FRACTURE FACES OF ZONULAE OCCLUDENTES FROM "TIGHT" AND "LEAKY" EPITHELIA

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ABSTRACT

Epithelia vary with respect to transepithelial permeability. In those that are considered "leaky", a large fraction of the passive transepithelial flux appears to follow the paracellular route, passing across the zonulae occludentes and moving down the intercellular clefts. In "tight" epithelia, the resistance of the paracellular pathway to passive flux is greatly increased. To see whether differences in the morphology of the zonula occludens could contribute to this variability in leakiness among epithelia, replicas of zonulae occludentes in freeze-fractured material from a variety of tight and leaky epithelia were examined. The junctions appear as a branching and anastomosing network of strands or grooves on the A and B membrane fracture faces, respectively. It was found that the zonula occludens from a "very leaky" epithelium, the proximal convoluted tubule of the mouse kidney, is extremely shallow in the apical-basal direction, consisting in most places of only one junctional strand. In contrast, the "very tight" frog urinary bladder exhibits a zonula occludens that is relatively deep (>0.5 μm) in the apical-basal direction, and consists of five or more interconnected junctional strands interposed between luminal and lateral membrane surfaces. Epithelia of intermediate permeabilities exhibited junctions with intermediate or variable morphology. Toad urinary bladder, mouse stomach, jejunum, and distal tubule, rabbit gallbladder, and *Necturus* kidney and gallbladder were also examined, and the morphological data from these epithelia were compared to physiological data from the literature.

INTRODUCTION

Simple epithelia vary greatly in their ability to maintain ionic and osmotic gradients between physiological solutions of different compositions. Some, such as amphibian urinary bladders, do maintain steep gradients, while others, such as the fluid transporting gallbladder and the proximal convoluted tubule of the kidney, do not (Frömter and Diamond, 1972). Such leaky epithelia allow large amounts of water and small solutes to flow passively from one face of the epithelium to the other. Because of the relatively low permeability of the epithelial cell plasma membranes, a large fraction of the transepithelial flux moves between the cells, rather than through them (Frizzell and Schultz, 1972; Frömter, 1972), taking the paracellular shunt (Boulpaep, 1971). To follow this paracellular route, molecules at the epithelial free surface must cross the sealing junctions between the cells (the zonulae occludentes or tight junctions), and move along the intercellular clefts. Macromolecular tracer studies (Miller, 1960; Farquhar and Palade, 1963; Goodenough and Revel, 1970) indicate that the zonula occludens is an important barrier in the paracellular route, although some studies indicate that in leaky epithelia ionic lanthanum may penetrate the

TABLE I

Several Simple Epithelia Classified According to Transepithelial Resistance with Measurements of Junctional Morphology

Tissue	Transepithelial resistance in Ω cm^2	Junctional Morphology				
		No. of strands		Depth in μm		
		Range	Mean ± SE	Range	Mean ± SE	N
Very leaky—Mammalian proximal convoluted tubule						
Dog, Rat	6 (5)	—	—	—	—	—
Mouse	—	1–2	1.19 ± 0.12	*	*	48
Leaky—*Necturus* proximal convoluted tubule	70 (5)	1–6	3.3 ± 0.15	0.1–0.8	0.46 ± 0.02	59
Rabbit gallbladder	30 (18)	2–6	4.1 ± 0.11	0.1–1.0	0.41 ± 0.02	90
Intermediate—*Necturus* gallbladder	300 (17)	4–8	6.2 ± 0.21‡	0.5–1.4	1.0 ± 0.05	23
Rat jejunum	>300 (2)§	—	—	—	—	—
Mouse jejunum	—	4–7	5.3 ± 0.17	0.25–0.75	0.39 ± 0.025	34
Intermediate to tight—Distal convoluted tubule	300–600 (5)					
Necturus		2–7	4.8 ± 0.36	0.1–1.0	0.38 ± 0.06	17
Mouse		4–7	5.8 ± 0.20	0.1–0.2	0.136 ± 0.006	18
Very tight—Mouse stomach		4–11	8.1 ± 0.34	0.3–0.9	0.63 ± 0.03	25
Amphibian urinary bladder						
Toad	1,000–2,000 (8)	5–11	8.1 ± 0.94	0.3–0.5	0.36 ± 0.03	7
Frog		5–14	7.9 ± 0.38	0.3–1.1	0.58 ± 0.03	34

* In most places the total junctional depth was equal to the width of one strand only.

‡ A. J. Hudspeth and A. G. Yee, personal communication.

§ Corrected for mucosal-serosal surface amplification (see Discussion).

N, number of measurements made (see Materials and Methods).

SE, Standard error of the mean.

The numbers in parentheses indicate the articles from which the physiological data were taken.

tight junction (Whittembury and Rawlins, 1971; Machen et al., 1972). In some cases, the paracellular permeability is also affected by the resistance of the intercellular cleft (Claude, 1968; Smulders et al., 1972; Wright et al., 1972).

The freeze-fracture technique reveals that the zonula occludens is made up of a complex network of anastamosing and branching strands which lie in the plane of the cell membrane (Kreutziger, 1968; Staehelin et al., 1969; Goodenough and Revel, 1970; footnote 1). These strands appear as ridges on the A or inner membrane face, and as grooves on the B or outer membrane face. We have examined zonulae occludentes in freeze-fractured material from a variety of tight and leaky epithelia and find that there are conspicuous and systematic differences in the depth and complexity of the junctions. These differences may be important in determining transepithelial permeability. A preliminary report of this work has appeared elsewhere (Claude and Goodenough, 1972).

MATERIALS AND METHODS

Specimens included mouse kidney, stomach, and jejunum, rabbit gallbladder, *Necturus* kidney, and gallbladder, frog (*Rana pipiens*) urinary bladder, and toad (*Bufo marinus*) urinary bladder. The kidneys were fixed by perfusion and then excised, and pieces were immersed in fixative. The other tissues were generally exposed to fixative *in situ* before pieces were

[1] Friend, D. S., and N. B. Gilula. 1972. Variations in tight and gap junctions in mammalian tissues. *J. Cell Biol.* **53**:758.

excised and dropped into fixative. Fixatives were made up in cacodylate buffer at pH 7.4 (0.1 M cacodylate for amphibia, 0.15 M for mammals) using glutaraldehyde or glutaraldehyde plus formaldehyde (Karnovsky, 1965). We are encouraged by recent results that indicate that aldehyde fixation does not greatly alter transepithelial permeability (Jard et al., 1966; Eggena, 1972; Bennett et al., 1972), and thus may not greatly alter the properties of the zonula occludens.

After fixation for 1–2 h at room temperature, pieces of tissue were rinsed in cacodylate buffer, soaked in 20% glycerol in cacodylate buffer, and frozen in liquid Freon cooled with liquid nitrogen. Platinum-carbon replicas were made in a Balzers freeze-etching device BA360M (Balzers High Vacuum Corp., Lichtenstein) and examined in Siemens Elmiskop Ia and 101, and Philips 200 electron microscopes.

To express the morphological data from different epithelia as quantitatively as possible we made a series of measurements of total junctional depth and the minimum number of junctional strands interposed between the luminal surface and the lateral cell surfaces. There were some difficulties with this procedure: some tissues, especially *Necturus* gallbladder and kidney, tended to fracture in such a way that very few zonulae occludentes were exposed, and in those few, the fracture plane frequently passed through only part of the junction. (This may have been a result of local interdigitations at the level of the junction). Therefore, measurements were made only in micrographs where the junction was exposed from its apical to its basal edge. In places where the extent of the junction exposed was less than 0.5 μm in length (measured parallel to the luminal surface), the measurements were made in the middle of the exposed junction. In junctions of which more than 0.5 μm was exposed, as in Figs. 1–7 and 9–10, measurements were made every 0.5 μm. Measurements were not made in regions where more than two cells come together, and the zonula occludens extends farther basally than usual. For each epithelium, data from all the micrographs were pooled and means and standard errors of the means were calculated.

OBSERVATIONS

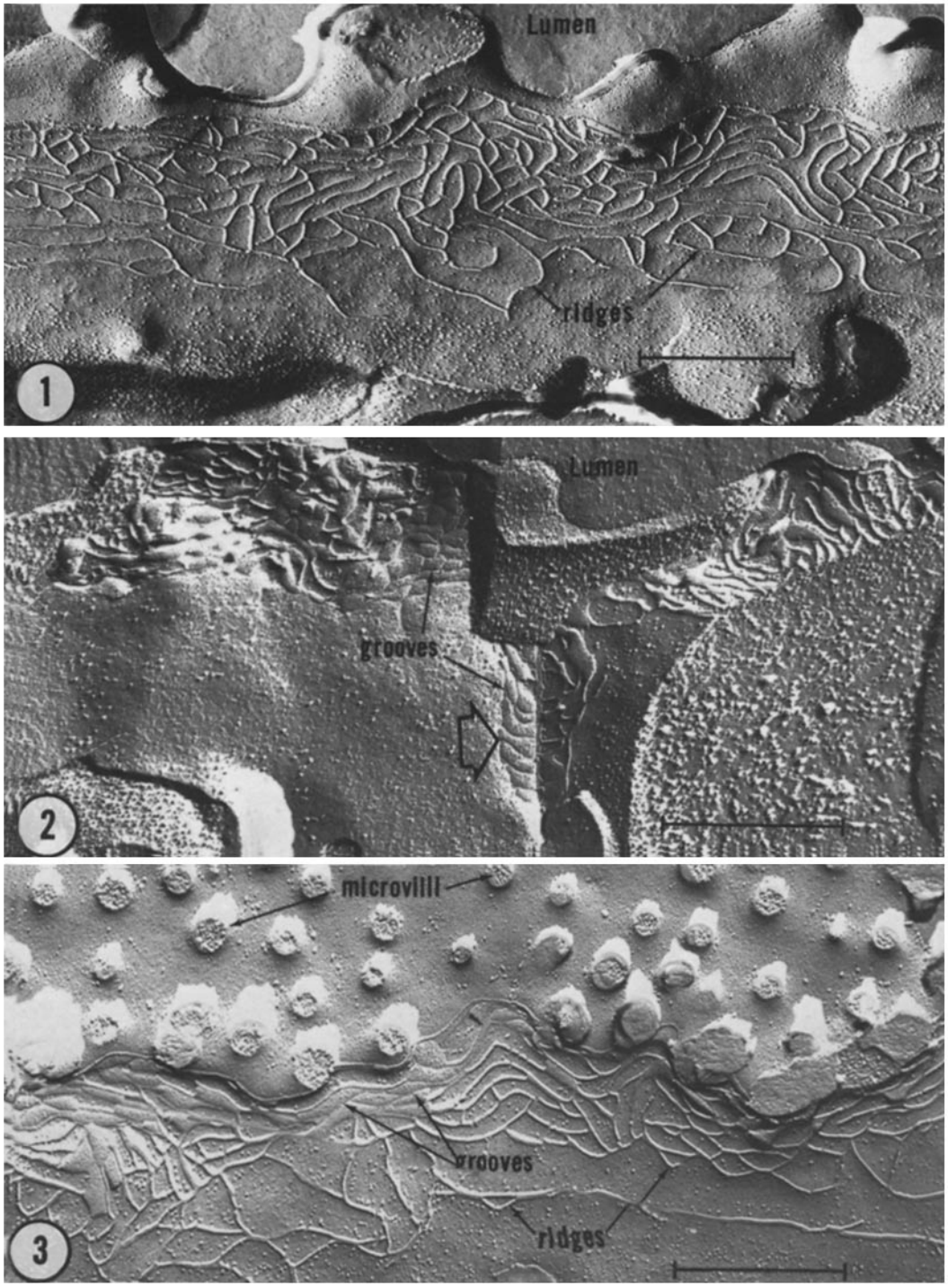
This study of the zonulae occludentes from various epithelia has revealed a qualitative correlation between junctional anatomy and permeability. The nomenclature system used in this paper, based on a survey of the physiological literature, classifies the epithelia as "very leaky", "leaky", "intermediate", "tight", or "very tight" on the basis of transepithelial permeability (Table I). The most dramatic morphological differences are seen between zonulae occludentes from very tight epithelia such as frog and toad urinary bladders (specific transepithelial resistance = 1,000–3,000 Ω cm²) and zonulae occludentes from very leaky epithelia such as the mammalian proximal convoluted tubule (6 Ω cm²). Epithelia of intermediate permeabilities have zonulae occludentes of intermediate or variable morphology.

Fig. 1 shows a replica from frog urinary bladder and illustrates the typical features of a tight zonula occludens. The junction itself is quite deep in the apical-basal direction (0.5 μm or more) and is composed of many (more than five) layers of interconnected strands interposed between lumen and intercellular space. The junction appears as a network of raised ridges on the A fracture face (inner membrane leaflet) as is illustrated in Figs. 1, 2, and 3, and as a series of interconnecting grooves on the complementary B fracture face (outer membrane leaflet), illustrated in Figs. 2 and 3. These two fracture faces have been shown to be

FIGURE 1 Frog urinary bladder, a very tight epithelium. The zonula occludens is seen as a series of interconnected ridges on the A fracture face. The overall junctional depth is 0.5 μm or greater, and there are five or more strands interposed between the luminal and the lateral membrane faces. Bar, 0.5 μm. × 45,500.

FIGURE 2 Toad urinary bladder, a very tight epithelium. The zonula occludens is seen as a series of ridges on the A fracture face as well as a series of grooves on the B fracture face (small arrows). Where the entire depth of the junction has been fractured, it can be seen to be about 0.4 μm in depth, and to be composed of seven or eight interconnected strands. The large arrow indicates an area where three cells come together, and the zonula extends basally along the facing lateral cell membranes. Bar, 0.5 μm. × 54,000.

FIGURE 3 Mouse stomach, a tight epithelium. The zonula occludens is seen in the A (ridges) and B (grooves) fracture faces. The junction is about 0.5 μm deep and is composed of at least six strands. At the top of the micrograph, the bases of cross-fractured microvilli can be seen. Bar, 0.5 μm. × 52,000.


Lumen
ridges
1
Lumen
grooves
2
microvilli
grooves
ridges
3

complementary by the double replica technique[2] and hence are the result of a single fracture plane. As described by Staehelin et al. (1969), the zonula occludens may take exceptionally long basal excursions in areas where more than two cells are joined; this phenomenon is illustrated in Fig. 2 (large arrow). Figs. 2 and 3, illustrating replicas from toad bladder and mouse stomach, respectively, are additional examples of physiologically tight epithelia. The zonulae occludentes are deep and display a complex morphology of at least five layers of interconnecting strands.

In sharp contrast to tight zonulae occludentes, the very leaky mouse proximal convoluted tubule displays an attenuated zonula occludens composed of only one or two strands. Figs. 4 and 5 show examples of zonulae occludentes from this epithelium. The junction in Fig. 4 is seen on both the A and B fracture faces; in Fig. 5, only the B face is evident. On the B face the junctional grooves are frequently studded with particles (Fig. 5, arrows): these particles may well represent remnants of the A face ridge still adhering to the B face, explaining the apparent discontinuities in some A face images (Fig. 4, heavy arrows).

These differences in junctional morphology do not simply reflect species differences. The tight (300–600 Ω cm²) mouse distal tubule zonulae occludentes (Fig. 6) may be contrasted with the junctions of the leaky mouse proximal tubule (Figs. 4 and 5). The distal tubule displays a zonula occludens with four or five interconnected strands and an overall depth of 0.1–0.2 μm. Proximal and distal tubule junctions from *Necturus* kidney may also be compared: the leaky (70 Ω cm²) proximal tubule junctions (Fig. 7) have two or three strands, with occasional interrupted strands (arrows), while the tight (300–600 Ω cm²) *Necturus* distal tubule junctions (Fig. 8) have four or five strands and a more complex branching and anastomosing network.

An interesting variation was seen in the rabbit gallbladder. This epithelium may be physiologically characterized as leaky (30 Ω cm²), yet some freeze-fractured replicas reveal a zonula occludens with an apparent tight morphology. As seen in Fig. 9, the rabbit gallbladder zonula occludens displays areas of many branching and anastomosing strands, with a deep apical-basal span. On close inspection, however, one can frequently find areas where the junction is reduced to only two or three strands (see arrows, Figs. 9 and 10). These latter areas may explain the physiological leakiness of the rabbit gallbladder.

Along the lateral surfaces of some epithelial cells one may encounter replicas of what appear to be elements of the zonula occludens (Fig. 11). Branching and anastomosing ridges may be seen (arrows) which are not organized into true zonulae, and which are described better as maculae occludentes. Since these junctional elements are discontinuous, they do not contribute significantly to the total transepithelial resistance. The maculae occludentes have no known function, but may be related to the genesis or degradation of the zonula occludens.

DISCUSSION

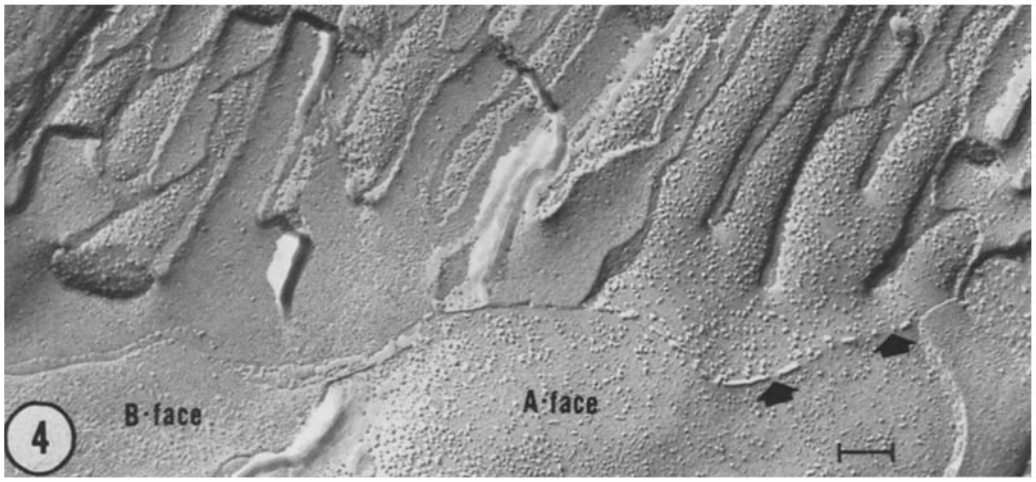
In this survey we have seen that tight and leaky freeze-fractured zonulae occludentes differ in ultra-

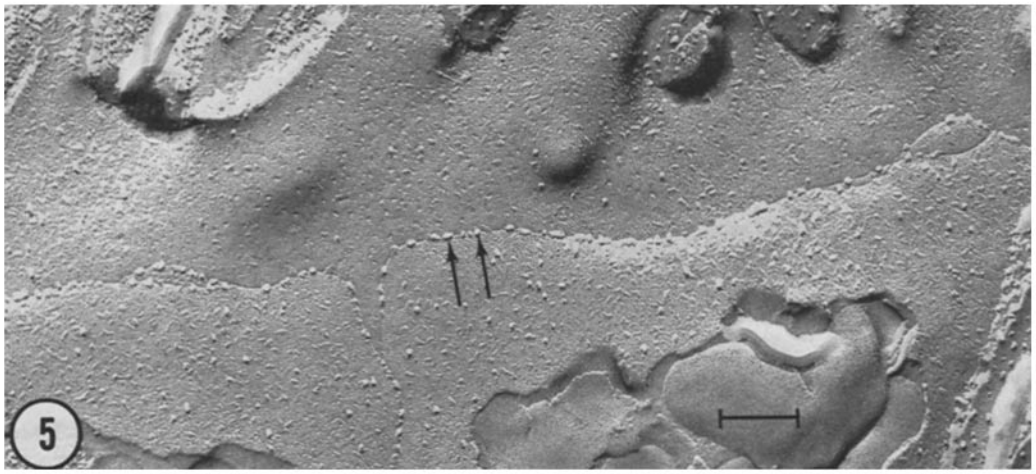
[2] Chalcroft, J. P., and S. Bullivant. 1970. An interpretation of liver cell membrane and junction structure based on observation of freeze-fracture replicas of both sides of the fracture. *J. Cell Biol.* 47:49.

FIGURE 4 Mouse proximal convoluted tubule, a very leaky epithelium. In most places the zonula occludens consists of only one strand, but there are occasional restricted areas where there are two or three strands. The junction is seen in both the A and B fracture faces. The heavy arrows indicate areas where pieces of the ridge appear to have broken away. Longitudinally fractured microvilli are seen in the upper part of the micrograph. Bar, 0.1 μm. X 70,000.

FIGURE 5 Mouse proximal convoluted tubule. Zonula occludens seen in the B fracture face. There are numerous particles lying in the groove (arrows), perhaps having broken from the complementary fracture face during cleavage. Bar, 0.1 μm. × 105,000.

FIGURE 6 Mouse distal convoluted tubule, a tight epithelium. The zonula occludens is deeper than in the proximal tubule and is composed of several (four or more) anastamosing strands, here seen as grooves in the B fracture face. Bar, 0.1 μm. × 70,000.


B-face
A-face
4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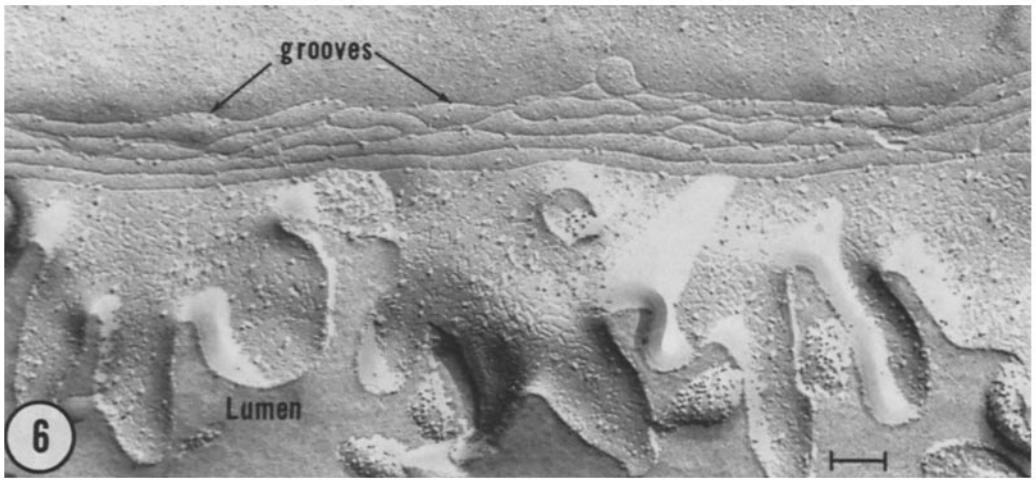
grooves
Lumen
6

FIGURE 7 *Necturus* proximal convoluted tubule, a leaky epithelium. Some of the strands in the zonula occludens are discontinuous (arrows). In places it looks as though portions of the ridges on the A fracture face are broken away, revealing grooves underneath (asterisks). Bar, 0.1 μm. × 68,000.

FIGURE 8 *Necturus* distal convoluted tubule, a tight epithelium. The zonula occludens, here seen in the B fracture face is deeper and more complex than in the proximal tubule. Bar, 0.1 μm. × 120,000.

FIGURE 9 Rabbit gallbladder, a leaky epithelium. In some places the zonula occludens is quite deep (>0.5 μm) and is composed of several strands. However, there are also areas (heavy arrows) where the junction is shallower and consists of only two or three strands; these attenuated areas may provide a favorable route for the transepithelial flow of ions. Bar, 0.5 μm. × 43,500.

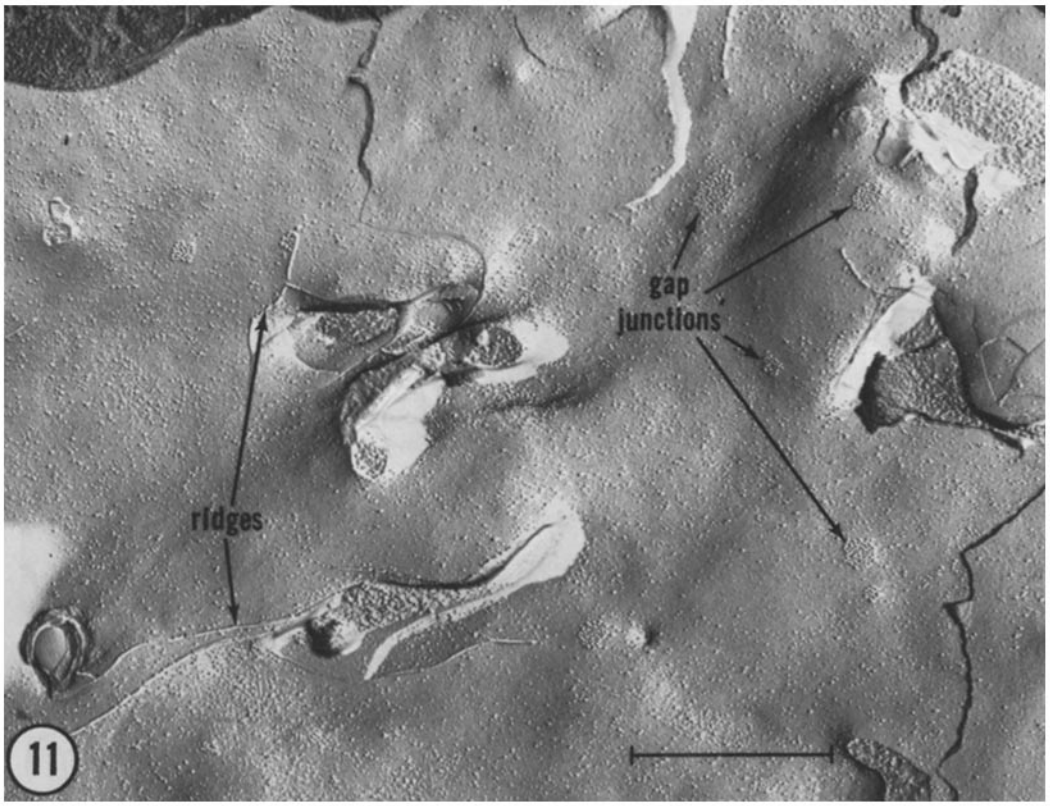

FIGURE 10 Rabbit gallbladder, a leaky epithelium. This is another example of a region of the zonula occludens where overall junctional depth is reduced (arrows) and there are only two to three strands making up the junction. Bar, 0.1 μm. × 64,000.

FIGURE 11 Mouse stomach, the lateral surface of an epithelial cell. As well as frequent small gap junctions, there are occasional anastomosing ridges that resemble elements of the zonula occludens, but are not associated with it. Since they are discontinuous, they do not contribute significantly to paracellular transepithelial resistance. Bar, 0.5 μm. × 55,000.

structure; very tight epithelia have deep zonulae with a complex network of anastamosing strands, while very leaky epithelia have shallower junctions composed of fewer strands. Epithelia with intermediate permeabilities generally have zonulae with intermediate, or variable morphology.

In comparing the data from different tissues as it is expressed in Table I, we can see that transepithelial permeability is correlated more closely to the number of strands that make up the junction than it is to total junctional depth. This is particularly evident in mouse distal tubule, where the zonula is made up of five or six strands which are compressed into a band less than 0.25 μm in depth, and in the toad urinary bladder, where approximately eight strands are compressed into 0.4 μm or less. If the tissues are grouped according to junctional morphology, the mouse stomach and the amphibian urinary bladders fall into one very tight category, with about eight strands, and the mouse proximal tubule represents the other extreme, very leaky, with only one strand in most places. *Necturus* proximal tubule and rabbit gallbladder look "fairly leaky", with three or four strands making up the zonula occludens, while *Necturus* distal tubule and gallbladder and mouse distal tubule could be grouped together as having a "fairly tight" morphology with five or six strands. Because the junctions can be quite variable in some tissues, such as the rabbit gallbladder, and because of difficulties inherent in making both the morphological and physiological measurements (see below), it seems premature at this time to try to make the correlations more precisely than this.

We found it advantageous to use freeze-fractured material rather than thin sections for two reasons. Although the difference in junctional depth between tight and leaky epithelia can often be quite striking in sectioned material (Farquhar and Palade, 1963; Claude, 1966, 1968), there is still the problem of orientation; an oblique section through a junction will make it appear deeper than it really is. This difficulty does not arise in freeze-fractured material. Another advantage of the technique is that the anastamosing strands that make up the junction are clearly visible. These strands, seen as ridges or as grooves in the A or B fracture faces, may be the intramembrane counterpart of the focal contacts (kisses) that are occasionally resolved in thin sections. Goodenough and Revel (1970) have presented negative-stain evidence that this branching and anastamosing network reflects the pattern of functional membrane contacts within the zonula occludens, and hence the site of the transepithelial permeability seal to macromolecular tracers such as hemoglobin (Miller, 1960; Farquhar and Palade, 1963). Our present results indicate that the number of strands, or barriers, in a junction and their geometrical arrangement may be more important to junctional permeability than overall junctional depth, so it is important to be able to resolve the individual strands; the freeze-fracture technique is ideal for this.

Current interpretations of freeze-fractured cell membranes (Branton, 1966; Pinto da Silva and Branton, 1970) postulate a splitting of the membrane in its hydrophobic interior. Thus the strands that appear in freeze-fractured zonulae occludentes probably represent structures lying within the junctional membranes rather than representing structures between the two membrane outer leaflets where they appear to fuse.

Additional resistances in the paracellular pathway must be accounted for in an attempt to correlate junctional morphology to transepithelial permeability. In some cases the interspaces basal to the zonula occludens are long and narrow enough to account for an appreciable part of the total resistance of the paracellular pathway. This situation arises in fairly leaky epithelia where junctional permeability is high and where the cells are tall and in close apposition to one another (Claude, 1968; Smulders et al., 1972; Wright et al., 1972). In epithelia where the junctions are very tight, however, the permeability of the paracellular pathway is reduced, and more of the flux is forced to pass through the cells. In these situations, the overall transepithelial permeability will tend to reflect the properties of the epithelial cell membranes, rather than properties of the paracellular pathway.

There are geometrical factors that will affect the contribution of the paracellular pathway to total transepithelial flux. The overall resistance of the paracellular pathway will depend not only on its specific resistance in the transepithelial direction, which will be determined by its structure, but also on the amount of this pathway available per unit area of luminal surface. Thus, although two epithelia may have junctions and intercellular clefts with the same specific resistance, the total flux carried by the paracellular shunt will be greater in the epithelium with more junctional element and intercellular cleft per unit area of luminal surface. For example, an epithelium made

up of small cells will have more zonula occludens per unit area of luminal surface than an epithelium with larger cells. Similarly, if there is much interdigitation between cells at the level of the junction, there will be more junction available per unit area of luminal surface.

A related geometrical problem also exists in epithelia where there is a large difference in surface area between serosa and mucosa. In the small intestine, for instance, the mucosal surface area may be an order of magnitude greater than the serosal surface area because of the villi (Wilson, 1962; Barry et al., 1965). The true mucosal transepithelial permeability would therefore be an order of magnitude lower than that calculated on the basis of serosal surface area alone (see Table I).

It is important to recognize that measurements of transepithelial flux represent the average flux per unit area, and do not reflect local variations in permeability. If the junctions are tight in some places and leaky in others, the measured permeability will be the sum of all the local permeabilities. This situation may exist in the rabbit gallbladder, where junctional morphology appears quite variable. Local variations may also arise where different cell types occur in a single epithelium. Examples may be found in the gastric mucosa, the small intestine, and in the proximal convoluted tubule of *Necturus* (Chase, 1923; Claude, 1967). Transient leaks may develop across epithelia such as the intestinal mucosa, where the rapid rate of cell turnover results in the sloughing off of cells with a concomitant breaking and rejoining of the zonula occludens.

As in any fine structural analysis, we are limited by the resolution of electron microscope techniques. Junctions from different epithelia may have different permeabilities based on variations in the ionic composition or degree of hydration of the junctional strands, even though they may not appear different in the electron microscope. These differences may be defined by chemical means or by studying the ion selectivity of the paracellular pathway (Barry et al., 1971; Machen and Diamond, 1972). In addition, it is possible that differences in permeability exist between strands in a single junction. Evidence suggestive of this has been obtained from osmotically stressed toad bladders where the zonula occludens responds asymmetrically to osmotic gradients (DiBona, 1972; Wade et al., 1973).

In conclusion, it appears possible to relate the morphology of the zonula occludens qualitatively to its transepithelial permeability. However, quantitative comparisons must depend on a careful assessment of all the possible geometrical and chemical variations that affect measurements of transepithelial permeability.

The excellent technical assistance of Miss Barbara L. Feldman is gratefully acknowledged. We are grateful for the helpful discussions and comments from Drs. E. L. Boulpaep, D. D. Potter, D. W. Fawcett, M. J. Karnovsky, J. B. Wade, R. D. Rodewald, and P. H. Patterson. We would like to thank Drs. A. J. Hudspeth and A. G. Yee for access to unpublished data.

This work was supported in part by Postdoctoral Fellowship No. 2-F02-NS32631-03 and Grant No. GM18974-01 from the National Institutes of Health, and a grant from the Charles A. King Trust-Medical Foundation, Inc. of Boston.

Received for publication 26 February 1973.

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