A FINE STRUCTURAL ANALYSIS OF INTERCELLULAR JUNCTIONS IN THE MOUSE LIVER

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ABSTRACT

Zonulae occludentes and gap junctions were examined both in the intact mouse liver and in a junction-rich membrane fraction from homogenized mouse liver. These preparations were visualized with the techniques of uranyl acetate staining en bloc, staining with colloidal lanthanum, negative staining with phosphotungstate, and freeze-cleaving. The zonula occludens is arranged as a meshwork of branching and anastomosing threadlike contacts sealing the lumen of the bile canaliculus from the liver intercellular space. The gap junction is characterized in section by a 20 A gap between the apposed junctional membrane outer leaflets, and permeation of this space with lanthanum or phosphotungstate reveals a polygonal lattice of subunits with a center-to-center spacing of 90–100 A. Freeze-cleaved gap junctions show a similar lattice. Extraction of junction-rich fractions with 60% aqueous acetone results in a disappearance of the 20 A gap in sectioned pellets and an inability to demonstrate the polygonal lattice with either the freeze-cleave or negative staining techniques. Extraction of the membranes with 50% acetone does not produce this effect. Thin-layer chromatography of the acetone extracts reveals a group of phospholipids in the 60% extract that are not detectable in the 50% extract. Acetone does not cause any detectable change in the structure of the zonula occludens, but the occluding junction becomes leaky to lanthanum following acetone treatment. The effects of other reagents on the junctions are reported.

INTRODUCTION

Farquhar and Palade (19, 20) describe the zonula occludens as an area of cell contact at the luminal border of epithelial cells in which there is apparent fusion of plasma membrane outer leaflets with consequent obliteration of the extracellular space. The zonula occludens, which forms a complete belt around some epithelial cells, has often been called a "tight junction," but this term has also been used to describe cell junctions in other locations on the cell surface in which there appears to be a "fusion" of plasma membrane outer leaflets.

Recent evidence, obtained with uranyl acetate staining en bloc, suggests that in some of these junc-

tions the extracellular space is actually not obliterated, but rather that a 20 A "gap" remains between the two closely apposed cell membranes (37). The "gap junction," in contradistinction to the zonula occludens, is permeable to colloidal lanthanum. In oblique sections through gap junctions containing lanthanum, a polygonal lattice of substructures is outlined by the heavy metal. A polygonal lattice has been observed with several techniques. The first description of the lattice was given by Robertson (41) in Mauthner cell synapses following a permanganate fixation. Benedetti and Emmelot (2–4) have described a similar substructure in isolated liver plasma membrane fractions

following negative staining with sodium phospho-tungstate (Na-PT), and Kreutziger (27, 28), using the freeze-cleave technique, has reported the presence of a polygonal lattice in liver cell junctions.

This paper will present evidence that the gap junction and the zonula occludens are distinct structures. In the mouse liver, which we have studied, the two junctions occupy characteristic positions between adjacent hepatocytes. The zonulae occludentes are found along the edges of bile canaliculi and form an extended linear seal between bile and intercellular spaces. The gap junction is found in macular or plaquelike areas along the boundaries of hepatocytes between the bile canaliculus and the space of Disse. The two junctions can be distinguished also by uranyl acetate staining en bloc, negative staining with Na-PT, staining with colloidal lanthanum, and by freeze-cleaving. Techniques are used which allow us to study the two junctions in isolated homogenates as well as in whole liver and to compare their resistances to several chemical reagents.

MATERIALS AND METHODS

Mature mice obtained from the Charles River Breeding Laboratory were used throughout.

Fixatives

All aldehyde fixation was carried out in 3% glutaraldehyde in 0.2 M s-collidine (5) pH 7.5. Postfixation was done with a 2:1 mixture of 2% aqueous OsO_4 and collidine buffer. If lanthanum was used, the fixative was a 1:1 mixture of fixative and 4% $LaNO_3$, buffered slowly to pH 7.7 with 1 N NaOH (37), the final concentration of aldehyde being 3%. Postfixation was done in a 1:1 mixture of the OsO_4-collidine used above and the 4% lanthanum solution. Recent experiments have shown that lanthanum at pH 7.7 is a colloid which is retarded by gel filtration on a Sephadex G-10 column (J. P. Revel and K. Wolken, 1969, unpublished observations).

Chemical Treatment

Whole livers and isolated membrane fractions were treated with sodium desoxycholate (1%, pH 8.0, from Sigma Chemical Co., St. Louis. Mo.), 4, 6, and 8 M solutions of urea, and 0.02 M EDTA (disodium salt). All of the above reagents were added to the primary perfusate for treatment of whole liver, or were added to a 0.001 M solution of $NaHCO_3$ for treatment of isolated membranes. Acetone treatment is described below.

Fixation of Whole Liver

Livers were fixed at room temperature by aldehyde perfusion through the portal vein at a pressure of 10 cm H_2O for 10 min. Perfusion with aldehyde was usually preceded by a 1-min perfusion with 0.5% $CaCl_2$ in isotonic saline. Chemicals perfused through the liver were added to this first perfusate. The calcium salt was omitted from the perfusate during the EDTA runs.

After the 10 min perfusion, livers were removed from the animals, cut into 1 mm cubes, and fixed in aldehyde for an additional hour before further treatment. Whole liver to be treated with acetone was then immersed in three 15-min changes of cold acetone (Fisher Scientific Company, Pittsburgh, Pa., electronic grade) at the appropriate concentration and was allowed to remain at 2°C in the third change for ½ hr. The tissue was then rapidly rehydrated by decanting the acetone and adding cold collidine buffer in two rinses. This treatment was followed by a 2 hr fixation in OsO_4 at 0°C. The tissue was usually stored overnight in collidine at 2°C.

The liver pieces were then stained en bloc with 2% uranyl acetate for 1 hr (25) and then rapidly dehydrated in a graded series of cold ethanols and embedded in Epon-Araldite (32). Sections were cut on a Cambridge Ultramicrotome (Cambridge Instrument Co., Inc., Ossining, N. Y.) with a diamond knife, picked up on uncoated 300 mesh copper grids, stained with lead citrate, and examined in a Siemens Elmiskop IA.

Freeze-Cleave

Freeze-cleaved replicas of liver were obtained by using the method of Bullivant and Ames (10). Aldehyde-fixed tissue was equilibrated for at least 20 min with 20% glycerol in collidine buffer before quick-freezing in Freon 22, precooled to a mixture of solid and liquid in liquid nitrogen (−158°C). Platinum carbon pellets were evaporated at high current on freshly cleaved tissue surfaces at an angle of 45° at a vacuum of 10^{-5} torr in a Kinney Vacuum Evaporator (Kinney Vacuum Co., Boston, Mass.). Carbon was then cast in a similar manner, normal to the cleaved surface. After thawing, the tissue was digested from the replica in a 10% Clorox solution. The replicas were washed in distilled water, picked up on unsupported 400-mesh grids, and examined in a Siemens Elmiskop IA.

Isolation of Junctions

The isolation procedure used was that of Neville (34), with the modification of an additional sucrose layer of d = 1.18 between layers d = 1.16 and d = 1.20, suggested by Emmelot et al. (18). The whitish-

tan layer at the interface between d = 1.18 and d = 1.16 was collected, washed free of sucrose, and resuspended in the appropriate chemical agent. Aqueous acetone concentrations of 40, 50, 60, 70, and 80% were routinely used on the membrane preparations. When thin-layer chromatography was to be performed on the acetone extract, 0.005% BHT (2,6-di-*tert*-butyl-4-methylphenol [Aldrich Chemical Co., Inc., Milwaukee, Wis.]) was added to the acetone as an antioxidant (53). After a 15 min treatment with a chemical, the plasma membranes were pelleted for 10 min at 10,000 rpm in a Sorvall SS-1 (Ivan Sorvall, Inc., Norwalk, Conn.). Supernatants were decanted and saved, and the pellets were fixed in aldehyde and OsO₄. Pellets were stained and prepared for electron microscopy as described above. All manipulations of the membrane fractions, up to and including fixation, were carried out at 0–2°C.

Thin-Layer Chromatography

30 g of silica gel, without CaSO₄ binder (Camag type D-0 [Arthur H. Thomas Co., Philadelphia, Pa.]), and 3 g of magnesium silicate (Absorbosil M-2, Applied Science Labs, Inc., State College, Pa.) were slurried with 80 cc of distilled water and spread 0.25 mm thick with a Desaga applicator (Brinkmann Instruments, Westbury, N. Y.) on five 20 × 20 cm glass plates. Dried plates were activated at 110°C for 1 hr before use. The supernatants that were recovered following the pelleting of the membranes described above were further centrifuged in a Spinco Model L (Beckman Instruments, Inc., Palo Alto, Calif.) at 40,000 rpm for 2 hr in the No. 40 rotor. The acetone in these supernatants was evaporated under N₂ at 37°C, and the residual water was evaporated *in vacuo* with Drierite and H₂SO₄ at 37°C. The dried residue was dissolved in 1 ml of redistilled petroleum ether (b. r. 40–60°C) for thin-layer chromatography (TLC), or in the alkaline copper reagent for protein determination by the method of Lowry et al. (30). For TLC, 50 µl of the sample was spotted on activated silica plates and developed under saturated conditions in Brinkmann tanks lined with No. 1 Whatman chromatography paper.

The solvent system used for one-dimensional separation of phospholipids was chloroform-methanol-water 65:25:4 (52). For two-dimensional phosphatide separation, the first direction was chloroform-methanol-water 65:25:4, and the second direction was *n*-butanol-acetic acid-water 60:20:20 (42). For separation of neutral lipids, the two step method of Skipski et al. (45) was used: develop first in isopropyl ether-acetic acid 96:4, dry plates for 45 min, then develop second step in petroleum ether-ethyl ether-acetic acid 90:10:1. Lipids were detected by spraying plates with 50% H₂SO₄ in saturated K₂Cr₂O₇ or 50% H₂SO₄ alone and heating for one hr at 110°C.

Negative Stain

Membranes were stained in suspension with 1% Na-PT adjusted to pH 7.0 with NaOH on parlodion-coated 200-mesh grids.

OBSERVATIONS

Morphological Characteristics of Gap Junctions

The gap junction in routine preparations, stained en bloc with uranyl acetate, is readily recognized even at low magnification in sectioned tissue (Fig. 2) as a darkly staining area of close cell-to-cell apposition, with a long, gently undulating contour. Higher magnification reveals a 20 A gap between the apposed plasma membranes of the junction (inset, Fig. 2). This gap is very constant in width along the length of the junction, and there appear to be no structures in the gap under these conditions.

When lanthanum is used to delineate the extracellular space, it permeates the 20 A wide space between the apposed junctional membranes of the gap junctions. Inspection of lanthanum-impregnated gap junctions in tangential views reveals regularly packed substructures, seen in Figs. 5 and 14, which lack intrinsic electron opacity, as previously described by Revel and Karnovsky (37). These substructures, or particles, may be seen in sectioned tissues only when outlined by an electron-opaque material like lanthanum. Since the lanthanum is limited to the 20 A space of the gap junction and does not penetrate beyond the outer leaflet of the "unit" membrane, as seen in cross-sectioned views, it follows that at least part of the particles outlined in tangential views are situated in the 20 A gap. The particles are closely packed, in most areas, into what appears to be an hexagonal lattice. The lattice is not regular, however, and shows interruptions and areas of nonhexagonal packing. Hence, the lattice is more correctly described as polygonal (9), as opposed to hexagonal (37). The particles themselves have been described as "hexagons," but we have been unable to confirm that the particles are actually hexagonal in outline. The subunits in the gap junction, as seen with lanthanum, show a center-to-center spacing of 90–100 A. Other dimensions of the lattice have been previously published (37).

Gap junctions as seen in isolated membrane pellets after sectioning have much the same appearance as those in intact tissue. In Fig. 1, the

junction appears to be a more rigid structure than the undulating profiles of nonjunctional plasma membrane with which it is continuous. In our material, the 20 A gap is maintained, despite the considerable changes in the environment of the junction as compared to its in vivo state. The inner leaflets of the junctional membranes, in contrast to the other profiles in the pellet, have very little associated amorphous material.

In preparations of the isolated membrane fractions, after negative staining with Na-PT, a polygonal lattice is present that is similar to the one detected with lanthanum in tissues. The dimensions of the lattice and of the particles are similar to those seen in lanthanum-stained preparations, and in freeze-cleaved specimens.

In nonjunctional areas of cell surface exposed by freeze-cleaving, plasma membranes display either one of two appearances, each of which is believed to represent different fracture faces of the membrane (for a review, see 26). One membrane surface is sparsely studded with particles and corresponds to the intracellular membrane face (IMF), while the other has a dense population of particles and corresponds to the extracellular membrane face (EMF). There is no general agreement as to the nature of these particles, and there is no reason to believe that they form a homogeneous population. In freeze-cleaved gap junctions, one can also recognize two faces, as described by Kreutziger (27, 28): a particulate polygonal lattice appears coplanar with the EMF, and a polygonal lattice of "pits" or depressions appears coplanar with the IMF. Both junctional faces may be seen in the replica in Fig. 3.

The particles in the freeze-cleaved polygonal lattice show the same distribution and 90-100 A center-to-center spacing as those revealed by negative and lanthanum staining. Tiny depressions (pits), seen in the alternate view of the junction, are smaller in diameter than the particles, and hence appear to be more widely separated, although they also show a center-to-center spacing of 90-100 A. Both the "pitted" and "particulate" lattices have the same density of subunits, approximately 6200 per square micron.

As the plane of cleavage passes from one face of the membrane to the other, the transition between the pitted and particulate views of the junction is seen as a serrated edge (single arrow in Fig. 4). No serrations can be seen in areas where the cleavage plane leaves the pitted surface but does not enter

the particulate polygonal lattice (double-headed arrow in Fig. 4), indicating that the serrated edge is related to the presence of the two lattices.

Close examination of the serrated edge between the two views of the junctional membranes suggests that the pits correspond to some of the spaces between the particles and not to the center of the particles themselves. In favorable views (single-headed arrow, Fig. 4), the pits closest to the serrated edge are seen in line with the spaces between the immediately adjacent particles of the particulate lattice. The pits, however, cannot be considered mere depressions in a plastic membrane which is molding itself into the clefts between the adjacent units of the particulate lattice. If this were so, one would expect twice as many pits per square micron as there are particles. In fact, one observes an equal density per square micron of the two kinds of subunits. The data available at the present do not indicate that the pits are openings of channels passing through the junctional membranes, but it is not possible to eliminate this suggestion.

Characteristics of the Zonula Occludens

In sections of whole liver, the zonula occludens is found immediately adjacent to the bile canaliculus (Fig. 2). Typically (19), one can see in it a series of punctate contacts where the outer leaflets of the apposed plasma membranes appear "fused" (Fig. 8). In sections of pellets of isolated liver cell membranes (Fig. 7), the occluding junctions are characterized by a similar series of punctate or focal contacts when seen in profile. Here, as in sections of intact liver, the cytoplasmic face of the junctional membranes is covered by an electron-opaque, fuzzy material, unique to this type of junction. This dense material was described by Farquhar and Palade (19) and may well be the hepatocyte's equivalent of the terminal web associated with zonulae occludentes of other epithelia.

Lanthanum treatment brings out marked differences between the gap junction and the zonula occludens. As already indicated, the gap junction does not present a barrier to the passage of lanthanum. The lanthanum passes readily around the macular gap junctions and also permeates the 20 A gap within the junction itself, delineating a typical polygonal lattice. The zonula occludens, however, does not permit the passage of the tracer beyond the first or second point of focal contact of the membrane's outer leaflets (except after chemical modifications which are described below). In-

troduction of the heavy metal tracer with the fixative via the portal vein results in staining of the extracellular space. The lanthanum does not enter the lumen of the bile canaliculus (Figs. 13 and 14), and high magnification views show that the metal is indeed stopped at the level of the punctate contacts of the zonula occludens (Fig. 14, *inset*) (39).

Freeze-cleave replicas of the zonulae occludentes appear as described by Kreutziger (27, 28). Again, as is the case with the gap junctions, there are two views of the zonulae occludentes which correspond to the two membrane faces. In Figs. 10 and 11, long branching and anatomosing "chains" of particles may be seen on the extracellular membrane face. In the alternate view, coplanar with the intracellular membrane face, the zonula occludens is seen as a network of grooves or troughs which have a geometry similar to that of the chains (Figs. 11 and 12). Chains or grooves are found in the same area adjacent to the bile canaliculus as the punctate contacts seen in sectioned material. If one considers each point of contact in sectioned material as a cross-section through one of the chains seen in freeze-cleaved preparations, one can easily appreciate the relationship between the different images produced by the two techniques. Staehelin has recently published a freeze-etch study of the zonula occludens in the mouse intestine (47). The images he has obtained are generally similar to the ones presented here, al-

though there are differences in the interpretation of some details.

Soaking the tissue blocks in acetone before fixation in osmium tetroxide seems to alter the permeability seal of the zonula occludens. Under these conditions, lanthanum can usually be found in the bile canaliculus and appears to surround the points of contact of cell membranes at the zonula occludens. In favorable tangential sections (Fig. 15) one can clearly recognize the threadlike nature of the cellular contacts. In fact, these "negatively stained" views of the zonula occludens resemble very closely the grooves or chains seen in freeze-cleaved preparations (see Fig. 12).

The threads or chains of the zonula occludens may be viewed by yet another technique. This consists of briefly treating the membrane pellets with DOC before negative staining with Na-PT, as suggested by Benedetti and Emmelot (3). Negatively stained surface views of the zonula occludens in such preparations reveals the long, anastomosing, threadlike structure of the zonula occludens, as illustrated in Fig. 9. Comparison of this figure with the freeze-cleaved replica in Fig. 10 shows that the anastomosing and branching pattern resulting from both techniques is similar. It is interesting that treatment of the pellet with DOC seems to be necessary to achieve this negatively stained view of the zonula occludens. It may be that the DOC causes a permeability change in the zonula occludens, allowing it to be permeated by the Na-PT.

FIGURE 1   An electron micrograph of a sectioned isolated membrane pellet. Both a zonula occludens (ZO) and a gap junction (GJ) may be seen, although at this magnification one cannot clearly recognize the details of plasma membrane contact. The zonula occludens has a dense, amorphous material associated with the inner leaflets of the plasma membrane. The adjacent zonula adherens has strands of filaments reminiscent of tonofilaments [to]. The gap junction (between the two large arrows) does not display these densities and appears smooth along its slowly undulating cytoplasmic faces. The insert shows part of a gap junction at much higher magnification. The electron-lucent space, characteristic of the gap junction after uranyl acetate staining en bloc is indicated by the two fine arrows. × 72,000; inset, × 420,000.

FIGURE 2   A low power view of sectioned mouse liver showing an area of apposition between two hepatocytes. At the top of the figure a bile canaliculus (BC) is seen. Immediately adjacent to this structure is a zonula occludens (ZO) which is always found separating the lumen of the bile canal and the liver intercellular space. At some distance from these structures a gap junction (GJ) is seen, recognizable at low power as a darker-staining area of close cell-to-cell apposition. Mitochondria (M) and lipid droplets (L) are seen in the cytoplasm of the hepatocytes. The inset is a high magnification view of a portion of a gap junction in tissue, after uranyl acetate staining en bloc. The uniform gap is delineated by the two fine arrows. There is a great similarity between the isolated and the in situ gap junctions, seen in the insets in Figs. 1 and 2. × 32,500; inset, × 420,000.
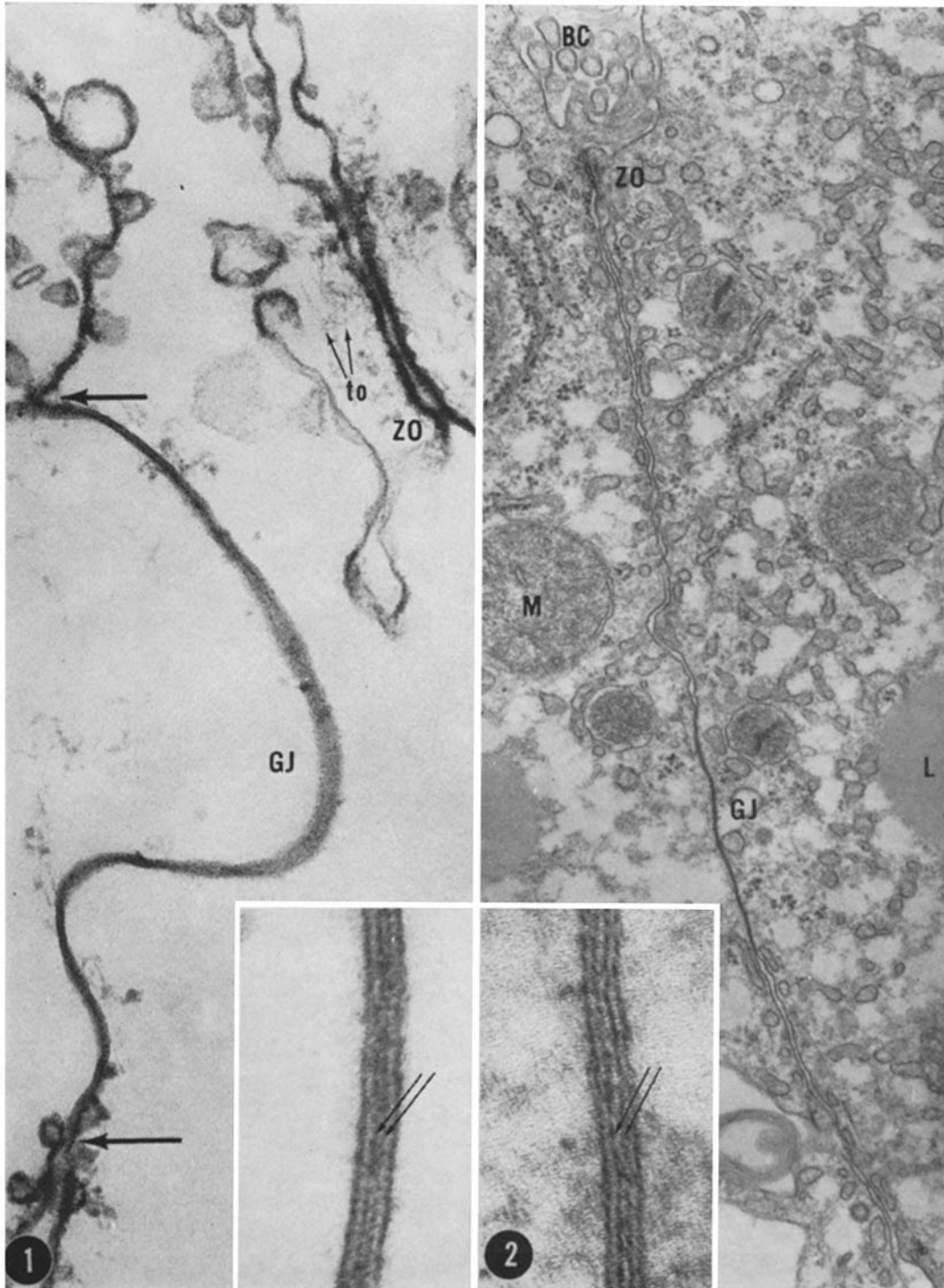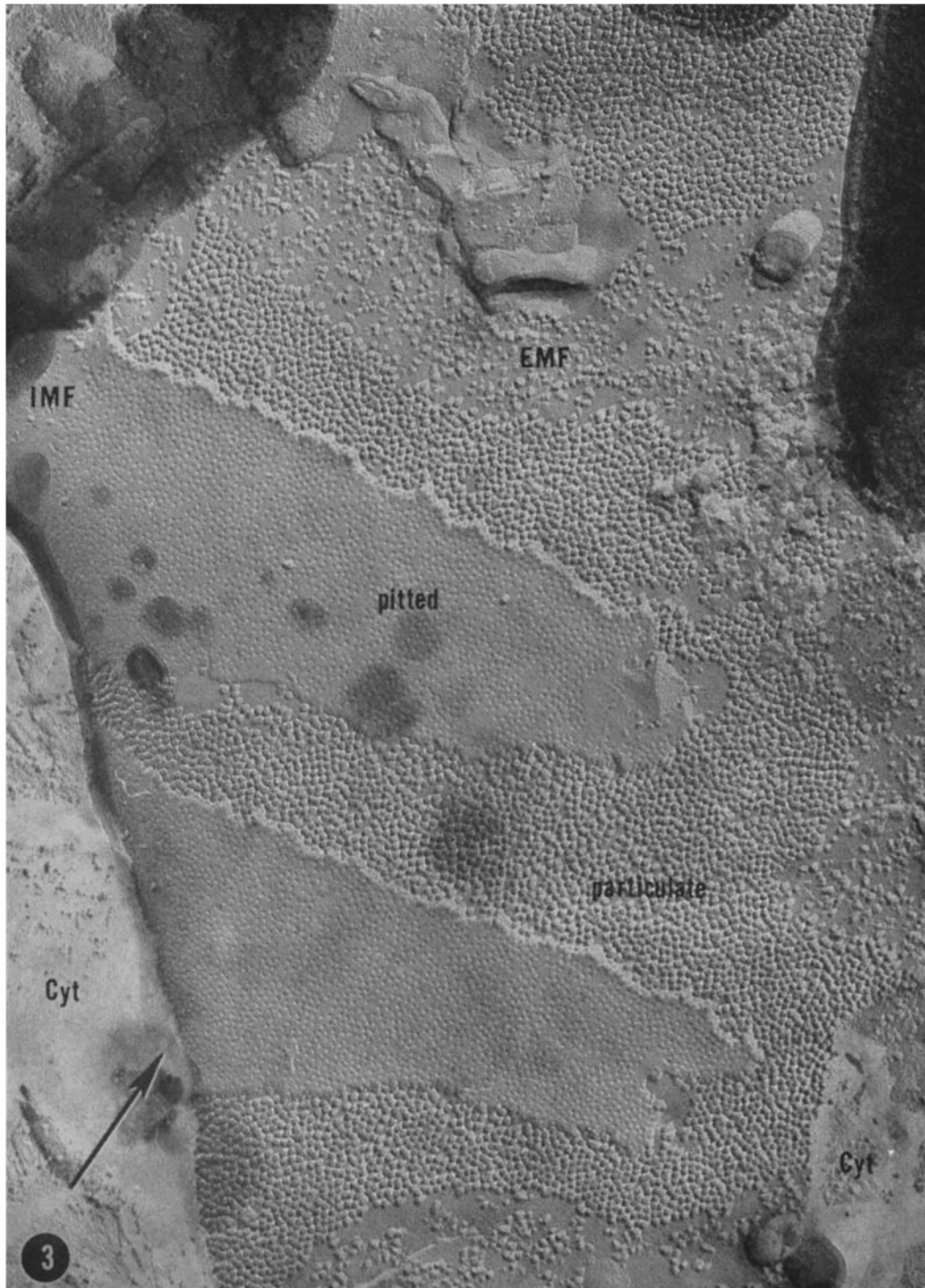
FIGURE 3   A replica of a freeze-cleaved gap junction from aldehyde-fixed whole mouse liver. Both the pitted and particulate lattices of the two fracture faces of the gap junction may be seen. The particulate lattice is seen to be coplanar with the extracellular membrane face (EMF) and the pitted lattice is coplanar with the intracellular membrane face, a small area of which can be seen at IMF. The cytoplasm (*Cyt*) of the two apposed hepatocytes can also be seen. The large black and white arrow indicates the direction of the shadow. × 102,500.

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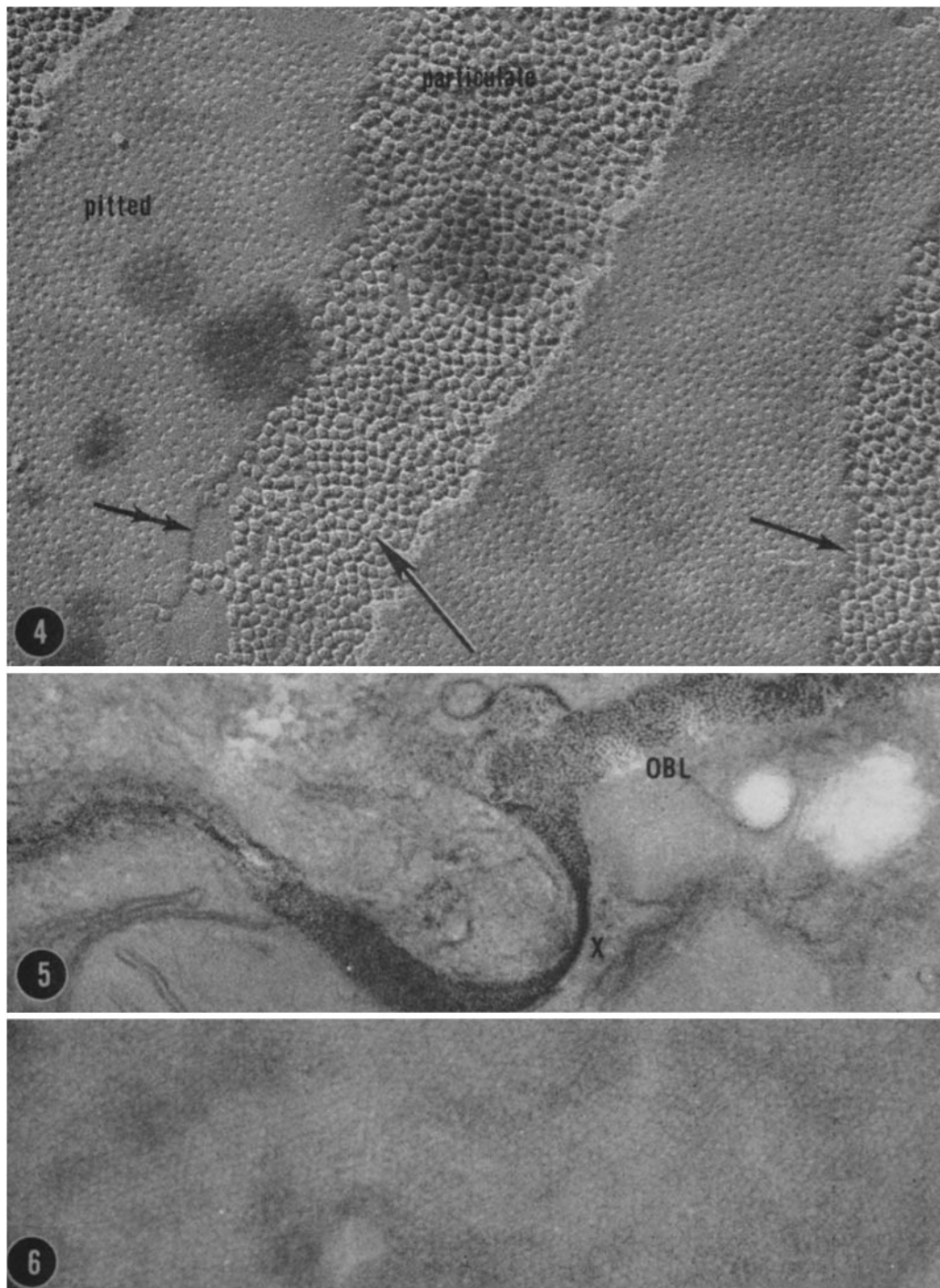
FIGURE 4 A higher magnification view of a portion of Fig. 3. The particulate and pitted views of the freeze-cleaved gap junction are seen. At the border between these two views of the gap junction one sees a serrated edge (single-headed arrow at right). When the two lattices are not contiguous (double-headed arrow), the border of the two planes is seen as a smooth edge instead of a serrated edge. The pits may be seen to line up with the spaces between the particles, and not with the particles themselves (single-headed arrow). The center-to-center distance between the subunits in both lattices is 90–100 A, similar to the dimensions of the lattice seen with lanthanum and Na-PT. × 190,000.

FIGURE 5 A view of a gap junction stained with colloidal lanthanum in a section of whole mouse liver. The junction is seen in cross-section at X, and as it turns obliquely in the plane of the plastic section at OBL, the polygonal lattice may be seen in near-tangential view. It is the spaces between the subunits of the lattice that are stained by the lanthanum. × 80,000.

FIGURE 6 A phosphotungstate (Na-PT) negatively stained gap junction from an isolated membrane pellet. The spaces between the particles of the polygonal lattice are stained, giving a picture similar to the lanthanum preparation in Fig. 5. Except for areas of uneven staining, the lattice appears homogeneous and uninterrupted. × 160,000.

Manipulations necessary to embed the DOC-treated pellet in plastic seems to result in a complete loss of the zonulae occludentes, for only the gap junctions can be found in sections of DOC-treated pellets.

The Effect of Acetone on Gap Junctions and Zonulae Occludentes

Treatment of either whole tissue or of membrane pellets with acetone causes a characteristic and reproducible change in the morphology of the gap junctions. As shown in Fig. 16, the 20 A gap disappears and the junction assumes a pentalaminar appearance. The over-all width of the extracted gap junction, as measured from the inner leaflets of the junctional membranes, remains approximately 140–150 A, the same as the dimensions of the controls. Concurrently with the obliteration of the gap, it becomes impossible to demonstrate the polygonal lattices usually seen in freeze-cleaved or negatively stained preparations. Attempts to freeze-cleave

acetone-extracted material were carried out on fixed tissue, extracted with 60% acetone. Under these conditions, membrane faces were still observed, including the freeze-cleave patterns of the zonulae occludentes, but no evidence of the polygonal lattice of the gap junctions was observed. Only the particles which characterize nonjunctional membrane faces could be found after 60% acetone extraction. Negative staining was carried out on isolated 60% acetone-extracted preparations which were rehydrated before application of Na-PT. As in the freeze-cleave preparations, numerous membranous elements could be visualized with the negative stain, but no areas with the distinctive polygonal lattice of subunits could be found. 95% ethanol or 70% methanol will produce the same loss of the 20 A gap when used in the same conditions as the acteone.

It is difficult to account for the disappearance of the gap and of the gap particles while the 140–150 A width of the junctions is maintained in section. This difficulty may well be due to the physical

FIGURE 7   A section of plastic-embedded, isolated membrane pellet. A zonula occludens (ZO) is seen immediately adjacent to part of a bile canal (BC). The junctional membranes of the zonula occludens approach one another closely at several points. Next to the occluding junction is a macula adhaerens (MA) which, like the zonula occludens, shows dense amorphous material on the cytoplasmic surface of the junction; this material is not seen on the other membrane profiles. The macula adhaerens also displays some intercellular material between the apposed junctional membranes. The insert shows a punctate contact (arrow) of an isolated zonula occludens at higher magnification. The isolated view looks similar to that seen in whole tissue in Fig. 8. × 96,000; insert, × 130,000.

FIGURE 8   A high magnification view of a zonula occludens in sectioned whole mouse liver. A series of four punctate membrane contacts (arrows) may be seen in this junction separating liver extracellular space (LES) from the lumen of the bile canal (BC). These punctate contacts are long, threadlike contacts in the dimension normal to the micrograph, and these are the threads which branch and anastomose to form the network seen in Figs. 9 and 10. × 240,000.

FIGURE 9   An isolated zonula occludens treated with desoxycholate (DOC) and negatively stained with Na-PT. There is an irregular branching and anastomosing network of threads or chains of punctate contacts which forms a belt across the micrograph (arrows). Damage to membranes has allowed the Na-PT to penetrate and outline the junctional elements. If the unstained areas were to represent regions of membrane fusion, one can easily appreciate how a section through such a structure could give the series of contacts seen in Fig. 8. × 43,000.

FIGURE 10   A replica of a freeze-cleaved zonula occludens from aldehyde-fixed whole liver, showing the chainlike view of the junction (fine arrows) which is coplanar with the extracellular membrane face (EMF). Note the similarity to Fig. 9. The strands correspond to the areas of junctional fusion. The discontinuities observed in the strands probably correspond to areas where the junctional material has remained with the reciprocal fracture face. The black and white arrow indicates the direction of shadow. × 71,500.
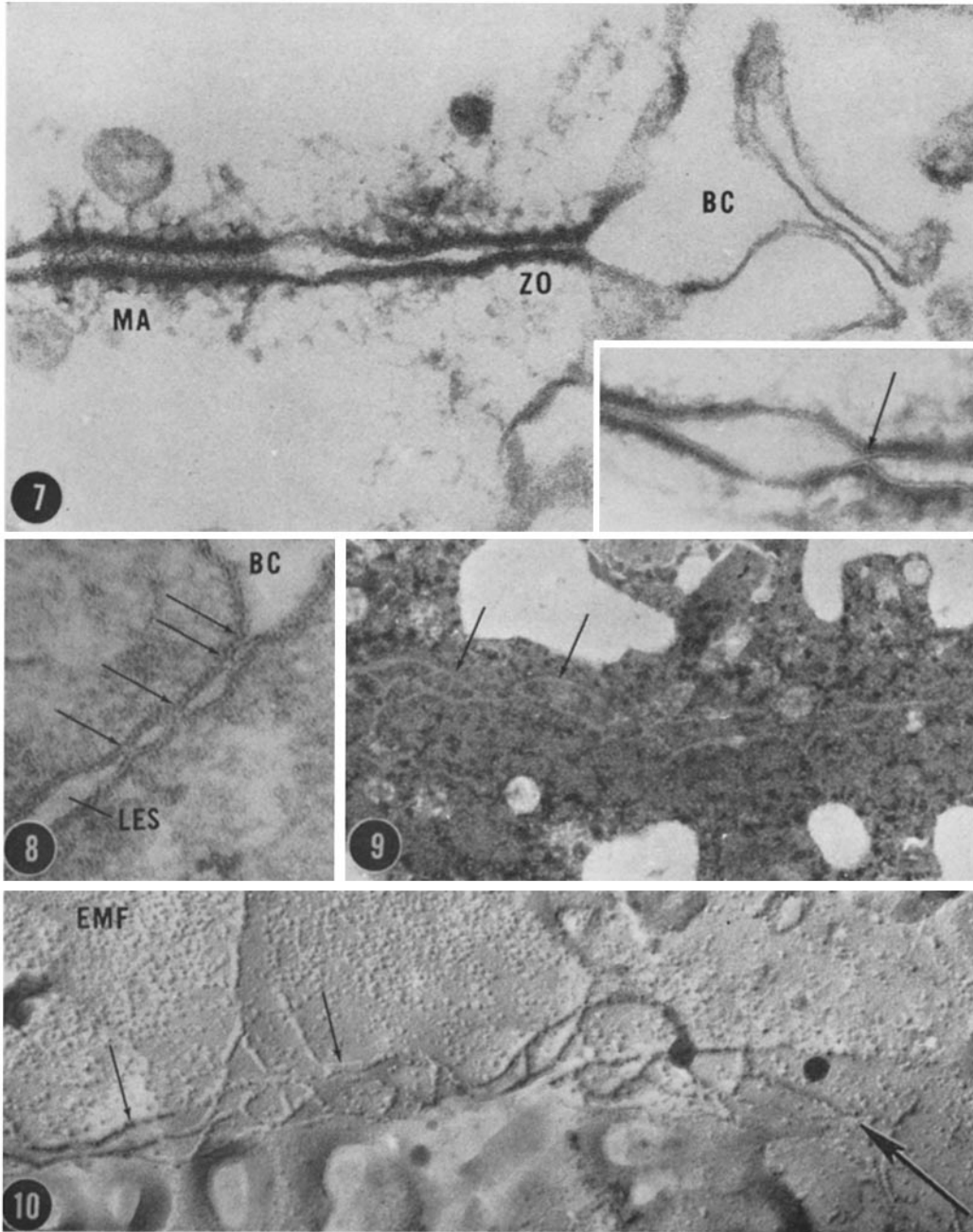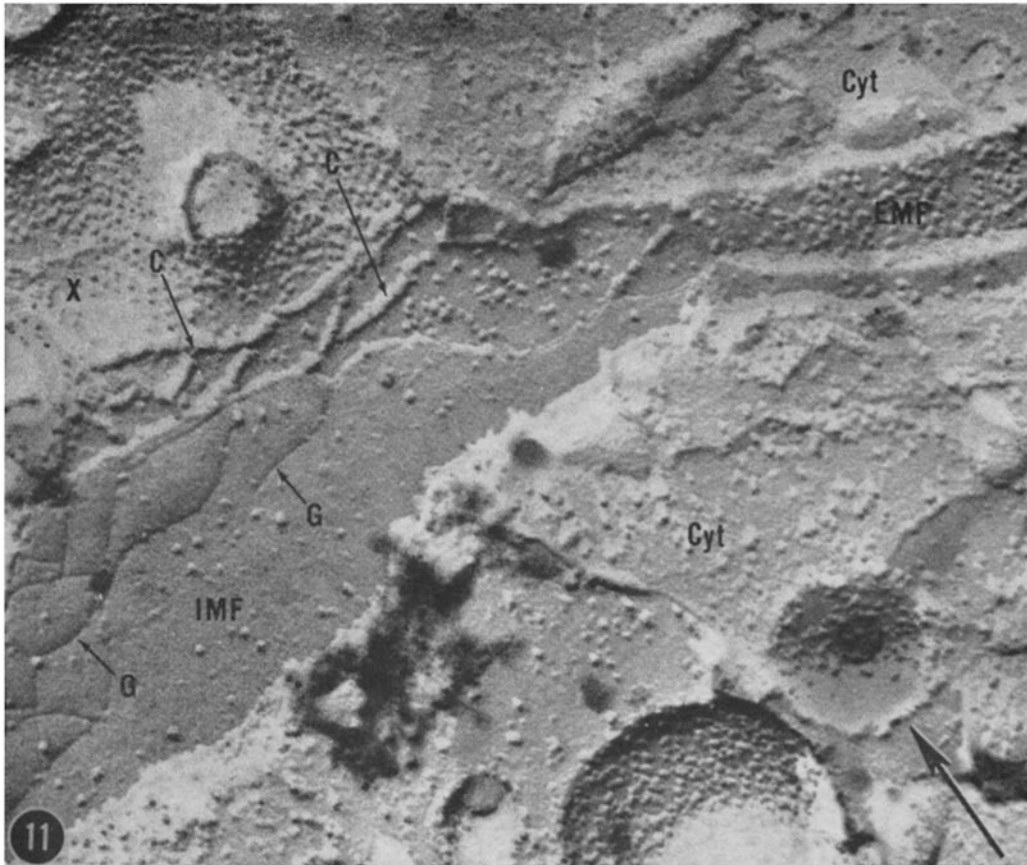
FIGURE 11 A replica of a freeze-cleaved zonula occludens showing both the chain aspect (C) of the junction, coplanar with the extracellular membrane face (EMF), and the grooved aspect (G), coplanar with the intracellular membrane face (IMF). The lumen of the bile canal cannot be seen in this replica. The extracellular membrane face at X borders the canalicular lumen, while the face at EMF borders the liver intercellular space. The cytoplasm of the two hepatocytes is indicated (Cyt). The black and white arrow indicates the direction of shadow. × 120,000.
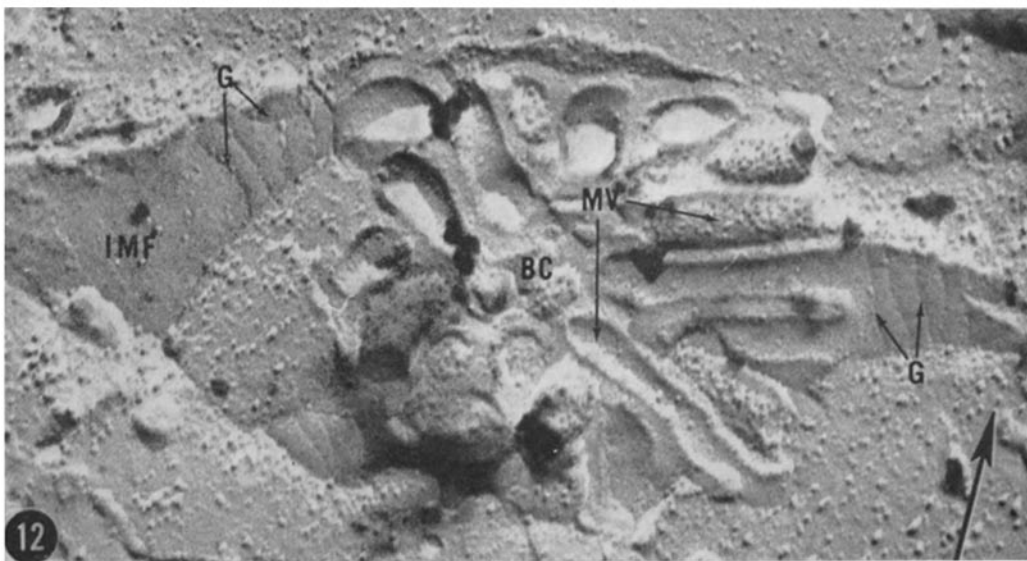
FIGURE 12 A replica of a freeze-cleaved bile canal (BC) showing numerous microvilli (MV) projecting into its lumen. Two grooved aspects of the zonulae occludentes which seal this bile canal are seen (G) to be coplanar with the intracellular membrane face (IMF). The grooves (and in other views, the ridges or strands) are found where sectioned material shows the membrane typical of the zonula occludens. The black and white arrow indicates the direction of the shadow. × 81,000.

measurements themselves, for the range of error spans the measured width of the intact gap (10%). Another possible explanation of this phenomenon, although not verified with measurements in our system, may be the membrane "swelling" reported by Fleischer et al. (21) in lipid-depleted mitochondrial inner membranes. These authors measure an increase in the width of the light band of the unit membrane, and this may be a property of other lipid-depleted membranes. It cannot be decided at this point whether the acetone has physically removed a component from the junction, or whether there has been a rearrangement or denaturation of a component, resulting in the observed change in heavy metal staining. It is interesting to note in this respect that nonjunctional membranes retain both their normal appearance (unit membrane) and 70–80 A width following treatment with 60% acetone.

The disappearance of the 20 A gap of the gap junction takes place even after fixation of the preparations with glutaraldehyde. Fixation with OsO_4, however, stabilizes the gap and the junction-associated particles, so that the junction will retain a gap through complete alcohol or acetone dehydration.

A critical concentration of acetone is necessary to effect this morphological alteration of the gap junction. Treatment of isolated junction pellets

with 50% acetone shows no effect, while 60% acetone results in a complete disappearance of the gap. We have attempted to approach the problem of the nature of the gap material by comparing the supernatant fluids recoverable after 50 and 60% acetone extractions of isolated membrane fractions. In thin-layer chromatograms (Fig. 19), three additional major phospholipids and several quantitatively less important phospholipids appear abruptly at an acetone concentration of 60%. These compounds have been tentatively identified in the two-dimensional chromatogram in Fig. 21. As shown in Fig. 20, two additional neutral lipids are detectable at the critical acetone concentration of 60%.

Protein, assayed by the Folin-phenol test as modified by Lowry et al. (30), was found in all of the extracts, and even in small amounts in non-acetone-treated controls. There was no evidence of appearance of new protein at the critical acetone concentration.

While the fine structure of the gap junction is altered by treatment with organic solvents, the zonula occludens appears morphologically unaffected in either sectioned material or in freeze-cleaved samples. It is clear, however, that some changes have occurred since the zonula occludens appears to become permeable to lanthanum, and the colloid can penetrate into the bile canaliculi
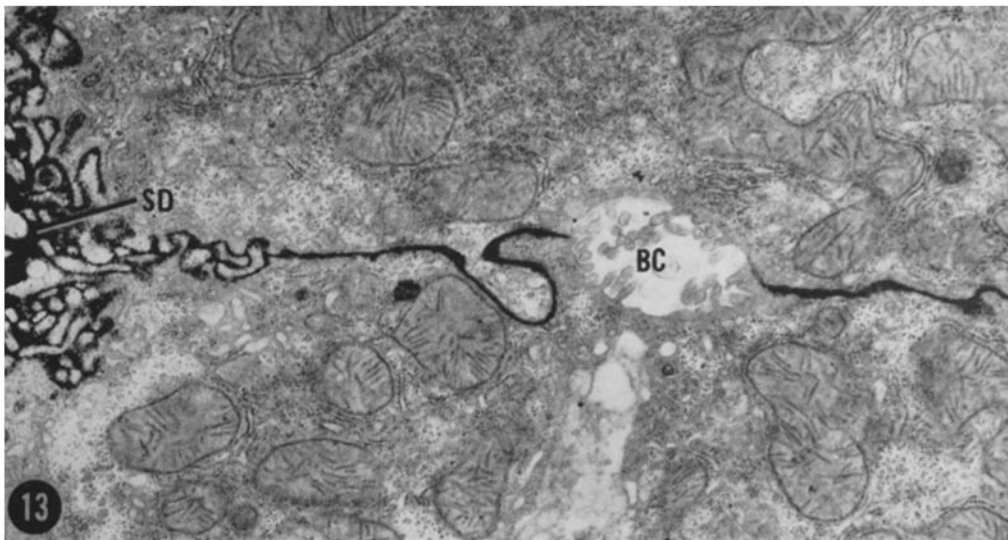

FIGURE 13   A low-power electron micrograph showing lanthanum filling the space of Disse (SD) and the intercellular cleft, and being abruptly stopped at the level of the zonula occludens, thereby leaving the bile canaliculus (BC) free of the tracer. × 14,000.
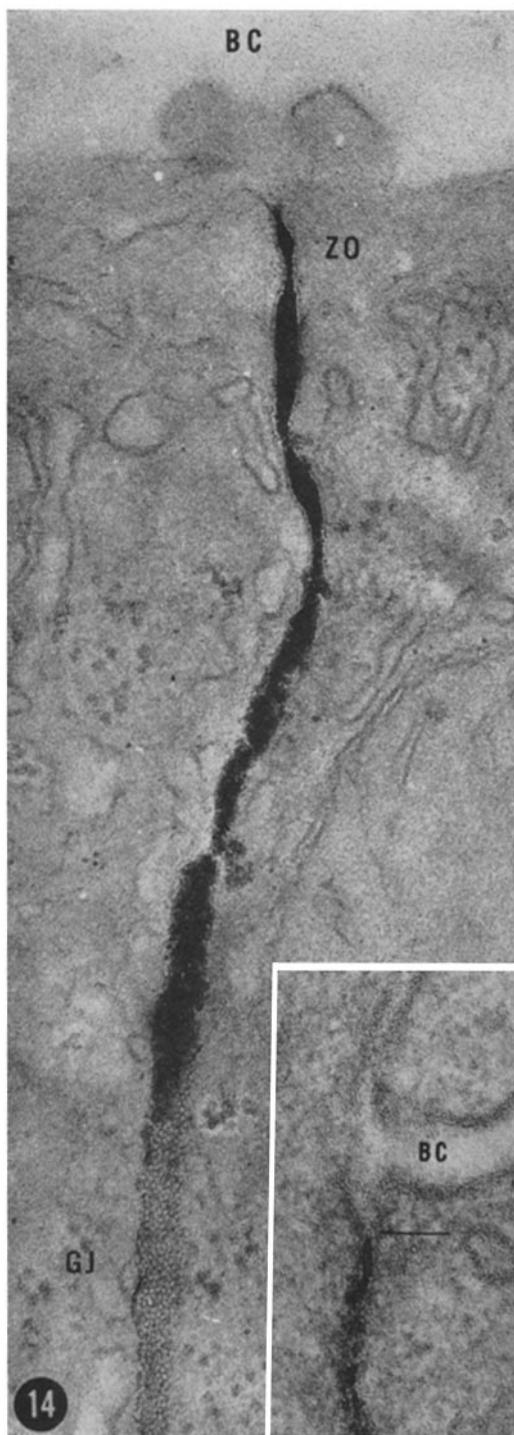
after 60% acetone extraction. Whether this is due to destruction of some component of the junction itself or to a general effect on membrane permeability is not easily answered at the present.

Effects of Other Reagents on the Junctions

Treatment of whole tissue and membrane pellets with either 0.02 M EDTA or 6 M urea produced no detectable morphological change in either the gap junction or the zonula occludens, as seen in section and in freeze-cleaved preparations. Treatment of the pellet with DOC had some effects on the gap junction. In section, this junction appears unaltered following a 15 min treatment, and the 20 A gap is still clearly visible (Fig. 18). But in negatively stained preparations, the polygonal lattice shows unusual patchy interruptions, appearing broken off in places as in Fig. 17. This type of interruption is not seen in the Na-PT stained controls (Fig. 6). Longer treatment with DOC can result in collapse of the 20 A gap in section, but this is usually variable. The interrupted images in negatively stained gap junctions may also be seen in Fig. 3 a in the paper by Benedetti and Emmelot (3). All other components of the membrane pellet, including the zonula occludens, disappear after treatment with DOC and subsequent embedding procedures, and one is left with the gap junctions and a fuzzy background material of unknown origin. The effects of DOC on the zonula occludens were described above.

DISCUSSION

While the term "tight junction" has been used to refer to both the zonula occludens and the gap junction, the evidence presented here, in addition to that already available in the literature, clearly indicates that these junctions are two different structures. The two junctions differ not only in their location between hepatocytes, but also in

Figure 14 An electron micrograph of a section of lanthanum-stained liver, showing the lanthanum stopped at the level of the zonula occludens (ZO). The bile canal (BC) is free of tracer. A gap junction (GJ) may also be seen in tangential view, showing its polygonal lattice of subunits. It is clear that the tracer is free to pass both around and through the gap junction, and that it is the zonula occludens which has the sealing role. The membrane contacts are oblique in this section, and hence the details of contact are not evident. The inset shows a high magnification view of a zonula occludens. At one of the punctate membrane contacts (arrow) the lanthanum is stopped from entering the lumen of the bile cana (BC). × 100,000; inset, × 140,000.
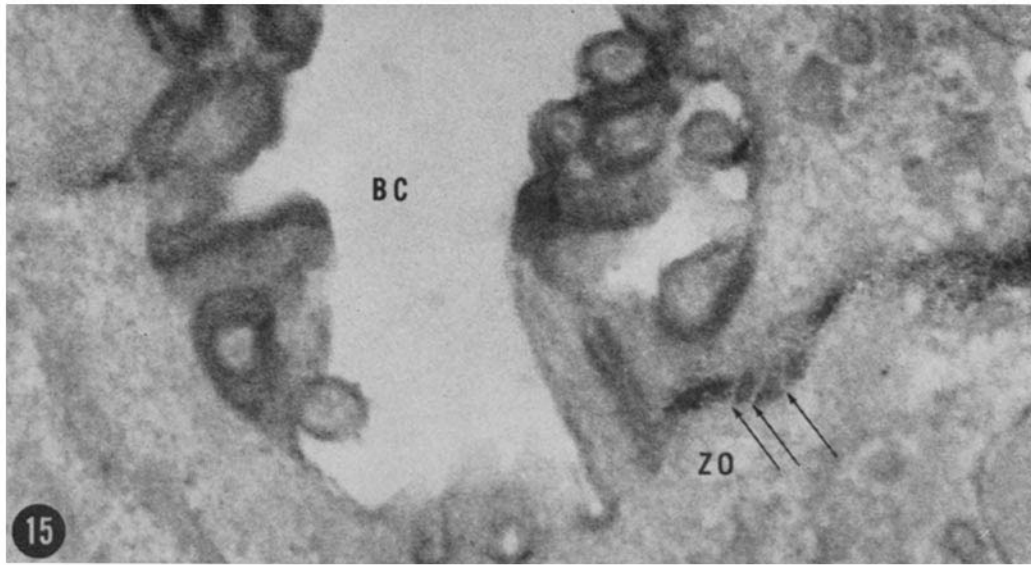
FIGURE 15 An electron micrograph showing a bile canaliculus permeated with lanthanum following treatment with 70% aqueous acetone; aldehyde-fixed mouse liver. The zonulae occludentes (ZO) seem to be leaky to lanthanum following acetone treatment, and the tracer is commonly found in the lumen of the bile canal (BC). Under these circumstances, the threadlike contact areas of the zonula occludens may be "negatively stained" by the lanthanum and are rendered visible in oblique views (arrows). Compare this view with the appearance in freeze-cleaved or in isolated fractions (Figs. 9–12). × 105,000.

their appearance after such diverse techniques as uranyl acetate staining en bloc, lanthanum staining, negative staining with Na-PT, and freeze-cleaving, as well as in their structural resistances to extraction with organic solvents. The macular gap junction is characterized by particles closely packed in a regular polygonal lattice, occupying a space 20 A wide, between the facing outer leaflets of the junctional membranes. The zonula occludens, on the other hand, consists of long, anastomosing threads or chains of membrane "contacts," where plasma membrane outer leaflets appear to fuse. This junction is disposed in a ribbon or band on either side of a bile canaliculus, thus forming a seal between liver intercellular space and the lumen of the bile canal.

The two junctions differ from each other also in their resistance to treatment with various chemicals. 60% acetone causes a characteristic loss of the polygonal lattice of subunits of the gap junction and a disappearance of the 20 A electron-lucent gap seen in sectioned material, while the zonula occludens appears morphologically unaltered. On the other hand, the zonula occludens seems more sensitive to DOC than the gap junc-

tion. Treatment of the membrane fraction with DOC, followed by negative staining with Na-PT, shows the polygonal lattice of the gap junction, but causes interruptions in the regularity of the lattice. In these negatively stained DOC preparations, the anastomosing thread surface view of the zonula occludens is also seen, but during subsequent dehydration and embedding for electron microscopy the DOC-treated zonulae occludentes are lost, and no evidence of these junctions can be found in the sectioned pellet. After DOC, the gap junction can be found in the sectioned pellet, but the 20 A gap occasionally disappears.

It is unclear how the gap junction is altered by the acetone treatment or by DOC. The inability to find the junction-associated lattices after acetone extraction in freeze-cleaved material may reflect a change in the cleaving properties of lipid bilayers, as suggested by Branton and Park (8) and Branton (7), but this would have to be a phenomenon taking place only at the gap junction itself since other membrane surfaces and the zonulae occludentes appear unchanged after 60% acetone treatment. The electron-lucent gap has
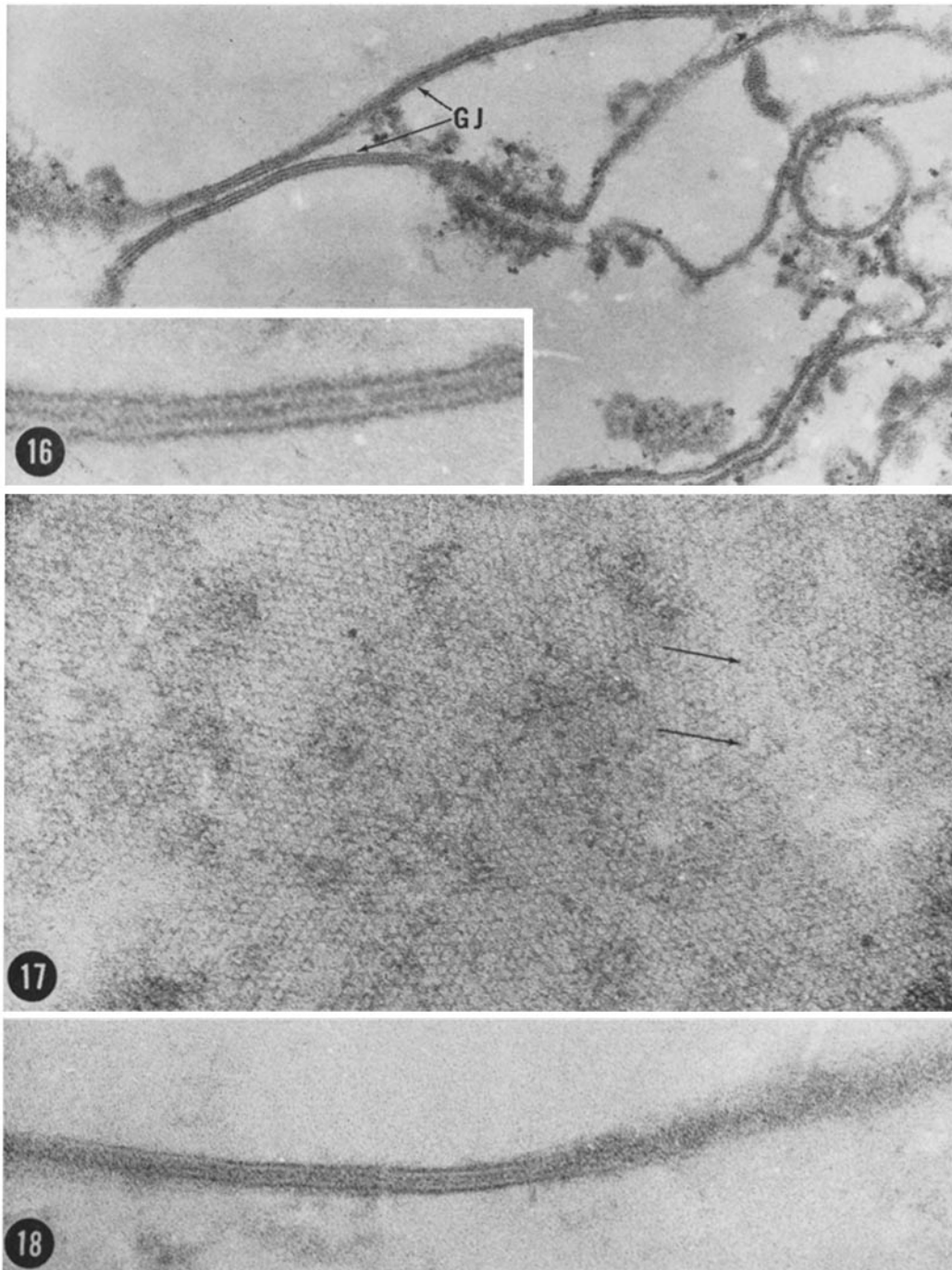
FIGURE 16   A micrograph of a sectioned membrane pellet following extraction with 60% acetone shows two gap junctions (*GJ*), still recognizable, although they have lost their electron-lucent gaps. Other membrane profiles in the micrograph show no obvious morphological alterations. The *inset* is a high magnification view of an acetone-treated gap junction. The junction appears pentalaminar or "tight." However, the over-all width of this altered junction is 140–150 A, similar to the control junctions illustrated in the *insets* of Figs. 1 and 2. × 100,000; *inset*, × 420,000.

FIGURE 17   An isolated gap junction treated for 10 min with DOC, and seen in surface view after negative staining with Na-PT. The polygonal lattice appears discontinuous in places (arrows), which might imply solubilization of some of the particles by DOC. × 200,000.

FIGURE 18   An electron micrograph of an isolated gap junction treated with DOC for 10 min and then embedded in plastic. The gap of this gap junction is visible, but this is not a constant finding following DOC treatment. DOC-treated gap junctions may, occasionally, appear pentalaminar. × 200,000.
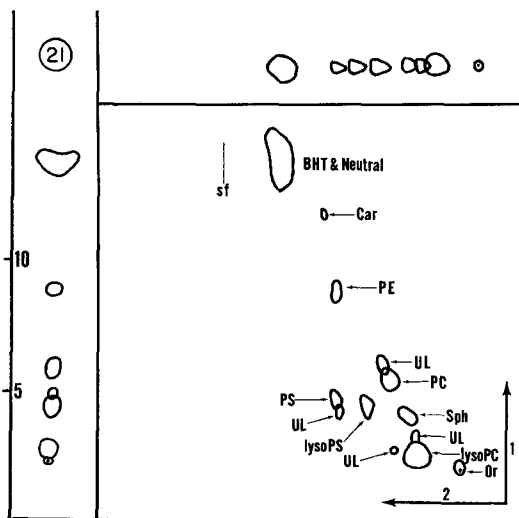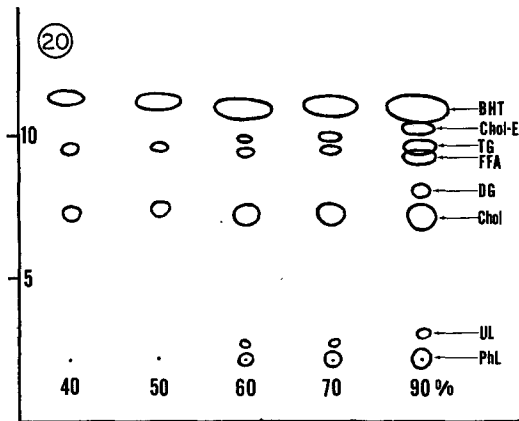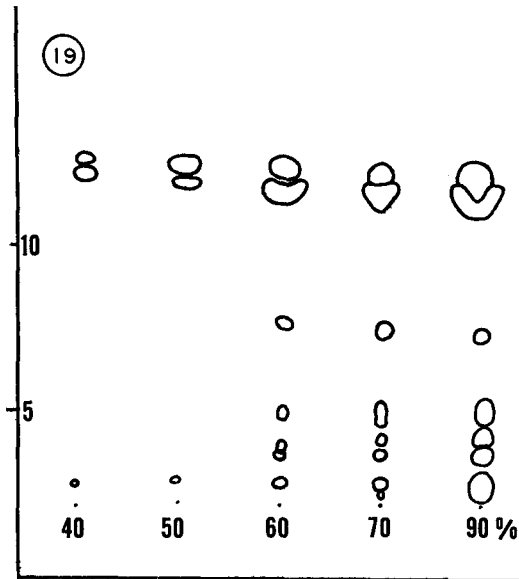
clearly disappeared from sectioned gap junctions, both in tissue and in the pellet following acetone, but since the electron microscope allows us to visualize only the heavy metal atoms which are bound to the junction after the extraction has been carried out, it is difficult to decide whether a component of the junction has been removed or simply altered in structure. It must suffice to say, at the present, that the gap junction has changed from its normal state, without implying any specific molecular details.

The results from studies with acetone and DOC do imply, at least, that lipids play a structural role in the polygonal lattice. This suggestion is supported by the thin-layer chromatography on the acetone extracts, which shows that several lipids, mostly phospholipids, appear in the extracts coincidentally with the observed structural alterations in the junction. The lipids extracted are similar to those reported for whole liver membrane extraction by Skipski et al. (46) and by Dod and Gray (16). Further support is gained from the observation that OsO_4 fixation, and not aldehyde fixation, will prevent both the structural alterations and the appearance of the phospholipids in acetone extracts. Osmium tetroxide has been reported to react with, and "fix,"

Figures 19, 20, and 21 Three tracings from thin-layer chromatograms on silicic acid plates of acetone extracts. Per cent values on the abcissa are per cent acetone in water. Centimeters are marked on the ordinate. Fig. 19 is a run with chloroform-methanol-H_2O (65:25:4), showing phospholipids extracted by increasing acetone concentrations. Note how a group of phospholipids appear at the 60% acetone concentration. Fig. 20 is a neutral lipid run with the two-step method outlined in the Methods section. An unidentified lipid and some triglycerides appear at the 60% acetone concentration. Fig. 21 is a phospholipid run with the two-dimensional separation described in the text. Some of the lipids have been tentatively identified on the chromatogram. The presence of a cardiolipin spot on the chromatogram indicates that the membrane pellet is contaminated with some mitochondria. The substances are PhL, phospholipids; UL, unidentified lipid; Chol, cholesterol; DG, diglyceride; FFA, free fatty acids; TG, triglycerides; Chol-E, cholesterol esters; Or, origin; lysoPC, lysophosphatidylcholine; Sph, sphingomyelin; lysoPS, lysophosphatidylserine; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Car, cardiolipin; sf, solvent front; BHT, 2,6-di-tert-butyl-4-methylphenol (Aldrich).

lipids (12, 13, 48), while aldehydes function poorly in this regard.

The suggestion that lipids play a structural role in the polygonal lattice of the gap junction does not, of course, exclude the possibility that protein, carbohydrate, or complex proteolipid or glycolipid may have a structural role in the gap junction. The Folin-phenol reaction indicates that that protein in varying amounts is present in the acetone supernatants. There does not appear to be a reproducible increase in the protein concentration in the 60% acetone extract, as compared to the 50% extract, but large amounts of nonspecific protein could well be masking the appearance of small quantities of specific junction protein under the conditions of the present quantitative method of protein detection. Structurally, 60% acetone extraction seems to cause no detectable alteration in the zonula occludens as seen in sectioned and freeze-cleaved material. DOC, however, causes complete disappearance of the zonula occludens in sectioned material, and acetone appears to render the aldehyde-fixed zonula occludens leaky to lanthanum in whole tissue. The tracer is routinely found in the bile canaliculi following acetone treatment. It must be concluded, then, that any alterations which occur in the zonula occludens following acetone extraction cannot, at this time, be visualized as structural changes in the electron microscope.

It must be emphasized that the presence of a narrow space between membranes does not necessarily indicate the presence of a polygonal lattice of subunits. In the case of nerve myelin, en bloc uranyl acetate staining has allowed one to recognize a tiny space at the level of the intermediate line which can be penetrated by lanthanum (40). In this case, however, the closely packed particles have not been found with either lanthanum or the freeze-cleave techniques. Myelin, of course, is also not an intercellular junction, since the close membrane associations have been shown to be complex infoldings of the plasma membrane of a single cell.

The gap junction and the zonula occludens differ from each other with respect to function as well as structure. Various reports in the literature indicate that the zonula occludens acts as a permeability seal in several epithelia. Miller (31) demonstrated the impermeability to hemoglobin of the terminal bar in mouse proximal convoluted tubule cells, and Farquhar and Palade (19)

showed that the zonula occludens was impermeable to hemoglobin in the kidney and impermeable to zymogen in the pancreas. Zonulae occludentes were further shown to be "tight" to horseradish peroxidase in the kidney and in the brain (36). More recently, zonulae occludentes have been shown to block the passage of lanthanum (9, 39). In the liver, the zonula occludens seals the bile spaces from the liver intercellular spaces. The function of the gap junction is not known, but lanthanum freely penetrates the 20 A gap, clearly showing that the junction has no sealing role. The gap junction's shape, coupled with its physical distance from bile canaculi, further excludes any sealing function.

There is evidence that the gap junction is the site of electrotonic coupling between cells. The gap junction and associated polygonal lattice have been described in mammalian heart (37), in smooth muscle (38), and in the Mauthner cell (9, 41). All of these tissues, including mammalian liver, have been reported to be electrically coupled (heart: 1, 51; smooth muscle: 6, 14, 15; Mauthner cell: 22; liver: 35). Electrotonic coupling between heart cells has also been demonstrated by Dreifuss et al. (17) and Muir (33), following disruption of the desmosome-like junctions at the intercalated discs. These studies show that synchronous beating of the myocardium continues after disruption of the desmosome-like junctions at the intercalated disc, but with the gap junctions left intact. There are other cell systems in which electric coupling has been reported between cells but in which electron microscopy has not revealed any gap junctions. In particular, the studies of Loewenstein (29) and Bullivant and Loewenstein (11) have suggested that septate desmosomes are involved in cell-to-cell electrotonic coupling in several invertebrates. In the clam, however, we have been able to localize gap junctions in addition to septate desmosomes. A. J. Hudspeth (personal communication) in a study of *Daphnia*, has also shown that one can find gap junctions as well as septate desmosomes. Lanthanum staining of this material reveals a polygonal lattice very similar to that observed in other tissues. These data indicate that both gap junctions and septate desmosomes can be found in at least some invertebrates, which raises the possibility that gap junctions may also exist in those invertebrate tissues in which electrotonic coupling has been demonstrated. On the other hand, the electrophysiological studies of

Sheridan (43, 44) and the electron microscope studies of Trelstad et al. (49, 50) on the early chick embryo indicate that "focal tight junctions" are responsible for the electrotonic coupling observed in these tissues. It must be concluded, therefore, that if the gap junction is capable of electrically coupling cells, it is not necessarily the only junction capable of doing so.

The experiments of Muir (33), the isolation studies of Benedetti and Emmelot (3), and the chemical treatments reported here show that the gap junction has a remarkable resistance to mechanical forces and to several chemical agents, implying that the junctional membranes are very firmly attached to one another. One could imagine, therefore, that the gap junction, in addition to being the possible mediator of electrotonic coupling, may play a role in intercellular adhesion.

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