polarized cell can be at a higher potential than the underlying segmentation cavity. Coupling was also observed when isolated cells in vitro are in contact. This coupling could take place by way of the apical junctional complex. It has been suggested repeatedly that tight junctions can be sites of electrical coupling between cells, and hence presumably sites of ion transfer (Loewenstein et al., 1965; Bennett et al., 1967; Furshpan and Potter, 1968). For this reason it was originally thought that coupling of *Fundulus* cells is by way of tight junctions. Since the apical junctions of the *Fundulus* enveloping layer are actually composites of tight and close junctions, however, we are in no position to decide which of these types of contact is responsible for the coupling, or indeed whether all are involved.

Inasmuch as it has not been established unambiguously that tight junctions are responsible for the electrical coupling, and since gap junctions possess polygonal subunits on the surfaces of the plasma membranes that bridge the gap (Revel and Karnovsky, 1967; Brightman and Reese, 1969), gap junctions also emerge as candidates for the coupling function. Gap junctions have been found in other electrically coupled cells, including neurons (Robertson, 1963; Brightman and Reese, 1969), cardiac muscle (Revel and Karnovsky, 1967), smooth muscle (Uehara and Burnstock, 1970), and liver (Goodenough and Revel, 1970). The deeply located gap junctions in *Fundulus* may supplement the apical junctions during gastrula stages, especially since they cover a more extensive area than the apical focal points of fusion. It should be noted, though, that cells of blastulae which lack the deeply situated gap junctions are also coupled (Bennett and Trinkaus, 1970).

It is also possible that coupling occurs where low-resistance plasma membranes are apposed but separated by an intercellular cleft of 100 Å or more. This possibility is suggested in *Fundulus* by the existence of communication by way of the extracellular space. The plasma membranes abutting on the extracellular space have low resistance but lack structural specializations as revealed by the electron microscope.

Focal tight junctions, similar to those in the

apical junctional complex of *Fundulus*, have also been described for cells of the early chick embryo (Trelstad et al., 1967), and these cells have also been shown to be electrically coupled (Sheridan, 1968). Although it has been proposed that the coupling in the chick blastoderm is mediated by way of these tight junctions (Sheridan, 1968), the *Fundulus* work (Bennett and Trinkaus, 1970) suggests that extracellular space and gap junctions may also be involved.

CONTACT INHIBITION: The kinds of contact made by contact inhibiting cells such as those of the *Fundulus* enveloping layer are of particular interest because they can be compared to those made by *Fundulus* deep cells which are noncontact inhibiting (Trinkaus and Lentz, 1967). Enveloping layer cells always form tight, gap, and close junctions with each other during gastrulation; deep cells form tight or close junctions much less frequently (Trinkaus and Lentz, 1967). In addition, enveloping layer cells invariably form desmosomes between one another during gastrulation; deep cells do not.

In another study, Martinez-Palomo et al. (1969) found that, whereas tight junctions occur more frequently between contact-inhibiting fibroblasts than between noncontact-inhibiting cancer cells, close junctions and desmosome-like structures are frequent in both. In a similar study, McNutt and Weinstein (1969) found that both nexuses (gap junctions) and desmosomes occur more frequently between normal cervical epithelial cells than between cervical carcinoma cells (which presumably are noncontact inhibiting). In contrast, cells of the 3T3 line, which are highly contact inhibiting, form few tight junctions in culture (Harris, 1970). Another recent study of contacts between contact-inhibiting cells (strain L) has led to inconclusive results (Flaxman et al., 1970). It seems either that the structural basis of contact inhibition varies for different cell lines, or that the contacts observed are not crucial for the process.

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