

Structural Integrity of Hepatocyte Tight Junctions

DAVID W. EASTER, JAMES B. WADE, and JAMES L. BOYER

Departments of Physiology and Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT The significance of discontinuities frequently found in freeze-fracture replicas of the tight junction was evaluated using complementary replicas of hepatocyte junctions from control and bile duct-ligated rats. An extensive analysis of complementary replicas using rotary platinum shadowing indicates that discontinuities in the protoplasmic (P) fracture face do not represent structural breaks in the tight-junctional network. In no case did P-face discontinuities correspond with interruptions in the groove network on the complementary extracellular (E) face. Quantitative analysis of replicas shows that P-face discontinuities result in part from "transfer" of material to the complementary E face (~7% of the junctional length). However, many P-face discontinuities (7-30% of the junctional length) are matched only by a groove on the complementary E face. This finding demonstrates that a significant amount of material can be lost during freeze-fracture. An analysis of junctions from bile duct-ligated rats, which are known to have an increased paracellular permeability, shows comparable transfer and loss of material. However, the number of junctional elements and the tight-junction network density was significantly reduced by bile duct ligation. These observations indicate that discontinuities in tight-junctional elements result during the preparation of freeze-fracture replicas and are not physiologically important features of the junctional barrier. Variation in the number of elements provides the best explanation for observed differences in tight-junction permeability.

The tight junction (zonula occludens) is a beltlike paracellular seal that limits the passage of extracellular tracers (11, 13, 23) and is responsible for the wide variation in the paracellular permeability of epithelia (2, 7, 12). Use of the freeze-fracture technique to split the membrane bilayer has provided a method to examine the intramembrane structure of the tight junction (5, 30, 32). The junction is seen as a network of anastomosing elements¹ on the protoplasmic membrane leaflet (P face) and complementary grooves in the extracellular membrane leaflet (E face). Junctional elements are occasionally seen lying in the E-face grooves and have been loosely termed "transferred particles," since intramembrane particles are more commonly associated with the P face of freeze-fractured membranes.

Since the recognition that the tight junction represents a region of very specialized intramembrane structure, there has been a continuing effort to correlate paracellular permeability with the details of junctional structure revealed by freeze-fracture. Claude and Goodenough (6, 7) have proposed that

paracellular permeability is governed by the number of sealing tight-junctional elements, a concept that has been supported by the observations of others (8, 10, 20-22). However, important exceptions to this correlation have also been reported (19, 25). These observations suggest that variation in paracellular permeability may not always be accounted for by the number of junctional elements. In addition, the discontinuous nature of the P-face elements had led to the suggestion that these areas of discontinuity may function as "pores" in the tight-junction network (3, 15, 22, 31).

This study examines the significance of discontinuities in the P-face network of rat hepatocytes by using complementary replicas to account for all junctional elements. Rotary shadowing (18) is employed to improve the resolution of junctional substructure. Junctions from bile duct-ligated rats are also evaluated, to assess junctional structure under conditions of altered paracellular permeability (1, 20).

MATERIALS AND METHODS

Bile Duct Ligation: Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing between 225 and 280 grams were used for all experiments. After intraperitoneal administration of 50 mg/kg sodium pentothal to achieve anesthesia, a 2- to 3-cm midline incision of the abdomen was made to expose the common bile duct. The bile duct of five rats

¹ The term "elements" is used throughout this paper to mean all P- and E-face junctional material, variously referred to as particles, strands, ridges, fibrils, or bars. Although it has been widely accepted that this material is intramembrane protein, recent observations raise the possibility that this material may be lipid in nature (16).

was doubly ligated with 5:0 silk above the entrance of the pancreatic ducts, and severed completely between the two ties. The bile duct of five control rats was similarly exposed and manipulated, but was not ligated or cut. The abdominal incision was then closed with 4:0 silk and skin clips, and the rats were allowed food and water ad libitum for a period of 24 or 96 h.

Specimen Preparation: After 24 or 96 h, rats were again anesthetized, and their livers exposed. A sample from the median lobe was rapidly excised and immediately diced into pieces $\sim 1 \text{ mm}^3$ after immersion in a fixative solution consisting of 2.5% glutaraldehyde buffered with 0.15 M sodium cacodylate and adjusted to pH 7.4. These small pieces of liver were then fixed by immersion for an additional 18–22 h and coded so that all subsequent procedures and measurements were carried out without knowledge of the treatment. After cryoprotection in 25% glycerol, tissue specimens were freeze-fractured and platinum-shadowed either by standard directional shadowing at 45° or by rotary shadowing with a 25° shadow angle. A Balzers BAF no. 301 freeze-fracture apparatus was used (Balzers Corp., Hudson, NH), and was equipped with a rotary stage and double-replica specimen table. Operating conditions were: fracture at -110°C stage temperature, a vacuum of at least 10^{-6} torr, no etching time, and a platinum thickness of 2.8 nm for unidirectional, 3.3 nm for rotary shadowing.

Measurements and Calculations: Complementary membrane fractures were obtained from three bile duct-ligated and three control rats (4–8 membrane pairs/rat were examined). Data was also compiled from noncomplementary fractures of an additional two bile duct-ligated and two control rats (7–10 membrane fractures/rat). Measurements of junctional characteristics were obtained at $\times 83,200$ either directly from photomicrographs, or from composite tracings of complementary membrane halves. Composite tracings were produced by overlaying complementary photomicrographs with clear plastic and tracing each junctional element with different colors for P-face elements, E-face elements ("transferred particles"), and E-face grooves not associated with P-face elements. In this way, a "complete" tight junction was reassembled on a single tracing from the data available on both complementary membrane leaflets. A digitizer (Bit pad one; Summagraphics Corp., Fairfield, CT) coupled to a programmed microcomputer (North Star Computers Inc., Berkeley, CA) was used to record the following five junctional parameters:

- Sum of P-face element lengths (P).
- Sum of E-face element lengths, i.e., "transferred particles" (E).
- Sum of junctional groove lengths not associated with either P-face elements or E-face elements (G).
- Sum of all tight-junctional element lengths (L), i.e., $L = P + E + G \pm$ measurement error.
- Length of canaliculus sealed by the measured junction, i.e., "canaliculus length" (C).

From these measurements, the following three calculations were made for control and bile duct-ligated rats:

- Tight-junction network density: L/C .
- Fraction of junctional elements found on the E-face fracture face (in percent), "% elements transferred": $(E/L) \times 100$.
- Fraction of junctional elements lost during fracture (in percent), "% elements lost": (i) as assessed with complementary tracings: $(G/L) \times 100$, or (ii) in noncomplementary micrographs: $[(L-P)/L] \times 100 - [(E/L) \times 100]$, i.e., fraction of elements missing from P face minus fraction of elements found on E face.

The accuracy of digitizing was estimated by several methods and was considered to give a $<1\%$ error within the range of lengths measured.

Also, another measure of the extent of the tight-junction network was obtained by counting the number of sealing tight-junction elements (excluding radially directed or loose-ended elements), at an interval of 2 cm along the junctional network, i.e., $\sim 0.2 \mu\text{m}$ at 83,200. This measure is called the "average number of elements."

Statistical comparison used one-way analysis of variance with calculation of the least significant difference. Since no significant differences were noted between the 24- and 96-h rats in any of the parameters measured, results are reported simply as control or bile duct-ligated rats.

RESULTS

Rotary vs. Unidirectional Shadowing

Rotary platinum shadowing was found to provide resolution of intramembrane junction substructure superior to that of unidirectional shadowing. Unidirectional platinum coating not only conceals particle boundaries in the platinum shadow but also tends to fill the shallow E-face grooves (particularly when shadowing is directed parallel to the direction of tight-junction elements). Also, unidirectional shadowing often produces very

large shadows from protruding structures, such as the canalicular microvilli, which can conceal large portions of the junctional network. This effect makes matching of complementary replicas from directionally shadowed specimens less productive than expected. At the lower platinum angle of rotary shadowing, protruding structures and even fracture plane contours rarely allow for a complete 360° shadowing (as can be appreciated on close examination of Fig. 2). While the resulting shadows can also obscure some E-face groove and particle detail, rotary platinum shadowing effectively minimizes these problems (compare unidirectionally shadowed Fig. 1 with rotary-shadowed Fig. 2).

Tight-junction Substructure

The freeze-fractured tight-junction network of both control and bile duct-ligated rat hepatocytes consists of discontinuous P-face elements, and continuous E-face grooves with occasional "transferred particles" (Figs. 1 and 2). Complementary replicas of 61 junctions from bile duct-ligated (28 junctions) and control (33 junctions) rats were assessed for structural completeness by the tracing method described above. Lengths of 232.2 and 302.5 μm of reassembled tight-junctional elements were measured over 63.5 and 58.6 μm of sealed bile canaliculus for bile duct-ligated and control animals, respectively.

These extensive tracings clearly showed that for both directional- (Fig. 1) and rotary-shadowed (Fig. 2) membranes some of the discontinuities in the P-face architecture resulted from occasional adherence of junctional elements to the E-face membrane leaflet (black arrows, Figs. 1 and 2). This E-face junctional element was invariably the size of the smallest junctional element commonly seen on the P face, hence the term "transferred particle." However, many areas of P-face junctional discontinuity did not have "transferred particles" on the complementary E face, but were always associated with an intact E-face groove (white arrows, Figs. 1 and 2). These specific areas of P-face discontinuity paired with an intact E-face groove indicate that junctional material was initially present, but most likely lost during the fracture process. In no case could a region of P-face discontinuity be matched with a discontinuity in the corresponding E-face groove.

Quantitative Analysis of Tight Junctions

From the analysis above, we conclude that the discontinuities in the P-face elements occur because junctional material is both transferred and lost during the freeze-fracture process. Quantitative data accumulated from five control and five bile duct-ligated rats demonstrate that a substantial amount of junctional material appears to be lost during fracture (Table I). Average values for "% elements lost" varied widely within controls (7–30%) and bile duct-ligated rats (11–20%). No significant difference was noted between treatment groups. Similarly, the amount of "% elements transferred" was also quite variable (1–11% in controls and 4–11% in bile duct-ligated rats), with no significant difference noted.

In contrast, measures of the extent of the tight-junctional seal, namely the average number of elements and the tight-junction network density, did differ significantly ($P < 0.001$ and $P < 0.01$, respectively) between bile duct-ligated and control rats (Table I). These changes in the tight junction can be seen qualitatively as the more dense junctional network of hepatocytes from control animals (Fig. 1) when compared with junctions from bile duct-ligated animals (Fig. 2).

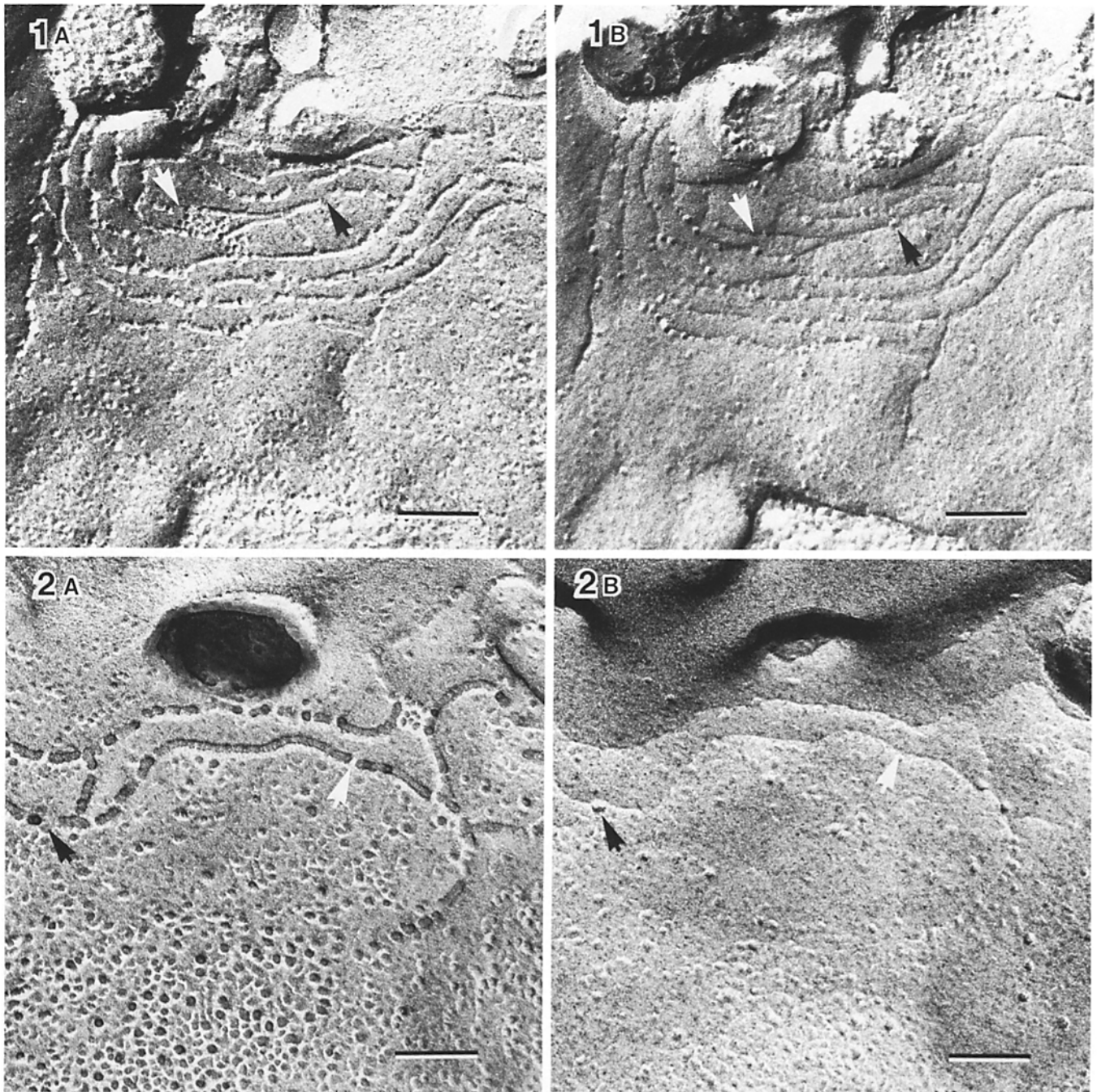


FIGURE 1 Freeze-fractured and unidirectionally shadowed complementary replicas from control hepatocyte. The tight-junction architecture is shown sealing the bile canaliculus (top), with discontinuous P-face elements (A) and continuous E-face grooves (B). Note "transfer" of junctional elements from the P to E membrane leaflet (black arrows), and loss of junctional elements associated with an intact E-face groove (white arrows). Bar, 100 μm . $\times 128,000$.

FIGURE 2 Freeze-fractured and rotary-shadowed complementary replicas from 24-h bile duct-ligated hepatocyte. As with controls, note "transfer" of junctional elements from P face (A) to E face (B) (black arrows), and loss of junctional elements (white arrows). Also, note improved resolution of junctional detail in this rotary-shadowed preparation when compared with the unidirectional shadowing of Fig. 1. Bar, 100 μm . $\times 128,000$.

DISCUSSION

Discontinuities in the P-face elements of tight junctions have been noted in numerous freeze-fracture studies (8, 15, 20-22, 24, 27, 28, 31, 33). Some authors have suggested that these discontinuities may represent structural pores or defects in the tight-junction seal. Regardless of whether the junctional ele-

ments are composed of intramembrane protein or cylindrical lipid micelles, as recently suggested (16), true discontinuities in the junctional network could provide a pathway for movement of solutes across the junction and thus have an important influence on epithelial permeability. However, the significance of the discontinuities observed can only be ascertained by examining the complementary E-face replicas in order to ac-

TABLE I
Quantitative Measurements of Tight-junction Structure

| | Average number of elements | Tight-junction network density | % Elements lost | % Elements transferred |
|-------------------------------|----------------------------|--------------------------------|-----------------|------------------------|
| | $P < 0.001$ | $P < 0.01$ | | |
| Control (5 rats) | 4.45 ± 0.52 | 5.36 ± 0.79 | 19.3 ± 8.9 | 7.3 ± 3.8 |
| Bile duct-ligated (5 rats) | 2.32 ± 0.16 | 3.59 ± 0.31 | 15.8 ± 4.6 | 7.2 ± 2.7 |

curately evaluate the complete junctional structure. Since most E-face fractures of tight junctions are grooves that correspond exactly to the elements on the P face, it is clear that these grooves represent complementary impressions of the junctional elements. Thus any discontinuity in the E-face grooves should represent an actual break in the tight-junction seal. In contrast, discontinuities in the P-face elements might occur not only because of true breaks but also because (a) elements of the junction have fractured with the E face ("particle transfer") or (b) elements of the junction have been lost during the fracture process ("particle loss"). Plastic deformation of intramembrane structures during the fracture process as described by Sleytr and Robards (29) may account for some variation in their appearance, but cannot reasonably explain actual displacement or loss of junctional elements. Analysis of complementary replicas as carried out in the present investigation can distinguish between these possible explanations of P-face discontinuities; P-face discontinuities due to actual breaks will be characterized by corresponding discontinuities in the E-face grooves, whereas discontinuities due to particle transfer will have particles at the corresponding site lying in the E-face grooves. This phenomenon has previously been demonstrated by Van Deurs and Koehler (31). That small portions of the junctional elements are actually lost during fracture has not been previously reported. However, we consider this the only reasonable explanation for the case when a discontinuity on the P face is matched by a clear groove on the complementary E face.

Although an extensive number of complementary replicas were examined in the present study of hepatocyte tight junctions, no examples of corresponding discontinuities on both fracture faces were encountered in adequately shadowed specimens. This indicates that the incidence of actual interruptions in the hepatocyte tight junction must be rare even though this epithelium is considered to be relatively leaky. Nor could such breaks be identified after bile duct ligation, which has been shown to radically increase paracellular permeability to ions and macromolecules (20). This indicates that structural breaks in the tight-junction network are unlikely to provide an adequate explanation in this model for variation in paracellular permeability.

The conclusion that bile duct ligation is not associated with discontinuities in the junctional network contrasts with reports by other groups (8, 20, 21). However, complementary replicas were not examined and published micrographs do not clearly demonstrate discontinuities in the E-face grooves. Van Deurs and Koehler (31) did examine complementary replicas in their analysis of choroid plexus and intestinal cell junctions and demonstrated that the "leakier" choroid plexus had more P-face discontinuities than did the small intestine. While they showed that some of these discontinuities resulted from transfer of particles to the E face, they did not evaluate the possibility

that loss of material could occur during fracture, nor did they use rotary shadowing. Our quantitative analysis indicates that loss of material can be significant, with 7–30% of the junctional element length apparently lost during fracture. Furthermore, we found that low-angle rotary shadowing as developed by Margaritis et al. (18) allows for more consistent and improved visualization of E-face grooves. In contrast, poor visualization occurs when unidirectional shadowing is aimed by chance parallel to the orientation of the grooves where there is little vertical relief for platinum shadowing. Thus most of our analysis used complementary freeze-fracture replicas that were rotary shadowed with platinum at a low angle of 25°. We found, however, that similar results could be obtained from quantitative analysis of P- and E-fracture faces from directionally shadowed specimens if analysis is restricted to junctions that by chance lie nearly perpendicular to the shadowing direction.

If, as suggested by our analysis, it appears that freeze-fracture replicas do not show the existence of pores within the elements of the tight-junction network, what factors might account for the wide range of paracellular permeabilities that occur in different epithelia and the physiological modulation of junctional permeability? The early work of Claude and Goodenough (7) demonstrated that a remarkable correlation exists between the number of junctional elements and the paracellular permeability of a variety of epithelia. The findings of many other investigators (8, 15, 20, 21, 26, 28), as well as the present study, support the basic concept that epithelia with a large number of junctional elements have a low paracellular conductance, whereas tissue that is characterized by few junctional elements has a high paracellular conductance. While for many purposes a simple count of the incidence of junctional elements at intervals along the junction is adequate, we believe that a more sensitive measure is provided by the "network density," which is the total length of junctional elements divided by the length of sealed membrane. This calculation, which is a straightforward measurement when performed using currently available digitizer-microcomputer systems, should better account for geometric variations in junctional architecture that occur in epithelia (14).

While the number of junctional elements provides for a reasonable structural explanation of junctional permeability in many instances, several authors have challenged this general relationship because of specific exceptions that have been described (19, 25). It is by no means clear that these exceptions necessarily imply that the junctional elements visualized by freeze-fracture are not the functionally important sealing structures. The exceptions cited, in which a large number of junctional elements are found in leaky epithelia, may instead be due to nonjunctional paracellular pathways, as has been suggested for the choroid plexus (24). It is also possible that regional heterogeneity in the tight-junction network is respon-

sible for some of the apparent discrepancies. Cereijido et al. (4) have recently shown by microelectrode studies that the tight-junctional conductance is not necessarily uniform along its length. Junctional structure is also known to vary along the length of the junction (4, 7, 17). It is possible that relatively few regions with a reduced number of elements that might not be encountered by investigators may in some cases be responsible for a relatively high paracellular conductance.

Regarding the effect of bile duct obstruction, others have reported qualitative reductions in element number as well as discontinuities in the element network (8, 20, 21). Although these reports do not take into account the possibility of lost particles during the process of fracture preparation, the significant reduction in both element number and density observed with quantitative techniques in the present study lends support to these observations. Furthermore, Metz et al. (20) have demonstrated that horseradish peroxidase can penetrate these junctional barriers in the bile duct-obstructed rat within 1 min after portal vein infusion, in contrast to control animals where this large protein is restricted in passage across the tight-junction barrier. A relationship between biliary permeability and tight-junctional structure has also been observed in several experimental models of cholestasis in rat including chronic phalloidin treatment (10) and administration of ethinyl estradiol (9). Altogether, these studies of junctional structure and permeability in the rat hepatocyte strongly suggest that tight junctions play an important role in regulating the transfer of water and solute between blood and bile.

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