

THE SPLITTING OF HEPATOCYTE GAP JUNCTIONS AND ZONULAE OCCLUDENTES WITH HYPERTONIC DISACCHARIDES

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ABSTRACT

Mouse livers were perfused *in situ* through the portal vein with the disaccharides sucrose, lactose, maltose, and cellobiose in hypertonic concentrations (0.5 M). This treatment resulted in plasmolysis of the hepatocytes and splitting of the gap junctions and zonulae occludentes. The junctions split symmetrically, leaving a half-junction on each of the two separated cells. The process of junction splitting is followed using the freeze-fracture technique, since the junctional membranes are indistinguishable from the nonjunctional membranes in thin sections once the splitting occurs. The split junctions are also studied using the freeze-etch technique, allowing a view of the gap junction extracellular surface normally sequestered within the 2-nm "gap." The monosaccharides sorbitol and mannitol did not split the junctions during the times studied (2 min), but substitution of the chloride ion with propionate in the perfusion mixture did result in junction splitting. An envelope of morphologically distinct particles surrounding freeze-fractured gap junctions is also described.

INTRODUCTION

Electrotonic coupling has been described in a wide variety of vertebrate and invertebrate tissues (7, 19, 33) and is characterized by a lowered resistance to the intercellular passage of ions. In many cases, gap junctions have been found between coupled cells in tissues (18, 23, 35), in embryos (9, 10, 32, 51), and between cells in culture (4, 19, 20, 27, 42, 48). Despite a vast literature of circumstantial evidence implicating the gap junction as the site of lowered intercellular ionic resistance, direct evidence is still lacking (see review by Bennett [7]).

Several studies have attempted to localize electron microscope tracers within junctions during transit from one cell to another. Reese et al. (46)

have reported intercellular passage of microperoxidase, although ultrastructural localization of the route followed by this glycoprotein tracer has not yet been published. The technical problems involved in demonstrating a stained individual junction at the fine structural level are overwhelming. Loewenstein (33) has reported the intercellular passage of horseradish peroxidase (HRP) between *Chironomus* salivary gland epithelial cells. Interestingly, the electron-opaque reaction product was localized in the septa of the septate junction, raising the possibility that these cell junctions play a role in electrotonic coupling. Since gap and septate junctions can coexist in a variety of arthropod epithelia (17, 21, 25, 26, 49), it would have been interesting if the gap junctions between the HRP-stained *Chironomus* cells had also been

studied. Due to the movement of HRP after aldehyde fixation (8), data from these kinds of experiments must be interpreted with caution.

There have been several studies which have correlated changes in junctional fine structure with electrotonic uncoupling. Barr et al. (2, 3) found the gap junctions (nexuses) "pulled apart" between myocardial and smooth muscle cells after perfusion with hypertonic sucrose solutions. In addition, they found that hyperosmotic treatments (with urea) had no effect on the gap junctions. McNutt et al. (34) reported that the polygonal lattice of particles in the nexus was still visible on the surface of junctions disrupted with hypertonic sucrose, but no micrographs have been published. Dreifuss et al. (16) also studied hypertonic effects on heart junctions and their results confirm those of Barr et al. (3). Following these earlier studies, Goode-nough and Gilula (22) perfused intact mouse liver with hypertonic sucrose and found that the freeze-fractured hepatocyte gap junctions split in half under these conditions, resulting in a separation of the gap junction membranes without an apparent perturbation of the fracture face lattice of particles.

Asada and Bennett (1) and Pappas et al. (38) exposed *Procambarus* axons to sodium propionate, resulting in the simultaneous uncoupling of the cells and a loss of gap junctions in thin-sectioned material. After return of the tissue to physiological saline, the coupling was restored and the gap junctions could again be found. A recent study by Peracchia (39, 40), however, provides some evidence that there may be two classes of gap junction at these crayfish synapses. In light of Peracchia's findings, it would be interesting to reexamine the treated *Procambarus* axons to see if one or both proposed junctional classes are sensitive to the propionate ion.

Attempts have been made to disrupt the zonulae occludentes, or true tight junctions, as well. Studies of Ussing (52), DiBona (14), Wade et al. (53), and DiBona and Civan (15) have demonstrated the ultrastructural sensitivity of zonulae occludentes to hypertonic and hyperosmotic solutions. Recently, Rapoport and his collaborators (43, 44) have succeeded in reversibly opening the blood-brain barrier with 2 M urea and DL-lactamide perfusions, although the ultrastructural basis for this increased vascular leakage has not been published.

In this paper, the effects of hypertonic solutions on the ultrastructure of both gap junctions and zonulae occludentes in the mouse liver are investi-

gated. In addition, a novel observation on the structure of the hepatocyte gap junction is presented.

MATERIALS AND METHODS

Mature male and female mice from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) were used throughout.

Mice were anesthetized by intraperitoneal injection (2.5 mg/g body weight) of Nembutal sodium (Abbot Laboratories, Chemical Marketing Div., North Chicago, Ill.). After opening of the peritoneal cavity, 50–100 U of heparin were injected directly into the inferior vena cava. The portal vein was then cannulated with a no. 22 needle, the inferior vena cava cut, and the experimental solutions perfused through the liver at 20 ml/min with 40 cm H₂O pressure for various time intervals at room temperature. Fixative containing glutaraldehyde and paraformaldehyde (28) was perfused through the same cannula for 5 min. If the livers did not become uniformly hard, the animal was discarded. Pieces of liver were excised from the left lateral lobe, cut into 1-mm square cubes, and fixed for an additional 10 min in aldehydes.

For freeze cleaving, the livers were equilibrated with 20% glycerol in 0.1 M cacodylate buffer, then frozen in liquid Freon on paper disks. Replicas were produced with a Balzers BA 360M according to standard techniques.

For sectioning, the tissue was postfixed in reduced osmium tetroxide (29) or in 1% osmium cacodylate. The livers were washed with distilled water, then stained with 1% uranyl acetate in distilled water for 30 min in the block. Tissues were embedded in Epon. Sections and replicas were examined with a Siemens Elmiskop 101 operating at 100 kV.

Solutions

Livers were perfused with 0.5 M and 0.25 M sucrose ("ultrapure", Schwartz Mann Div, Becton, Dickinson & Co., Orangeburg, N. Y.) in 0.9% bicarbonate- or phosphate-buffered saline, pH 7.4. In propionate experiments, NaCl and sucrose were omitted and 0.9% sodium propionate was substituted. Perfusion times lasted from approximately 5–10 s to 2 min. Livers were also perfused with 0.5 M β -lactose, grade III, maltose hydrate, grade II, β -D(+)-cellobiose, D-sorbitol, and D-mannitol in bicarbonate-buffered 0.9% saline, pH 7.4. All these sugars were obtained commercially from Sigma Chemical Co. (St. Louis, Mo.) and were filtered through activated charcoal before use.

Measurements

Since the length of shadow in freeze-fracture replicas will vary as a result of shadow angle, all measurements of particles made in this study were done normal to the direction of shadow. These measurements are not absolute, however, since they will vary with the amount of platinum evaporated from run to run. For this reason, not only the diameter of the particles has been measured,

but also the center-to-center distance between the regularly arranged gap junction particles is measured. This latter measurement will not be affected by either shadow angle or amount of platinum deposited.

RESULTS

The Gap Junction

Normal gap junction morphology has been well documented in a variety of animal species (18, 23, 30, 35, for a review, see 36). Characteristically, the freeze-fractured hepatocyte gap junction displays a polygonal lattice of particles on the A fracture face, and a complementary (13) lattice of pits on the B fracture face. Frequently, the fracture plane moves from the gap junction membrane of one cell across the 2-nm "gap" to the junctional membrane of the adjacent cell, revealing both A and B fracture faces in one replica (see Fig. 3). Assuming that these junctional membranes fracture in a central hydrophobic region similar to plasma membranes (12, 41), then the 2-nm gap characteristic of thin-sectioned gap junctions (23, 47) is not normally apparent in the freeze-fractured image, although it must lie between the A and B fracture faces (see solid arrows, Figs. 3 and 5). The close apposition of the A and B junctional fracture faces, without an apparent intercellular space, is a characteristic feature of intact freeze-fractured gap junctions. The 2–4-nm gap is a characteristic feature of the gap junction in appropriately stained thin sections, whether the junction is observed *in situ* (47), attached to enzymatically disrupted cells (11), or in isolated junction preparations (23, 24).

Figs. 3–5 also demonstrate a new ultrastructural detail of gap junction morphology. In these figures, an unusually high shadowing angle has rendered the characteristic lattice of pits on the gap junction B fracture face somewhat indistinct, although the pitted lattice may still be seen on the B face of the smaller junction in Fig. 3. Due to the high shadowing angle, and probably also to uncoupling conditions, a row of large (diameter approximately = 9.5 nm) particles, which cast longer apparent shadows than the usual gap junction particles (diameter approximately = 7.7 nm), may be seen to surround the edge of the junctional A face (see arrows, Fig. 4). The range of center-to-center distances of these particles (9.5–12 nm) is also larger than the range of center-to-center distances between the usual gap junction particles (6–10 nm). The perimeter particles leave correspondingly deeper pits on the B fracture face (see

black and white arrows, Fig. 5). Even at the low magnification in Fig. 3, the loose aggregation of large pits forms a striking line, demarcating the boundary between junctional and nonjunctional membrane B faces. Particle-free zones have been described surrounding myocardial gap junctions (35); these large perimeter particles may be the counterpart in the liver.

The characteristic gap junction morphology may be altered by intravascular perfusion of 0.5 M sucrose. Initially, a rapid plasmolysis of the hepatocytes occurs, forming widened foci of intercellular space (see Fig. 1). The zonulae occludentes adjacent to the bile canaliculi appear initially unaffected, as do the gap junctions (inset, Fig. 1). Within 5–10 s, however, the gap junctions take on a more irregular appearance (Fig. 2) sharply contrasting to the junction's smooth contour in control specimens. Occasionally, a widening of the 2-nm gap is observed (see *B* Fig. 2 and inset, Fig. 2). Further description of the breakdown of gap junction morphology in thin sections has not yet been possible because the separated junctional membranes are indistinguishable from the nonjunctional plasma membrane. It must be emphasized that the membrane appositions shown in Fig. 2 and the inset, Fig. 2 cannot be identified with certainty as gap junctions. Identification of gap junctions in thin sections relies on the presence of the 2–4-nm gap; loss of this gap due to the experimental conditions makes gap junction identification ambiguous. However, the process of junctional breakdown may be followed unambiguously using the freeze-fracture technique. After extremely short perfusions with 0.5 M sucrose (approximately 5 s), all junctions still appear intact (Fig. 3). The 2-nm gap, separating the A and B gap junction fracture faces (see solid arrows, Figs. 3 and 5), does not yet appear to have widened.

Longer perfusion times result in a splitting of the gap junction along a central plane, and each cell retains one of the two junctional membranes (22). Initially, as shown in Figs. 6–9, the A and B fracture faces of the junctional membranes are seen pulling apart (arrows), separated by intercellular space. In control specimens perfused with 0.25 M sucrose, this separation of the two fracture faces is never seen. The two junctional membranes ultimately separate as the cells pull apart (Figs. 10 and 11). It is possible to identify gap junction fracture faces on widely separated cells, where the junctional particles are seen directly abutting the intercellular space (Figs. 12, 13, and 17). A

diagram of the stages of junction splitting is shown in Fig. 19.

There are two possible ways to interpret the replica in Fig. 17, which produces an ambiguity. The first interpretation, which we favor in this paper, would hold that the array of junctional particles would continue beneath the surface of the extracellular ice, along the edge indicated by the arrows. This would mean, then, that the replica is of a half a gap junction, still organized into a lattice within the membrane matrix, but no longer contacting another cell. The second interpretation would assert that the edge abutting the extracellular ice (arrows, Fig. 17) is also the free edge of the junction. If this were so, and the adjacent cell completely cleaved away, then the junction might be intact and unsplit. This ambiguity, not present in Figs. 6–11 which show both the A and B fracture faces, has made the study of the subsequent fate of the split junctions very difficult.

The ambiguity of interpreting half-junctions, without the proximity of the adjacent cell, may be avoided by using the freeze-etch technique. The results of such an experiment are shown in Fig. 18. The tissue pictured in this micrograph was perfused with 0.5 M sucrose, then fixed as usual. Instead of equilibrating the tissue with 20% glycerol, however, the liver was washed exhaustively with distilled water and frozen in distilled water. The tissue was then etched at -100°C for 60 s (41), then replicated with platinum and carbon. This resulted in a fracture face of the junction (Fig. 18) and an etch face, separated by a ridge (41).

The lowering of the water table in Fig. 18 has

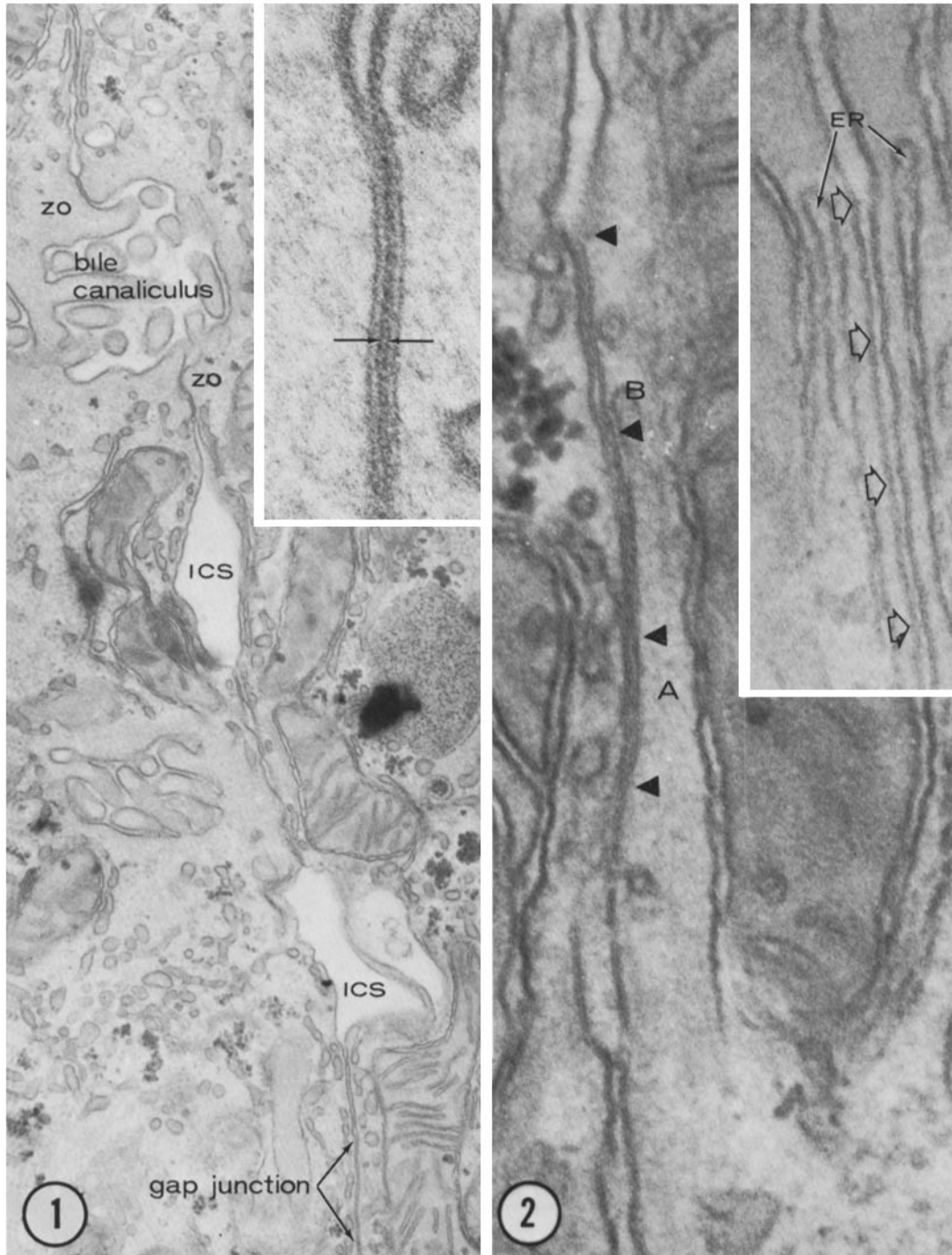
exposed a new view of the gap junction: the lattice of subunits within the experimentally widened 2-nm gap. This shallow (1–2 nm) lattice on the etch face may also be seen in Fig. 18 on an adjacent gap junction, which was exposed during the etching process. This second junction, well below the surface of the ice during the fracturing process, displays no fracture face.

The new lattice of particles exposed on the gap junction etch face (designated the “D face” by McNutt and Weinstein [35]) probably is half of the lattice outlined by colloidal lanthanum (47). The new etch lattice has the same center-to-center dimensions as the fracture lattice (approximately 8 nm). Since a lattice of particles is seen on all the gap junction etch faces and never a lattice of complementary pits, one must conclude that the gap junction is splitting centrosymmetrically, leaving an etch-face lattice of particles on each of the separated junctional membranes. No etch faces of zonulae occludentes have been observed.

It must be emphasized that the junction splitting phenomenon is not a synchronous event throughout the entire liver. Even after relatively long (2 min) perfusion times, some junctions can be found which display no apparent ultrastructural changes, while others split completely after short exposure. In any given area of the liver, during one experiment, those junctions which are splitting appear to do so in synchrony; completely split and partially split junctions are never found side by side. Totally unaffected junctions, however, can always be found in the vicinity of those which are split or splitting.

FIGURE 1 This micrograph shows an area of association between two liver cells near a bile canaliculus after a very short (5 s) portal perfusion with 0.5 M sucrose. Plasmolysis has resulted in areas of focal enlargement of the intercellular space (*I*CS), but there have been no apparent ultrastructural changes in the zonulae occludentes (*Z*O) adjacent to the bile canaliculus or in the gap junction. The *inset* shows a gap junction at higher magnification; the arrows delineate the 2-nm gap. Despite the treatment with hypertonic sucrose, this junction appears identical to control specimens. This may be due to nonuniformity in the liver vasculature or to the existence of several junctional types. $\times 30,000$, *Inset*, $\times 266,000$.

FIGURE 2 After somewhat longer perfusion times, or in other areas of liver treated as in Fig. 1, some gap junctions may be found which display an altered morphology suggestive of junction breakdown. This micrograph shows a gap junction (arrowheads) which has lost its smooth contour characteristic of control images. In some areas (*A*), the junction appears to have its usual 2-nm gap, while in other areas (*B*), the junction shows local areas of enlargement (4 nm at *B*). The *inset* is a micrograph which is interpreted to show a gap junction (arrows) flanked on both sides by cisternae of the endoplasmic reticulum (*E*R). This junction displays a greatly enlarged gap along its entire length. There is, however, considerable ambiguity in interpreting these images, since the junctional membranes are indistinguishable from nonjunctional plasma membrane once the 2-nm gap is lost. $\times 125,000$, *Inset*, $\times 145,000$.



The Zonula Occludens

In liver, the zonula occludens is found immediately adjacent to the bile canaliculus, sealing the bile spaces from the hepatic intercellular fluid (Fig. 1). The normal morphology of the zonula occludens in freeze-fractured specimens is characterized by a branching and anastomosing network of ridges on the A fracture face, and a complementary network of grooves on the B fracture face (13, 18, 23, 30, 50). Negative stain evidence has been presented which suggests a correspondence between the freeze-fracture ridges and the points of apparent membrane fusion revealed at the zonula occludens in thin section (23).

After perfusion with 0.5 M sucrose for 30 s or longer, it is possible to find zonulae occludentes which have separated in a manner apparently similar to the gap junctions. Fig. 14 shows a bile canaliculus in thin-sectioned material which has split in two. This suggests that the zonula occludens, like the gap junction, has split at some central point rather than tearing away from the adjacent cell, as is the case with calcium-free or enzymatic disruption of tissues (11, 31, 37).

In freeze-fracture replicas, the branching and anastomosing network characteristic of the zonula occludens may be seen on the disrupted junctional membrane fracture face (see Figs. 15–17). It would appear, then, as with the gap junction, that hypertonic sucrose indirectly triggers the separation of the zonula occludens junctional membranes. Each cell retains one of the two membranes, rather than the entire junction tearing away from one cell and remaining with the other. In order to be stated with

absolute certainty, however, etch faces of the split zonulae occludentes must be found.

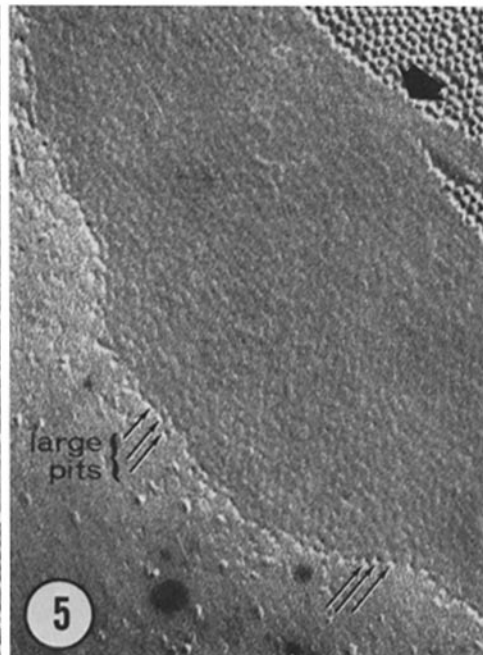
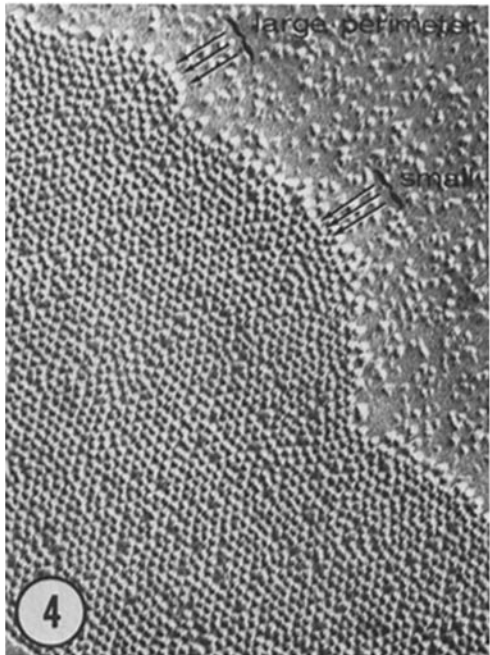
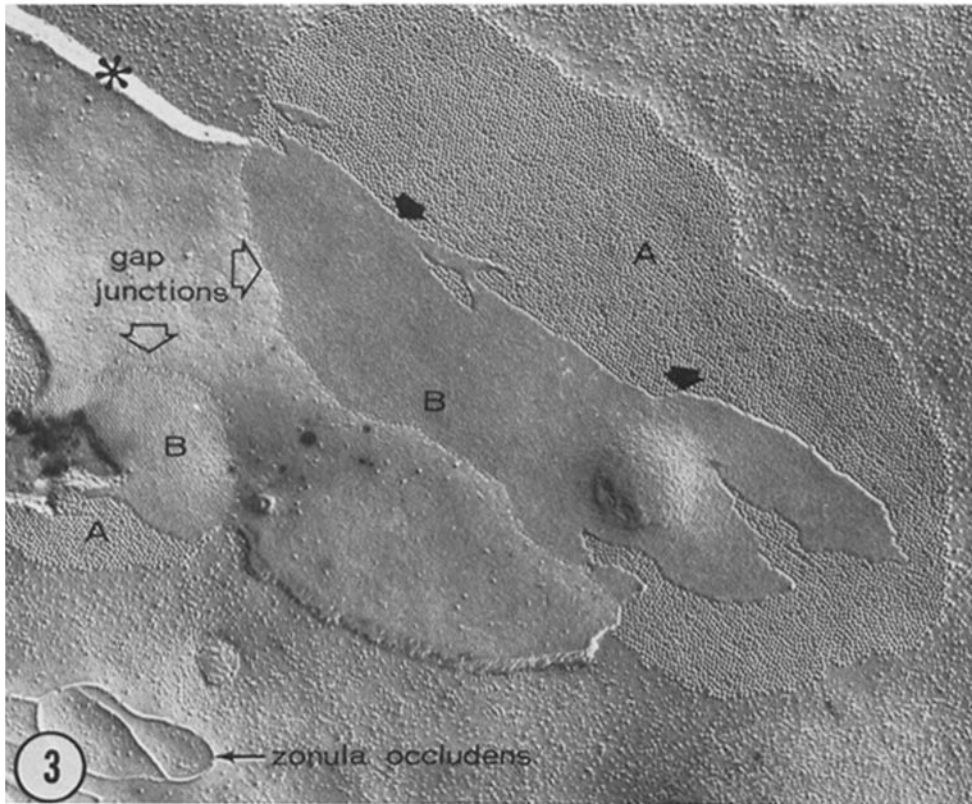
The branching and anastomosing ridges of the zonula occludens appear to be affected by the experimental procedure. In control specimens, or after short perfusion times (Fig. 3), the ridges on the A fracture face are smooth and continuous, interrupted only occasionally by discontinuities. After separation of the junction, the strands are more discontinuous and particulate in nature (see Figs. 15 and 17), suggesting an additional disorganization of the junction in its hydrophobic interior.

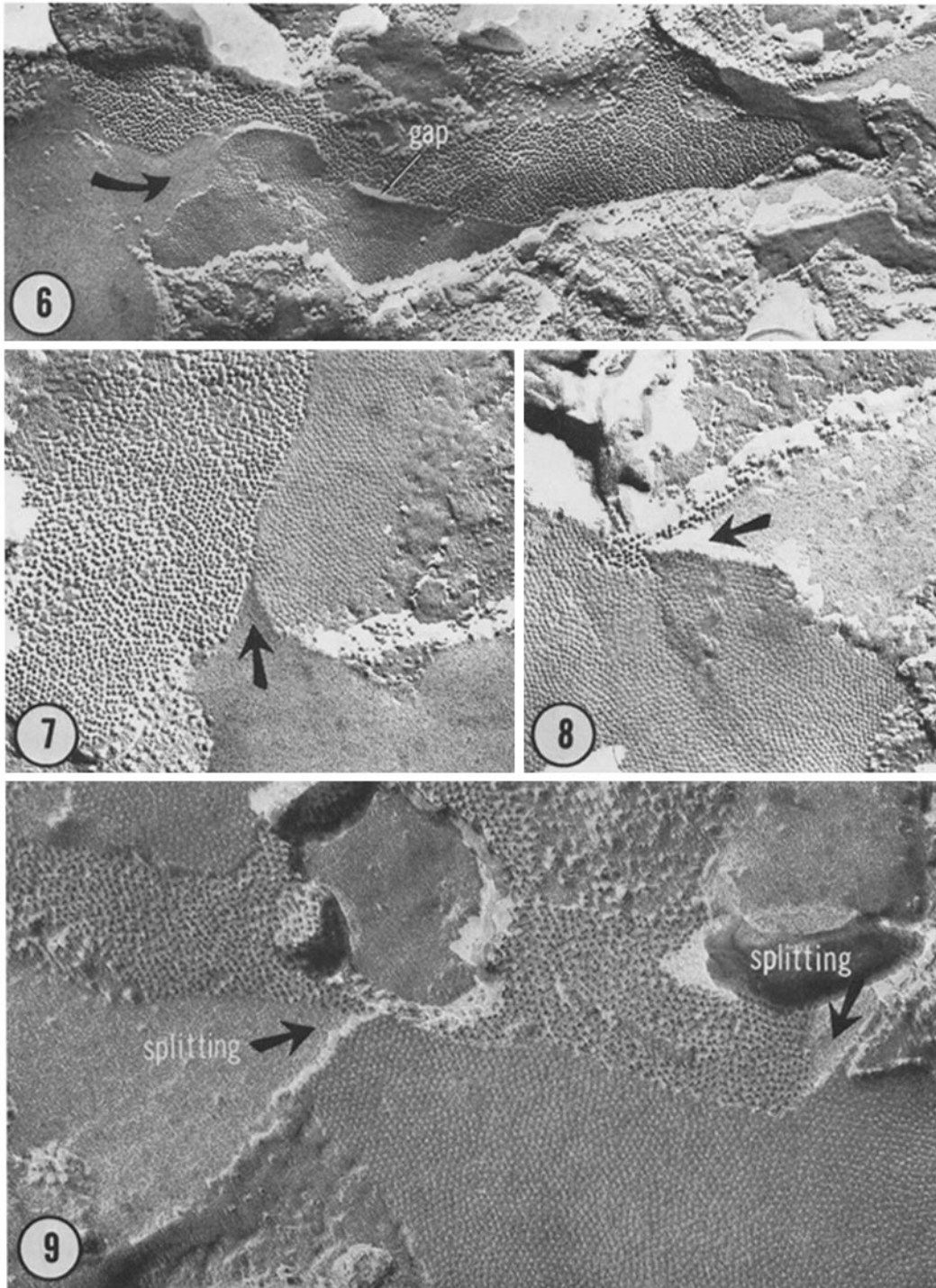
The effects of hypertonic sucrose are mimicked by portal perfusion with hypertonic (0.5 M) solutions of the disaccharides lactose, maltose, and cellobiose. Perfusions with 0.5 M sorbitol and mannitol result in neither the plasmolysis nor the junctional disruption caused by the disaccharides during the time-course of experiment (30 s). We were able to find a few examples of both gap junctions and zonulae occludentes, which have apparently split after treatment with 0.9% sodium propionate, in freeze-fractured material. These results support the findings of Asada and Bennett (1) and Pappas et al. (38). There may also have been a decrease in the absolute numbers of junctions, or in their combined total surface area, which we would not have detected since no morphometric analyses were attempted.

DISCUSSION

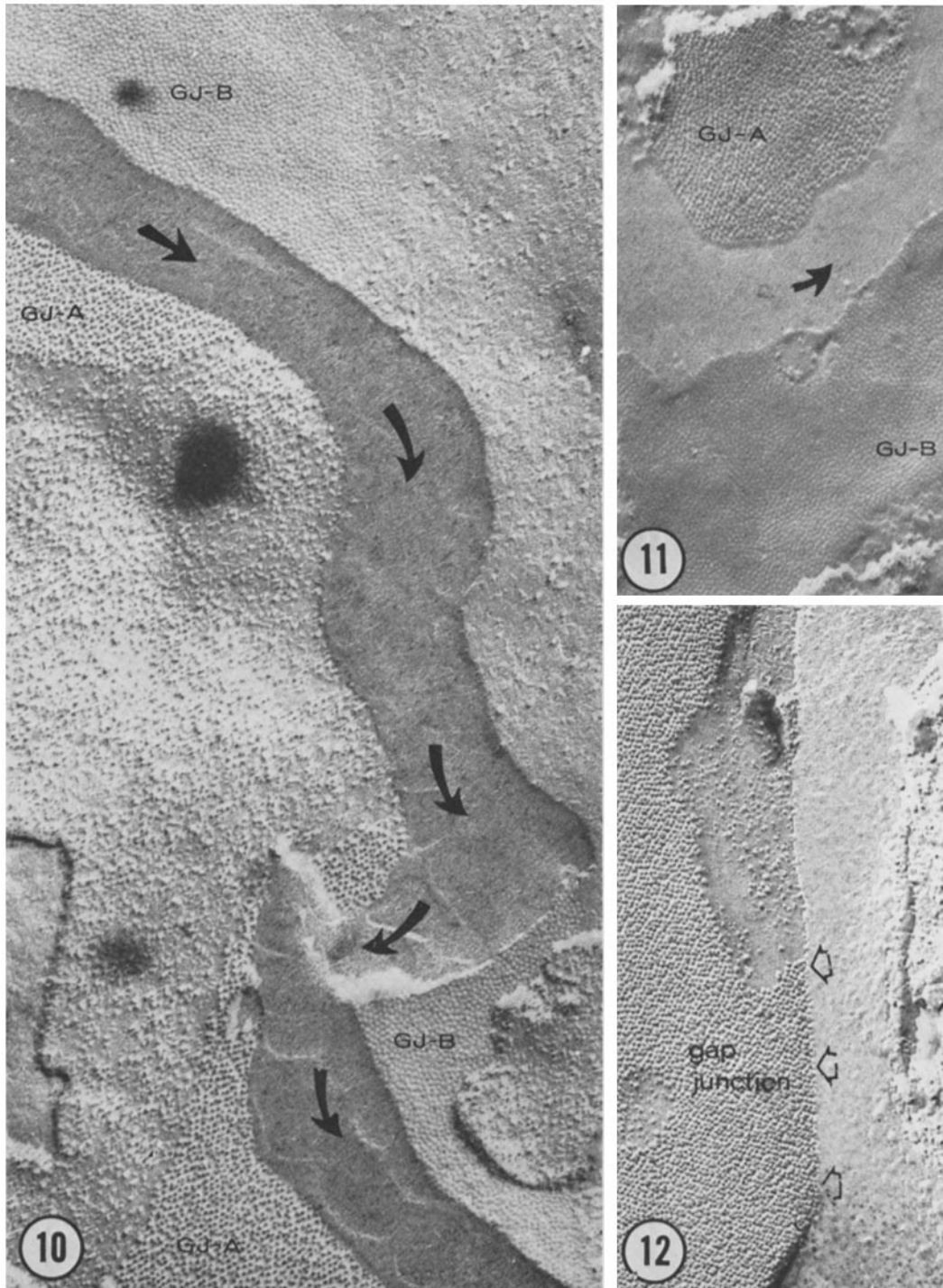
Exposure of hepatic parenchymal cells to hypertonic conditions results in a rapid plasmolysis of the cells with subsequent separation of the gap

FIGURES 3–5 These electron micrograph of freeze-cleave replicas show an area of association between two liver cells treated with very short exposures to hypertonic sucrose. Fig. 3 shows both the A and B fracture faces of two gap junctions, and a small portion of the A face ridge of a zonula occludens. Figs. 4 and 5 show details of Fig. 3 at higher magnification. The intercellular space (*) is seen in nonjunctional areas. The gap junction B face closely overlies the A face (large, solid arrows Figs. 3 and 5) with no apparent enlargement of the 2-nm gap, an image indistinguishable from control specimens. The lattice of pits on the functional B face is poorly resolved due to the high angle of shadow. These micrographs also illustrate a new ultrastructural detail of the gap junction. Due to the high-angle conditions of shadowing, or to the sucrose uncoupling procedure, a ring of morphologically distinct particles and pits may be described encircling the gap junctions. On the B face (Figs. 3 and 5), a boundary of deeper, more prominent pits can be seen (small arrows, Fig. 5) which are easily distinguished from the poorly resolved pits of the remaining junctional B face. On the A face (Figs. 3 and 4), a population of perimeter particles may be seen forming an envelope applied closely to the main body of more densely associated small junctional particles. These perimeter particles cast a longer apparent shadow than the smaller particles, and have a larger diameter (9.5 nm compared to 7.7 nm) measured normal to the direction of shadow. The center-to-center distance of the perimeter particles (9.5–12 nm) is larger than that of the rest of the lattice (6–10 nm). Fig. 3, $\times 65,000$; Figs. 4 and 5, $\times 165,000$.





FIGURES 6-9 These micrographs of freeze-cleave replicas show gap junctions in the process of "splitting": a separation of the junctional A and B fracture faces. All the livers shown here were perfused in the 5-10 s range. In each case, the intercellular space can be seen penetrating (arrows) between the junctional lattices of pits and particles. In Fig. 6, a localized separation of the two fracture faces may be seen occurring within the matrix of the junction (gap), suggesting that splitting does not occur exclusively at the junction edges. Fig. 6, $\times 85,000$, Figs. 7 and 8, $\times 96,000$, Fig. 9, $\times 124,000$.



FIGURES 10-12 Micrographs of freeze-fracture replicas of mouse liver treated with hypertonic sucrose. In Figs. 10 and 11, the intercellular space (arrows) is seen penetrating between the gap junction A face (*GJ-A*) and the gap junction B face (*GJ-B*). Fig. 12 shows a gap junction whose A face particles abut directly on the intercellular space (arrows); the adjacent cell does not present a surface-fractured membrane. Unlike Figs. 6-11, images such as Fig. 12 do not prove junction splitting since the B face is not seen. Fig. 10, $\times 65,000$; Fig. 11, $\times 70,000$; Fig. 12, $\times 83,000$.

junctions and zonulae occludentes. The junctional separation is unique since it involves splitting down a central plane, rather than a tearing away of the whole junction from one of the cells (11, 28, 34). Once the junctions have split, the junctional membranes are indistinguishable from adjacent non-junctional membranes, denying further study with thin-sectioned material. In freeze-fractured specimens, the process can be followed to better advantage, and a complete separation of the junctional membranes can be described. Once the hepatocytes have separated to the degree that only A or B junctional faces are found, interpretation of the freeze-fracture replicas becomes difficult since split, half-junctions remaining on the cell surface cannot be unambiguously differentiated from intact junctions presenting only an A or B fracture face.

The junction splitting must be due to the hypertonic conditions, and not directly to the chemicals perfused. Several different disaccharides in hypertonic concentrations will produce the splitting phenomenon, ruling out the uniqueness of a sucrose effect. In addition, gap junctions and zonulae occludentes can be isolated on sucrose gradients (5, 6, 20, 21): exposure of the junctions to these hypertonic conditions after cell homogenization does not result in junction splitting. Thus, the hypertonic conditions must stimulate secondary or tertiary reactions within the cell, which results in the separation of the junctional membranes.

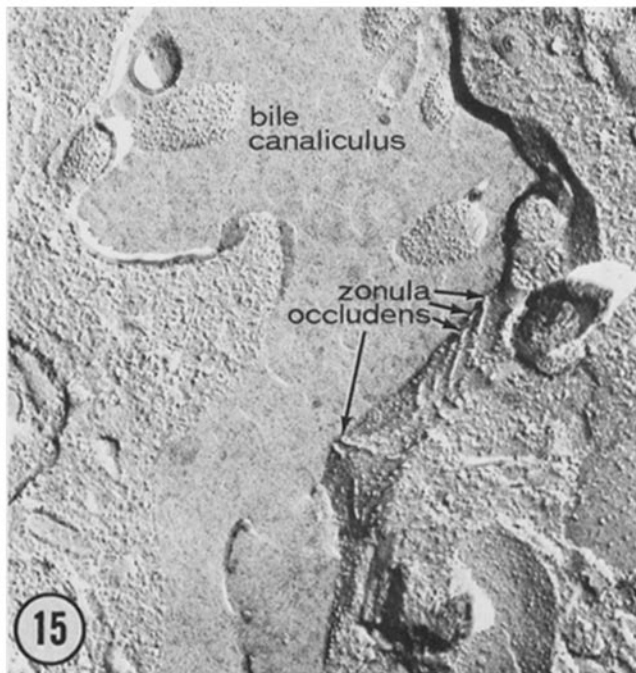
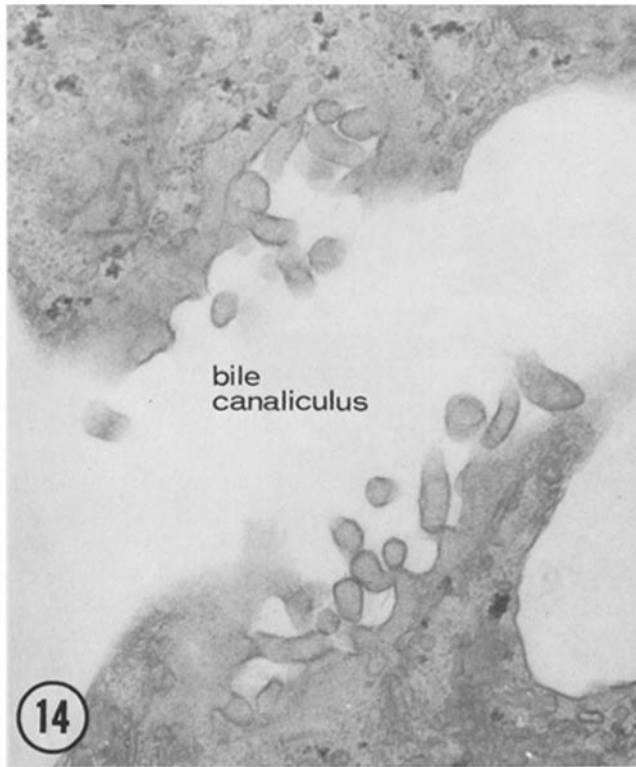
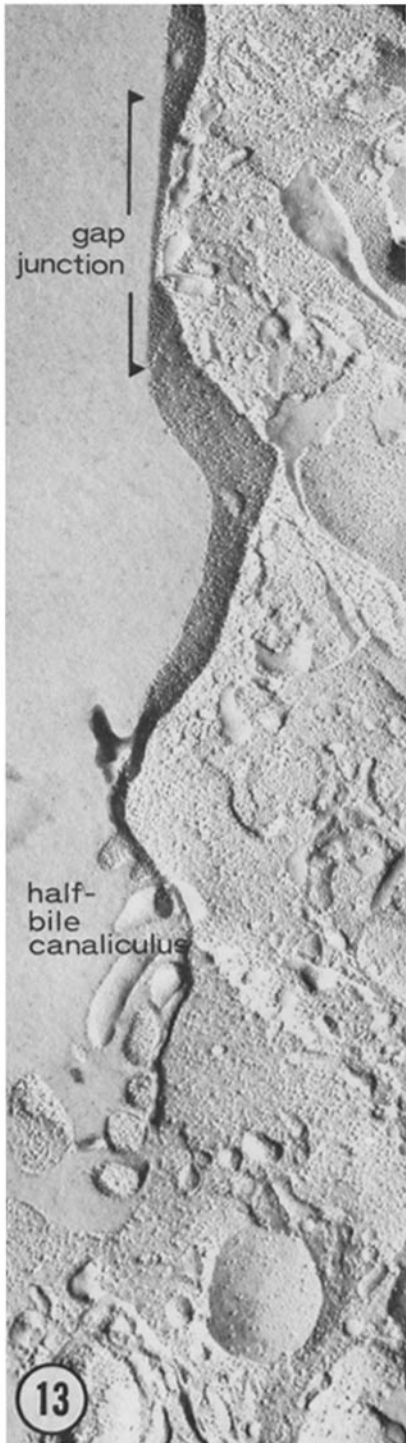
Establishing a time-course for the effects observed is very difficult with the perfusion system currently used. Due to irregularities in the vasculature of the liver, dependent upon local physiological conditions, different liver lobules will not have a uniform vascular irrigation during the experiment. In addition, there may be differences in irrigation from cell to cell within a given lobule, depending upon the cell's functional location (45). The freeze-fracture method, as used, gives random sampling through the tissue, and hence the time-

course of perfusion and the absolute hypertonicity in any given area are not accurately known. In the range of 5 s, plasmolysis and a limited amount of junctional splitting occur. In the range of 10–20 s, many splitting and split junctions may be found, in addition to junctions which do not appear to have been affected at all. After 30 s of perfusion, only completely split or still intact junctions are found; affected cells are usually very widely separated. It is not known if the junctions which remain unsplit, even after 2-min perfusion, represent areas which were protected from the hypertonic conditions, or if they represent a second functional class of gap junctions which are unaffected by the perfusion procedure.

Electrotonic uncoupling in response to chloride substitution (1) and glutaraldehyde fixation (9) is known to take place with a time-course of seconds to minutes, and the junction splitting described here has a time-course in seconds. The junction splitting, then, may result directly in electrotonic uncoupling, or the splitting may be a secondary event to uncoupling, perhaps necessary for the breakdown and synthesis of new, coupled junctions at some later time period. One must constantly bear in mind, while examining any micrographs of intercellular junctions, that the junction prepared for electron microscopy is of necessity uncoupled. Cell death is a rapid and potent uncoupler of cells, and the uncoupling effect of electron microscope fixatives has been carefully studied (9). Thus, unless one uses rapid freezing techniques, without fixatives or cryoprotective agents, the junctions are all probably in the uncoupled state. Therefore, the data presented in this paper do not prove either that the junction splitting represents the actual event of electrotonic uncoupling, or that the splitting represents complex secondary reorganization of the junctional structure. A detailed electrophysiological study of hypertonically shocked liver may shed some light on these problems.

Deep etching of freeze-fractured split gap junc-

FIGURES 13-15 These micrographs demonstrate the effects of portal perfusion of hypertonic sucrose upon the zonulae occludentes. Fig. 13 is a replica of a liver cell which has separated some distance from adjacent cells. A half-bile canaliculus is seen with microvilli projecting into the canalicular lumen, now confluent with the extracellular spaces. A half-gap junction is also seen directly facing the extracellular space. Figs. 14 and 15 are micrographs of the initial splitting of the bile canaliculi, and both halves of the canaliculi can be seen. Fig. 14 is a thin-sectioned view; there is no evidence of torn membranes at the obligatory sites of the zonulae occludentes. Fig. 15 is a freeze-fractured view of a split canaliculus, showing the A face strands still present on the split junctional membrane. Figs. 13 and 14, $\times 35,000$; Fig. 15, $\times 50,000$.



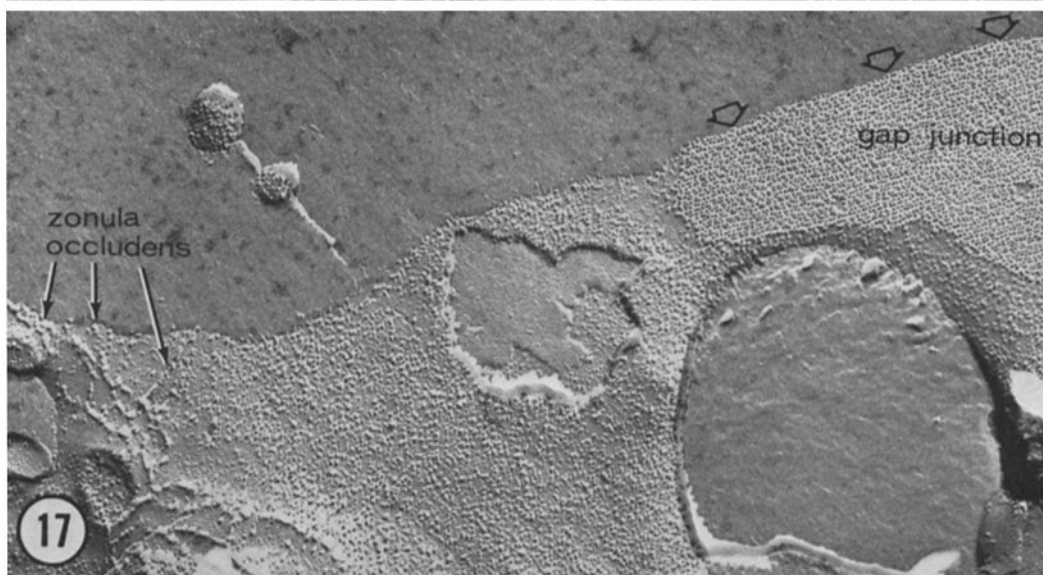
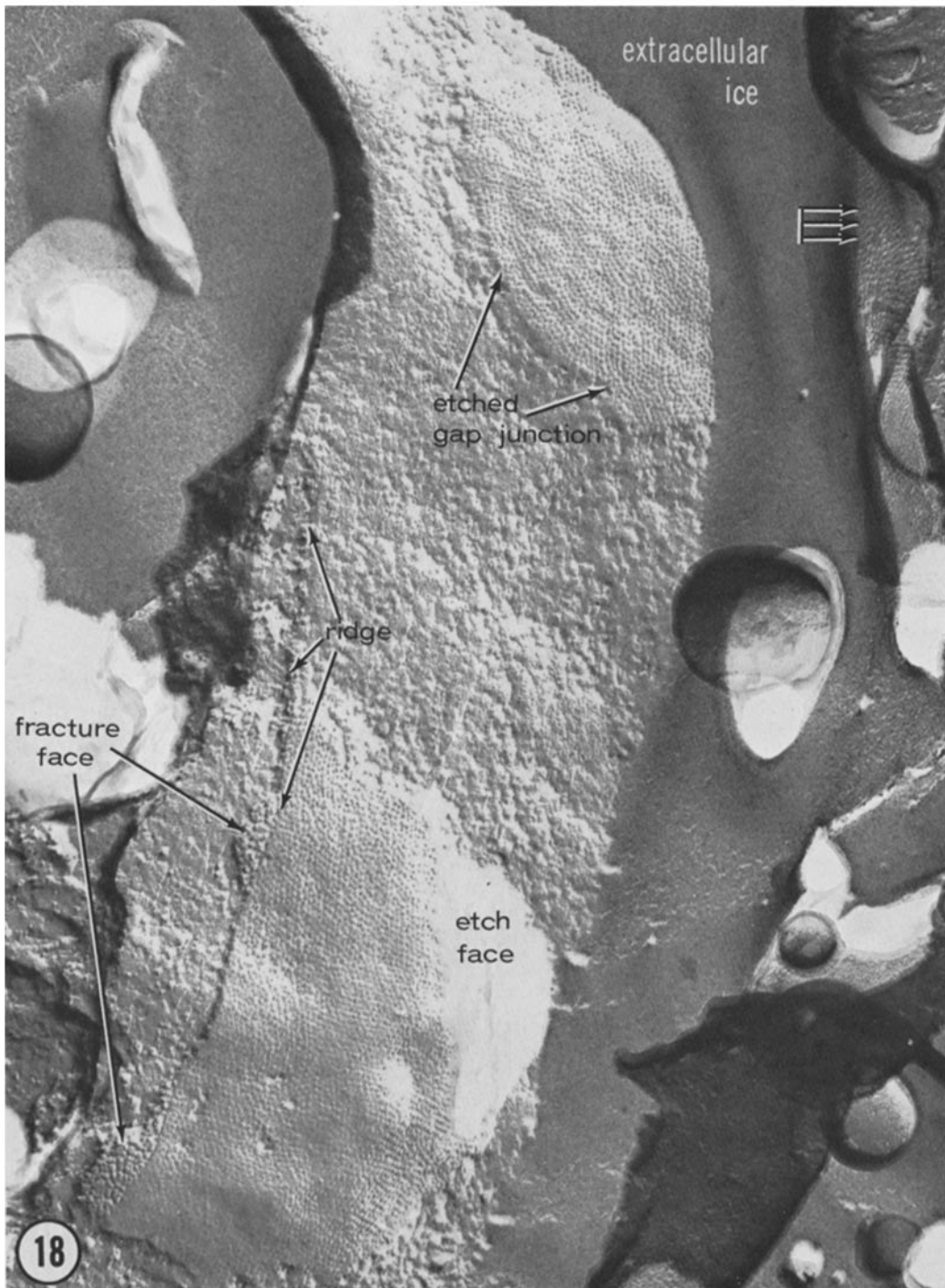


FIGURE 16 This micrograph of a freeze-fracture replica of a half-bile canaliculus shows the B face of the zonula occludens adjacent to the extracellular space. The top half of the micrograph shows extracellular ice: the adjacent cell is not visible in the micrograph. The canalicular microvilli now project directly into this extracellular space, the physiological compartmentalization of bile and blood spaces having been lost. A small patch of gap junction B face is also seen (large arrow). $\times 63,000$.

FIGURE 17 This figure shows a portion of the surface of an hepatocyte separated from adjacent cells by portal perfusion with hypertonic sucrose. A faces of both a gap junction and a zonula occludens are seen directly abutting the extracellular space (large arrows). The fate of the half-junctions subsequent to this stage is not yet known. $\times 56,000$.

FIGURE 18 A replica of freeze-etched mouse liver after splitting of the junctions with 0.5 M sucrose for 30 s. The tissue was frozen directly in distilled water without cryoprotectants after fixation in aldehydes. The fracture process exposed two small areas of gap junction fracture face where the lattice of particles may be discerned. The preservation of the fracture face lattice is poorer than in other specimens due to the distilled water freezing without cryoprotectants. The etching process (-100°C for 60 s) has lowered the water table from the level demarcated by the ridge to the level shown in the replica (extracellular ice). The lowering of the water table has exposed the etch face of two gap junctions. The junction etch face is characterized by an extremely shallow lattice of particles, which has the same center-to-center interparticle distance as the lattice seen on the fracture face (8-9 nm). The etched lattice on the adjacent cell representing the other half of the junction may also be seen (triple arrow). Thus the gap junction appears to split centrally in the 2-nm gap in response to hypertonic sucrose administered *in vivo*, leaving etch lattices on each of the adjacent cells. $\times 110,000$.



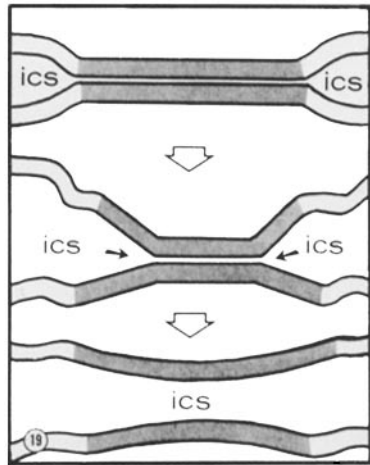


FIGURE 19 This diagram depicts three stages in the splitting of the gap junction with hypertonic disaccharides. The local differentiation of the membrane at the junction is schematically represented by stippling the membrane profile. In the upper diagram, a control gap junction is depicted with its characteristic gap separating the apposed stippled junctional membranes. At this point, the intercellular space (*ics*) becomes severely narrowed. The middle drawing depicts a diagrammatic junction in the process of splitting response to hypertonic treatment of whole liver. The intercellular space invades the 2-nm gap (arrows), splitting the junction, although the junctional membrane differentiation (stippling) still remains localized in the membrane. The bottom diagram illustrates the gap junction completely split by hypertonic disaccharides. A wide intercellular space (*ics*) now separates the two junctional membranes, which still reveal the characteristic lattice of particles in freeze-fractured specimens (stippling). It is not known how long the junctional membranes remain organized in a lattice after splitting. The zonula occludens appears to split in a similar manner.

tions reveals the surface of the junction normally sequestered within the 2-nm gap. A shallow lattice of particles is revealed on both halves of the split junctional membranes which has the same center-to-center distance as the more commonly seen fracture face lattice. The presence of a similar etch lattice on both halves of the gap junction suggests that the split has been centrosymmetric, occurring within the 2-nm gap.

The observation that hypertonic conditions might also split the zonulae occludentes sealing the bile spaces in the liver has physiological and pharmacological interest. If the breaking of the junctions can be reversibly controlled, the prospect

of vascular injection of pharmacological agents past blood-tissue barriers may have useful applications.

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