JUNCTIONAL COMPLEXES IN VARIOUS EPITHELIA

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ABSTRACT

The epithelia of a number of glands and cavitary organs of the rat and guinea pig have been surveyed, and in all cases investigated, a characteristic tripartite junctional complex has been found between adjacent cells. Although the complex differs in precise arrangement from one organ to another, it has been regularly encountered in the mucosal epithelia of the stomach, intestine, gall bladder, uterus, and oviduct; in the glandular epithelia of the liver, pancreas, parotid, stomach, and thyroid; in the epithelia of pancreatic, hepatic, and salivary ducts; and finally, between the epithelial cells of the nephron (proximal and distal convolution, collecting ducts). The elements of the complex, identified as *zonula occludens* (tight junction), *zonula adhaerens* (intermediary junction), and *macula adhaerens* (desmosome), occupy a juxtaluminal position and succeed each other in the order given in an apical-basal direction.

The *zonula occludens* (tight junction) is characterized by fusion of the adjacent cell membranes resulting in obliteration of the intercellular space over variable distances. Within the obliterated zone, the dense outer leaflets of the adjoining cell membranes converge to form a single intermediate line. A diffuse band of dense cytoplasmic material is often associated with this junction, but its development varies from one epithelium to another.

The *zonula adhaerens* (intermediate junction) is characterized by the presence of an intercellular space (~200 A) occupied by homogeneous, apparently amorphous material of low density; by strict parallelism of the adjoining cell membranes over distances of 0.2 to 0.5 μ; and by conspicuous bands of dense material located in the subjacent cytoplasmic matrix.

The desmosome or *macula adhaerens* is also characterized by the presence of an intercellular space (~240 A) which, in this case, contains a central disc of dense material; by discrete cytoplasmic plaques disposed parallel to the inner leaflet of each cell membrane; and by the presence of bundles of cytoplasmic fibrils converging on the plaques.

The *zonula occludens* appears to form a continuous belt-like attachment, whereas the desmosome is a discontinuous, button-like structure. The *zonula adhaerens* is continuous in most epithelia but discontinuous in some. Observations made during experimental hemoglobinuria in rats showed that the hemoglobin, which undergoes enough concentration in the nephron lumina to act as an electron-opaque mass tracer, does not penetrate the intercellular spaces beyond the *zonula occludens*. Similar observations were made in pancreatic acini and ducts where discharged zymogen served as a mass tracer. Hence the tight junction is impervious to concentrated protein solutions and appears to function as a diffusion barrier or "seal." The desmosome and probably also the *zonula adhaerens* may represent intercellular attachment devices.

Specialized intercellular junctions, known as desmosomes and terminal bars, have been studied extensively during the last few years (*cf.* 1–4) by electron microscopy. As originally suggested by the light microscopical observations of Bizzozero (5) and Schaffer (6), these junctions are now recognized as local modifications of the surface of adjacent yet separate cells, rather than as intercellular bridges (*cf.* 4). As far as their functional significance is concerned, two distinct aspects should be considered: first, their function in cell-to-cell adhesion, and, secondly, their effect upon epithelial permeability. Earlier studies (2–4, 7, 8) have primarily or exclusively stressed the first aspect, as evidenced by the frequent use of the terms "adhesion plate" (9) and "attachment belt" (10) as synonyms for desmosome and terminal bar. The second aspect has only recently received some attention (11–15) in several laboratories, including ours (16, 17).

For this study we have surveyed a number of epithelia, particularly those of the mucosae of cavitary organs, and in all cases investigated we have found a characteristic junctional complex between adjacent epithelial cells. The arrangement varies in detail from one epithelium to another but typically consists of three successive components to be described as tight junction (*zonula occludens*), intermediate junction (*zonula* or *fascia adhaerens*), and desmosome (*macula adhaerens*). The tight junction is located the closest to the lumen and the desmosome the farthest away from it.

Experiments in which concentrated solutions of hemoglobin were used as a mass tracer indicate that it is the tight junction, rather than the desmosome, which acts as a barrier to the diffusion of the tracer, and suggest that this element of the junctional complex seals off the lumen from the intercellular spaces.

MATERIALS AND METHODS

Materials

Our observations were carried out on the following species and epithelia:

RAT: The absorptive epithelia of the stomach, jejunum, and colon; the glandular epithelia of the stomach, pancreas, liver, parotid, and thyroid; the duct epithelia of the liver, pancreas, and parotid; and the nephron epithelium (proximal and distal convolution, and collecting ducts).

GUINEA PIG: The absorptive epithelia of the segments of the digestive tract mentioned above for the rat, plus that of the gall bladder; the glandular epithelia of the stomach, liver, and pancreas; the duct epithelia of the pancreas and liver; the epithelia of the mucosae of the uterus and oviduct.

Tracer Experiments

The experiments in which hemoglobin was used as a mass tracer were carried out on 3 normal and 2 nephrotic (*cf.* 16) rats. Two injections, each of 1 gm of 2 × crystallized bovine hemoglobin (Pentex Inc., Kankakee, Illinois) in ~5 ml saline, were administered intraperitoneally, the first at 24 and the last at 16 hours before collecting kidney specimens. Two additional rats (one normal, the other nephrotic) received hemoglobin infusions of approximately 4 gm in 15 ml saline over 15 minutes, kidney specimens being fixed 7 to 8 minutes after the completion of the infusion. Similar results were obtained in all cases (see page 396).

Fixation and Processing of Tissue

For intestine, stomach, gall bladder, uterus, and oviduct, fixation was started by injecting the fixative into the corresponding central cavity as recommended by Palay and Karlin (18). In the case of the pancreas, liver, parotid, and thyroid, pieces of tissue were removed directly from the anesthetized animals, and immersed in a drop of fixative in which they were subsequently trimmed. The fixation of the kidney was initiated by injecting the fixative *in situ* as previously described (19). All tissues were fixed for 1 to 1½ hours in 1 or 2 per cent osmium tetroxide (OsO_4) buffered at pH 7.6 with either acetate-Veronal (20) or phosphate buffer (21). Several tissues (intestine and kidney) were also fixed in potassium permanganate (22). Most specimens were subsequently dehydrated in graded ethanols and finally embedded either in methacrylate (1/5, methyl/butyl), Epon (23), or Araldite (23).

Some blocks of each tissue were stained by placing them for 30 minutes in 1 per cent phosphotungstic acid in absolute ethanol prior to infiltration (*cf.* 23). Other specimens were dehydrated in acetone and stained in potassium permanganate before embedding (24). Thin sections prepared from all these blocks were stained with uranyl acetate (25) or lead hydroxide according to Karnovsky (26). Finally some preparations were "doubly stained," first in uranyl acetate then in lead hydroxide.

Sections cut from Epon- or Araldite-embedded specimens were examined directly after staining, whereas those cut from methacrylate-embedded tissues were "sandwiched" with carbon prior to examination in the electron microscope (27).

Microscopy

Micrographs were taken at original magnifications of 12,000 to 40,000 with a Siemens Elmiskop I, operating at 60 or 80 kv, with a double condenser, and a 50 μ objective aperture.

OBSERVATIONS

Structure of the Junctional Complex

In all epithelia studied we have found a tripartite junctional complex located along the lateral surfaces of adjacent cells, in close proximity to the lumen. Although there are characteristic variations in detailed arrangement from one situation to another, similar complexes have been regularly encountered in all the epithelia listed under Materials and Methods. The complex occurs in a typical form in the epithelium of the intestinal mucosa (jejunum) where its detailed arrangement will be described. Since many of the structural details are similar to those encountered in other epithelia, they will be illustrated with micrographs taken not only from the intestine but from other organs as well.

Junctional Complex of the Intestinal Epithelium

Three morphologically distinct types of surface modifications can be identified along the sides of adjoining intestinal epithelial cells. Beginning nearest the lumen and proceeding away from it along the intercellular spaces, there is first a tight junction, followed by an intermediate junction, which in turn is frequently followed by a typical desmosome (Fig. 1).

TIGHT JUNCTION (ZONULA OCCLUDENS): The first element of the complex is located immediately behind the line of reflexion of the plasma membrane from the apical to the lateral surface of the cell body. Hence it is situated close to the lumen at the base of the striated border (Figs. 1, 2, and 4). At relatively low magnification, this junction is seen as an area of what appears to be an extreme narrowing of the intercellular "gap" which at this level measures only 70 to 90 A and sometimes is bisected by a faint intermediate line (Figs. 1 and 2). At higher magnifications it is apparent that the tight junction is actually a region in which the membranes of adjoining cells come together and fuse,[1] with resultant obliteration of the intercellular space, the intermediate line representing the merged outer leaflets of the adjoining cell membranes (Figs. 3 to 5). As originally described by Zetterqvist (29), and Sjöstrand and Zetterqvist (30), the plasma membrane on the luminal surface of the intestinal epithelial cell has a triple-layered structure readily visible in OsO_4-fixed tissues whenever the cell membrane is sectioned perpendicularly to its surface (Figs. 1, 2, and 4). More precisely, the membrane is composed of two dense layers ~40 A each, separated by a lighter ~30 A layer. The outer layer is slightly less dense and in places thinner than the inner one (Fig. 1). This slight asymmetry of the membrane is almost completely removed by $KMnO_4$ fixation (31, 32) or by $KMnO_4$ staining (see Fig. 2). The total thickness of the structure is ~110 A, or slightly greater than that of the usual "unit membrane" of Robertson (31, 32). In thin sections cut normally to the plane of the junction, the three layers of the cell membrane on the luminal surface of each cell can be followed to their point of mergence, usually located where the cell membranes turn from the apical to the lateral surfaces of the adjacent cells (Figs. 3 to 5). At this point the two outer leaflets of the converging membranes join to become the intermediate line of the junction. Thus each tight junction has a structure comparable to that of a single myelin lamella and corresponds to an "external compound membrane" of Robertson (33). Moreover, as in the case of myelin lamellae, in OsO_4-fixed tissues the intermediate line of the junction, which represents the fused outer leaflets of the two membranes, appears thinner and less dense than the inner leaflets on either side (Fig. 4). It becomes, however, as thick and as dense as the latter after $KMnO_4$ staining. Rather frequently this fusion line is discontinuous: it appears more as a series of dots than as a continuous structure, a zone of discontinuity frequently occurring immediately behind the point of mergence.

In normal sections, the tight junction follows a straight or slightly wavy course and extends in depth from 0.2 to 0.5 μ. At the basal end of the junction, the fusion line splits again at a low angle

[1] By membrane fusion we mean the mergence of the outer leaflets of the apposed membranes into a single leaflet of about the same thickness (20 to 30 A) as each of the contributory layers. The term is therefore used in a more general sense than by Robertson (28) for whom membrane "fusion" implies extensive elimination of the material of the outer leaflets (supposedly protein or mucoprotein).

into two lines: the emerging outer leaflets of the lateral cell membranes (Fig. 5)

Variations in this general arrangement are sometimes encountered and consist of one or several focal splittings of the fusion line within the junction followed by their refusion (Fig. 5). In some cases, the space separating the inner leaflets from one another is narrowed in places to <90 A with no intermediate line visible (Fig. 7). In such instances a reorganization of the fused membranes may be involved (see footnote 1).

In general there are no visible fibrillar differentiations in the subjacent cytoplasmic matrix along this element of the junctional complex although sometimes a thin accompanying zone of diffuse densification is encountered (Figs. 3 and 4).

Abbreviations for Figures

D, desmosome, *macula adhaerens*
L, lumen
Za, *Zonula adhaerens*
Zo, *Zonula occludens*
bb, brush border
cm, cell membrane
fl, fusion line in *zonula occludens*
il, inner leaflet of the cell membrane
ol, outer leaflet of the cell membrane
mv, microvilli
v, vacuole
z, zymogen granule

FIGURE 1

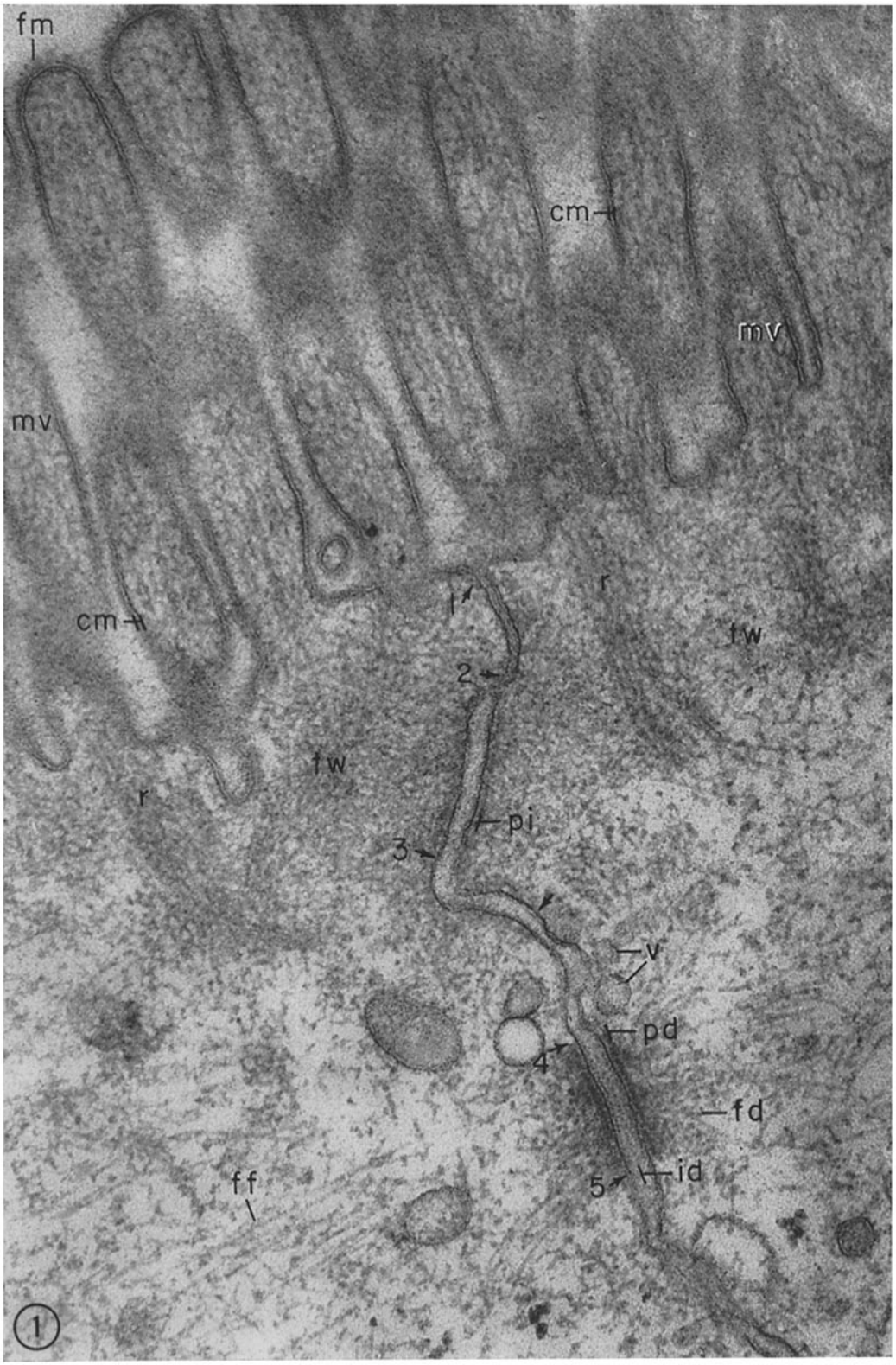
Junctional complex between two cells in the epithelium of the intestinal mucosa (rat). The tight junction (*zonula occludens*), located nearest the lumen, extends from arrow *1* to arrow *2*. The narrowing of the apparent intercellular "gap" ($\sim$90 A) is clearly visible, but the fusion line of the two apposed membranes cannot be clearly distinguished at this magnification. Note that there is relatively little accumulation of dense cytoplasmic material along this part of the complex.

The intermediate junction (*zonula adhaerens*) extends from arrow *2* to arrow *3*. A relatively wide intercellular space ($\sim$200 A) is maintained throughout the junction. Extensive condensation of cytoplasmic fibrils occurs as a fine feltwork along either side of the junction. This condensation is continuous with the terminal web (*tw*) into which the filamentous rootlets (*r*) of the microvilli penetrate. Plate-like densifications within the cytoplasmic feltwork can be seen along part of the junction, especially along the right side (*pi*).

The limits of a desmosome are marked by arrows *4* and *5*. This element is characterized by a wide intercellular space ($\sim$240 A) bisected by an intermediate line (*id*). Bundles of cytoplasmic fibrils (*fd*), coarser (diameter $\simeq$80 A) and more distinct than those of the terminal web, converge into dense plates (*pd*) on each side of the desmosome. These plates are separated from the inner leaflets of the cell membrane by a zone of low density. Similar fibrils (*ff*) appear throughout the remainder of the field below the terminal web.

Between the intermediate junction and the desmosome, the two apposed cell membranes are separated by an irregular space of varying width and show membrane invaginations and associated vesicles (*v*). The trilaminar structure of the cell membrane (*cm*) shows clearly along the microvilli (*mv*), (wherever the membrane is sectioned normally), and within the desmosome. It can also be made out, though less regularly, along the lateral cell margins (*e.g.*, at unnumbered arrow). Note that the luminal membrane is nearly symmetrical, the outer leaflet being only slightly thinner and less dense than the inner leaflet, whereas the lateral membrane is definitely asymmetrical. The total thickness of all three layers is about 110 A along the apical surface of the cell but measures only about 70 to 80 A along the lateral intercellular spaces. Note also the fluffy dense material (*fm*) (probably mucus) associated with the tips and sides of the microvilli.

Specimen fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6); and embedded in Epon. $Pb(OH)_2$-stained section. $\times$ 96,000.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Tight junctions are seen in the described location whenever the plane of section approaches normal to the plane of the intercellular boundary; moreover, oblique or grazing sections, cutting close to the luminal surface of the epithelium (Figs. 2 and 6), reveal that such junctions run without interruption for considerable distances along the apical perimeter of the cells. Accordingly it can be assumed that these junctions are continuous structures which surround each cell and bind together the plasma membranes of the entire epithelium. Admittedly neither a complete "belt" around a given cell, nor belt continuity throughout the epithelium has yet been demonstrated; however, it should be stressed that a "loose" type of junction has never been seen so far in the location described, and that points of continuity from one belt to another are frequently seen in favorably oriented sections (Fig. 10).

In view of these findings we propose that the first element of the junction complex be called *zonula occludens*, which means closing belt in Latin, the language used in the international morphological nomenclature. The term describes better than "tight junction" the main structural features of this element.

INTERMEDIATE JUNCTION (ZONULA ADHAERENS): The second element of the junctional complex is located immediately behind the tight junction, between the latter and the desmosome—hence the name here used (Fig. 1). It extends in depth for ~0.3 to 0.5 μ, and is characterized by the presence of a true intercellular space, which measures ~200 A across, and by a conspicuous densification of the subjacent cytoplasmic matrices. In normal sections the plasma membranes of the adjoining cells clearly show a triple-layered structure. However, here as well as along the rest of the lateral surface of the cells (Fig. 1), the outer leaflet is less dense and less clearly outlined than the inner one, to the extent that it is sometimes difficult to demonstrate. In addition, at this level the cell membrane measures ~80 A, the inner, middle, and outer layers accounting for ~30 A, ~25 A, and ~25 A, respectively; these dimensions remain essentially the same along the entire lateral cell surface (*cf.* Fig. 1). The cell membrane is, therefore, thinner and more asymmetrical on the lateral than on the apical aspect of the cell (*cf.* 34). Throughout the intermediate junction, the apposed membranes run strictly parallel to one another over a straight or only slightly wavy course. This arrangement contrasts markedly with the meandering and

FIGURE 2

Section cutting normally through a tight junction (*zonula occludens*) close to the luminal surface of the intestinal epithelium (rat). The junction extends diagonally across the micrograph from the lower left to the upper right corner (arrows) over a distance of 6 μ. The narrow apparent gap, (*i.e.* the space separating the inner leaflets of the apposed cell membranes), is visible all along the junction, but the fusion line cannot be clearly distinguished at this magnification. The marker to the right indicates the dimensions of a usual intercellular space at the same magnification.

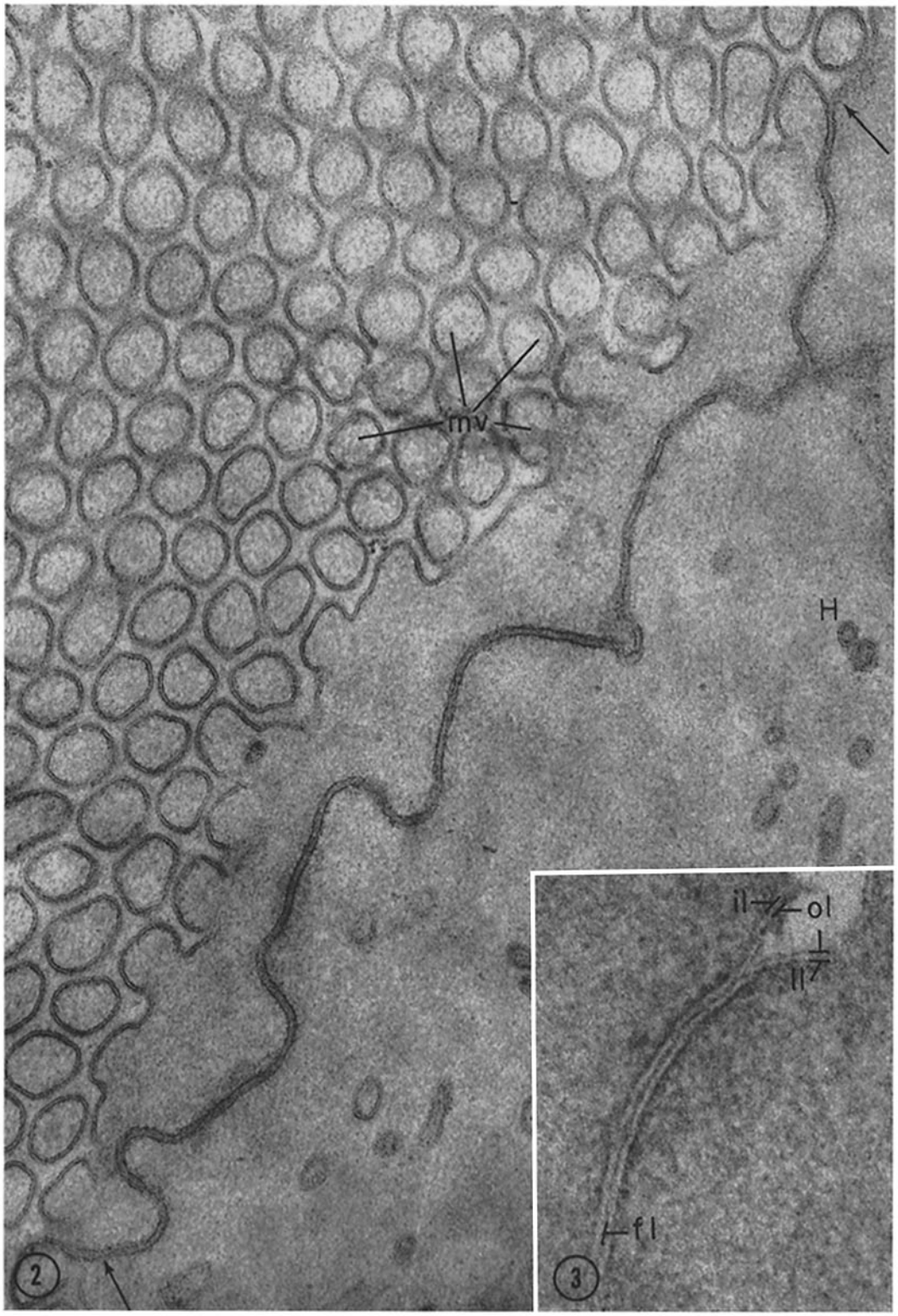
The triple-layered structure of the epithelial cell membrane (total thickness 110 A) is visible along the free cell margin and around the microvilli (*mv*) which are cut mostly in cross-section. Note that the usual slight asymmetry of the apical cell membrane has been virtually removed by $KMnO_4$-staining (compare with Fig. 1).

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6), dehydrated in acetone, stained in block with $KMnO_4$, and embedded in Epon. $Pb(OH)_2$-stained section. × 60,000.

FIGURE 3

Tight junction between two epithelial cells (mucous surface cells) of the gastric mucosa. The fusion line (*fl*) which represents the fused outer leaflets (*ol*) of the adjoining cell membranes is clearly visible all along the junction. In the case of the gastric epithelium, the outer leaflet of the cell membrane is slightly denser and thicker than in other epithelia, and accordingly the fusion line is more readily visible. Note the slight condensation of cytoplasmic material along the junction.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. $Pb(OH)_2$-stained section. × 230,000.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sometimes divergent course followed by the same membranes in the basal direction beyond the junction.

The intercellular space of the intermediate junction is occupied by a homogeneous, apparently amorphous material of moderate density occasionally bisected by a faint band of higher density.

The zones of cytoplasmic densification along this junctional element have a tightly matted fine fibrillar texture (Fig. 1) with most of the fibrils seemingly running parallel to the cell surface. Occasionally the mat is further condensed into a plate running parallel to the cell membrane from which it is separated by a narrow light zone (Figs. 1 and 20). The fibrillar material in these dense zones appears to be a local condensation of the terminal web described Puchtler and Leblond (35).

Like the tight junction, this second element of the junctional complex can be readily identified in its characteristic position whenever the section approaches a plane normal to the boundary of the cell. It can also be followed without interruption over relatively long distances in oblique or grazing sections (*cf.* Figs 17 and 20). Hence we assume that it also forms a continuous belt around each cell and a continuous two-dimensional network throughout the epithelium, at least in the intestine.

We propose to call this element of the junctional complex *zonula adhaerens, i.e.* adhering-belt, or *fascia adhaerens, i.e.* adhering band. The first form applies to situations similar to one described in the intestinal epithelium; the second to those encountered in other epithelia (see below) in which the intermediary junction is not continuous around the perimeter of each cell. The new terms refer to structural and functional rather than topographical details, hence their application is more general. The intermediate position of the junction, stressed by the term originally used, is not apparent in many sections, since the desmosomes (see below) are discontinuous and frequently sparse structures. Moreover sometimes they seem to be altogether absent.

THE DESMOSOME (MACULA ADHAERENS): The third element of the junctional complex is usually located at a distance of 0.2 μ or more from the basal end of the intermediate junction. It is characterized by the presence of laminar densities in the intercellular space, and by high local concentrations of dense amorphous and fibrillar material in the subjacent cytoplasmic matrix (Fig. 1).

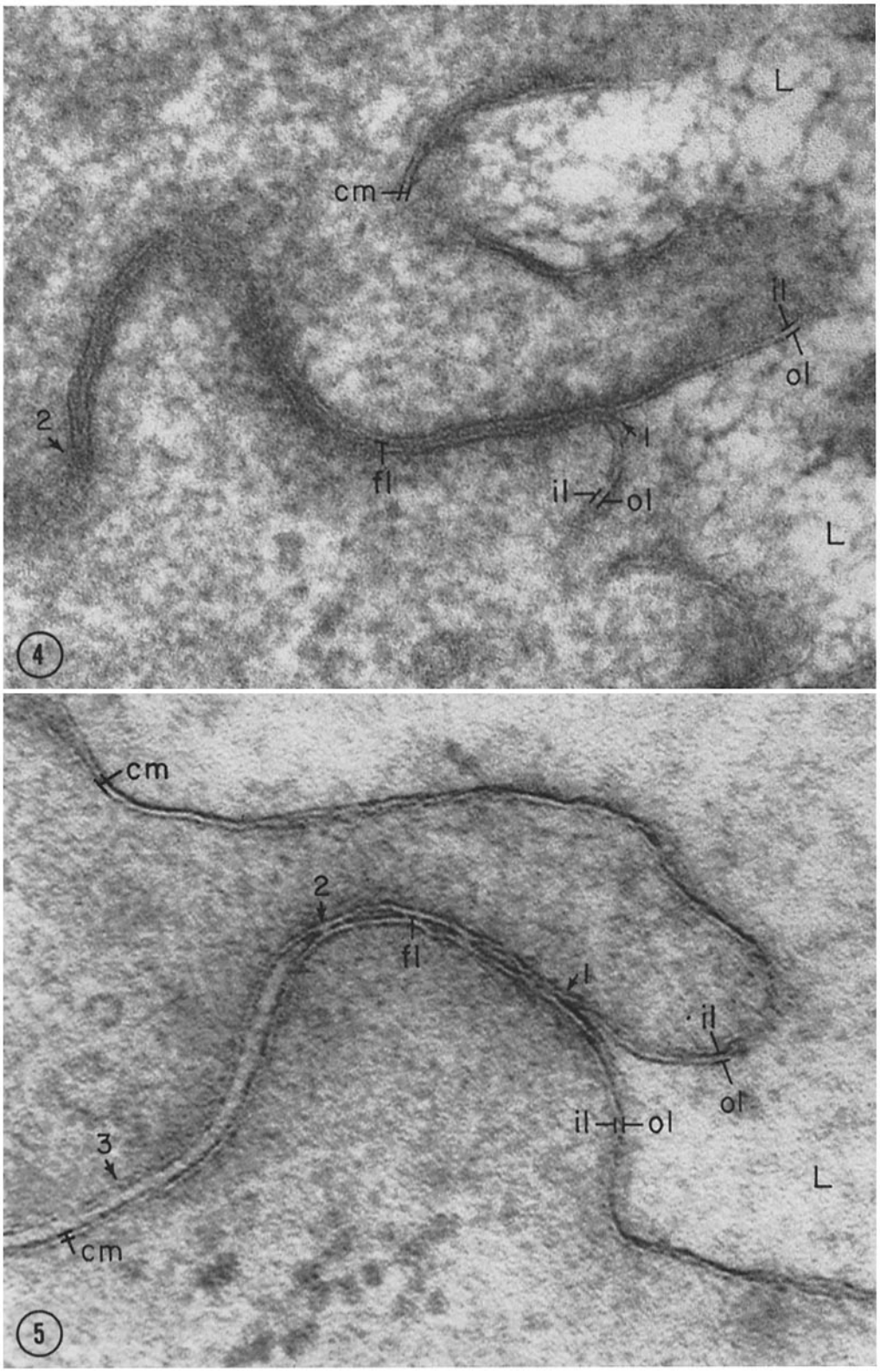
Normal sections show that the desmosome con-

FIGURES 4 AND 5

Detailed structure of tight junctions (*zonulae occludentes*) in the epithelium of the intestinal (Fig. 4) and gastric (Fig. 5) mucosa (rat) shown at high magnification. Each tight junction (between arrows *1* and *2*) can be recognized as an area in which the lateral cell membranes of two adjoining cells come together and fuse with resultant obliteration of the intercellular space. Because of the favorable orientation of the sections, the three-layered membranes which cover the luminal surface of the two adjoining cells can be followed to their point of mergence (arrow *1*), located near the point where the membranes turn from the apical to the lateral surfaces of the cells. At this point the two outer leaflets (*ol*) of the converging membranes fuse to form the intermediate or fusion line (*fl*) of the junction. In Fig. 4 the fusion line appears thinner and less dense than the inner leaflets on either side. In Fig. 5, the fusion line is denser and thicker presumably because the outer leaflet of the cell membrane of gastric epithelial cells is noticeably thicker and denser than the corresponding leaflet in the intestinal epithelium. In Fig. 4 the membranes remain fused throughout the junction while in Fig. 5 there are several focal splittings of the fusion line within the junction followed by their refusion. The apparent discontinuity in the outline of the leaflets ~30 mμ before the merging point in Fig. 5 is due to the twisting of the membranes within the thickness of the section.

An intermediate junction (*zonula adhaerens*) (arrows *2* to *3*) is included in Fig. 5. At its level the intercellular space is widened to ~200 A, and is occupied by a moderately dense, amorphous material.

Specimens fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. $Pb(OH)_2$-stained sections. Fig. 4, × 200,000; Fig. 5, × 220,000.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sists of 2 straight plaques of dense material each disposed parallel to the inner leaflet of the corresponding plasma membrane (*cf.* 1, 3, 7, 36–38) and separated therefrom by a light space of ~40 A. The two apposed plaques are aligned in perfect phase, measure 0.2 to 0.3 μ in length, and appear to be of circular or oval shape. They are arranged in strict parallelism with respect to one another and seem to be rather rigid; they are rarely curved, but the cell membrane may be sharply bent at their margins. The unit membrane can be clearly followed through the desmosome, the only visible modification being a densification of the inner leaflet in phase with the plaques (Figs. 1, 8, and 9).

At this level the intercellular space measures ~240 A across and is usually occupied by a disc of moderately dense material bisected by a denser central layer referred to in the literature as "intercellular contact layer" (3)[2] or "median stratum" (38).

Bundles of cytoplasmic fibrils, coarser and more distinct than those of the terminal web, converge on the inner aspect of each desmosomal plaque (Fig. 1), sometimes obscuring its outlines (*cf.* Fig. 12). Most of these fibers approach the plaque at a high angle, but occasionally the bundles run parallel to it and fray out into the cytoplasmic matrix at its margins. Within each convergent bundle of fibrils one or two plate-like zones of further densification are frequently seen running parallel to the main plaque.

In contradistinction to the first and second element of the complex, the desmosome is not present in all normal sections, is not continuous over long distances, and therefore appears to be a discontinuous, button-like—rather than a continuous, belt-like—type of attachment (Fig. 12). In fact more than one row of desmosomes probably occur along the sides of the cells, since several such structures can frequently be detected along a given intercellular space.

In keeping with the rest of our nomenclature, we propose the term *macula adhaerens*, *i.e.* adhering spot, as an alternate for desmosome. It has the advantage of describing one of the main features of the structure, *i.e.* its discontinuous button-like character, and of distinguishing it from other "bodies" or structural entities involved in cell-to-cell attachment.

GENERAL REMARKS: The junctional complex described is encountered among all cells of the intestinal epithelium irrespective of their differentiation: for instance, it binds absorptive epithelial cells to goblet cells (Fig. 18) or to other glandular cells in the crypts. So far, no evidence has been obtained about the fate of the junctional complex during sloughing off of the epithelium or leucocyte migration. As a rule, the cell membrane directly involved in junctional elements is free of invaginations or associated pinocytic vesicles; however, such structures are encountered along

[2] Originally Odland (3) assumed that the "intercellular contact layer" was flanked by two additional extracellular dense bands referred to as "intermediate dense layers." However, as shown by Karrer (38) and confirmed by us, the latter actually correspond to the outer leaflet of the apposed cell membranes which may appear slightly denser at this level than along the rest of the lateral aspect of the cell.

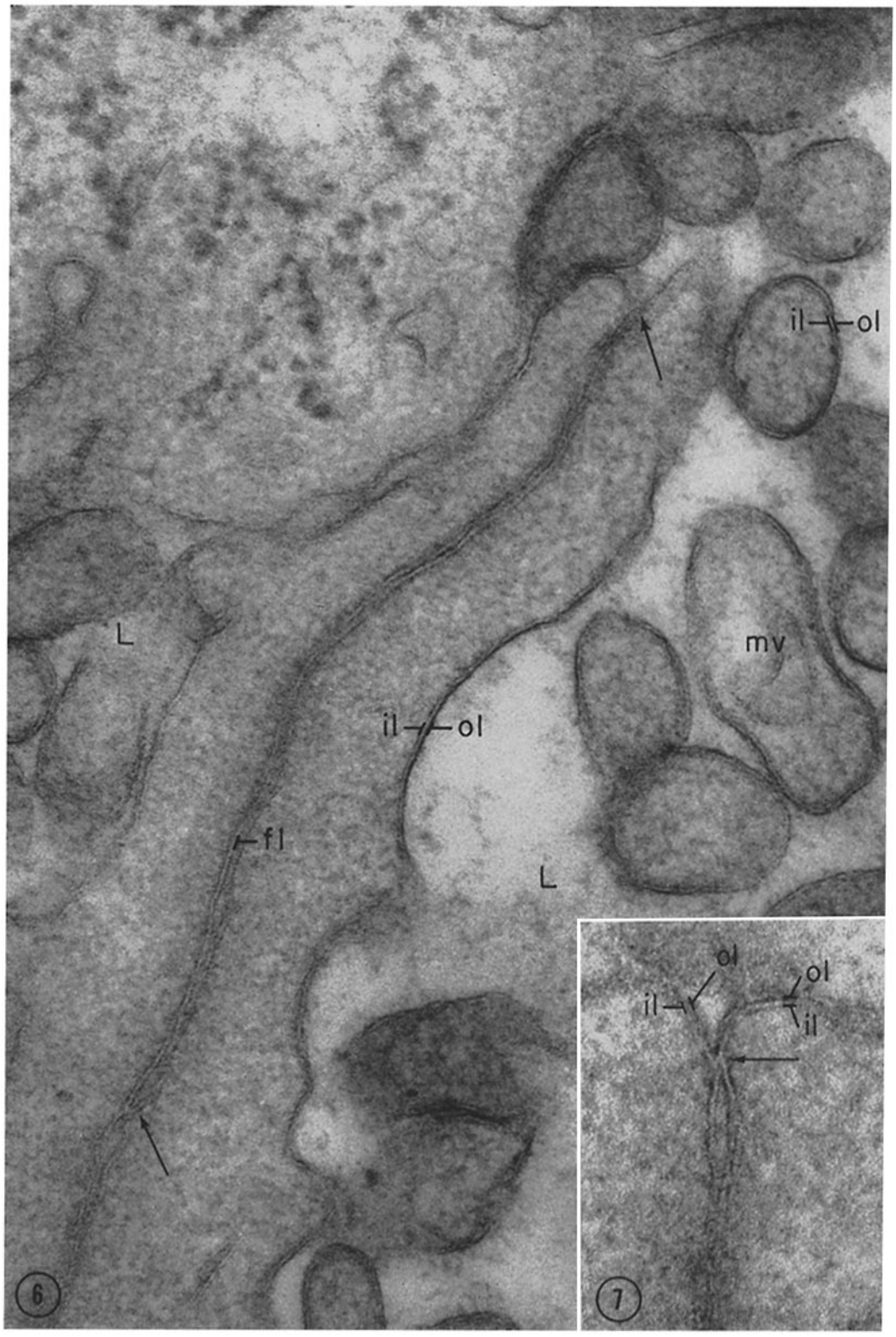
FIGURE 6

Oblique section through a gastric gland (rat) near the luminal surface of two parietal cells. A tight junction is cut over a long distance and extends diagonally across the field from the lower left to upper right (arrows). The fusion line (*fl*) can be followed in most places throughout the junction. The upper left corner is occupied by a zymogenic cell. Note that in this case, as in that of the covering epithelia of the gastric mucosa (Fig. 5), the usual asymmetry of the apical cell membrane is reversed; *i.e.*, the outer leaflet appears denser and thicker than the inner one.

FIGURE 7

Tight junction between two epithelial cells of the gastric mucosa (rat) showing an area (arrow) at the initial point of membrane fusion where the space separating the inner leaflets of the fused membranes is narrowed to 100 A and no intermediate line is visible.

Specimens fixed in 1 per cent OsO_4 phosphate buffer (pH 7.6) and embedded in Epon. $Pb(OH)_2$-stained sections. Fig. 6, × 145,000; Fig. 7, × 185,000.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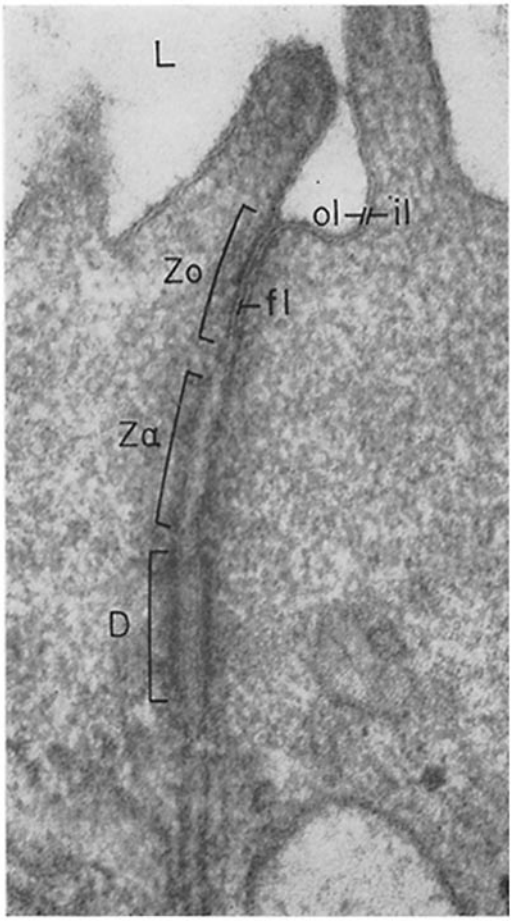
the membrane which connects one element of the complex to another (Fig. 1).

Hypotonic treatment, carried out by injecting distilled water into the lumen of an isolated intestinal loop for 15 to 30 minutes prior to OsO_4 fixation, does not cause opening of the tight junctions. It is noteworthy that in such specimens the outer leaflet of the apical cell membrane appears thicker and denser than in untreated specimens; consequently the fusion line of the tight junctions is more readily visible.[3]



Junctional Complexes in the Epithelia of Other Segments of the Digestive Tract

The complexes joining the epithelial cells of the gastric or colonic mucosae are entirely similar to those described in the epithelium of the jejunal mucosa, only the fusion line of the *zonula occludens* is more easily demonstrated (Figs. 3 and 5). This

[3] In a systematic study of the effect of hypotonic treatment upon the membrane of the microvilli, Millington and Finean (39) noted an increase in the thickness of the membrane to 140 A after longer intervals than used by us. They do not mention the increase in thickness of the outer leaflets which in their case may have been obscured by PTA staining. Their observations indicate that after PTA staining the asymmetry of the membrane is actually reversed, the outer leaflet being slightly denser and thicker (by ~10 A) than the inner one.

FIGURE 8

Junctional complex between two epithelial cells of the mucosa of the gall bladder (guinea pig), showing the occluding zonule (*Zo*) followed by adhering zonule (*Za*), followed by the desmosome (*D*). The trilaminar structure of the apical cell membrane can be clearly distinguished and traced into the occluding zonule. The fusion line (*fl*) is also visible within the junction.

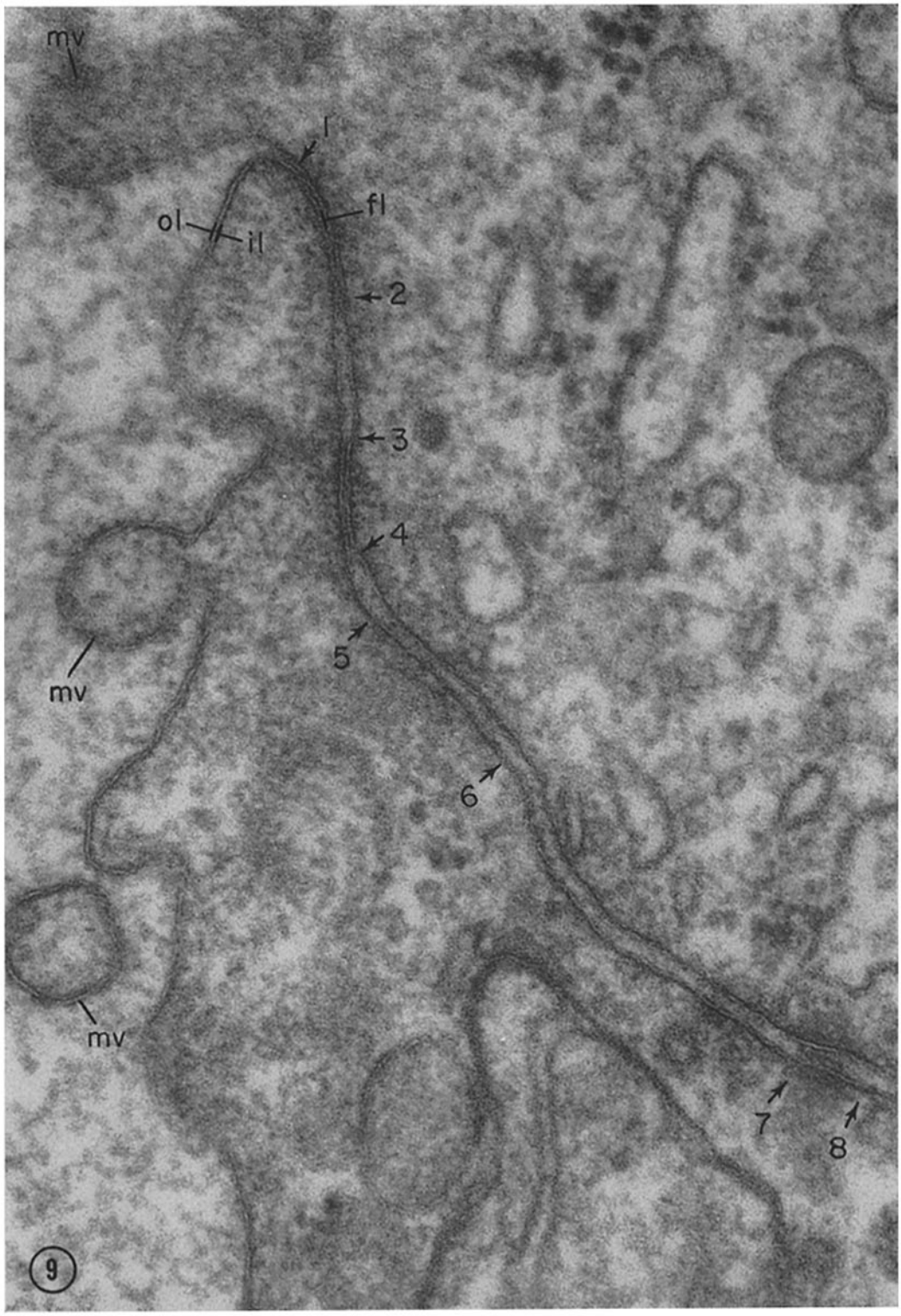
Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Araldite. $Pb(OH)_2$-stained section. × 155,000.

FIGURE 9

Junctional complex between two epithelial cells of a thyroid follicle (rat).

The occluding zonule stretches from arrow *1* to arrow *4* with an interruption between arrows *2* and *3*, probably due to the fact that at this level the zonule is bent and the section cuts "below" its basal margin. The fusion line (*fl*) is visible within both segments of the zonule but the merging of the outer leaflets (*ol*) of the cell membranes into this line is not clearly shown (see Fig. 11). An adhering zonule can be recognized between arrows *5* and *6*, and a desmosome between arrows *7* and *8*. A few mμ to the left of arrow *7* the section probably touches the margin of another desmosome.

Specimen fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6), dehydrated in acetone, stained in block with $KMnO_4$, and embedded in Epon. Section stained in uranyl acetate and $Pb(OH)_2$. × 145,000.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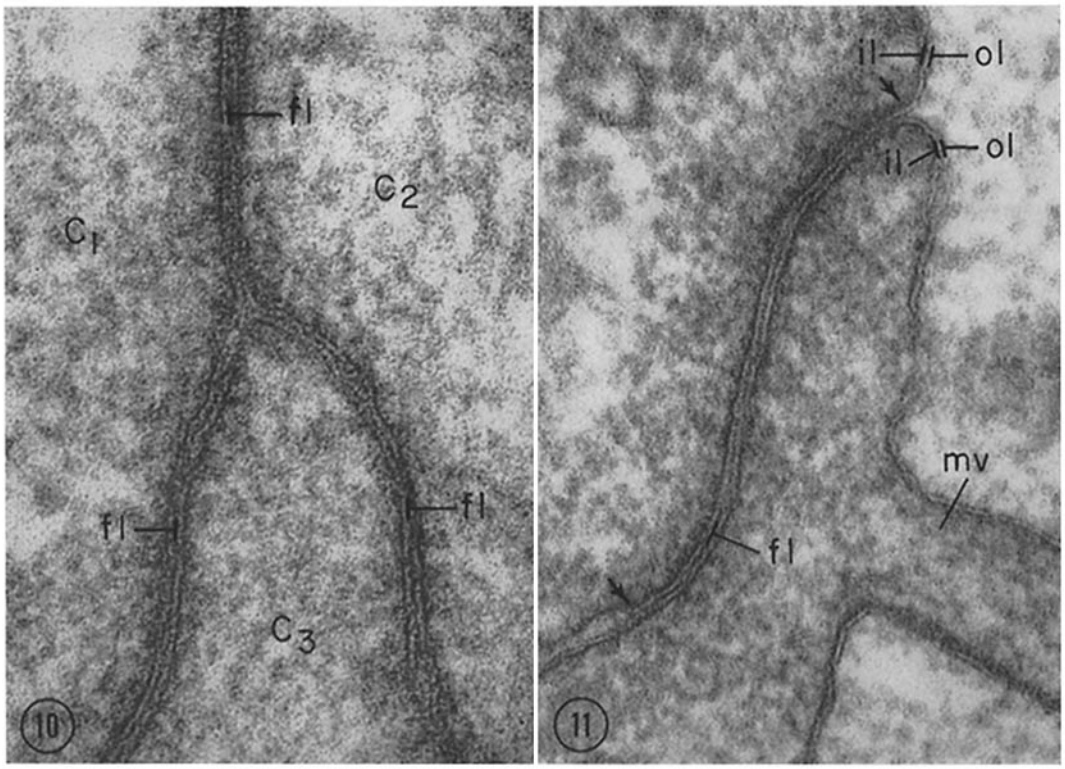


FIGURE 10

Thyroid follicle (rat). The section cuts through the epithelium parallel and immediately basal to the luminal surface.

The occluding zonules of three adjacent cells (C_1, C_2, C_3) merge into an inverted Y figure. Fusion lines (*fl*) are seen in each branch of the Y and appear to be continuous from one branch to another. × 190,000.

FIGURE 11

Zonula occludens in a thyroid follicle (rat).

The tight element of the junctional complex extends between the arrows. The mergence of the outer leaflets (*ol*) of the cell membranes into a fusion line (*fl*) is clearly shown. Note the bands of condensed cytoplasmic matrix associated with the occluding zonule and continuing beyond it. Specimens and sections prepared as for Fig. 9. × 130,000.

is due to the fact that, especially in the epithelium of the gastric mucosa, the outer leaflet of the apical cell membrane is slightly denser and thicker than the inner leaflet. Hence the asymmetry of the membrane is reversed in comparison to that already described for the apical membrane of intestinal epithelia. The lateral cell membrane in the gastric epithelium shows the same type of "reversed" asymmetry (Fig. 5).

JUNCTIONAL COMPLEXES IN GLAND AND DUCT EPITHELIA

Entirely similar complexes are encountered in the gastric glands (Fig. 6) and the gall bladder epithelium (Fig. 8), except that in the latter the depth of the *zonula adhaerens* is more variable.

A slight variant occurs in the thyroid (Figs. 9 to 12), the liver (Figs. 13 and 14), the pancreas (Figs. 15 to 17), and the parotid. In all these cases,

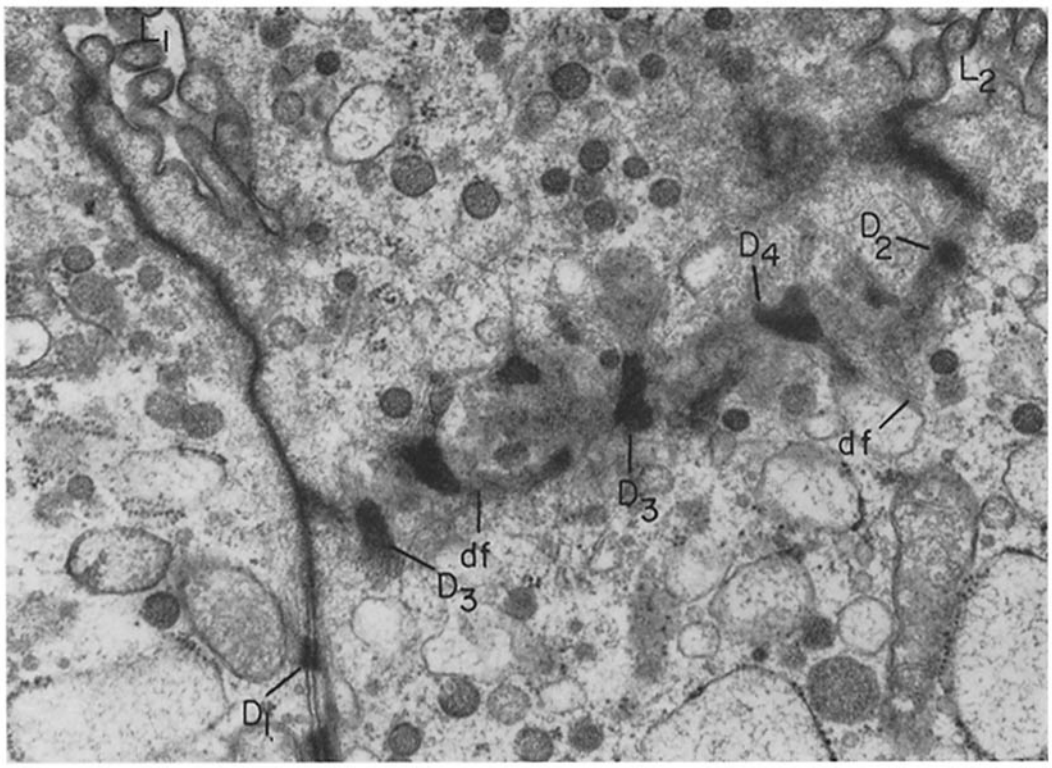


FIGURE 12

Thyroid follicle (rat).

Oblique section which touches the follicular lumen at L_1 and L_2; cuts normally through a junctional complex from L_1 to the desmosome marked D_1; and grazes the surface of one of the cells joined in the complex (upper part of the field).

The dense more or less circular (D_2), oval (D_3) and "lobated" (D_4) dense regions are desmosomal plates partially or entirely included in the thickness of the section. The micrograph shows the discontinuous, "macular" character of the desmosomes, and the bundles of fibrils (*df*) that converge on their plates.

Specimen and section prepared as for Fig. 9. × 26,000.

the outer leaflet of the plasma membrane appears as a faint discontinuous band after OsO_4 fixation, and correspondingly the fusion line of the tight junction is either poorly outlined or entirely invisible (Figs. 15 and 16). Staining the tissue in block with phosphotungstic acid or in sections with uranyl or lead does not substantially improve the visualization of the unit membrane; however, staining in block with $KMnO_4$ (24) or double staining the sections (first with uranyl and then with lead) increases the density of the outer leaflet and fusion line (Figs. 9 to 11, 14, 29, and 30). From such preparations it can be clearly appreciated that the luminal membrane of these cells differs from the apical membrane of the intestinal epithelia: it is more asymmetrical, for the two dense leaflets are more unequal in thickness and density; and it is finer, since the over-all thickness of the unit does not exceed 70 A.

In the case of most glandular epithelia (Figs. 9 to 11 and 13 to 17) as well as the epithelium of the gall bladder (Fig. 8), a high concentration of dense material is visible in the cytoplasmic matrix along the *zonula occludens* and continues without noticeable change along the *zonula adhaerens*.

In glandular epithelia, adhering zonules al-

though generally present show considerable variation in the extent of their development. It is probable that in some cases they are discontinuous, therefore *fasciae* rather than *zonulae*. Occasionally they are missing and in such cases the occluding zonule may be immediately followed by a typical desmosome. Desmosomes are present in all these epithelia but their size and frequency vary noticeably from one epithelium to another: they are relatively small (Fig. 13) and infrequent in the liver, and unusually large and frequent in the thyroid (Figs. 9 and 12) and the parotid.

In all these cases, the same complex is used to join together all types of cells in a given epithelium irrespective of their differentiation. In the liver, for example, the same complex appears between parenchymatous liver cells on each side of the bile capillaries; between parenchymatous cells and duct cells at the confluence of bile capillaries and bile ducts; and between duct cells along the duct epithelium. In the gastric glands, typical junctional complexes bind parietal cells to either zymogenic cells, or mucous neck cells; and in the pancreas, they join—in succession—acinar cells to centroacinar (Fig. 16) and centroacinar to duct cells.

Junctional Complexes in the Epithelium of the Uterine and Oviductal Mucosae

The junctional complexes in the columnar epithelia of the mucosae of the uterus and oviduct are similar to those found in the gland and duct epithelia described above, except for a more pronounced variability in the development of the adhering zonules. In many cases the latter are rudimentary or altogether missing.

Junctional Complexes in the Nephron

Considerably more variation is found in the nephron epithelium in which the organization of the junctional complexes varies typically from one segment to another.

PROXIMAL TUBULE: In the proximal convolution, the occluding zonule is extremely shallow, being as a rule reduced to 200 to 400 A in depth (Figs. 19, 20, 24 to 26). Even so, it is clearly

FIGURE 13

Bile capillary between two parenchymatous liver cells (guinea pig). On each side, an occluding zonule can be easily recognized by the apparent narrowing of the intercellular "gap" between arrows *1* and *2*. Each tight junction is followed by an adhering zonule extending between arrows *2* and *3* and, farther away, by a desmosome marked *D*. A condensation of finely fibrillar material occurs mainly along the adhering zonules on each side of the bile capillary.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. Section stained in uranyl acetate and $Pb(OH)_2$. × 65,000.

FIGURE 14

Guinea pig liver, bile capillary.

An occluding zonule, with a distinct fusion line (*fl*) and associated bands of dense cytoplasmic material (*db*), closes the luminal end of an intercellular space. Microvilli protrude into the lumen, and small vesicles (*v*) limited by a distinct unit membrane, occur in the cytoplasm.

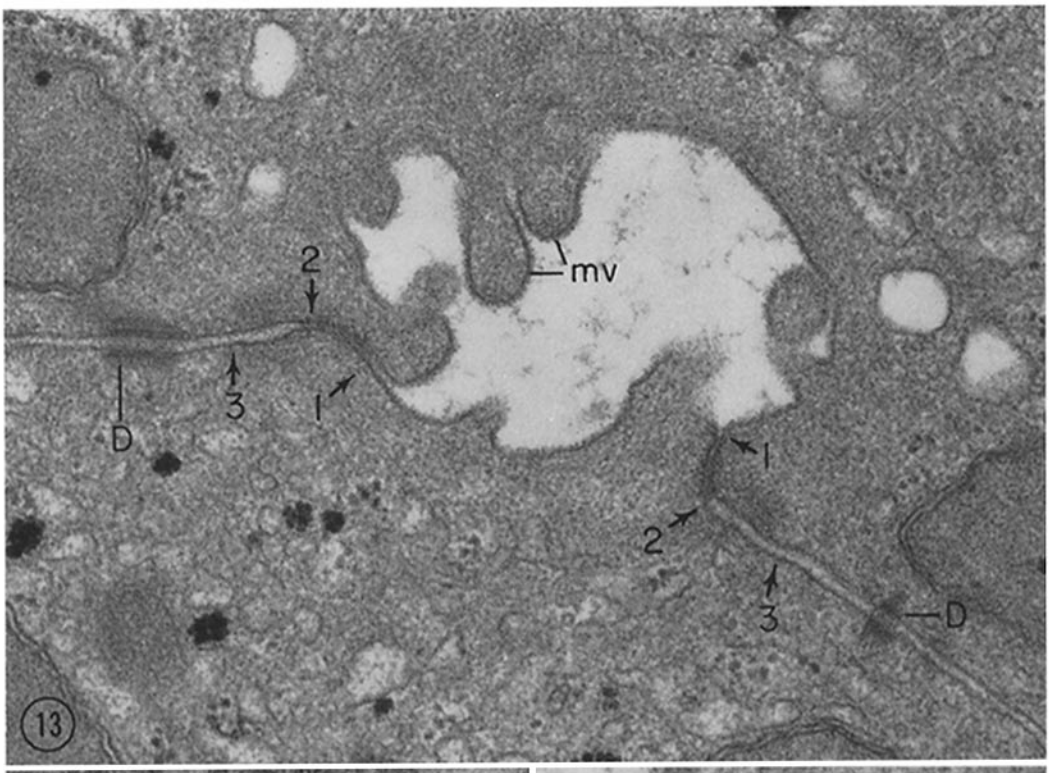
Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6), dehydrated in acetone, stained in block with $KMnO_4$, and embedded in Epon. Section stained with uranyl acetate and $Pb(OH)_2$. × 145,000.

FIGURE 15

An occluding zonule extends from arrow *1* to arrow *2* and closes the luminal end of the intercellular space in a pancreatic acinus (guinea pig).

The convergence of the outer leaflets (*ol*) of the two merging cell membranes can be seen quite clearly, but the fusion line within the zonule is barely visible in this preparation stained only with $Pb(OH)_2$ (compare with Fig. 14). The two wider spots within the zonule probably represent focal splittings.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. × 135,000.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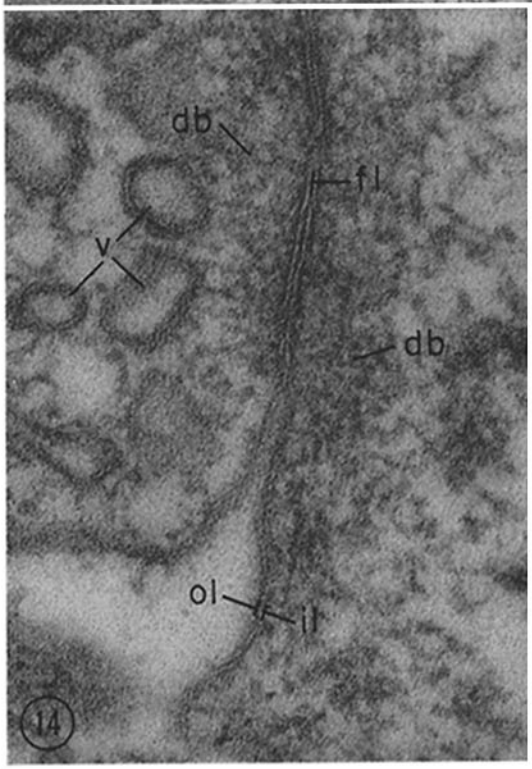
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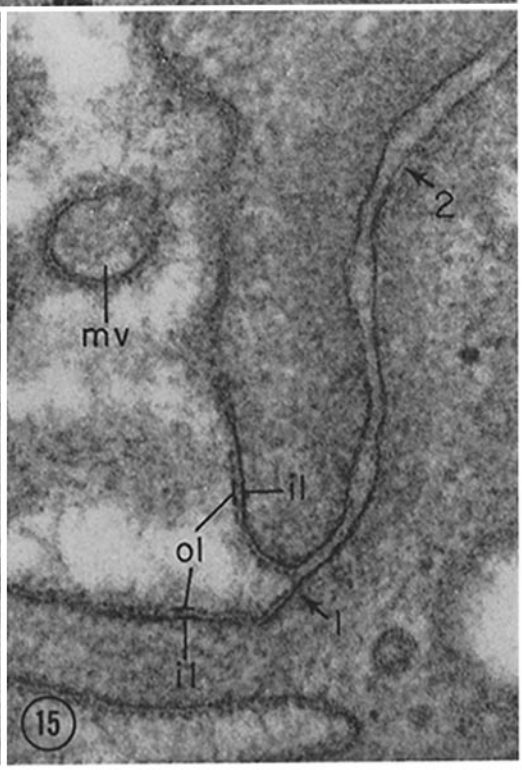
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recognizable, especially in sections normal to the luminal surface, as a localized stricture (~90 A) occurring near the lumen along the otherwise relatively open intercellular spaces. This stricture represents the space between the inner leaflets of the apposed cell membranes. The fusion line in this miniature tight junction is difficult to demonstrate, for as already noted by Miller (11), the outer leaflet of the apical plasma membrane is only rarely visible in OsO_4-fixed proximal convolutions. It can, however, be demonstrated in $KMnO_4$-fixed or stained specimens (Fig. 22). In such preparations the total thickness of the membrane, like that of gland and duct epithelia, does not exceed ~70 A. The adhering zonules, which follow immediately behind the tight strictures, are deep and usually associated with a continuous, relatively broad band of dense cytoplasmic material (Figs. 19, 20, and 24 to 26). At their level the intercellular space is occupied by an amorphous or finely fibrillar material of moderate density, whereas beyond the junctions the space appears "empty" (Figs. 24 to 26).

DISTAL TUBULE AND COLLECTING DUCT: The situation is almost reversed in the distal convolution and in the collecting tubules: the occluding zonule is generally well defined and extensively developed. It measures up to 0.3 μ in depth (Figs. 21, 23, and 27), and its fusion line is readily demonstrated (Figs. 23 and 27), for the outer leaflet of the apical cell membrane is well outlined in OsO_4-fixed specimens. Moreover the apical membrane is relatively thick: it measures ~110 A like the corresponding membrane in intestinal epithelia. Bands of dense cytoplasmic material accompany the occluding zonule along its entire course (Figs. 21 and 23) and continue along the second element of the complex when present (Figs. 23 and 27) (see below). This second element, however, shows marked variation in its development from one intercellular space to another: in some instances it is well defined, measures up to 0.2 μ in depth and contains, as in the proximal convolution, a moderately dense material in its gap (Figs. 23 and 27); in others, the junction is shallow and poorly developed; and finally in many cases no distinct differentiation of the cell surface can be recognized in the expected location (Fig. 21). It follows that in the distal convolution the second element of the junctional complex is not a continuous structure around each cell and throughout the epithelium; *i.e.*, a zonule. Accordingly it deserves the designation *fascia adhaerens*. As already noted, a similar situation seems to prevail in the epithelium of the mucosae of the uterus and oviduct and in that of the thyroid.

All along the epithelium of the nephron tubule, and especially in the distal convolutions, desmosomes are small and definitely less frequent than in other epithelia examined; the interval between

FIGURE 16

Section through the lumen of a pancreatic acinus (guinea pig) showing three junctional complexes between contiguous cells. Each occluding zonule runs from the lumen to the first arrow along the intercellular space and is immediately followed by an adhering zonule whose end is marked by the second arrow. Small desmosomes (D_1, D_2, D_3) complete each junctional complex.

The clearest image is given by the complex that includes D_1; the adhering zonule that precedes D_3 shows a "gap" narrowing in its middle portion, where the section presumably cuts again through the occluding zonule. The obliquity of the section blurs the image of the adhering zonule and of most of the occluding zonule that precede D_2; hence the position of the first arrow on this complex is uncertain.

Associated bands of dense cytoplasmic material begin at the occluding zonules and continue uninterrupted along the adhering zonules, becoming thicker at the level of the latter.

Note that although the upper cell is centroacinar, and the lower cells acinar (as indicated by the zymogen granules (Z) they contain), they are attached to one another by similar complexes.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. Section stained in uranyl acetate and $Pb(OH)_2$. × 75,000.


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D2
16

them and the adhering zonules or fasciae is more variable and generally greater (Fig. 19).

GLOMERULAR EPITHELIUM: A far reaching exception to the junctional pattern we have described is encountered at the beginning of the nephron where the cells of the visceral glomerular epithelium appear to be joined together in an entirely different manner: the junction zone is

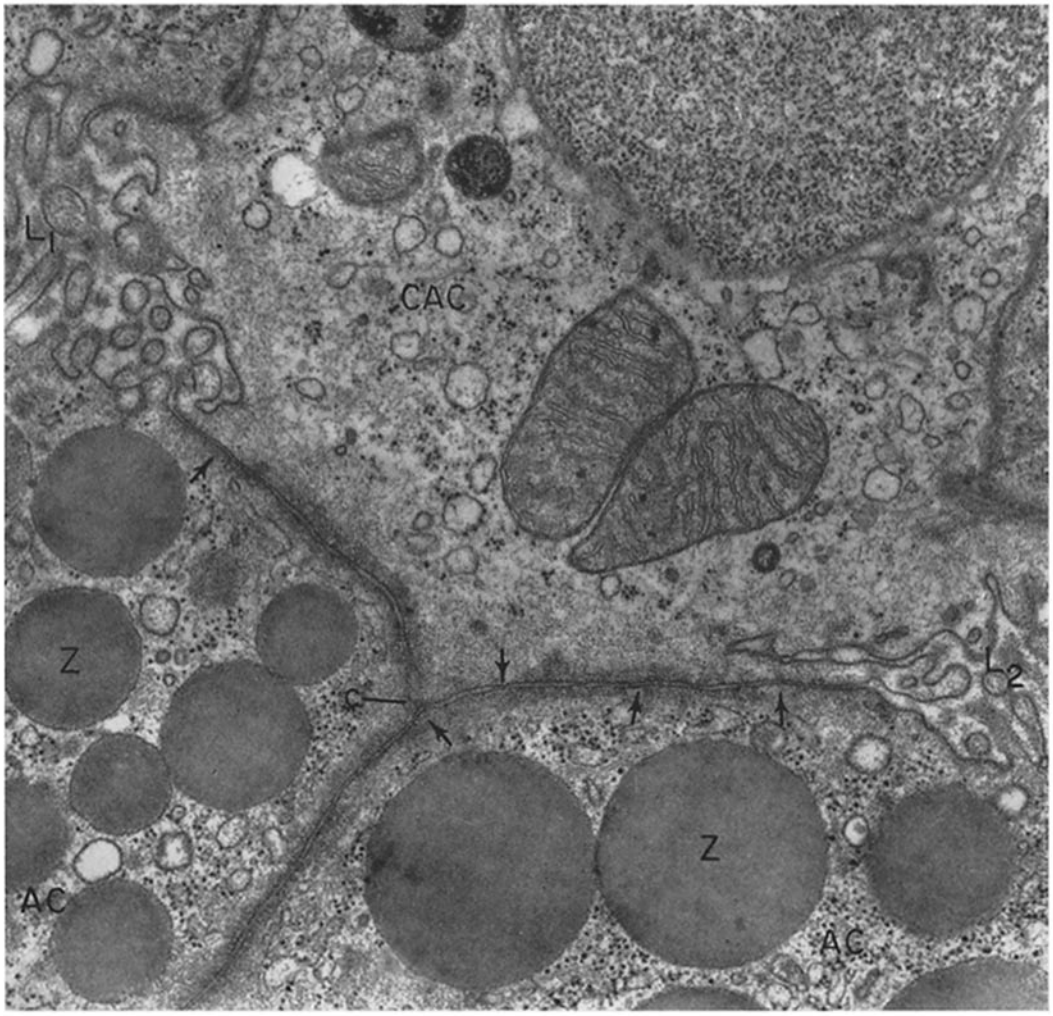


FIGURE 17

Pancreatic acinus (guinea pig).

Between the acinar lumina L_1 and L_2, the section cuts broadly through junctional complexes at the level of occluding and adhering zonules. The latter can be recognized by the apparent narrowing of the intercellular gap (arrows). The micrograph shows clearly the broad band of dense cytoplasmic material associated with both the occluding and adhering zonules. Note the continuity of these elements of the complex in the plane of the epithelium and the continuity of the associated bands of condensed cytoplasmic material over the point of confluence of three intercellular spaces (*c*). *AC*, acinar cell; *CAC*, centroacinar cell.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. Section stained in uranyl acetate and $Pb(OH)_2$. × 26,000.

reduced in depth (19),[4] and the junction line is extremely tortuous, because of the well known extensive interdigitation of the foot processes of the epithelial cells. No junctional elements directly comparable to an occluding or adhering zonule are encountered in mature glomeruli. The zone of closest apposition, *i.e.* the urinary slit, has some structural features reminiscent of desmosomes: in grazing sections it appears as a relatively large gap (200 to 250 A) bisected by a distinct intermediate line as previously described by us in the rat (19) and more recently confirmed by Rhodin (40) in the mouse and by Trump and Benditt (41) in the human glomerulus. However, this intermediate line is a filamentous two-dimensional structure, not a plate, as in the case of the desmosome. Moreover, the type of associated cytoplasmic fibrillar differentiation and the continuous character of the urinary slit are comparable to those of the adhering zonules.

[4] We have previously considered this junction to have a depth of about 40 A—that is, the thickness of the slit membrane. Another possible interpretation is that the junction extends from the level of the basement membrane to the slit membrane (~500 A).

Results of Experiments Utilizing Mass Tracers

To obtain some information on the permeability characteristics of the various elements of the junctional complex, we took advantage of the progressive concentration undergone by hemo-

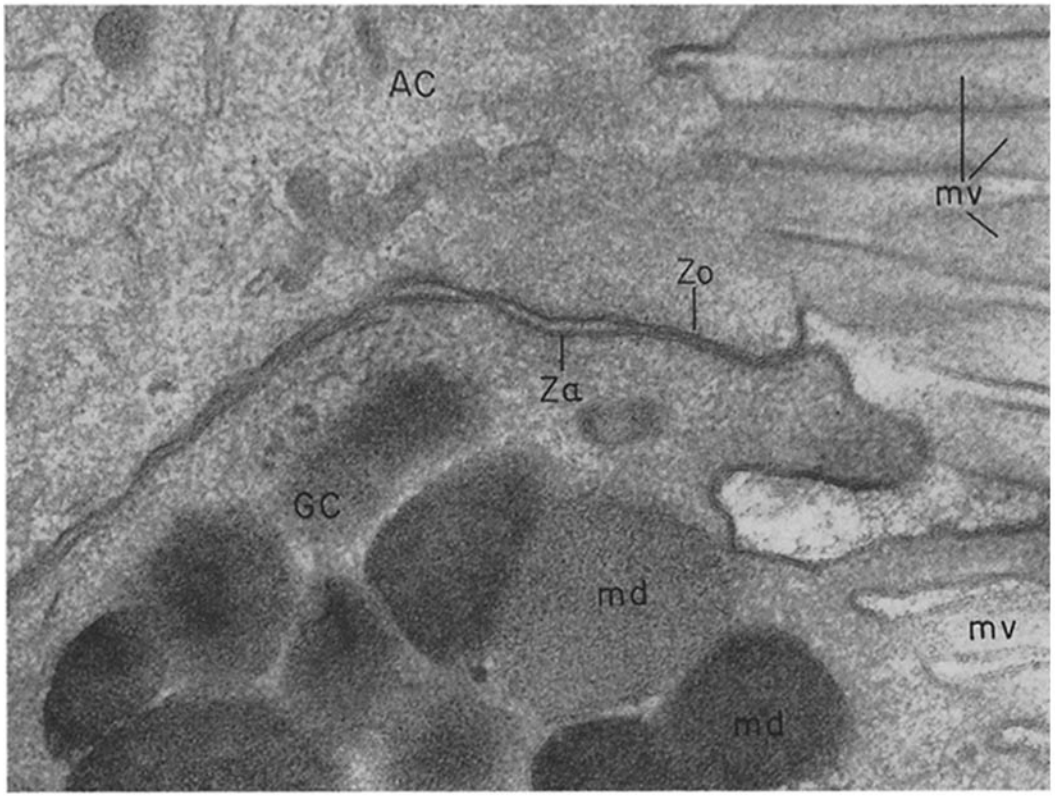


FIGURE 18

This figure illustrates a junctional complex with an occluding zonule (*Zo*) and an adhering zonule (*Za*) between an absorptive epithelial cell (*AC*) and a goblet cell (*GC*) in the epithelium of the intestinal mucosa (rat). The dense mucus droplets of the goblet cell are marked *md*.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. $Pb(OH)_2$-stained section. × 90,000.

globin in the renal tubules during experimental hemoglobinuria. Hemoglobin injected intravenously or intraperitoneally filters through the glomeruli, is concentrated by reabsorption of water in the tubules, and eventually forms a dense homogeneous mass that fills the tubular lumina (*cf.* Miller, 11). The concentration of the intraluminal hemoglobin—and therefore its electron opacity—varies considerably not only along a given tubule, but also among equivalent segments of different nephrons. In extreme instances, when the hemoglobin-containing filtrate is concentrated in the lumen of the proximal segments, distal segments, and collecting ducts to a high density, it can be used as a mass tracer to explore the outline of the tubule lumen and its connections (11). In all segments of the tubule examined the dense mass can be followed along the cellular margins down to the level of the occluding zonules (Figs. 24 to 27). Beyond this level, the intercellular spaces appear free of the tracer. Since the density of the material in the junctional gaps is considerably lower than that of the luminal hemoglobin, and since there is no evidence of a concentration gradient down the junctional complex, it can be assumed that the penetration of hemoglobin molecules is effectively stopped along the line of fusion of the adjoining cell membranes in the tight junction (Fig. 27). In any case it is clear that the concentrated tracer does not reach the adhering zonules or fasciae, the desmosomes, and the intercellular spaces beyond the level of the occluding zonules.

Seeking another situation in which the permeability characteristics of the junctional complex could be investigated, we studied pancreatic acini and ducts during zymogen discharge, occurring either spontaneously during prolonged fasting (hunger secretion); or induced physiologically, upon chyme entry into the duodenum; or experimentally, upon the administration of carbamylcholine. The discharged zymogen acts as a natural mass tracer which frequently fills the acinar and duct lumina, clearly outlining every surface detail with its continuous, homogeneous, electron-opaque substance (Figs. 28 to 30). In our material, this mass tracer was found to stop regularly at the point of mergence of the cell membranes that marks the beginning of the tight junctions (Figs. 29 and 30). No dense material has been seen so far in the intercellular spaces beyond the occluding zonules of either acini or ducts (Figs. 28 to 30).

Thus in the two examples provided it is demonstrated that the *zonulae occludentes* are impervious to concentrated protein solutions.

DISCUSSION

Significance of Results

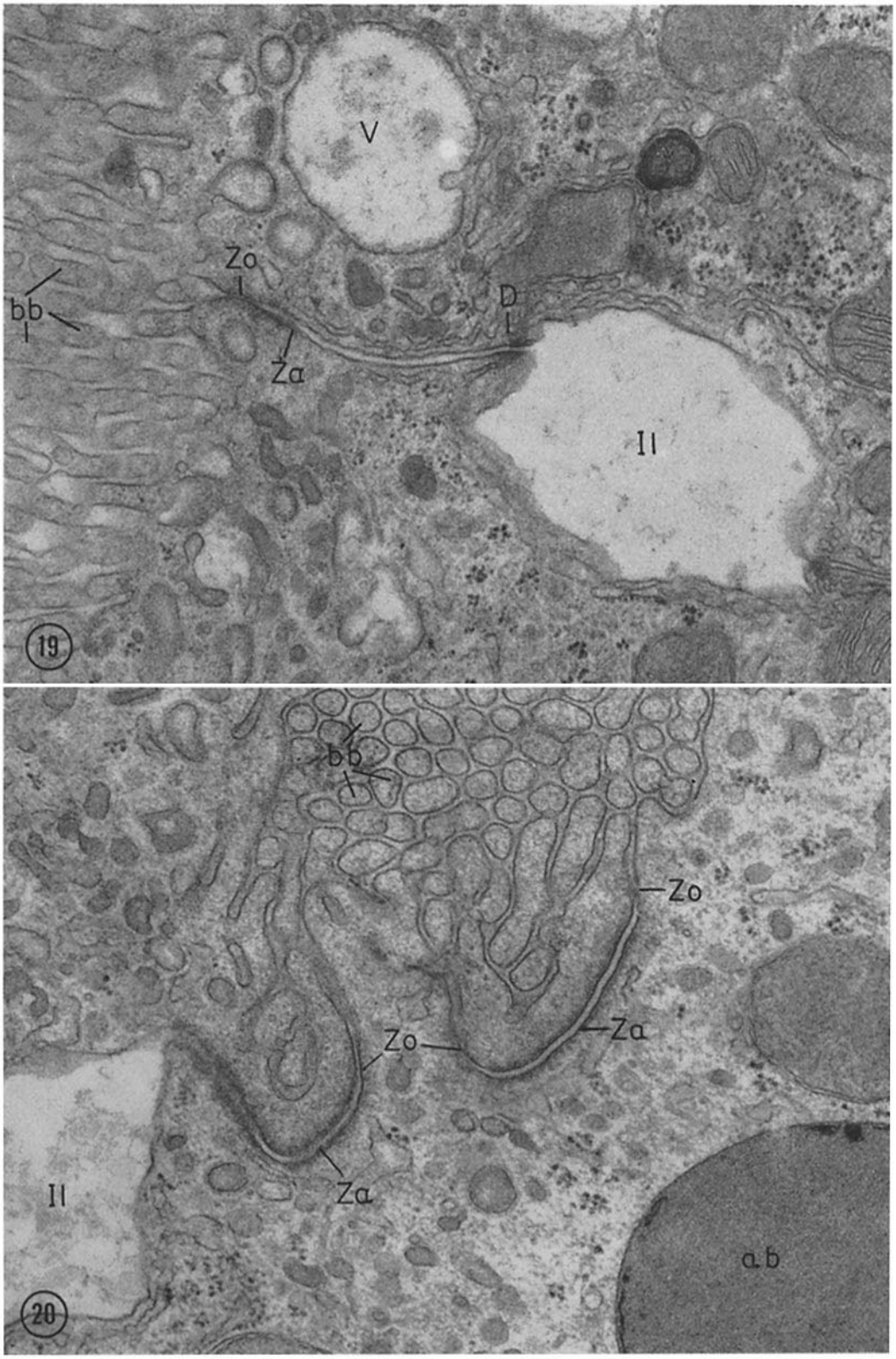
From a morphological standpoint, our findings demonstrate the regular occurrence in various epithelia, especially in those of the mucosae lining cavitary organs, of a characteristic junctional complex whose components bear a constant relationship to each other and to the lumen of the

FIGURES 19 AND 20

Junctional complexes in the epithelium of the proximal convolution (rat kidney). In this part of the nephron, the tight junction is very shallow and appears only as a short stricture along the otherwise "open" intercellular space. (See also Figs. 24 to 26.) In Fig. 19 an occluding zonule (*Zo*) is followed by an adhering zonule (*Za*) which in turn is followed at some distance by a small desmosome (*D*). Luminal to the desmosome, the cell membranes are relatively closely apposed and follow a more or less parallel course, whereas basal to it the intercellular space expands greatly to form a large intercellular lake (*Il*).

In Fig. 20, where the plane of the section is oblique and cuts close to the luminal surface of the cells, two adhering zonules (*Za*) are cut broadly and the prominent condensation of cytoplasmic material found along them is clearly demonstrated. A large absorption droplet is marked *ab* and the microvilli of the brush border *bb*.

Specimens fixed in 1 per cent OsO_4 in acetate-Veronal buffer (pH 7.6) and embedded in Epon. $Pb(OH)_2$-stained sections. Fig. 19, × 42,000; Fig. 20, × 46,000.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organ. Indeed, with some variations already discussed, we have found this complex in all organs so far examined. It can therefore be considered to be of widespread, probably universal occurrence in such epithelia. From a functional standpoint, we have demonstrated that it is the occluding zonule of this complex which acts as a barrier to the free passage of concentrated protein solutions from the lumina to the intercellular spaces.

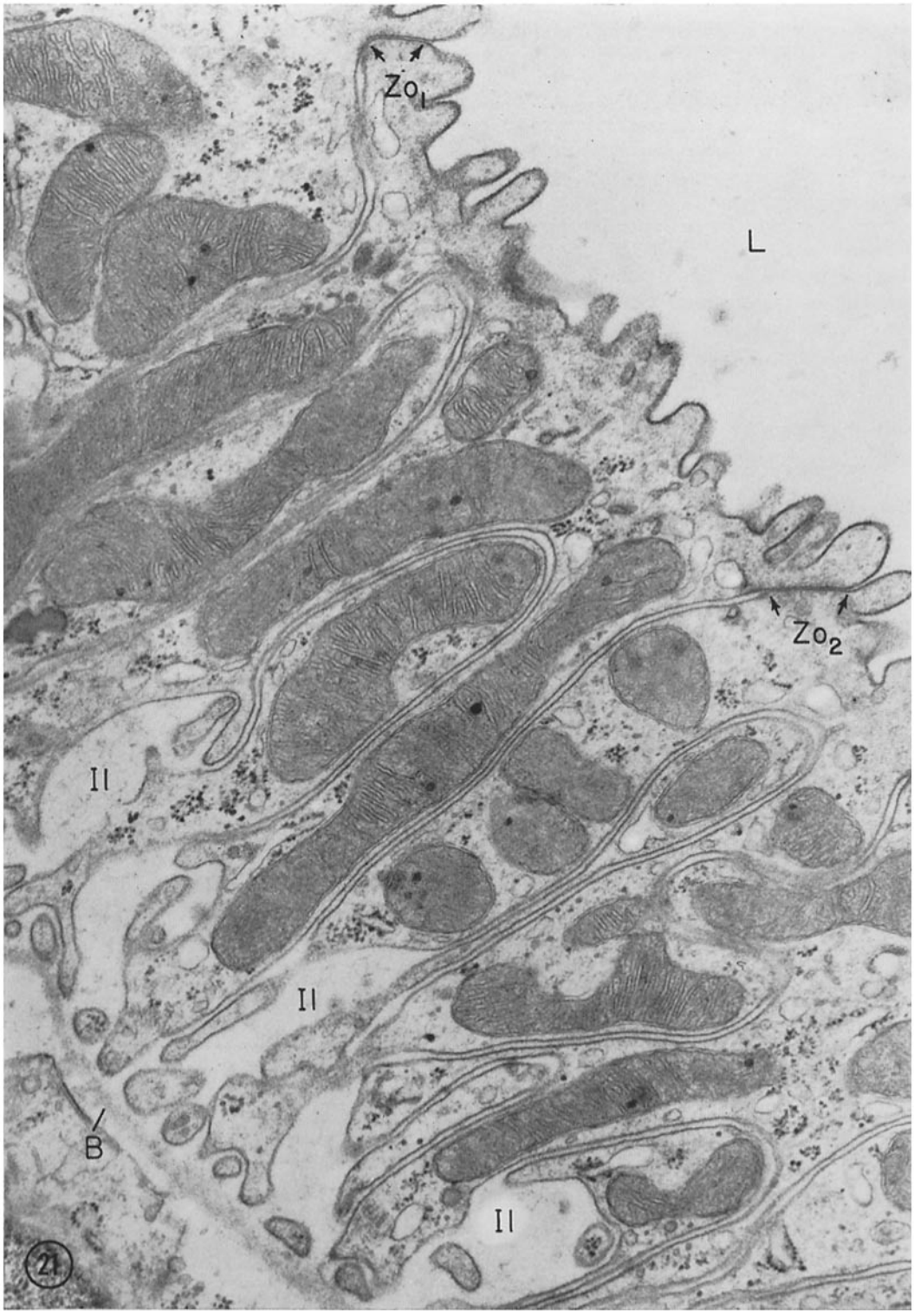
Morphology

ZONULA OCCLUDENS (TIGHT JUNCTION): We have already pointed out that the "tight junction" corresponds to Robertson's "external compound membrane" and is basically similar in structure to a single lamella of either compact (31, 32) or loose (42) myelin. However, the occurrence of such areas of membrane fusion in other locations has not been generally appreciated, probably because the existing descriptions have appeared mostly as incidental observations. For example, in the epithelium of Brunner's glands, Moe (43) noted that in some places, particularly near the apical part of the cells, "the lateral cell membranes seem to fuse, the outer dense layers of the membranes merging into each other." Choi (44) also recognized "areas of fusion of the lateral cell membranes" immediately luminal to "terminal bars" in the epithelium of the toad bladder. Junctions characterized by fusion of the lateral cell membranes have also been found in other locations, not necessarily connected with visceral lumina, and given a variety of names. They were described by Karrer, under the name of "quintuple-layered cell interconnections," between cells of the human cervical epithelium (38), and between striated muscle fibers in the wall of thoracic veins (45); by Robertson as "external compound membranes" near the luminal boundary of intestinal epithelial cells (31), and between endothelial cells in the blood capillaries of developing brain (32); by Gray as areas of "closed contact" between glial processes surrounding cerebral capillaries (46); by Muir and Peters as "quintuple-layered membrane junctions" between endothelial cells of blood capillaries in a number of tissues (13); by Peters as "quintuple-layered units" between glial cells and between myelin sheaths and glia in the optic nerve (47); by Devis and James as "quintilinear regions" between fibroblasts in tissue culture (48); and, most recently, by Dewey and Barr as "nexuses" between smooth muscle cells in the jejunum (49). Finally, we have occasionally encountered "close contacts" of the same type between erythrocytes and endothelial cells in the blood capillaries of the rat and guinea pig. In all these locations the structure described is characterized, like our *zonula occludens*, by fusion of the cell membranes and obliteration of the intercellular gap with the formation of an intermediate line representing the fused outer leaflets of the adjoining membranes; hence it is clear that, regardless of the name applied, all these appearances represent basically the same structure,

FIGURE 21

Section through a distal convolution (rat kidney) showing two junctional complexes between contiguous cells. In both cases the only element of the junctional complex encountered along the intercellular spaces is the occluding zonule (Zo_1, Zo_2) which in this epithelium usually extends, as it does here, for distances of 0.1 to 0.2 μ. Adhering zonules appear to be absent, and no desmosomes are visible throughout this entire field. Note that the intercellular space closed by Zo_2 can be followed throughout the section all the way from the luminal to the basal surface of the cell. The latter is covered by a basement membrane (*B*). The apposing cell membranes are closely approximated (~200 to 250 A) and run a virtually parallel course from the end of the occluding zonule nearly to the cell base where they expand to limit a broader, more irregular space (*Il*). The deep infoldings of the cell membrane, the basal compartments they form, the high frequency of mitochondria, and their relationship to the compartments are well demonstrated.

Specimen fixed in 1 per cent OsO_4 in an acetate-Veronal buffer and embedded in Epon. $Pb(OH)_2$-stained section. × 34,000.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although in three dimensions many of them are probably discontinuous and hence maculae or fasciae, rather than zonulae.

ZONULA ADHAERENS (INTERMEDIATE JUNCTION): We can find in the literature no clear description of the adhering zonule as a distinct entity. However, junctions of similar structure were clearly illustrated by Fawcett (2) in a renal carcinoma of the frog under the name of "terminal bars." In fact, from a perusal of the literature it is clear that, although this type of junction has been illustrated and sometimes designated as "terminal bar" by many microscopists (*e.g.* 18), it has not been clearly distinguished from the usual desmosomes.

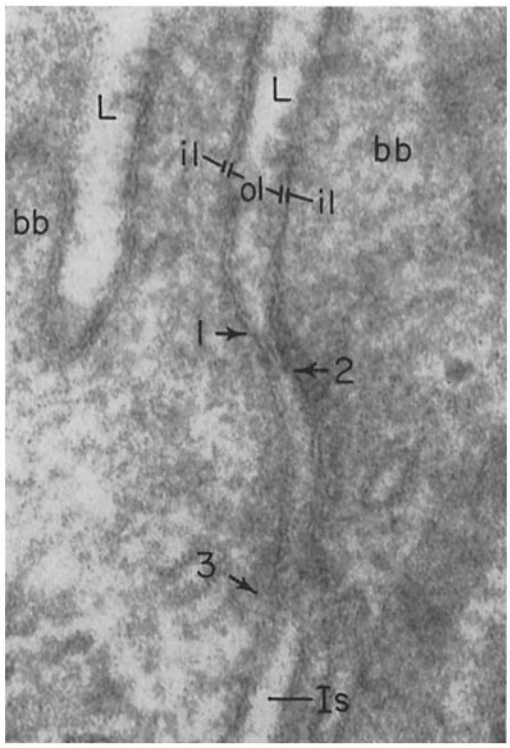


FIGURE 22

Junctional complex in the proximal convolution (rat kidney). The occluding zonule extends from arrow *1* to arrow *2*, is very shallow, but shows quite clearly a fusion line along its entire length. The adhering zonule, seen between arrows *2* and *3*, is partially blurred by the obliquity of the section. The intercellular space beyond the adhering zonule is marked *Is*.

Specimen fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6), dehydrated in acetone, stained in block with $KMnO_4$, and embedded in Araldite. Section doubly stained with uranyl acetate and $Pb(OH)_2$. × 150,000.

MACULA ADHAERENS (DESMOSOME): Desmosomes have been described in nearly all epithelia examined with the electron microscope (*cf.* Fawcett, 4). Their detailed structure has been studied in a number of tissues, particularly the epidermis (3, 36), cervical epithelium (38), mesothelium (7), and vascular endothelium in the swim bladder of the toad fish (4). An examination of the micrographs published by others shows, however, that this term has not been restricted to junctions having the structure of classical desmosomes. At various times it has been used to designate elements having the morphology of each of the types of junctions we have described in this paper. Further confusion stems from the fact that during the past few years the terms "desmosome" and "terminal bar" have been used more or less interchangeably. This leads directly to the next question in our discussion, as to which of the three elements of our junctional complex corresponds to the "terminal bar" of classical histology.

TERMINAL BARS: In the histological literature, it is generally assumed that terminal bars are bands of condensed intercellular cement which hold together the cells of an epithelium and seal up the intercellular spaces, thereby preventing or hindering matter from reaching or leaving the corresponding lumen along these spaces (50, 51). The concept has an exclusively morphological basis: the demonstration—by iron hematoxylin staining—of a chromophil material located around the cells, presumably in the intercellular spaces. In grazing sections this material is seen as a nearly hexagonal network outlining the cells, whereas in normal sections it appears as a series of dots located between adjacent cells, immediately below the free surface of the epithelium (*cf.* 6, 2). Although not experimentally proved, the functional implications of the concept turned out to be strong enough to affect the nomenclature. The bars were originally named *Schlussleisten* (50), *bandelettes de fermeture* or *bandelettes obturantes* (*cf.* 52), *i.e.* closing or locking bands, a meaning still implied, though less clearly expressed, in the English equivalent "terminal bar."

From the beginning, some histologists have regarded the bars as a special type of desmosome (6, 53) and recently this view has been restated and further developed by Fawcett (3, 4) and Fawcett and Selby (54) who showed that at the

sites where terminal bars are observed in the light microscope, a structure similar to that of desmosomes could be resolved by electron microscopy.[5] For this reason they concluded that terminal bars and desmosomes are indistinguishable except for their shape: the former are strips or bands that "may extend the whole width of the cell," whereas the latter take the form of round plaques or discs (54). During the last few years this view has been widely accepted and, as a result, the terms desmosome and terminal bar have been used more or less interchangeably in describing attachment structures in columnar epithelia (*cf.* 12, 18, 29, 43, 44). We believe that this trend should be reexamined in view of a number of recent findings, including the ones here reported. It is clear that the occluding zonule is the element of the complex best suited to close up the intercellular spaces, a function originally ascribed to the terminal bar. It is also reasonably certain that the hematoxylin staining and silver impregnation of these bars are primarily due to the associated dense band of cytoplasmic material, rather than to any special property of the cell membrane or intercellular material involved (2, 55). Our survey shows that this dense band is characteristically associated with either the adhering zonule or the occluding zonule or both, depending on the epithelium. Finally electron microscope evidence, contributed by many (*cf.* 2, 4), including us, clearly indicates that the desmosomes are discontinuous structures located away from the lumina, and varying greatly in relative frequency. Hence it is unlikely that they contribute regularly or frequently to the terminal bar image seen in the light microscope. It follows that in the large majority of cases the electron microscopical equivalent of the terminal bar must be limited to the first two elements of the junctional complex, and that the respective contribution of each of these elements must vary from one epithelium to another. For instance, in the proximal convolution of the nephron the adhering zonule undoubtedly represents the terminal bar, for the occluding zonule is extremely shallow: its depth is

[5] Fawcett showed that adjoining cells differentiate peripheral bands (in three dimensions, plates) of high density along desmosomes as well as along terminal bars. He originally ascribed this appearance to a thickening of the apposed cell membranes, but on improved preparations recently established that the bands actually represent an accumulation of dense material in the subjacent cytoplasmic matrix (4).

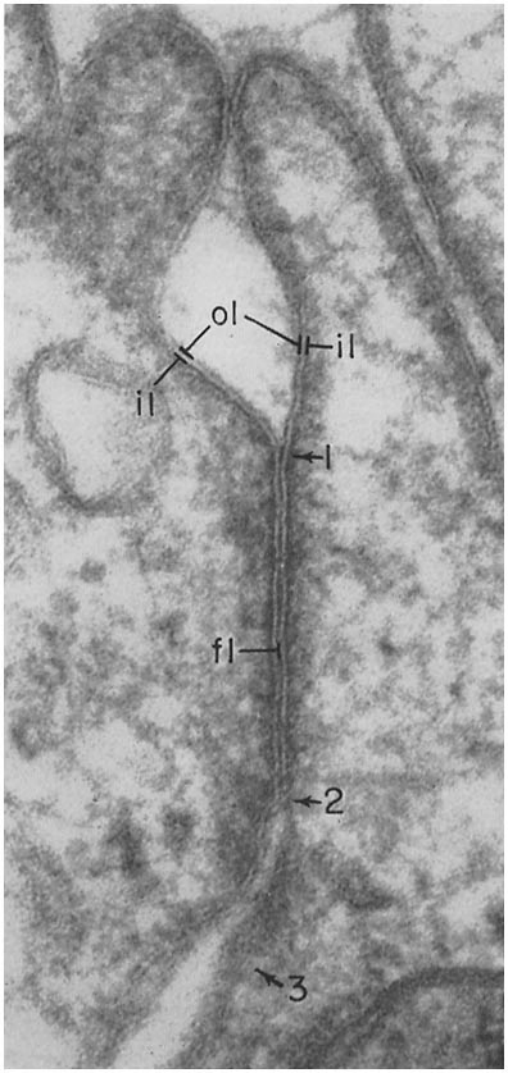


FIGURE 23

High magnification showing details of a junctional complex in the distal convolution (rat kidney). The tight junction is quite deep, extending between arrows *1* and *2*. The fusion line (*fl*) is visible along most of its length. The triple-layered structure of the apical cell membrane can be seen along most of the luminal cell surface. Part of a shallow adhering zonule appears between arrows *2* and *3*. Dense cytoplasmic material extends along the occluding as well as the adhering zonule.

Specimen fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6), dehydrated in acetone, stained in block with $KMnO_4$, and embedded in Araldite. Section doubly stained with uranyl acetate and $Pb(OH)_2$. × 150,000.

actually below the limit of resolution of the light microscope. In the intestinal epithelium the main component of the terminal bar is probably also the adhering zonule, although some contribution by the tight junction is not excluded: the tight component has enough depth to be seen in the light microscope, but only a limited amount of associated dense cytoplasmic material. In many other epithelia, especially in those of the glands and ducts examined and in those of the distal segments of the nephron, the contribution of the occluding zonule to the terminal bar must be substantial if not predominant, for the band of associated cytoplasmic material has considerable depth as well as density. Moreover, in certain cases the second element of the complex is poorly developed (*e.g.* gall bladder, thyroid, liver, uterus, and oviduct) and in some instances it can be entirely missing (distal convoluted tubule, thyroid, and uterus). Our survey suggests that a poor development of the adhering zonule occurs concomitantly with an extensive development of the occluding element, but further work is needed to establish this possible correlation.

Our observations indicate, therefore, that the term "terminal bar" does not designate a single, well defined structure at current levels of microscopical resolution. Moreover, they show that the function implied by this term is carried out by a structure (belt of membrane fusion) other than that responsible for chromophilia (belt or band of cytoplasmic condensation) and that these two structures are not necessarily coincidental. Since this situation can only end in confusion, we suggest that the use of the term "terminal bar" be discontinued in electron microscopical cytology and histology, and propose that the old and the newly recognized elements of the junctional complex be designated by the English names or their Latin equivalents we have already introduced and discussed under Results.

Junctional Complex and Epithelial Permeability

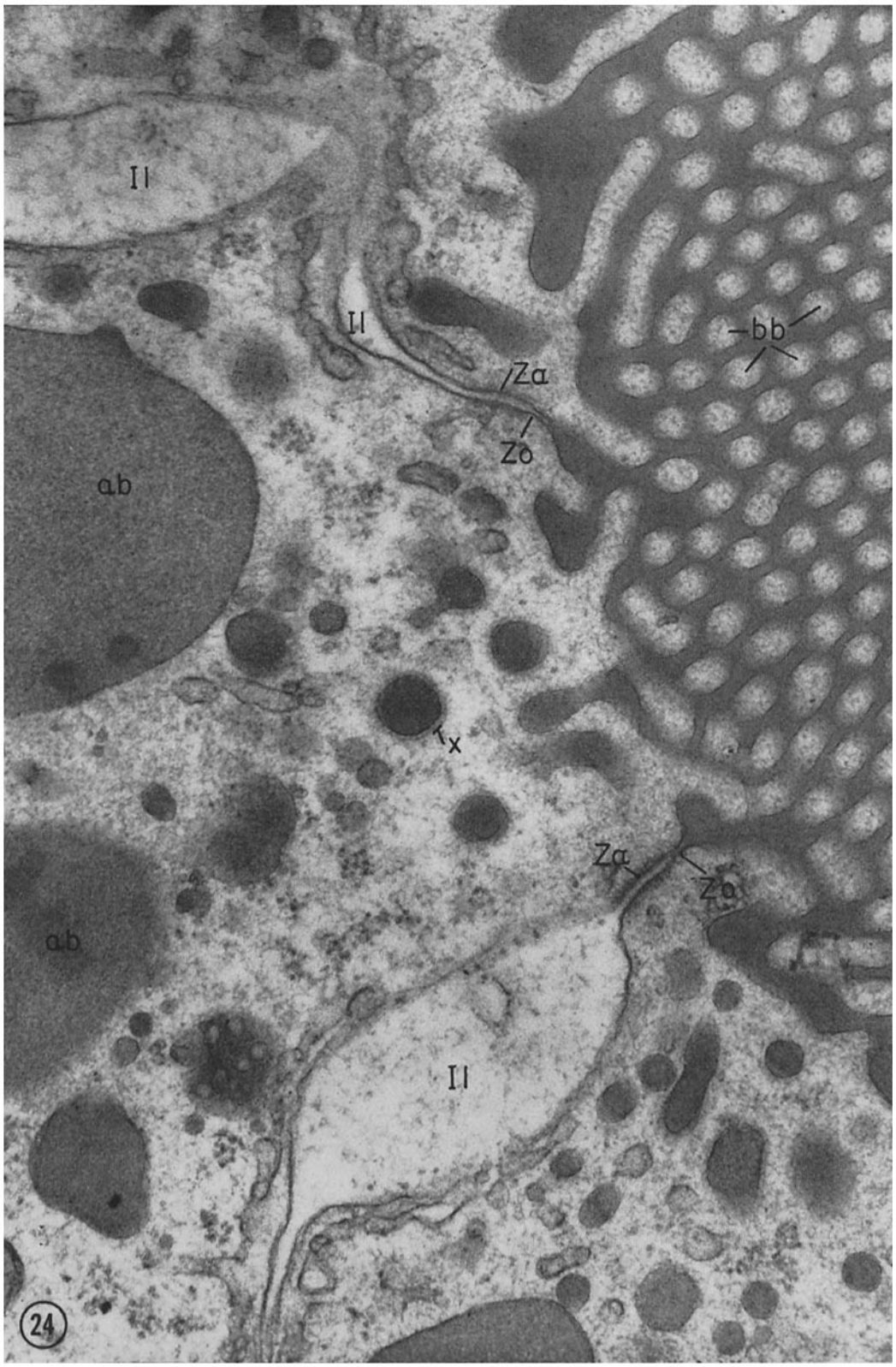
The assumption that intercellular junctions play a role in epithelial permeability can be traced back to Bonnet (50) and Zimmermann (51) who suggested that terminal bars may act as a diffusion barrier between the corresponding lumen and intercellular spaces. Direct support for this assumption was recently provided by Miller (11) who showed that in experimental hemoglobinuria the hemoglobin concentrated in the nephron lumina does not penetrate beyond the "terminal bars." Comparable evidence was obtained by Kaye *et al.* (14, 15) on a simple squamous epithelium (the corneal endothelium) using particulate (and, as such, less favorable) tracers which also did not penetrate the "terminal bars." In neither instance was the structure of this barrier further defined. Our studies clarify Miller's results by demonstrating that the *zonula occludens* is the barrier that restricts the flow of the mass tracer along the intercellular spaces, and by providing a reasonable structural basis, *i.e.* the fusion of the apposed cell membranes, for his and our observations. Moreover, we extended this type of evidence to other epithelia, namely those of the pancreatic acini and ducts, using discharged zymogen as a natural mass tracer.

FIGURE 24

Portion of a proximal convoluted tubule from a rat with experimental hemoglobinuria. A dense continuous mass of concentrated hemoglobin completely fills the tubular lumen and outlines the microvilli of the brush border (*bb*) and the various invaginations of the cell surface. Some of these invaginations, and the vesicles they form, appear surrounded by a distinct layer (*x*) of condensed cytoplasmic material. Where two cells meet, the dense mass can be followed along the cellular margins only down to the level of the occluding zonules (*Zo*); it does not appear in the adhering zonules (*Za*) or in the distended intercellular spaces (*Il*) below this level.

A number of membrane limited bodies (*ab*) of varying sizes and with a density similar to, or somewhat greater than that of the hemoglobin in the lumen are present in the cytoplasm. They undoubtedly represent protein absorption droplets filled with ingested hemoglobin.

Specimen fixed in 1 per cent OsO_4 in acetate-Veronal buffer (pH 7.6), dehydrated in alcohol, stained in block with PTA, embedded in Epon. $Pb(OH)_2$-stained section. × 50,000.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Indirect evidence suggests that occluding zonules may also be impermeable to small molecules and possibly water. They seal up the intercellular spaces of the epithelium in organs which, as part of their function, maintain marked chemical and electrical potential gradients between the lumen and the subepithelial spaces. Recent work on amphibian kidney (56, 57) and urinary bladder (58), for instance, suggests that such gradients are created by the activity of the corresponding epithelia.[6] It should be added that they are successfully maintained, notwithstanding the multitude of intercellular spaces that break the continuity of the epithelium. It is, therefore, reasonable to assume that back diffusion along the intercellular spaces is minimized or prevented. In the interpretation of physiological data, the spaces are either ignored or tacitly assumed to be closed at their luminal end by a cement substance, an assumption which follows old morphological ideas about the nature and topography of the terminal bars. Our observations indicate that the *zonula occludens* must be the site of maximal constraint to diffusion along the intercellular spaces, for the latter appear to be obliterated at the level of this junctional element, at least according to morphological evidence. It should be stressed, however, that the intercellular spaces are closed off near the lumen by the fusion of the adjoining cell membranes, and not by the interposition of a resolvable layer of cement, as previously assumed in the light microscopical literature. This finding is timely for, according to recent work (2, 55), the basis for the whole intercellular cement hypothesis seems to be topographically in error; the hematoxylin-stained or silver-impregnated material of the terminal bar appears to be intra-, not extracellular in location.

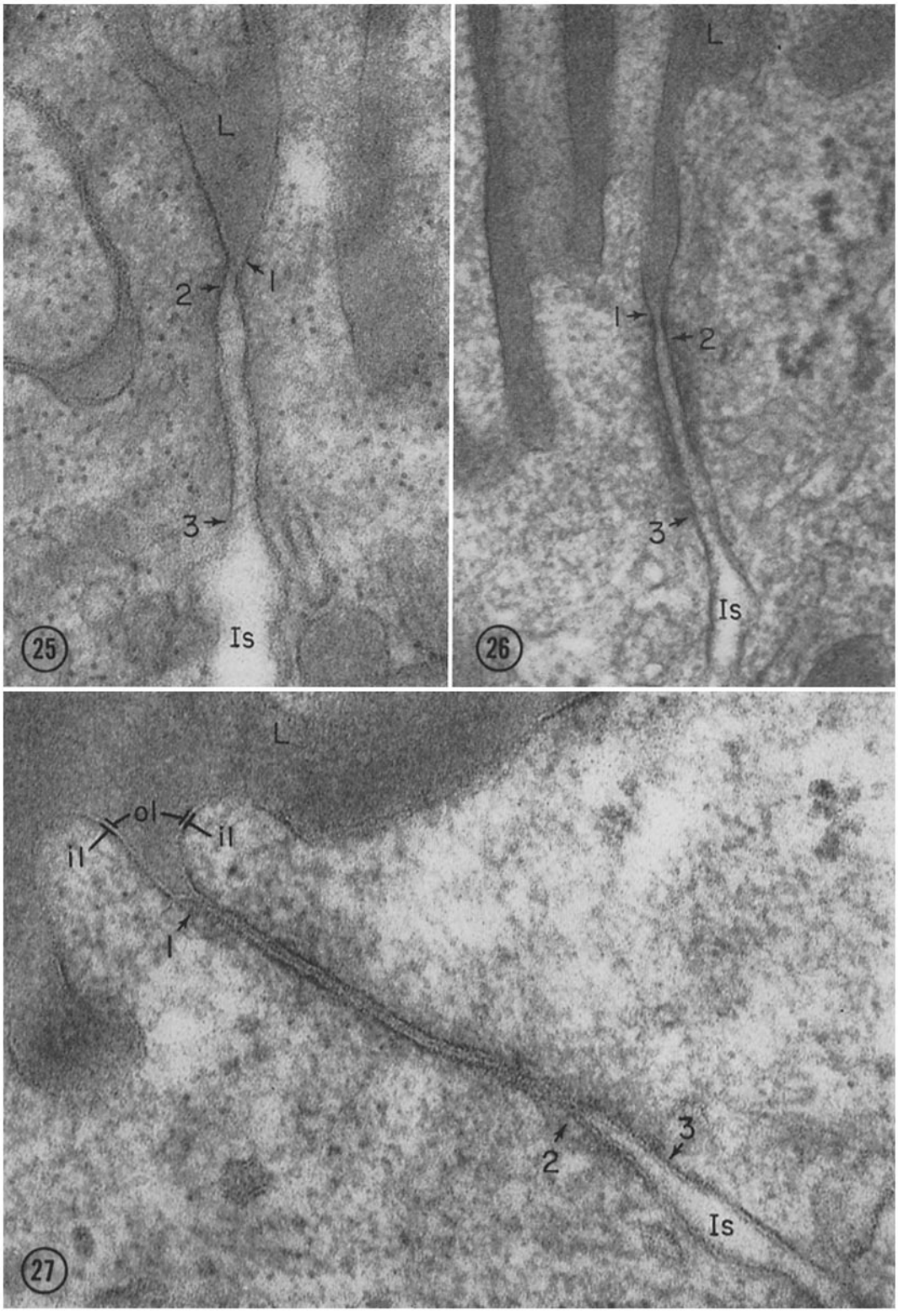
Within the past few years a number of researchers have speculated on the possible influence of intercellular junctions, characterized by membrane fusion and obliteration of intercellular spaces, upon epithelial permeability. Robertson suggested that such junctions may serve to regulate diffusion along the intercellular spaces (32). Peachey and Rasmussen (12) postulated that the zones of extreme narrowing found along the intercellular spaces in the epithelium of the toad bladder may assure an "almost leakproof bladder." Gray (46) suggested that similar junctions ("close contacts") among the glial cells surrounding cerebral capillaries serve to seal off the extracellular spaces and route water and metabolites across

[6] A similar situation applies in the frog skin (59). In this situation, however, the geometry of the epithelium is complicated by stratification, and information about intercellular junctions is still incomplete (*cf.* 1, 60).

FIGURES 25 TO 27

Higher magnifications of intercellular junctions between kidney tubule cells from rats with experimental hemoglobinuria. Figs. 25 and 26 are from the proximal convolution, and Fig. 27 is from a distal tubule. In all the figures it can be clearly seen that the hemoglobin concentrated in the tubular lumen penetrates only down to the level of the occluding zonule and does not reach the adhering zonule or intercellular space (*Is*) beyond. The penetration of the tracer appears to be effectively stopped at the initial fusion point of the adjoining cell membranes that marks the beginning of the occluding zonule (Fig. 27). Note that in the case of the proximal tubule (Figs. 25 and 26) the occluding zonule is very shallow and appears only as a focal (~300 A) stricture (between arrows *1* and *2*) along the otherwise open intercellular spaces, whereas the adhering zonule (between arrows *2* and *3*) is quite deep (~2500 A) and is associated with a dense condensation of cytoplasmic material. In the distal convolution (Fig. 27) the situation is reversed, the occluding zonule (arrows *1* to *2*) is deep (~3000 A) and is followed by a relatively shallow (~800 A) adhering zonule (arrows *2* to *3*). Dense cytoplasmic material is associated with both the occluding and adhering zonules. In the case of the proximal convolution the outer leaflet of the cell membrane and the fusion line are rarely visible in this type of preparation, whereas in the distal segment both leaflets can be clearly seen and followed into the occluding zonule.

Specimens in Figs. 25 and 26 fixed in 2 per cent OsO_4 in acetate-Veronal buffer (pH 7.6) with sucrose and embedded in Araldite. $Pb(OH)_2$-stained sections. The specimen preparation for Fig. 26 is the same as for Fig. 24. Fig. 25, × 135,000; Fig. 26, × 96,000; Fig. 27, × 150,000.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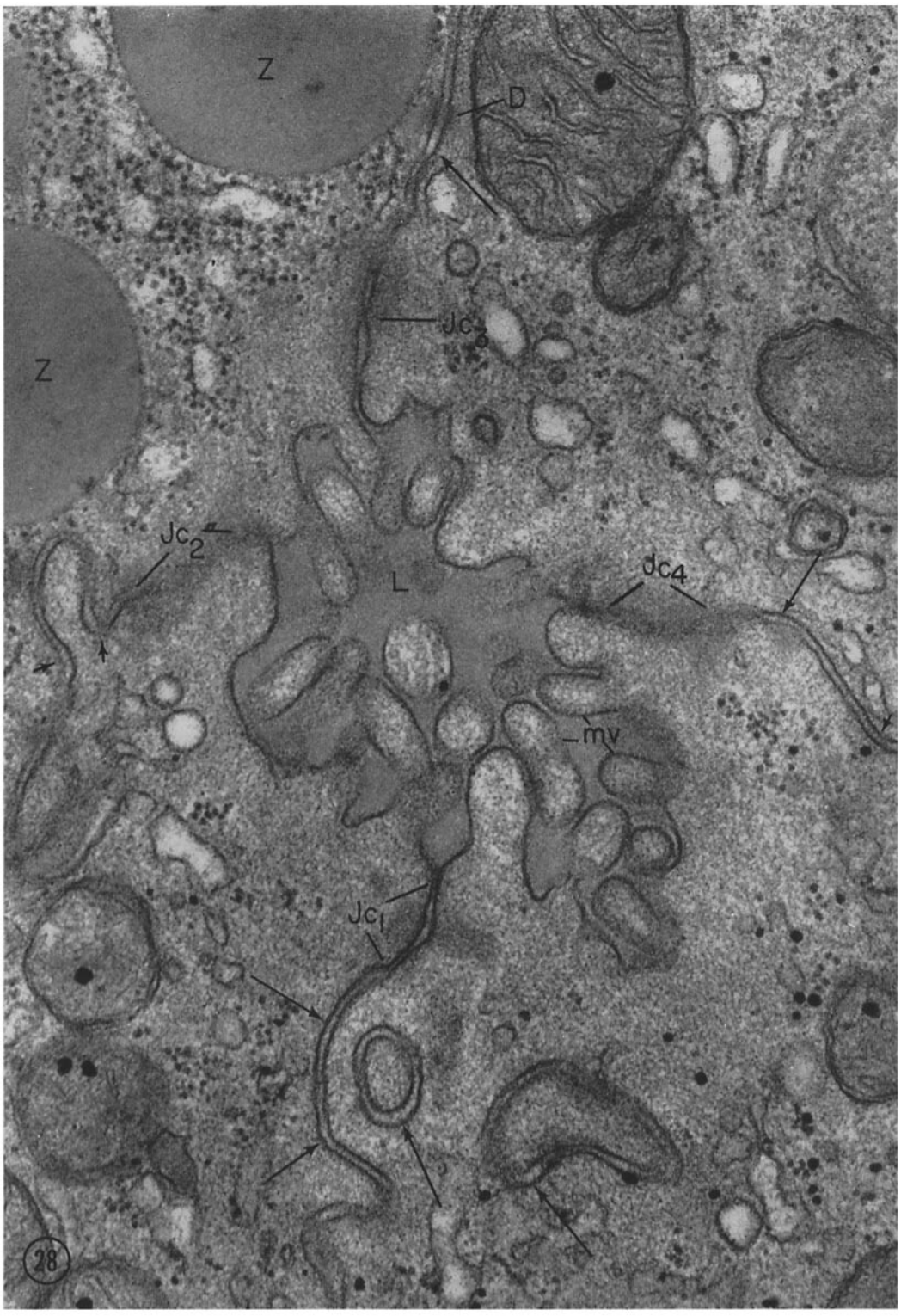
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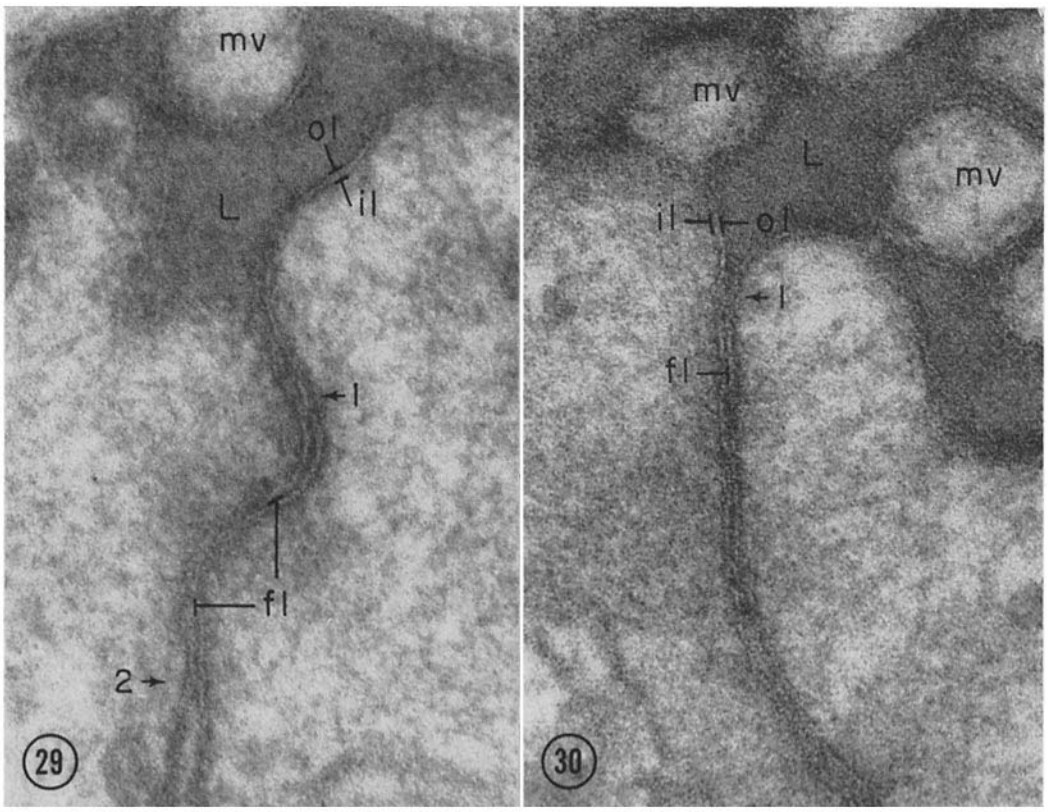

FIGURES 29 AND 30

In these figures the dense mass of discharged zymogen is stopped at the point of mergence of the membranes of two apposed cells into an occluding zonule (arrow *1*). The fusion line (*fl*) is visible in both cases. In Fig. 29 the intercellular space beyond the occluding zonule (arrow *2*) appears free of discharged zymogen.

Specimens fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6), dehydrated in acetone, stained in block with $KMnO_4$, and embedded in Epon. $Pb(OH)_2$-stained sections. × 200,000.

FIGURE 28

This figure together with the following ones (Figs. 29 and 30) illustrate the behavior of discharged zymogen in the acinar and duct lumina of guinea pig pancreas.

In Fig. 28 discharged zymogen, which matches in density the content of zymogen granules (*Z*) completely fills a glandular lumen (*L*) bounded by an acinar and three centroacinar or duct cells. The material that occupies the intercellular spaces beyond the junctional complexes marked Jc_1 to Jc_4 is of noticeably lower density (arrows); the difference shows to better advantage in normally or nearly normally sectioned spaces (long arrows). A single junctional complex (Jc_1) is normally cut. At its level it is clear that the mass of discharged zymogen stops at the occluding zonule. The situation at the other junctional complex is obscured by the obliquity of their section.

Specimen fixed in 1 per cent OsO_4 in phosphate buffer (pH 7.6) and embedded in Epon. Section stained in uranyl acetate and $Pb(OH)_2$. × 58,000.

glial cells. Finally, Muir and Peters (13) recently found similar junctions in the endothelium of blood capillaries and predicted that comparable arrangements will be detected "wherever a sheet of cells separates two zones of different constitution in order to prevent intercellular diffusion of ions and small molecules."

Our studies, initiated and carried out independently of those of Muir and Peters, confirm their hypothesis as far as morphological aspects are concerned, and provide preliminary evidence for the physiological speculations of the other authors (12, 32, 46, 47) previously mentioned. Moreover, our findings suggest that the elements described in the junctional complexes of epithelia are functionally specialized: the occluding zonule as a diffusion barrier or "seal," and the desmosomes (adhering maculae) as intercellular attachment devices, with the adhering and probably the occluding zonules participating also in the latter function.[7] Further work on the junctions found in simple and stratified squamous epithelia, especially in vascular endothelia[8] may further clarify this assumption.

Finally, it should be mentioned that at least in one case there is evidence that one type of junction can be replaced by another in relation to certain changes in function. Our previous studies (16, 19) have shown that a special type of junction, *i.e.* the urinary slits of the renal glomerular epithelium, are progressively replaced by "tight junctions" during the development of an experimental nephrosis in rats. In a reversed situation, Vernier and Birch-Andersen (61) have recently shown that the "tight junctions" present in the visceral epithelium of immature human glomeruli, are replaced during maturation by normal urinary slits. These findings are consistent with the assumption that these slits, whose partial resemblance to desmosomes and adhering zonules has already been discussed, are permeable to the glomerular filtrate, whereas "tight junctions" are not.

[7] Preliminary observations indicate that epithelial cells remain attached to one another at the level of the desmosomes, as well as at that of the adhering and occluding zonules, when the cell bodies retract and the intercellular spaces greatly enlarge, under the influence of hypertonic treatment prior to or during fixation.

[8] Our findings indicate the presence of tight junctions in the peritoneal mesothelium and, in confirmation and extension of references 32, 13, and 47, in the endothelium of all types of blood capillaries. In the latter case the occluding zonule frequently takes the form of a focal stricture or "pinch" similar to the situation described above in the proximal convolution, the adhering zonule is missing or poorly developed, and desmosomes are generally absent, at least in mammalian material.

Comments on Structural Variations Encountered in Cell Membranes

The current prevailing tendency is to regard all cell membranes (as well as those of most intracellular organelles) as similar if not identical in structure. This trend is due mainly to the extensive studies of Robertson (28, 31–33, 62) who has shown that all cellular membranes have a similar trilaminar appearance in $KMnO_4$-fixed tissues, and hence postulated that they are all composed of a single bimolecular leaflet of polar lipids, sandwiched between two dissimilar layers of non-lipid material. His concept has derived additional strength from its agreement with older, indirect evidence concerning the molecular architecture of the cell membrane (*cf.* 63). So strong has been the concentration on Robertson's hypothesis of the "unit membrane" that relatively little attention has been paid to variations in membrane structure visible after OsO_4 fixation.

Our survey, carried out primarily on OsO_4-fixed tissues, confirms that the cell membrane has the same trilaminar structure in all epithelial cells investigated, but in addition reveals that this basic pattern shows significant variations primarily in respect to the total thickness of the unit and the thickness and density of its outer leaflet. These differences seem to us of sufficient importance to warrant separate discussion.

Based on their appearance after OsO_4 fixation, the cell membranes we have studied can be separated into two distinct types. The first is limited in distribution to the luminal (apical) aspects of some absorptive epithelia (intestine, stomach, colon, and gall bladder) and certain segments of the nephron (see below); it is relatively thick ($\sim$110 A), and its outer layer shows up clearly, especially after lead staining, wherever the membrane is normally sectioned. As a result, the membranes in this category appear nearly symmetrical, the outer leaflet being only slightly less dense than the inner one. The other type of membrane occurs more widely: it is found along the lateral and basal surfaces of the absorptive epithelia just

mentioned, and along the entire perimeter of all other epithelial cells studied, *i.e.* gland and duct epithelia of the liver, pancreas, and thyroid, epithelia of the uterus and oviduct, mesothelia and capillary endothelia. In these locations the total thickness of the plasmalemma, like that of Schwann cells (28, 31, 32), is significantly less (70 to 80 A); furthermore, the membrane is highly asymmetrical: even after lead or uranyl staining, the outer leaflet is generally much finer and less dense than the inner one. In certain cases it is indeed hardly visible. The same layer is readily visualized, however, in tissues that have been fixed in OsO_4 and stained in block with $KMnO_4$ prior to embedding (24) or in sections which have been doubly stained, first with uranyl and then with lead. It should further be noted that within each of these groups there is a certain amount of further variation according to cell type: in the first group, for instance, the luminal membrane of gastric and, in some cases, colonic epithelia has an outer leaflet which is thicker and denser than the inner one; hence in this situation the usual asymmetry of the cell membrane is reversed. In the second group, the cell membrane of gland and duct cells is more asymmetrical than the membrane covering the lateral and basal aspects of absorptive epithelia; furthermore, the luminal membrane of many gland and duct epithelia (*e.g.* thyroid, liver, and parotid) is less asymmetrical than the lateral and basal membranes of the same cells (*cf.* Fig. 15.)

It will be noted that while membranes of the first type were found to occur, so far, mainly along the luminal surfaces of absorptive epithelia, not *all* absorptive epithelia possess this type of luminal membrane. In this respect, the most remarkable variation is encountered along the various segments of the nephron where the luminal cell membrane of glomerular epithelia (*cf.* 19) and the luminal membrane of the cells of distal tubules and collecting ducts are of the first (thick, nearly symmetrical type) whereas the entire membrane of the cells lining the proximal tubule and the lateral and basal membrane of the cells lining the distal tubule and collecting duct are of the second, (thin, symmetrical) type.

There are in the literature several comments on structural differences encountered in OsO_4-fixed cell membranes: Sjöstrand (34) has stressed the fact that the plasma membrane can appear rather different along various parts of the surface of the same cell. Wissig (64) has also called attention to differences between the apical and basal or lateral plasmalemmata in various types of nephron epithelia; moreover he has utilized these differences to classify pinocytic vesicles into different groups, based on their type of limiting membrane. Mercer (65) has noted that a typical stratification is regularly seen in the surface membrane of amebae, but is less apparent in other membranes of the same cell. We have called attention to the fact that the cell membrane of one cell type, the glomerular epithelial cell, differs from that of other cell types in the same section (19). Several investigators (19, 66–69) have shown that the plasmalemmata of certain cell types (or of certain aspects of the same cell, 67, 69) respond selectively to staining with PTA.

Although Robertson has attached little importance to such differences and attributed them to deficiencies in OsO_4 fixation,[9] it seems to us that they deserve closer scrutiny. The different patterns described are consistent and, as such, probably valid. Such findings do not challenge the unit membrane hypothesis; they suggest only that the composition of the membrane layers varies with the cell type and the environment the cell faces. It is hoped that the morphological differences described will eventually be correlated with distinctive functional characteristics of the plasmalemma in different locations (and of the limiting membranes of various cellular organelles), but for the moment such a correlation is not yet possible.

Part of this work was presented at the First Annual Meeting of the American Society for Cell Biology, Chicago, November 2 to 4, 1961 (17).

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[9] In the case of OsO_4-fixed intestinal epithelium, Robertson agreed (*cf.* 32) that the differences in the appearance of the membranes lining the microvilli, on the one hand, and the remainder of the cell surface, on the other, "no doubt reflect underlying chemical differences in the components of the membranes."

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