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Freeze-etch Appearance of the Tight Junctions in the Epithelium of Small and Large Intestine of Mice

L. Andrew Staehelin, T. M. Mukherjee, and A. Wynn Williams

Biophysics Section, Physics and Engineering Laboratory, D. S. I. R., Lower Hutt, New Zealand, and Electron Microscope Laboratory, Pathology Department and Medical Research Council of New Zealand, University of Otago Medical School, Dunedin, New Zealand

With 20 Figures

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Summary

The ultrastructure of the central layer and the contributing plasma membranes of tight junctions has been studied in epithelia of the jejunum and colon of mice.

Examination of freeze-etched plasma membranes of epithelial cells has revealed that they consist of a central layer, with fracturing characteristics similar to bimolecular lipid leaflets, which is covered on both sides with a layer of particles.

The "fusion" of the outer membrane surfaces of adjacent cells in the region of the tight junction leads to the formation of a new common structure consisting of a meshwork of fibrils embedded in a matrix substance. The fibrils probably contain protein. They have a diameter of 65 ± 10 Å and are linked together so that they form around the distal end of each cell a continuous belt-like meshwork which is extended proximally at the joints where three cells meet. As the fibrillar mesh appears to be strongly attached to the central lipid layer of the two adjoining membranes, in contrast to the weakly bound surrounding matrix, it is believed that the fibrils forming the continuous meshwork could be the mechanical coupling and the sealing elements of the tight junction. Their arrangement in the form of a concertinalike mesh would make the whole structure very flexible. In the region of the junction the membranes are constricted along the lines of attachment to the fibrils and bulge outwards, *i.e.* towards the cytoplasm, in the areas of the matrix material. In the resulting grooves on the cytoplasmic side of the plasma membranes regularly spaced particles with a diameter of 90 ± 10 Å can be detected. Various observations suggest that these particles could be connected through the central layer of the membranes to the fibrils on the other side. This would offer a possible explanation for the known abhesion properties of tight junctions. The described structures are also evaluated in terms of current theories of cell communication.

1. Introduction

The *zonulae occludentes*, tight junctions or closing belts have been found in a number of different epithelial tissues [10, 11, 12]. They form the outermost component of the junctional complex between adjacent epithelial cells and are presumed to be continuous around each cell, acting as barriers where

exchanges along the intercellular spaces are restricted or prevented. It has been demonstrated that the *zonulae occludentes* are impermeable not only to macromolecules such as haemoglobin and enzymes [11, 17] but also to water, ions, and small water soluble molecules [12, 20].

Sections of fixed epithelial cells reveal the *zonulae occludentes* situated on the lateral surfaces of the cells immediately below the luminal border. In these regions the intercellular space is obliterated by the coming together and fusion of the membranes of the adjoining cells. The fusion appears as a mergence of the outer leaflets of the apposed unit membranes into a single leaflet of about the same thickness as each of the contributory layers [11]. However, variations in the characteristics of the cross-sectioned central layer or fusion line are also not uncommon. FARQUHAR and PALADE [11] have reported that in some regions the fusion line may be discontinuous and appears often as a series of dots rather than as a continuous structure. Yet in other places the fusion line may exhibit one or several focal splittings within the junction.

Such variability in the appearance of the central elements of the tight junction makes it difficult to interpret correctly the exact relationships between the contributing structures. One of the essential factors responsible for this confusion is a limiation inherent with sectioning of chemically-fixed and embedded specimens since the plane of sectioning usually cuts across this region rendering it difficult to see the surface structures of the epithelial cells.

An alternative preparation method, the freeze-etching technique, based on an idea of STEERE [32] and developed by MOOR and MÜHLETHALER [21], offers the possibility of visualising surface structures of cell membranes by fracturing and replicating tissues in the frozen state. It appeared therefore that the application of this technique to the problem of the ultrastructure of the tight junction could result in a better understanding of the structure and function of the junction as a whole.

Fig. 1. Cross-fractured junctional complex between two epithelial cells of the small intestine showing the desmosome (*D*), followed by the *zonula adherens* (*Za*), followed by the *zonula occludens* (*Zo*) or tight junction. The central ridge of the desmosome can be clearly seen, while the tight junction exhibits very irregular fracture planes. Axial filaments can be detected in the cross-fractured microvillus (*M*). ×98,000

Fig. 2. Cross-fractured junctional complex between two epithelial cells of the small intestine revealing the desmosome (*D*) with a central ridge and converging fibrils in the adjacent cytoplasm, followed by the adhering zonule (*Za*) at the upper end of which the plasma membranes merge and fuse to form the occluding zonule (*Zo*). No details of the latter can be seen due to the long shadow, which is printed white as in all micrographs. Microvilli (*M*). ×45,000

Fig. 3. Obliqueli fractured tight junction (*Zo*) indicating the underlying pattern inside the junction, which causes the rough irregular fracturing observed in cross-fractured occluding zonules (see Fig. 1). Cross-sectioned microvilli (*M*). Specimen prefixed in glutaraldehyde. ×105,000

Figs. 1–3

Another important factor which influenced us greatly in embarking on this investigation was the fact that the micrographs of frozen etched plasmalemmas of various animal, plant and bacterial cells always reveal membrane surfaces covered with particles [2, 4, 16, 21, 22, 25, 26, 29, 30, 31]. The interpretation of these particle covered surfaces is still controversial. MOOR and MÜHLETHALER [21] and STAEHELIN [30, 31] describe the particles as being located on the plasma membrane surfaces and favour an arrangement of the membrane components according to the model of DANIELLI and DAVSON [7, 8]. BRANTON [2, 3], however, advocates a splitting of nearly all membranes during the fracturing procedure of the freeze-etching process and places therefore the particles into the interior of the membranes, a suggestion which is more in line with the micellar membrane theories of GREEN and PERDUE [15] and BENSON [1].

Based on their interpretation that the particles are located on the surface of a carrier membrane MÜHLETHALER, MOOR and SZARKOWSKI [24] explained the appearance of the unit membrane structure in sections [27] by a denaturation of the globular surface proteins during fixation. They also believe that a zipper-like interlocking of the surface particles of adjacent membranes could account for the observed adhesion of certain membranes, such as the thylakoids in grana regions of chloroplasts. Under these conditions it seemed possible that an investigation of the *zonulae occludentes* with the freeze-etching technique could reveal whether the tight junctions are formed by a close interlocking or a real fusion of the adjacent membrane surfaces. In addition, it was thought that the study of "fused" membranes might provide some more information on the structure of the membranes involved.

2. Materials and Methods

To prevent the formation of ice crystals during the freezing process 20% to 30% glycerol was added either to a Ringer solution or to 2% glutaraldehyde buffered at pH 7.2 with phosphate buffer, and injected into 2–3 cm long ligated loops of the small or large intestines of anaesthetised NZCW mice. The animals were kept under anaesthesia for about 15 minutes, following which small pieces of epithelium were removed, cut into cubes of less than 1 mm^3, and rapidly frozen on copper discs in Freon 12 held at — 150° C. The "freeze-etching" was performed according to MOOR and MÜHLETHALER [21] on a freeze-etch apparatus produced by Balzers. Etching of the specimens varied from 30 seconds to 2 minutes at — 100° C. The replicas were studied with a Philips EM 200 and a Hitachi HU-11 A electron microscope. The encircled arrows on the micrographs indicate the shadowing direction.

3. Results

3.1. Cross-fractures

In cross-fractured and frozen-etched specimens, the elements of the junctional complex appear in much the same order as seen in sections of chemically fixed material (Figs. 1 and 2). The desmosomes (*maculae adherentes*) are

characterized by the parallel apposing cell membranes, the presence of a granular central ridge in the intercellular space and the occurrence of cytoplasmic fibrils converging on this specialised region. The next element of the

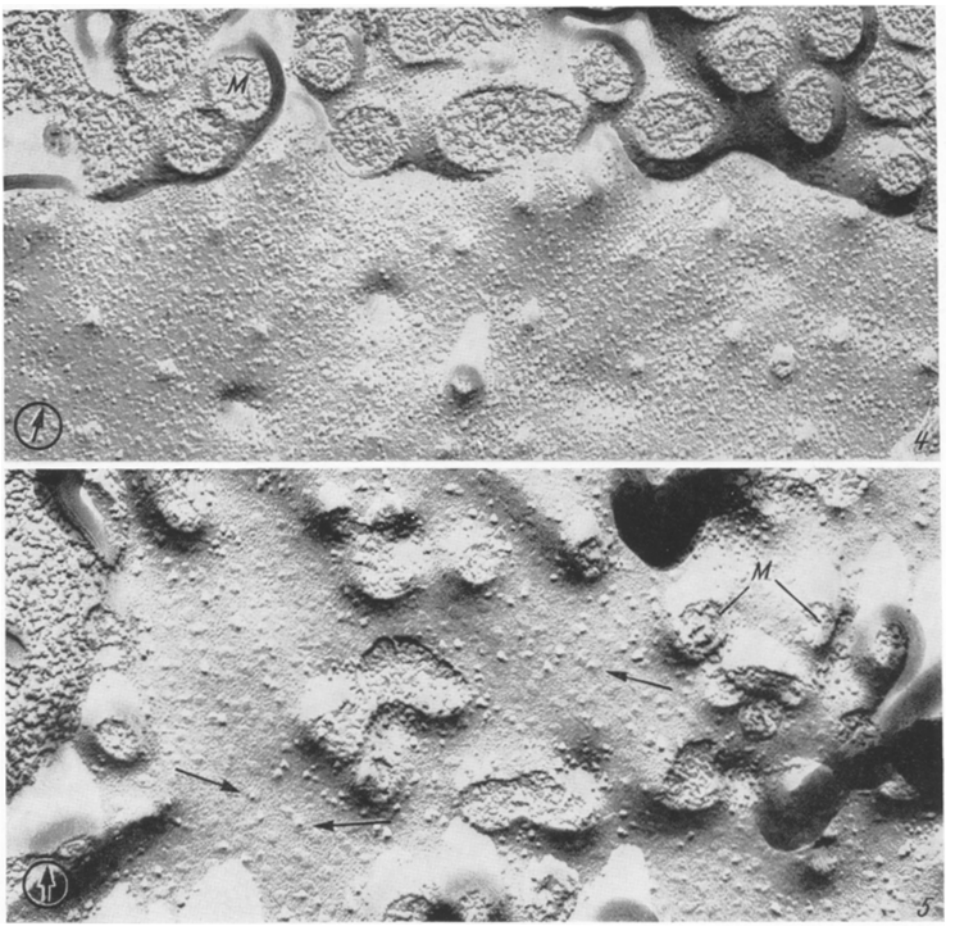


Fig. 4. Micrograph showing the outer surfaces of two epithelial cell plasma membranes of the small intestine. They are covered with randomly distributed particles of various sizes. The truncated cones are microvilli (*M*) broken off at their base ×80,000

Fig. 5. Surface view of a partly split plasma membrane of an epithelial cell of the large intestine. The exposed membrane face is covered with plaque structures indicating the presence of a bimolecular lipid leaflet. Some of the plaques (arrows) carry surface particles that have not been split away. Cross-fractured bases of microvilli (*M*). ×80,000

junctional complex, the intermediate junction (*zonula adherens*) does not show anything strikingly different from what we know already from our observations of sectioned specimens. At the luminal end of the intermediate junction the band of intercellular material narrows and the cell membranes

appear to come together and fuse to form the third element of the junctional complex, the tight junction (*zonula occludens*). In contrast to the desmosomes and the intermediate junctions where cross-fracturing exposes a smooth surface showing almost the same picture as obtained in sectioned material,

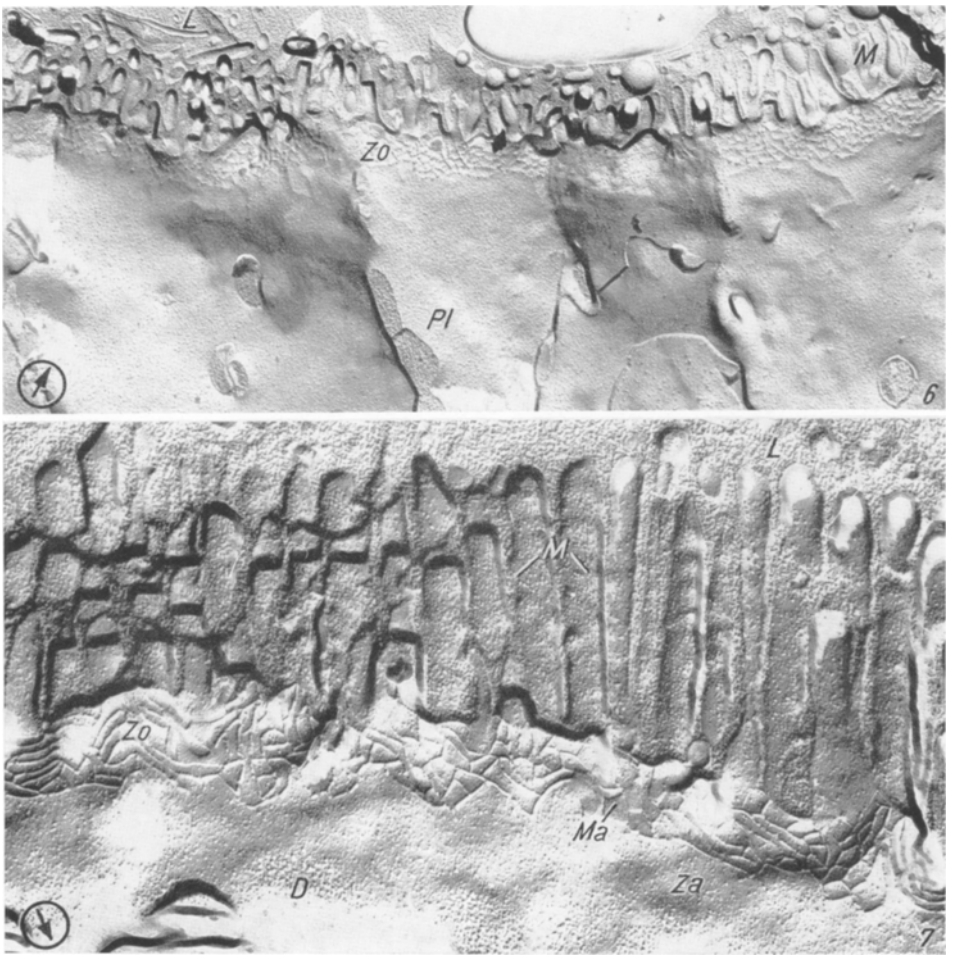


Fig. 6. Low power micrograph of epithelial cells split along the intercellular space and seen in surface view. The continuity of the band-like meshwork of fibrils (*Zo*) located at the base of the microvillous border (*M*) and belonging to the tight junction is clearly visible. Plasma membrane surface (*Pl*), lumen (*L*). ×18,000

Fig. 7. Surface view of a junctional complex split along the intercellular space. At the base of the microvillous border (*M*) the *zonula occludens* or tight junction (*Zo*) appears as a band-like structure consisting of a meshwork of fibrils with diameters of 65 ± 10 Å embedded in a matrix (*Ma*), which has been nearly completely broken away in this micrograph. The adjacent particle covered outer plasma membrane surface belongs to the adhering zonule (*Za*), while the split desmosomes (*D*) can be recognized as clusters of large particles, lumen (*L*). Specimen pre-fixed in glutaraldehyde. ×45,000

the tight junction invariably exposes a rough and irregular surface when cross-fractured (Figs. 1 and 3). This difference in fracturing characteristics seems to be caused by the complex internal structure of the tight junctions, which is described below.

3.2. Tangential Fractures

3.2.1. Structure of the Plasma Membrane

In order to understand fully the structure of the tight junction it is necessary to examine first the appearance of the frozen-etched plasma membranes contributing to the *zonulae occludentes.*

The plasma membranes of epithelial cells reveal two different types of membrane surfaces. In the first case (Fig. 4) the exposed membrane faces are covered with numerous randomly distributed particles with diameters ranging from 50–90 Å. Frequently, however, a slightly different picture is revealed (Figs. 5 and 11) where the particle-carrying layer is discontinuous and appears as a series of discrete islands or plaques which themselves rest on an essentially smooth surface. In an earlier publication STAEHELIN [30] has demonstrated that plaque covered membrane faces are characteristic of partly split frozen-etched bimolecular lipid leaflets the plaques representing the island-like remnants of the partly broken away upper-lying layer of the bilayers. The observation of similar plaques underlying particles on the surface of the partly split plasma membranes of some intestinal epithelial cells therefore indicates that these membranes could possess a central bimolecular lipid leaflet sandwiched between two layers of particles as postulated previously by DANIELLI and DAVSON [7, 8] in their pauci-molecular membrane model. In conformity with these earlier findings the particle covered membrane face in Fig. 4 is interpreted as representing the real plasma membrane surface while in Fig. 5 the partly split central bimolecular layer of the plasmalemma is exposed due to the fracturing away of most of the surface particles.

3.2.2. Fine Structure of the Tight Junction

Our findings are summarized in the diagram Fig. 19, which may help the interpretation of the complex three dimensional structures seen on the micrographs.

In specimens where the fracture plane develops along the apposed intercellular border thereby exposing a surface view of the junctional region of several cells in a row, a band, 0.2–0.3 μ in width, consisting of a meshwork of ridges may be observed (Fig. 6). This meshwork is located immediately below the microvillus border in the tight junction region and seems to form a continuous belt-like structure around all cells (Figs. 6, 8, and 9). Since the plasma membrane surface seems continuous in Fig. 11 where the ridges have been broken away, and since in Fig. 13 it appears that sections of the

ridges have been pivoted out of their original position it appears that these ridges are applied surface decorations rather than for example folds of the membrane surface. They are therefore interpreted as a meshwork of fibrils

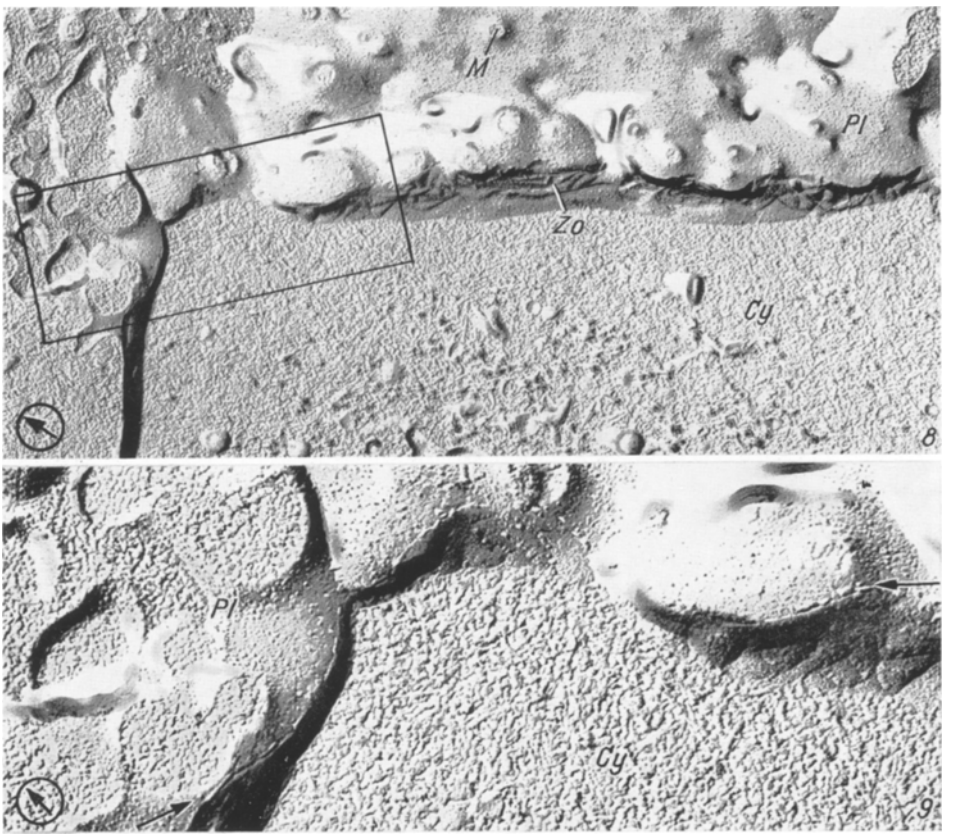


Fig. 8. Low power micrograph demonstrating the location and the continuity of the tight junctions between adjacent epithelial cells. The plasmalemma (*Pl*) of the upper cell is seen as from the side of the lumen and bears remnants of broken off microvilli (*M*). Immediately below the line of reflexion of this plasma membrane from the apical to lateral cell surface the tight junction (*Zo*) can be observed due to the fracturing away of the plasmalemma and some of the cytoplasm (*Cy*) of the lower adjacent cell. In the left corner of this cell the tight junction is revealed in an edge-on-view (see also Fig. 9). ×30,000

Fig. 9. Higher magnification of the tight junction segment seen in edge-on-view on the left side of Fig. 8. The tight junction is delineated towards the lumen by a fibril (arrows), which has been partly broken away during the fracturing procedure. Cytoplasm (*Cy*), plasmalemma (*Pl*). ×74,000

which is attached to the surfaces of both the adjoining cell membranes. In Figs. 7, 12, 13, and 14 the characteristics of this complex tight junction structure may be examined in detail. The interlinked fibrils have a diameter of 65 $\pm$ 10 Å and are embedded in an amorphous substance termed matrix

(Figs. 12 and 14). The matrix, which presents a smooth, fine textured appearance, may often partly or completely tear away during the freeze-etching process (Figs. 7, 12, 13, and 14). After glutaraldehyde fixation the fibrils of the meshwork are usually less fragmented (Figs. 7, 12, and 13) than in material without prefixation (Figs. 10 and 11). Occasionally the inter-

Fig. 10. Micrograph showing microvilli (*M*) and a small portion of a split tight junction of a specimen, which was not prefixed with glutaraldehyde. The fracturing has broken away large portions of the meshwork leaving only small fibril pieces attached to the underlying membrane. ×53,000

Fig. 11. A further example of a tight junction from a specimen not fixed with glutaraldehyde before freezing. The only remnants of the fibrillar meshwork are small segments of fibrils some of which are arranged in rows. The underlying exposed plasma membrane has been partly split and exhibits a plaque (*P*) covered surface. This indicates the presence of a central bimolecular lipid layer in the regions of the plasma membranes contributing to the junctional complex. The slight ridges (*R*) seen on the membrane surface are caused by the constrictions of the plasmalemma along the meshwork fibrils, most of which have been torn away from the membrane surface in this picture. Microvilli (*M*). ×80,000

linked fibrils expose a beaded substructure with a repeating distance of 120 to 150 Å (Fig. 13, arrows) or are fragmented into smaller pieces some of which appear to be twisted out of the normal fibril axis (Fig. 13, right lower corner). The fibrillar meshwork does not seem to be organized according to any specific and regular pattern, however, the overall arrangement of the mesh could be compared with a concertina, which can be stretched or compressed and twisted. Only where three epithelial cells form a joint may some degree of orderly arrangement be detected. In such circumstances the meshwork is

extended along the triple junction in a ladder-like arrangement with a central common fibril connected to parallel fibrils laid down between the adjacent cells (Figs. 14, 16, 18, and 20).

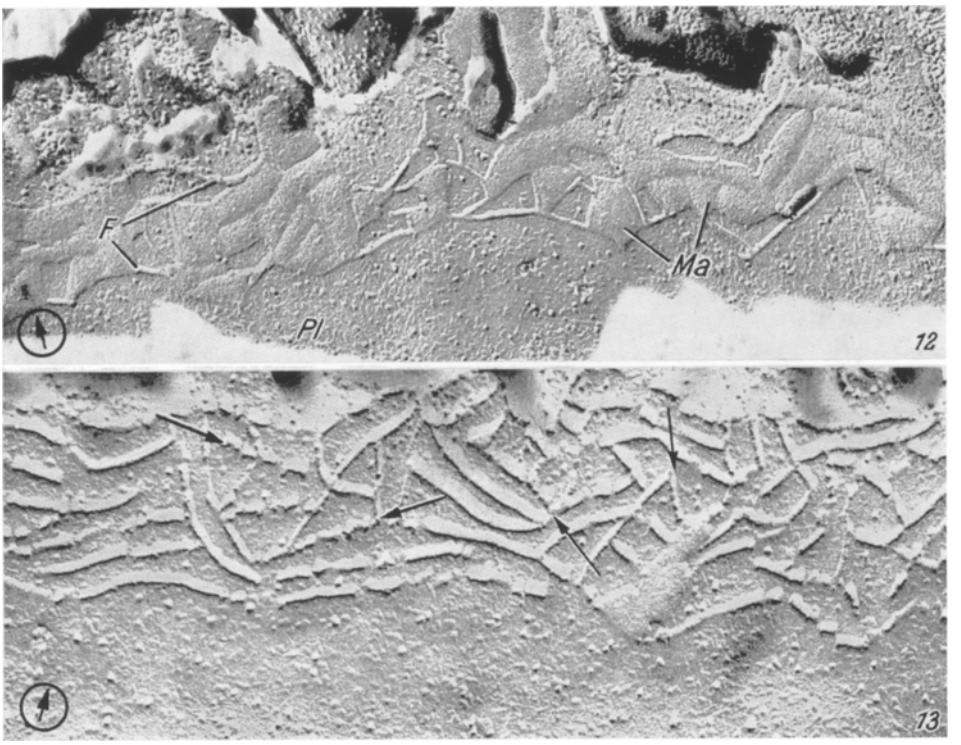


Fig. 12. Surface view of the central layer of a tight junction exposed by the removal of the upper-lying plasma membrane. Both the sealing fibrillar meshwork and the surrounding matrix (*Ma*) can be seen. The irregular polygons reveal where matrix material has been torn away between the fibrils. Grooves are present in the matrix along the lines of the meshwork. The delineation of the tight junction towards the lumen and the intercellular space is formed by fibrils (*F*). Outer particle covered surface of the underlying plasma membrane (*Pl*). Specimen prefixed in glutaraldehyde. ×80,000

Fig. 13. Fibrillar meshwork of a tight junction. The mesh reveals no regular pattern. A number of fibrils exhibit a beaded substructure with a repeating distance of 120 to 150 Å (arrows), while others are fragmented into smaller pieces some of which appear to be twisted out of the normal fibril axis (right lower corner). Specimen prefixed in glutaraldehyde. ×105,000

Several of our observations suggest a very strong attachment of the fibrillar meshwork to the lining membranes. In Fig. 7 the meshwork shows little sign of damage and seems to be still firmly fixed to the membrane surface, despite the fact that the matrix has been almost completely removed. Even where the plasma membranes in the region of the junctional complex have been partly split during the fracturing procedure, numerous fragments of the

fibrils may still be found adhering to the exposed membrane faces (Fig. 11). Further evidence of the strong attachment of the fibrils to the plasma membrane comes from the observation that in the region of the tight junction the paired membranes are constricted along the paths of the fibrils and appear to bulge outwards towards the cytoplasm in the intervening spaces occupied by the matrix. In surface views these constrictions of the plasma membranes appear either as low ridges (Fig. 11) where the fibrillar meshwork has been removed and the outer surface of the plasmalemma is laid bare or as grooves (Figs. 8, 12, 14, 15, and 19) on the cytoplasmic side of the plasma membrane.

In the region of the tight junction the plasma membrane shows slight alterations in the characteristics of the particles. On the outer surface, or the surface taking part in the formation of the central layer of the tight junction, relatively few particles are present (Figs. 7 and 13). Instead we have the meshwork of fibrils and the matrix material taking their place. Along the lines of attachment of the broken away fibrils ridges are seen on the plasma membrane surface (Fig. 11). The cytoplasmic side of the plasma membrane shows nearly the same sort of distribution of particles as mentioned earlier (Fig. 15). In addition, however, the membrane surface is traversed by grooves marking the distribution of the underlying meshwork fibrils (Figs. 8, 14, and 15). In the grooves particles with a diameter of 90 ± 10 Å may be observed at intervals of 120 to 150 Å distributed throughout their length (Figs. 15, 17, and 19). Occasionally the grooves may contain only a few 90 Å particles or none at all (Fig. 14). This is presumed to result from the removal of the particles during the fracturing process.

4. Discussion

4.1. Structure of the Plasma Membrane

In order to discuss the significance of our observations on the structure of the tight junction it seems necessary to elaborate first the present knowledge regarding the interpretation of frozen-etched membrane surfaces. This has been a matter of controversy since the initial publication of Moor and Mühlethaler [21] on the plane of fracturing in relation to biological membranes. Basing their interpretation of frozen-etched membranes on the membrane model of Danielli and Davson [7, 8], they came to the conclusion that the fracture planes follow the outer particle covered surface of biological membranes. This view has been challenged by Branton in a number of publications [2, 3, 5, and 9]. He postulates that freeze-etching does not normally expose the true outer membrane surfaces, but demonstrates two hitherto unseen inner membrane faces obtained by splitting of the membranes in half. His proposed membrane model [2] shows particles located in the interior of an extended bilayer, which is similar to the micellar membrane

theories of BENSON [1] and GREEN and PERDUE [15] for chloroplast and mitochondrial membranes. It must, however, be mentioned that BRANTON has not yet elaborated his theory for plasma membranes. In a recent report STAEHELIN [30] demonstrated that frozen-etched plasma membranes of animal, plant and bacterial cells exhibit a variety of surface images which seem to be caused by the fracture planes developing at different levels through or along the uper-lying halves of these membranes. STAEHELIN [30] believed that his observations could be better explained according to the paucimolecular membrane theory of DANIELLI and DAVSON [7, 8] than with any of the existing micellar membrane models. The work reported in this paper tends to support this view. For example if Fig. 5 is examined it will be seen that many particle carrying plaque structures are present on the exposed membrane surface. As the plaques probably represent island-like remnants of the partly broken away upper-lying half of a bilayer [30] it is suggested that the plasma membranes of the intestinal epithelial cells consist of a central bimolecular lipid layer covered on both sides with particles (Fig. 19).

4.2. Identification of the Tight Junction

We have based our assumption that the complex structures observed in this study belong to the tight junction on the following points.

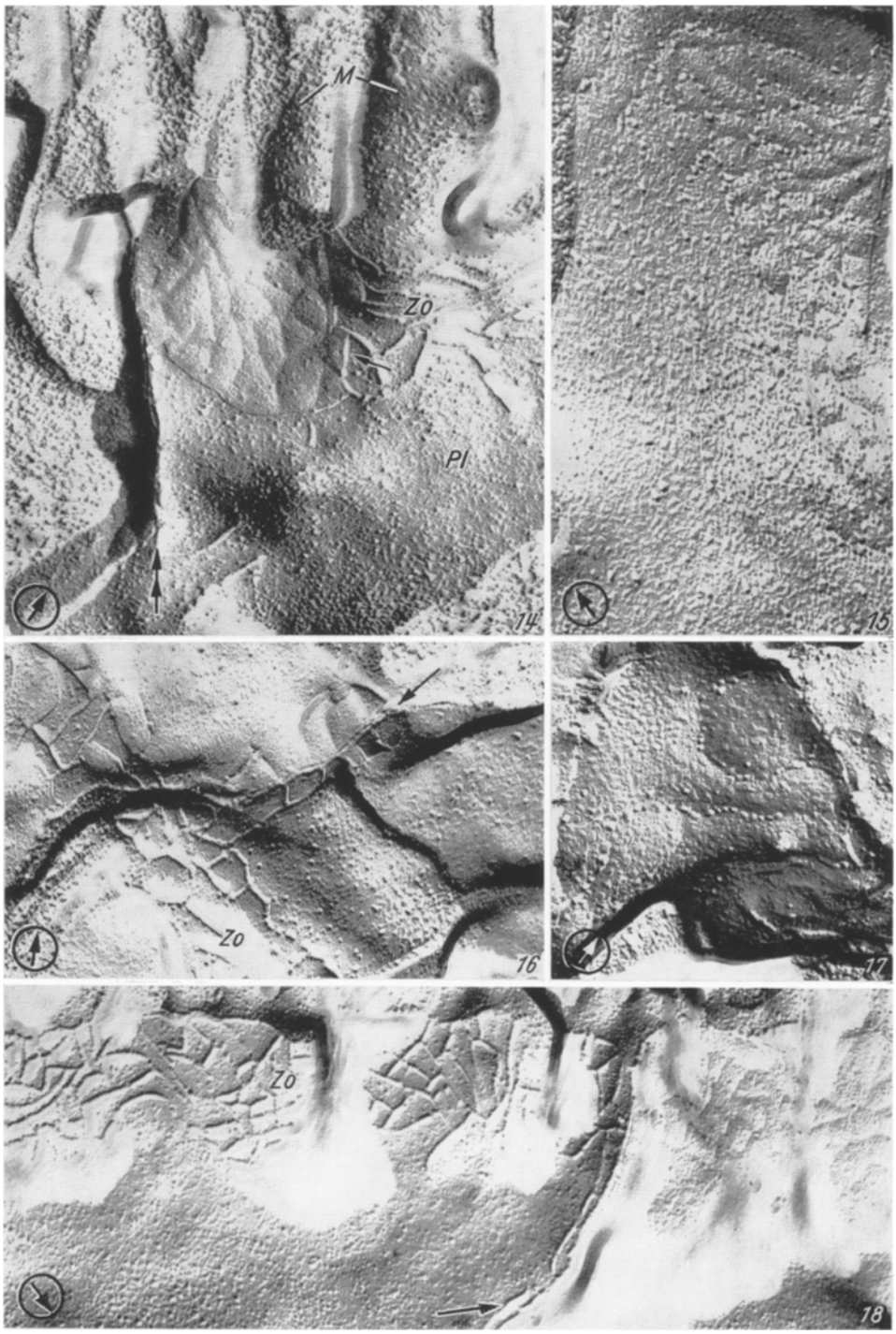
Fig. 14. Surface view of a tight junction (*Zo*) in the region of three adjoining cells. One of the cells has been broken away to allow a view of the two others. The relationship between the exposed meshwork fibrils on the right and the partly cross-fractured (arrow) matrix containing the fibrils on the left can clearly be recognized. It is also noticed that the grooves in the surface of the matrix appear as continuations of the fibrils. In the adjoining region of the three cells (double arrow) the partly cross-fractured tight junction is extended along the joint. The exposed particle covered outer surface of the plasmalemma (*Pl*) belongs to the adhering zonule. Microvilli (*M*). Specimen prefixed in glutaraldehyde. ×80,000

Fig. 15. Cytoplasmic surface of a plasma membrane in the tight junction region showing surface particles. Rows of particles with diameters of approximately 90 Å can be observed in the grooves marking the attachment sites of the meshwork fibrils to the other side of the membrane. ×80,000

Fig. 16. Micrograph showing the extension of the *zonula occludens* (*Zo*) along the line of contact of three adjoining cells. The meshwork is developed between each pair of adjacent membranes in a ladder-like way with all crossbars attached on one side to a central fibril (arrow) lying in the common axis. Specimen prefixed in glutaraldehyde. ×80,000

Fig. 17. Surface view of a partly split plasma membrane in the tight junction region as seen from the side of the cytoplasm. In the areas where none have been torn away, the 90 Å particles in the grooves marking the membrane constrictions are fairly regularly spaced at intervals of 120 to 150 Å. ×105,000

Fig. 18. Junction of three cells showing the ladder-like extension of the fibrillar meshwork of the tight junction (*Zo*) toward the intercellular space (arrow). Specimen prefixed in glutaraldehyde. ×59,000



Figs. 14–18

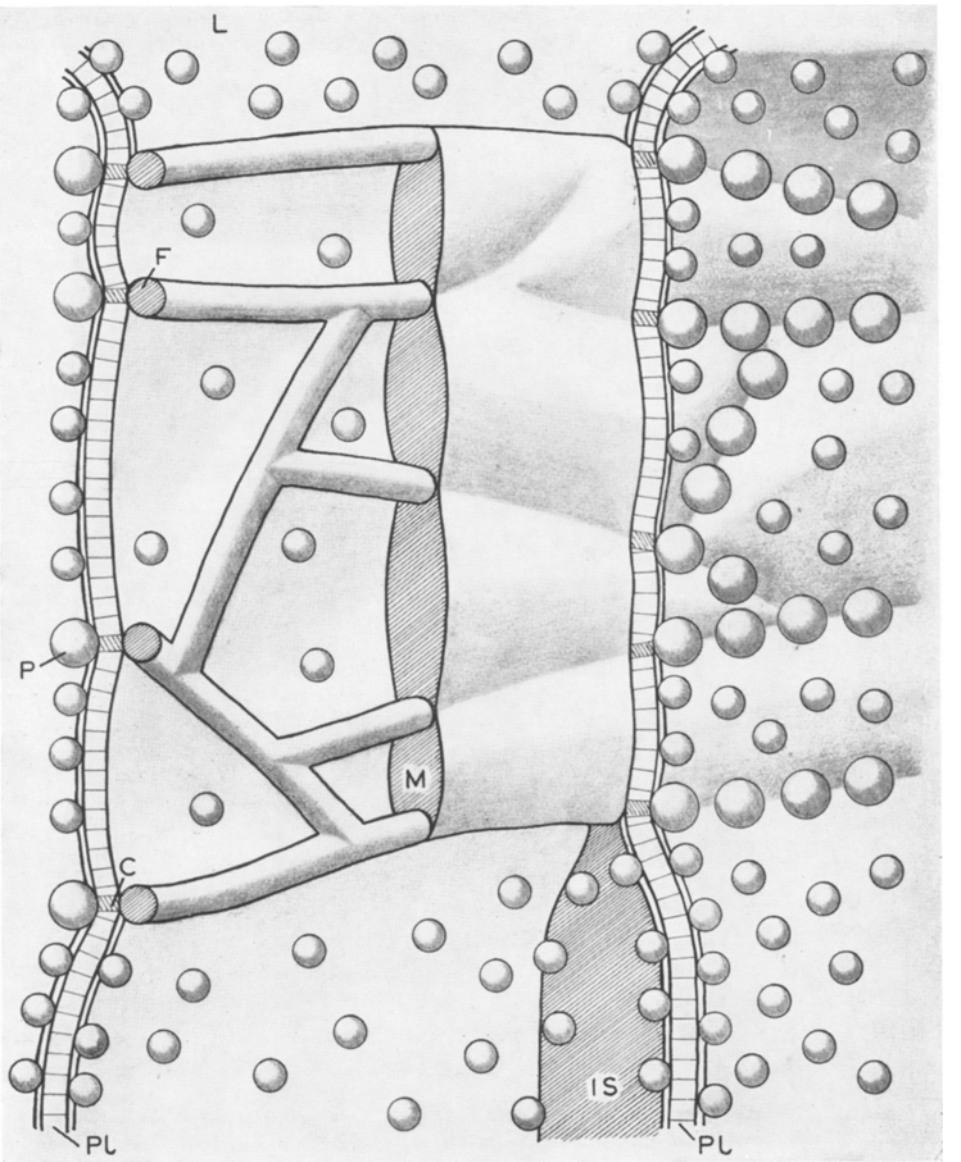


Fig. 19. A schematized drawing depicting a cut-away view of an intestinal tight junction. The plasma membranes (*Pl*) of the adjacent epithelial cells have been visualized as modified pauci-molecular membranes consisting of a central bimolecular lipid layer covered on both sides with particles and monolayer of non-lipid material. In the region of the tight junction the outer surface layers of the opposing membranes are replaced by a joint structure made up of a meshwork of interlinked fibrils (*F*) embedded in an amorphous matrix (*M*) with fibrils delineating the whole structure towards the lumen (*L*) and the intercellular space (*IS*). This central layer of the tight junction and the closely opposed plasma membranes are constricted along the fibrils and bulge outwards where matrix material is present. In the resulting grooves on the cytoplasmic side of the plasma membranes larger particles (*P*)

4.2.1. In pictures where the intercellular surface of the plasma membrane is visible the meshwork of fibrils appears to be located immediately below the microvillus border (Figs. 6, 7, 8, and 9). It is known from conventional sectioning studies that this is the site of the tight junction.

4.2.2. The distribution of the fibrillar mesh in the form of a band along the whole length of the upper lateral border of the epithelial cells in Fig. 6 suggests that this structure probably forms a continuous girdle around every epithelial cell. This is the essential characteristic of the tight junction [11, 12].

4.3. Possibility of Artifacts

Although the possibility of artifacts appearing during the process of freeze-etching cannot be ruled out, despite a freezing rate of -1000° C/sec (unpublished results), the observation of the same tight junction structures with or without chemical prefixation with glutaraldehyde seems to point to their actual existence in living tissues. This leads to the question why the fibrillar meshwork has not yet been detected in sectioned material. The "normal" quintuple layered structure of tight junctions as seen in sections depends on the unit membrane appearance of the two adjoining plasma membranes, the outer layers of which "fuse", according to the theory, to form the junctions. MÜHLETHALER *et al.* [24] have argued that the unit membrane as seen in fixed sectioned material itself probably represents a fixation artifact. They suggest that fixatives such as potassium permanganate and osmium tetroxide produce the unit membrane appearance of plasma membranes by denaturing globular proteins and causing them to uncoil and spread out, forming artificial surface laminae which take up heavy metal stains and appear as dark lines in cross-sections. Because the fibrils of the tight junction mesh probably contain protein is seems possible that a similar denaturation and staining could account for the uniform density of the "fused" central layer in sectioned material. This blurring of the fibrils could be enhanced by similar staining properties of the matrix material lying between the fibrils. MÜHLETHALER *et al.* [24] have also reported that frozen-etched chloroplast lamellae are considerably thicker than those in sectioned material. They attribute the shrinkage of the membranes to the denaturation of the proteins and to the extraction of lipids during dehydration and embedding of the specimens. We believe that the same factors could influence the dimensions of tight junctions.

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can be seen at regular intervals. These are possibly connected (C) through the central lipid layer of the membranes to the meshwork fibrils thus holding both structures in close contact with each other and enabling the fibrils to function as mechanical coupling and sealing elements of the tight junctions

A possible freeze-etch artifact, which has to be considered, is the effect of the facturing on the arrangement of the meshwork fibrils in the tight junctions. It could be argued that a displacement of the fibrils during the fracturing procedure might be responsible for the irregular appearance of the fibrillar meshwork. However, the fact that where we can obtain an idea of the fibrillar arrangement while viewing through the cytoplasmic side of the plasma membranes (Figs. 14, 15, and 19) the arrangement of the fibrils does not show any difference from regions where they have been laid bare (Figs. 13 and 14).

4.4. Structure and Function of the Tight Junction Elements

It seems to be established, then, that the tight junction, which appears in sectioned material usually as a fusion of two adjoining cell membrane surfaces is in fact a complex structure consisting of a meshwork of interlinked fibrils sandwiched between two adjacent membranes (summarized in Fig. 19). The spaces of the mesh appear to be filled with an amorphous, not further identified, substance termed matrix. After fixation with glutaraldehyde the individual fibrils of the meshwork become more resistant to fracturing (Figs. 7, 10, 11, 12, and 13). Since glutaraldehyde is known to cross-link proteins but not polysaccharides [14, 28] it is suggested that the fibrils may contain protein.

A number of observations such as the preferential breaking away of matrix material from split tight junctions (Fig. 7) and the constriction of the membranes along the fibrils (Figs. 14 and 15) support the idea of a strong attachment of the fibrils to the adjacent membranes. The fracturing behaviour of the fibrils and their relation to other junctional components suggests a possible mode of attachment to the central lipid layer of these membranes. In numerous specimens the fibrils reveal a beaded appearance or are broken up into smaller units by the fracturing forces (Fig. 13). The repeating distance of the beaded segments and of some of the fractured fibrils is in the order of 120 to 150 Å. This corresponds to the intervals found between 90 Å particles which are distributed along the grooves on the cytoplasmic side of the membranes (Figs. 15 and 17), and it is suggested that the particles could perhaps be connected to the fibrils of the mesh by members extending through the central lipid layer (Fig. 19). Additional evidence for this theory comes from the following findings. In Fig. 13 (right lower corner) some of the fibril pieces appear to be twisted out of the normal fibril axis as if they had been turned around an attachment point of the fibril to the underlying membrane. Under these circumstances it seems not unreasonable to believe that the fibrillar mesh could act as the mechanical coupling element of tight junctions by serving as a link between the sets of 90 Å particles disposed along the

length of the grooves on the cytoplasmic sides of the two adjoining membranes. This structural continuity between adjacent cells could explain the known adhesion properties of occluding junctions [13].

Another functional role of tight junctions, which has been especially stressed in recent years, is that of a permeability barrier [11, 17, 20]. The fibrillar meshwork appears very suitable for this function. The fibrils of the mesh seem to be not only closely applied to the central lipid layer of the junctional

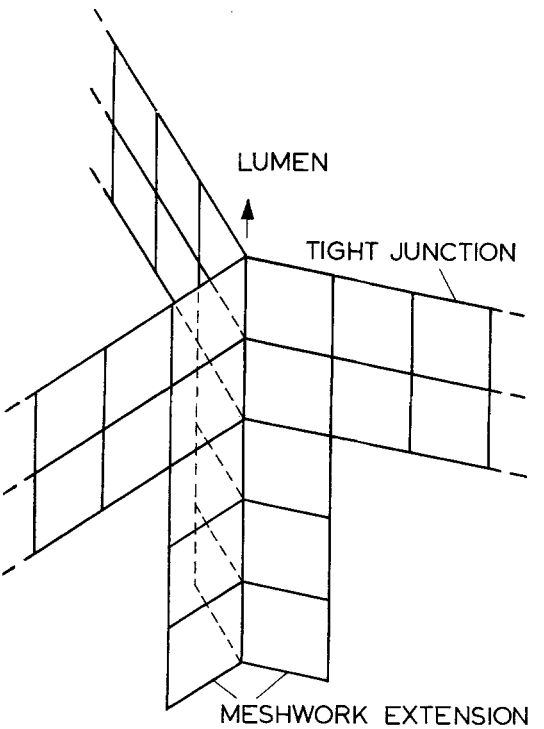

Fig. 20. Schematic diagram showing how the tight junction fibrillar mesh is modified where three epithelial cells join together. The meshwork is extended along the triple junction in a ladder-like arrangement with a central common fibril connected to parallel fibrils laid down between each pair of adjacent cells

membranes, which would make the attachment "leak-proof", but they also appear to form a continuous belt around all epithelial cells (Figs. 6 and 8). If we assume a low permeability for the fibrils then they may very well perform the sealing function of the tight junctions as well. Their arrangement in the form of a concertina-like mesh (Figs. 7, 12, and 13) embedded in a matrix would provide the *zonulae occludentes* with a highly flexible coupling and sealing element which could be expected to maintain its integrity and properties during the great variations in tension caused by the passage of food materials and faecoliths, and also by peristaltic movements. The changing conditions of tension may also be the reason for the irregular appearance of the meshwork pattern as seen in freeze-etch replicas.

Until now we have been discussing the structure of the tight junction when only two adjoining cells are taking part in its formation. However, as the tight junction system appears to be continuous around all epithelial cells we must also consider the structure where three cells join together. In these regions we can observe how an extension of the meshwork and a rearrangement of the fibrils can produce a simple and probably highly effective sealing element between three cells. Freeze-etch micrographs of these joints (Figs. 14, 16, 18, and 20) suggest that there exists a central common fibril from which other fibrils arise in a ladder-like manner to form the meshwork between each pair of cells. This observation provides further evidence for the continuity of the tight junction throughout the epithelial tissues.

Let us now consider how far the structure of the tight junction presented in this paper fits in with the intercellular communication theory of LOEWENSTEIN [18]. One of the essentials for an intercellular communication system is the possibility for the transport of substances from one cell to another without losses into the intercellular spaces. Again the tight junction structure as described in this paper seems to be suitable to serve for such a purpose for the following reason. In an earlier paragraph we came to the conclusion that the interlinked fibrils of the mesh probably represent the sealing elements of the tight junctions. If this is true then we have between the fibrils and the plasma membranes, where the matrix material lies, closed compartments through which substances could be transported from cell to cell without leakage into the intercellular spaces. A necessary condition for the functioning of such a system would be a mosaic-like membrane in which permeable and impermeable areas follow the outline of the meshwork fibrils. As permeability changes of membranes can be brought about by various molecular mechanisms [19, 23] and because the presence of local differentiations on plasma membranes has been demonstrated [31], it seems not unreasonable to assume the existence of such a system. Furthermore BULLIVANT and LOEWENSTEIN [6] have put forward a similar theory for the exchange of substances through septate junctions.

The basic structural similarity between the regular "honey-comb" mesh of septate junctions [6] and the irregular fibrillar meshwork of tight junctions is striking. This makes it tempting to speculate that a more or less regular mechanical coupling and sealing meshwork could represent a common feature of all truely tight junctions.

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Authors' address: Dr. L. A. STAEHELIN, Biophysics Section, Physics and Engineering Laboratory, D. S. I. R., Lower Hutt, New Zealand.