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Structural Basis for Physiological Regulation of Paracellular Pathways in Intestinal Epithelia

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Summary. Isolated segments of hamster small intestine were perfused with oxygenated salt-fluorocarbon emulsions with or without 10–25 mM glucose, alanine or leucine. Resistances of intercellular occluding junctions and of lateral spaces and the distributed capacitance of epithelial plasma membranes were estimated from steady-state transepithelial impedances at frequencies from 0.01–10 kHz. The segments were then fixed *in situ* with isorheic 2.5% glutaraldehyde while continuing to measure impedance. This method of fixation increased the resistance of lateral spaces but had little effect on the resistance of occluding junctions or on membrane capacitance. The large decreases of impedance induced by glucose or amino acids were preserved in fixed tissue and could therefore be correlated with changes in structure. The observed changes of impedance were interpreted as decreased resistance of occluding junctions and lateral spaces together with increased exposed surface of lateral membranes (capacitance). Glucose, alanine or leucine induced expansion of lateral intercellular spaces as seen by light and electron microscopy. Large dilatations within absorptive cell occluding junctions were revealed by electron microscopy. Freeze-fracture analysis revealed that these dilatations consisted of expansions of compartments bounded by strands/grooves. These solute-induced structural alterations were also associated with condensation of microfilaments in the zone of the perijunctional actomyosin ring, typical of enhanced ring tension. Similar anatomical changes were found in epithelia fixed *in situ* at 38°C during luminal perfusion with glucose in blood-circulated intestinal segments of anesthetized animals. These structural changes support the hypothesis that Na-coupled solute transport triggers contraction of perijunctional actomyosin, thereby increasing junctional permeability and enhancing absorption of nutrients by solvent drag as described in the two accompanying papers.

Key Words tight junction · cytoskeleton · intestine · absorption

I. Introduction

In accompanying papers [13, 14] evidence is provided that activation of Na-coupled solute transport induces (i) increased fluid absorption, (ii) increased intestinal clearance of hydrophilic solutes, (iii) decreased electrical resistance of occluding junctions and lateral intercellular spaces and (iv) increased capacitance (functional surface area) of epithelial plasma membranes. It was proposed that primary functions of transcellular active transport of glucose and amino acids are to supply the osmotic force for absorption of fluid and to regulate the permeability of intercellular occluding junctions, thus providing the conditions needed for absorption of nutrients by solvent drag. Madara, Barenberg, and Carlson [8] previously showed that contraction of the perijunctional actomyosin ring and increased permeability of occluding junctions can be induced pharmacologically by Cytochalasin D.

The magnitude of changes in electrical impedance and permeability induced by Na-coupled solute transport led us to expect that concomitant changes in epithelial dimensions should be readily detectable. This expectation has been realized, and in the present paper we show that activation of glucose or amino acid transport induces structural pertubation of absorptive cell occluding junctions, widening of lateral intercellular spaces and condensation of elements of the perijunctional actomyosin ring. These structural changes in response to glucose can be demonstrated in intact, blood-perfused intestine as well as in isolated, perfused segments.

II. Materials and Methods

A. Perfusion Techniques and Impedance Analysis

Perfusions of hamster small intestine were carried out in vitro and in vivo by the techniques described in the accompanying papers [13, 14]. Transmural impedance measurements were made at frequencies from 0.01–10 kHz and analyzed as de-

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scribed previously [13]. The impedance of the epithelial layer at any frequency was determined by subtracting the impedance of the sub-epithelial layers (the sheath) from the measured transmural impedance. The sheath impedance was determined separately after removal of the epithelium by incubation for 1 hr in Ca-free Ringer's solution containing 5 mM EGTA; the epithelium could then be squeezed out of the segment by a roller. The frequency-impedance characteristics of the remaining tubular sheath were then determined, after which the tissue was fixed and prepared for light microscopy. As shown in Fig. 2*F*, after removal of the epithelium by this process, the subepithelial component of the intestine remains and displays its usual geometric form as judged by light microscopy. The impedance of the subepithelial layers was only 6 ± 1 ohm per cm length of segment at all frequencies [13]. The impedance of the sheath, unlike that of the epithelium, was unaffected by addition of glucose to the lumen.

B. Morphologic Techniques

Primary fixation of intestinal segments, both in vitro and in vivo, was carried out by changing the normal perfusate to an isorheic solution containing 2.5% glutaraldehyde with sufficient NaCl to make the conductivity equal to that of normal perfusion fluid at 38°C (48 Ω cm). The impedance changes caused by fixation (*see* Fig. 1 of Results) were complete in less than 5 min. Using this technique it was found that the impedance changes caused by glucose or amino acids were preserved in the fixed tissues as described in detail in the Results section. In contrast, when the tissues were fixed by conventional procedures (e.g., by excising the tissue and cutting it into small sections under cold fixative) the structural changes induced by Na-coupled solute transport were absent. Doubtless, it is for this reason that the profound structural changes which accompany Na-coupled solute transport in the small intestine have been overlooked. After primary fixation, all tissues used for thick and thin sections were washed in 0.1 M sodium cacodylate buffer, postfixed for 1 hr in 1% osmium tetroxide, dehydrated in a graded series of alcohols and embedded in epoxy resin. 1-μm sections were obtained with glass knives and stained with toluidine blue. Thin sections were obtained with diamond knives, mounted on copper mesh grids and finally stained with uranyl acetate and lead citrate. Tissues used for freeze fracture (after primary fixation) were washed in 0.1 M cacodylate buffer and processed as previously described [8]. Briefly, after embedding in 3% agar, 150-μm slices were prepared using a Smith-Farquhar tissue chopper. Tissues were cryoprotected by exposure to 25% glycerol for 1 hr and subsequently mounted between gold discs, frozen in a partially solidified slurry of Freon 22 and stored in liquid nitrogen. Replicas were prepared under a vacuum of 10^{-7} torr using a Balzer's 300 freeze-etch device with stage temperature set at −110°C. Replicas were cleaned as described previously [7] and mounted on 200-mesh hexagonal grids, which were coated with Formvar for additional replica support.

C. Morphometric Analysis

1. Occluding Junctions

Quantitative analysis of occluding junction structural changes induced by Na-coupled solute transport were made using replicas from 10 in vitro perfused segments. Replicas used for analyses were selected on the basis of quality of replication and orientation of fractured villi. All images of occluding junctions within replicas associated with villus absorptive cells were photographed and printed at a final magnification of 50,000×. Photographs were then analyzed for the presence of dilated interstrand compartments and for interstrand compartments having concave or convex faces. Compartments were considered to be enlarged if their smallest width was at least 2× that of controls. Control dimensions were derived from morphometric analyses of 10 absorptive cell occluding junctions from tissues perfused without organic substrates. The total *length* of the occluding junction in each replica analyzed was also measured so that the frequency of enlarged compartments could be expressed per unit length of occluding junction. Total lengths of occluding junction which were measured for separate groups ranged from 180 to 400 μm.

2. Perijunctional Actomyosin

Condensation of perijunctional actomyosin was quantified by measuring optical transmission of electron micrograph negatives taken at 26,000×. Measurements were made with a Photovolt Model 520-M photomultiplier densitometer using an aperture of 2 mm^2. The negatives were placed over a diffuse light box and the light adjusted to 100% transmission with the aperture over an unexposed portion of the negative. Relative transmissions were then measured over the perijunctional actomyosin ring (T_P) and over adjacent portions of the terminal web (T_W). Results were expressed as the ratio T_P/T_W.

3. Mucosal Surface Area

Lastly we used previously described computer-assisted morphometric techniques [11] to determine the surface area amplification present in segments mounted in vitro. Segment diameters were 5 mm and surface amplification was 5.2×. Thus each cm length of intestine mounted in vitro contained approximately 8 cm^2 of mucosal surface area.

III. Results

A. Effects of Fixation on Impedance, With and Without Addition of Glucose to Luminal Perfusion Fluid

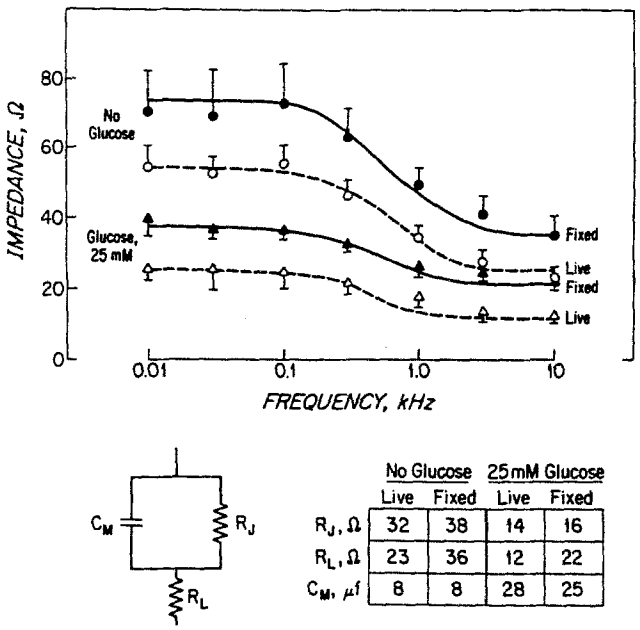
1. Segments Perfused in Vitro

Figure 1 shows steady-state impedance-frequency plots for epithelia before and after fixation, with and without glucose. The lines drawn are theoretical plots for the equivalent circuit in the inset having values of R_J, R_L and C_M shown for each condition. The experimental points fit the theoretical curves within their standard errors. The data indicate that fixation increased lateral space resistance, R_L, but had little effect on either junctional resistance or membrane capacitance. The effects of glucose on R_J and C_M were preserved in the fixed tissue. Results similar to those shown in Fig. 1 were ob-

tained using amino acids instead of glucose. Thus it was possible, in these isolated perfused segments, to fix the tissues in either the closed, high impedance state (i.e., without transportable organic substrates) or in the open, low impedance state during Na-coupled transport. The anatomical correlates are discussed in Section B below.

2. *Segments Perfused in Vivo*

In several experiments the small intestine was fixed in living, anesthetized animals by sudden addition of 2.5% glutaraldehye to the perfusion fluid. This was done using long segments (20–40 cm) or short segments (3 cm); the latter were mounted in the tubular frame used for impedance measurements [13] without interfering with the mesenteric circulation. The time required for fixative to penetrate the epithelium and paralyze underlying smooth muscle was less than 2 min as judged by the continuously recorded intraluminal pressure; circulation of blood in the mesenteric vessels stopped in the oxygenated state within this time. To our surprise, fixation of long lengths of the small intestine had no obvious effects on the anesthetized animal, even after 30 min of perfusion with 2.5% glutaraldehyde at 4 ml min^{-1}. In contrast to segments fixed during in vitro perfusions, the impedances of segments fixed in vivo were extremely low at all frequencies, whether or not glucose was present in the luminal perfusion fluid at the time of fixation. The impedance-frequency function of segments fixed in vivo did not fit the electrical analog of Fig. 1, and the tissue behaved as though the resistance of the apical plasma membranes was abnormally low. We are unable to explain this interesting difference between fixation in vivo and in vitro; similar differences have been noted by previous investigators using gall bladder epithelium or other tissues (*see* discussion by Wright and Goodenough in reference [2]). Although the transepithelial impedances of in vivo preparations were not preserved in fixed tissues as they were in vitro, the principal structural changes caused by transportable organic solutes were nevertheless present as described in Section B below.



	No Glucose		25mM Glucose	
	Live	Fixed	Live	Fixed
R_J, Ω	32	38	14	16
R_L, Ω	23	36	12	22
C_M, μf	8	8	28	25

Fig. 1. Effects of fixation on intestinal epithelial impedance in in vitro segments perfused with or without glucose. Impedances of live and fixed perfused segments were measured at seven frequencies from 0.01 to 10 kHz. The experimental points were fitted to the analog electrical circuit shown below the abscissa and described in detail in the accompanying paper on impedance [13]. The lines are theoretical curves describing the model and having values of R_J, R_L, and C_M obtained from the experimental data. The experimental points fit the theoretical curves within the standard errors of measurements. The tissues were fixed by suddenly changing luminal perfusion fluid to isorheic 2.5% glutaraldehyde at 38°C while continuing measurement of impedance. Fixation increased the resistance of the lateral spaces but had little effect on occluding junction resitance, R_J, or on membrane capacitance, C_M. The effects of glucose on impedance were therefore preserved by fixation, and this made possible correlations with structural changes

B. Effects of Glucose or Alanine on Structure of Epithelial Absorptive Cells

1. *Effects Detected by Light or Low Magnification Electron Microscopy*

Epithelial structure was well maintained in in vitro preparations perfused for an hour or more with oxygenated fluorocarbon, with or without added substrates. Specifically, villus absorptive cells were columnar, nonvacuolated and displayed a prominent microvillus brush border (Figs. 2*A*,*B*, 3 and 4). This general appearance of structural integrity was comparable to that of villus epithelium obtained from in vivo perfused segments (Figs. 2*C*,*D*, and 5). Sections obtained from preparations which had been perfused with glucose (or amino acids) could easily be distinguished from those perfused without substrates by the appearance of the intercellular lateral spaces. In the absence of substrates, the intercellular spaces were collapsed with closely apposed lateral membranes (Figs. 2*A* and 3), whereas glucose elicited expansion of the intercellular spaces and exposure of lateral membranes (Figs. 2*B*, 4 and 5). The expansion of the intercellular spaces was more variable in in vivo preparations than in in vitro ones. The expansion of lateral spaces was most prominent on the upper one-half to two-thirds of

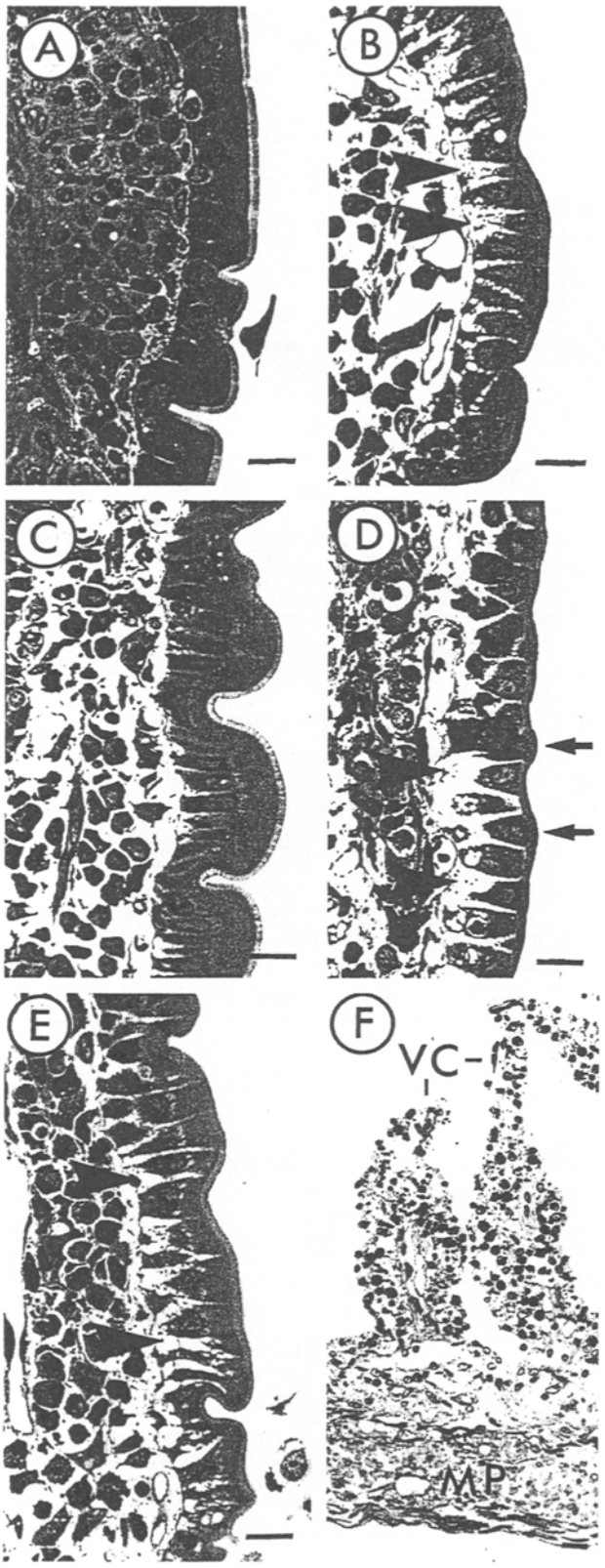


Fig. 2. Light micrographs of 1 μm sections of villus epithelium. (*A*) After perfusion in vitro without substrates and (*B*) with 25 mM glucose. The general morphological appearance of villus epithelial cells is similar in both conditions, but exposure to glucose results in marked dilatation of the lateral (paracellular) spaces (arrowheads). The thin extensions that span the dilated lateral spaces represent cytoplasmic processes at sites where adjacent cells are tethered together by desmosomes. (*C*) After perfusion in vivo without substrates and (*D*) with 25 mM glucose. The morphological state of the villus epithelium with and without glucose exposure in vivo is comparable to the analgous in vitro states. In addition to lateral space dilatation (arrowheads) the surfaces of glucose-exposed absorptive cells may display a subtle convexity (arrows). (*E*) In vitro perfused intestinal segment which was exposed to luminal glucose but also to luminal ferrocyanide at a concentration that greatly reduces fluid absorption (*see* accompanying paper, ref. 14). Despite reduction of fluid absorption (not measured in these experiments), lateral spaces are dilated (arrowheads) and appear comparable to those of villus epithelium exposed to glucose alone (*see B* above). This finding supplements evidence that lateral space dilatation may not be a direct result of increased fluid absorption (*see* Discussion). (*F*) Low magnification view of an intestinal wall from a segment "de-epithelialized" by exposure to 5 mM EGTA. The structure of the nonepithelial component of the wall remains intact. Thus these preparations may be used to determine the impedance contributed by the subepithelial portion of the intestine. (*VC* = de-epithelial villus cores; *MP* = muscularis propria); bars = 20 μm

villi. Fine linear densities which spanned the dilated spaces were present between cells (Fig. 2*B*); these thin cytoplasmic processes often abutted at the site of spot desmosomes. In tissues fixed in vivo, the apical surfaces of absorptive cells exposed to glucose were often subtly rounded (Figs. 2*D* and 5). This response was not apparent in tissues taken from in vitro preparations or from in vivo preparations perfused without glucose. Other structural properties of the epithelial cells such as the height of the cells or their microvillus brush borders were unchanged by addition of substrates. However, the appearance of the underlying lamina propria cells in the upper regions of villi was clearly altered by the presence of glucose. Without substrates the extracellular space of the lamina propria was often collapsed (Fig. 2*A*). In contrast, exposure to glucose in vitro resulted in uniform separation of individual cells and increased extracellular space (Fig. 2*B*). This difference is to be expected since fluid absorption stimulated by glucose in in vitro preparations accumulates in subepithelial tissue instead of being absorbed by capillary blood or lymph as it is in vivo [5, 12]. In tissues fixed by conventional methods, the differences in lateral space dimensions between tissues perfused with glucose compared to those perfused without glucose were not marked.

2. *Effects of Glucose on Occluding Junctions*

In the absence of glucose, occluding junctions between adjacent villus absorptive cells consisted of zones in which the lateral membranes were closely

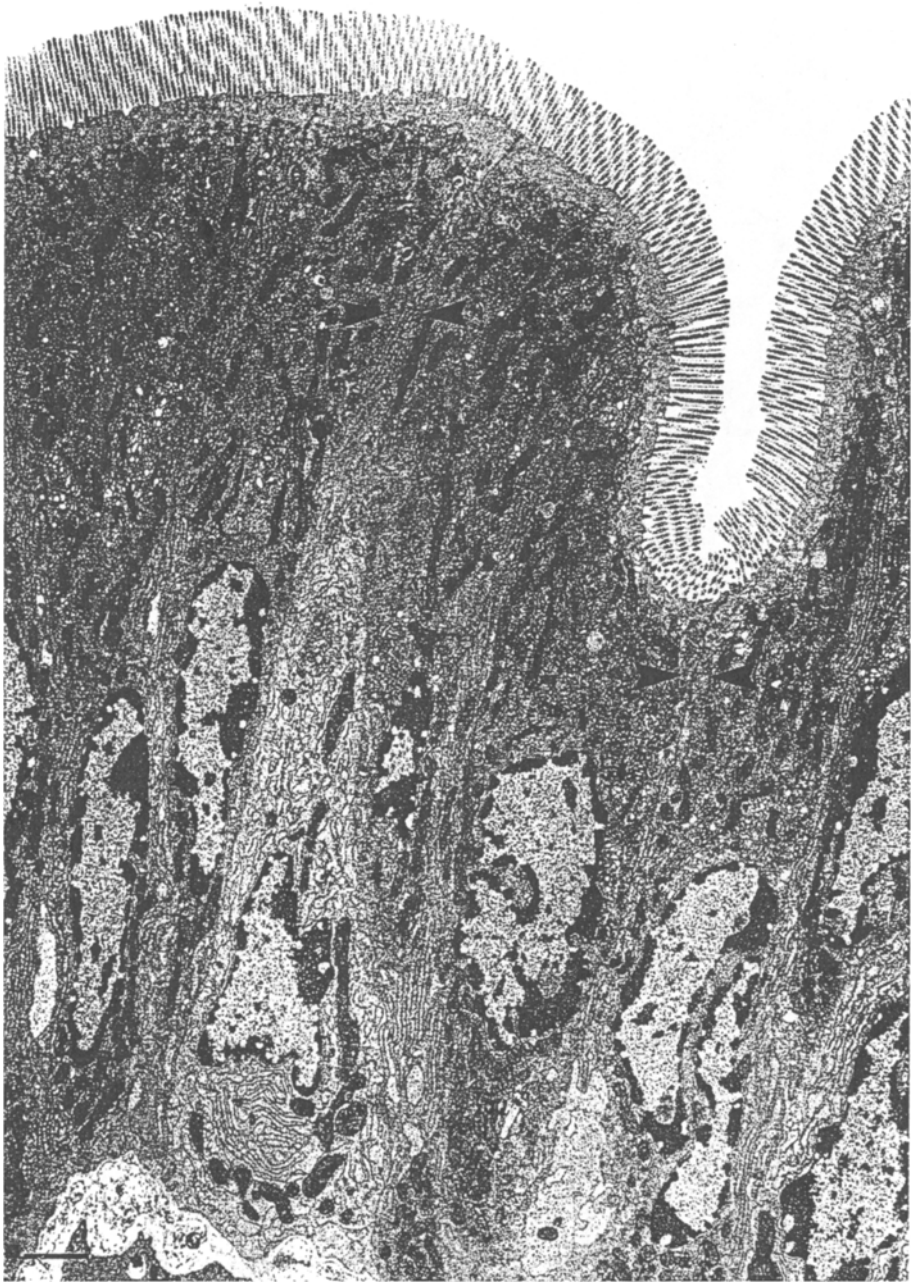


Fig. 3. Low magnification electron micrograph of absorptive cells fixed during perfusion in vitro without glucose or amino acids. Lateral spaces (arrowheads) are collapsed. Although not seen well at this magnification, lateral interdigitations classically described for these cell types are present. There is no evidence of a large pre-existing intracellular pool of membrane vesicles which might be rapidly incorporated into and expand either the apical of lateral membrane domains. Bar = 2 μm

apposed as is typical for this epithelium (Fig. 6*A*) [10]. In contrast, the occluding junctions in segments perfused with glucose or amino acids in vitro (Figs. 6*B* and 7) or in vivo (Fig. 11*B*) contained numerous expanded regions or intrajunctional dilatations. Such dilatations ranged from less than 0.1 to 0.5 μm in width (Fig. 7) and they occurred not only between absorptive cells but also at sites where absorptive cells adjoined goblet cells. Where observed, junctional dilatations usually appeared singly within any vertical section plane, but two or more stacked dilatations were occasionally identified (Fig. 7); large junctional dilatations sometimes contained vesicle-like particles (Fig. 7). In in vitro preparations perfused with substrate-free solutions junctional dilatations were never detected. Occasional junctional dilatations of small diameter (0.5 μm) were found in segments perfused in vivo with glucose-free solution. It is probable, however, that epithelial cells in segments perfused in vivo have access to some glucose from blood even though the lumen may be glucose-free [13, 14].

Freeze-fracture analysis revealed that the glucose-induced junctional dilatations consist of expanded interstrand compartments. In substrate-free tissues, absorptive cell occluding junctions ap-

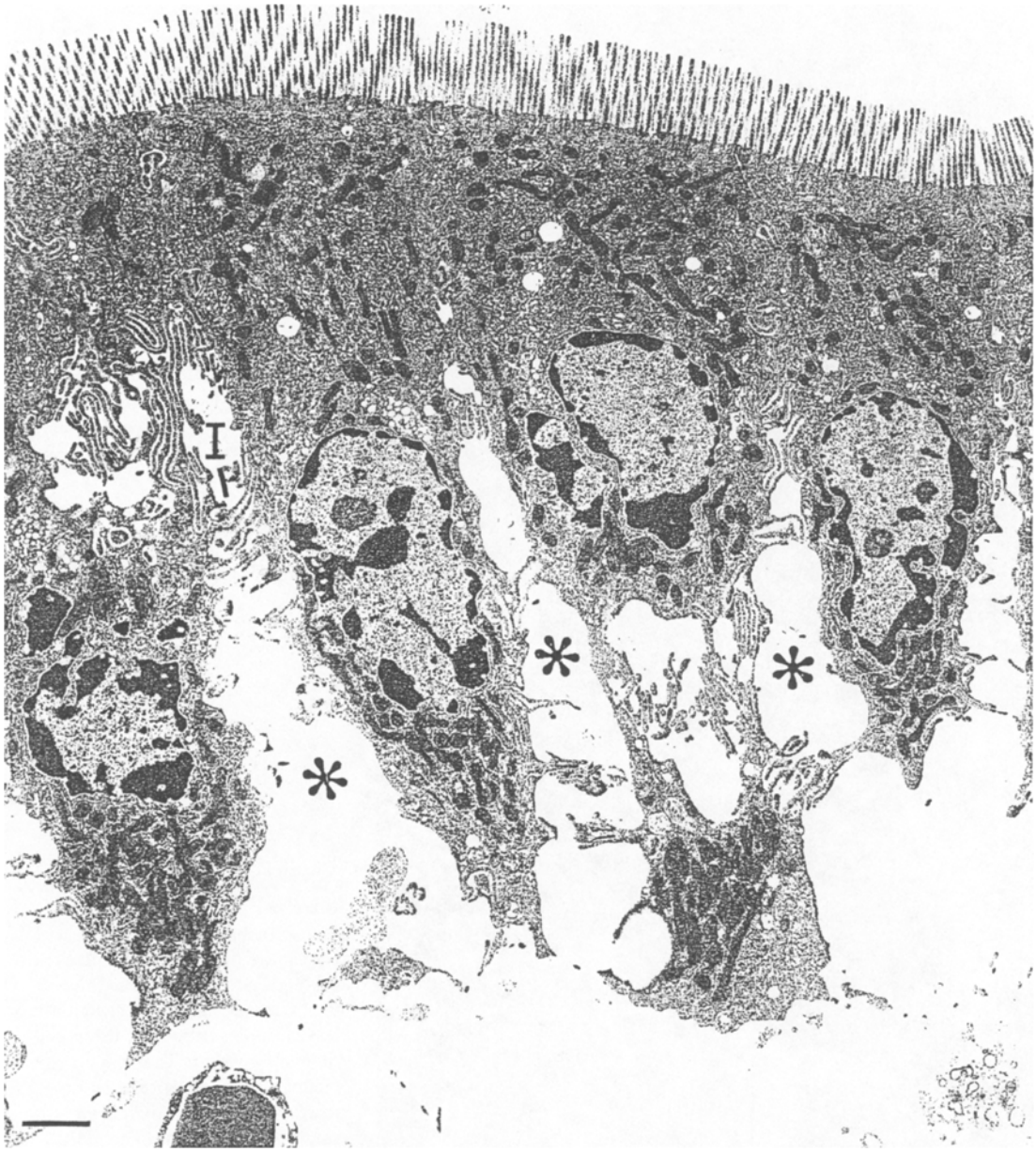


Fig. 4. Low magnification electron micrograph of absorptive cells fixed during perfusion with glucose (in the low impedance state). Lateral spaces (asterisks) are markedly dilated. Such dilatations highlight the lateral interdigitations (*I*) which normally are present between these cells [10]. Comparison with Fig. 3 shows that the area of the apical membrane (microvillus surface) is not detectably altered by glucose; in contrast, the amount of basolateral membrane exposed to intercellular fluid markedly increases as a result of lateral space expansion. These anatomical changes correlate well with the observed decrease of lateral resistance and increase of membrane capacitance induced by glucose. Bar = 2 μm

peared as they are usually described [10], namely in the form of interlinked P face strands and E face grooves (Fig. 8*A*). In contrast, the occluding junctions of glucose-perfused segments contained many distorted interstrand compartments corresponding to junctional dilatations seen in thin section (Fig. 8*B*). The membrane faces associated with such compartments were often concave or convex and occasionally displayed a secondary elevation or depression (Fig. 8*B*). A perpendicular drawn across occluding junctions in glucose-free preparations intersects 4–6 strands (Fig. 8*A*); in contrast, only 1–3 strand/grooves would be encountered at or near the junctional dilatations induced by glucose (Fig. 8*B*). A quantitative analysis of the effects of glucose on interstrand compartments is presented in Table 1. Obvious dilated interstrand compartments such as those illustrated in Fig. 8*B* were never seen in glu-

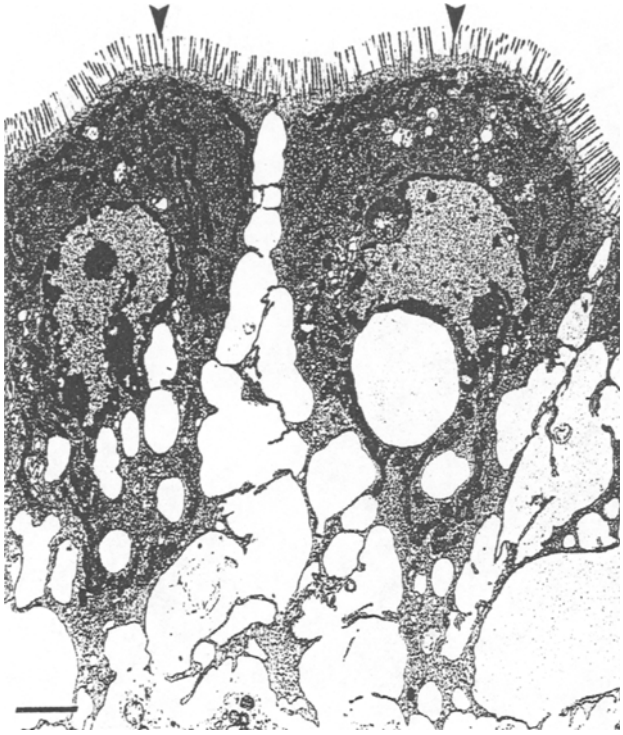


Fig. 5. Effects of glucose on expansion of lateral space in vivo. Perfusion with glucose in vivo induced expansion of lateral spaces and appears to lead to subtle, multifocal rounding of the absorptive cell apices (arrowheads). Bar = 2 μm

cose-free tissues from in vitro preparations and were always seen multifocally in preparations fixed during perfusion with glucose or amino acids. However, our criteria for identification of enlarged compartments were designed to detect even minor dilatations (*see* Materials and Methods), and such minor dilatations were occasionally found in glucose-free preparations as shown in Table 1. Using these criteria, we found that enlargement of interstrand compartments was 9× more frequent when glucose was present and approximately one enlarged compartment was present in each micrometer length of absorptive cell junction. Since such compartments had diameters from about 0.1–0.5 μm, it follows that in the glucose-activated state from 10–50% of total absorptive cell junctional length contains enlarged interstrand compartments. Stated another way, a single absorptive cell having a circumference of 35 μm may have about 35 junctional dilatations around its apical circumference during absorption of glucose (or amino acids).

3. *Condensation of Perijunctional Actomyosin*

Alterations of terminal web cytoskeletal structure were found consistently in segments that had been

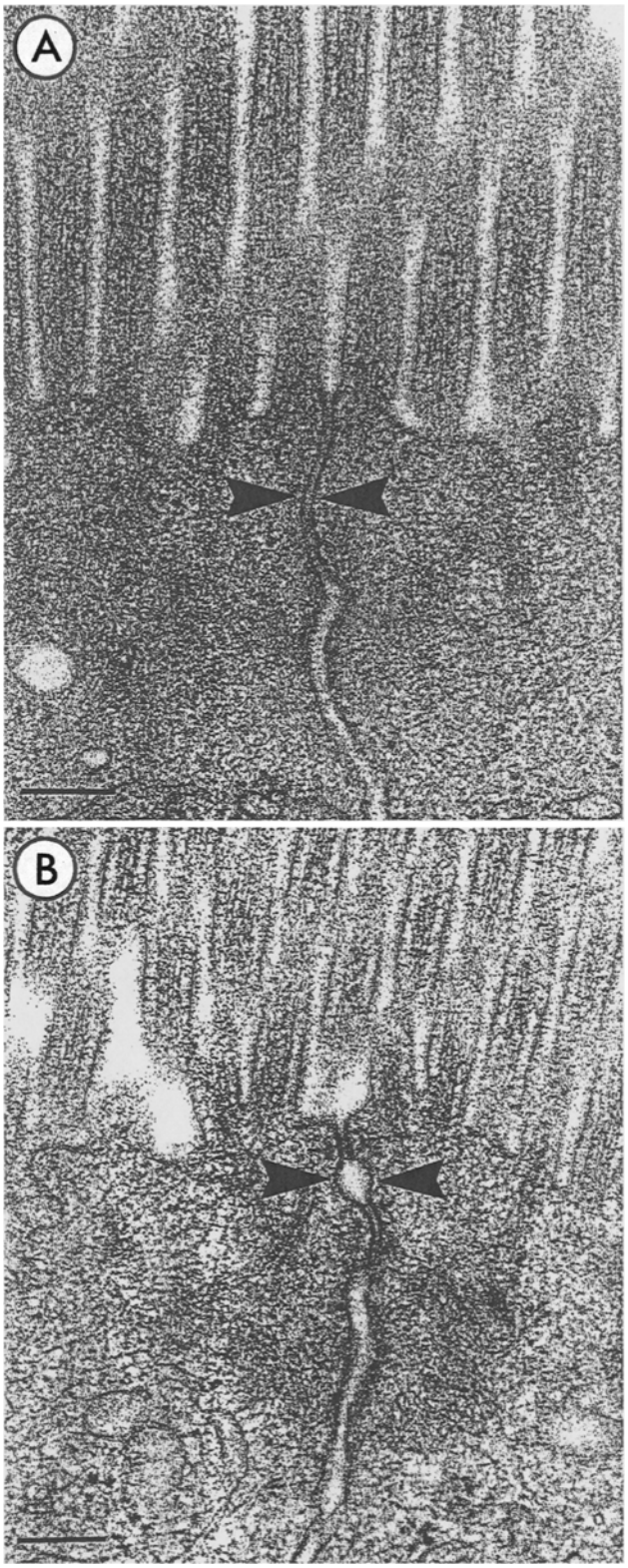


Fig. 6. Electron micrographs of absorptive cell occluding junctions obtained from tissues perfused (*A*) without substrates and (*B*) with 25 mM luminal glucose. Occluding junctions in the glucose free, high impedance state revealed closely apposed lateral membranes (arrowheads). In contrast, dilatations within the zone of the occluding junction (*B*, arrowheads) are induced by perfusion with glucose. Bar = 2 μm

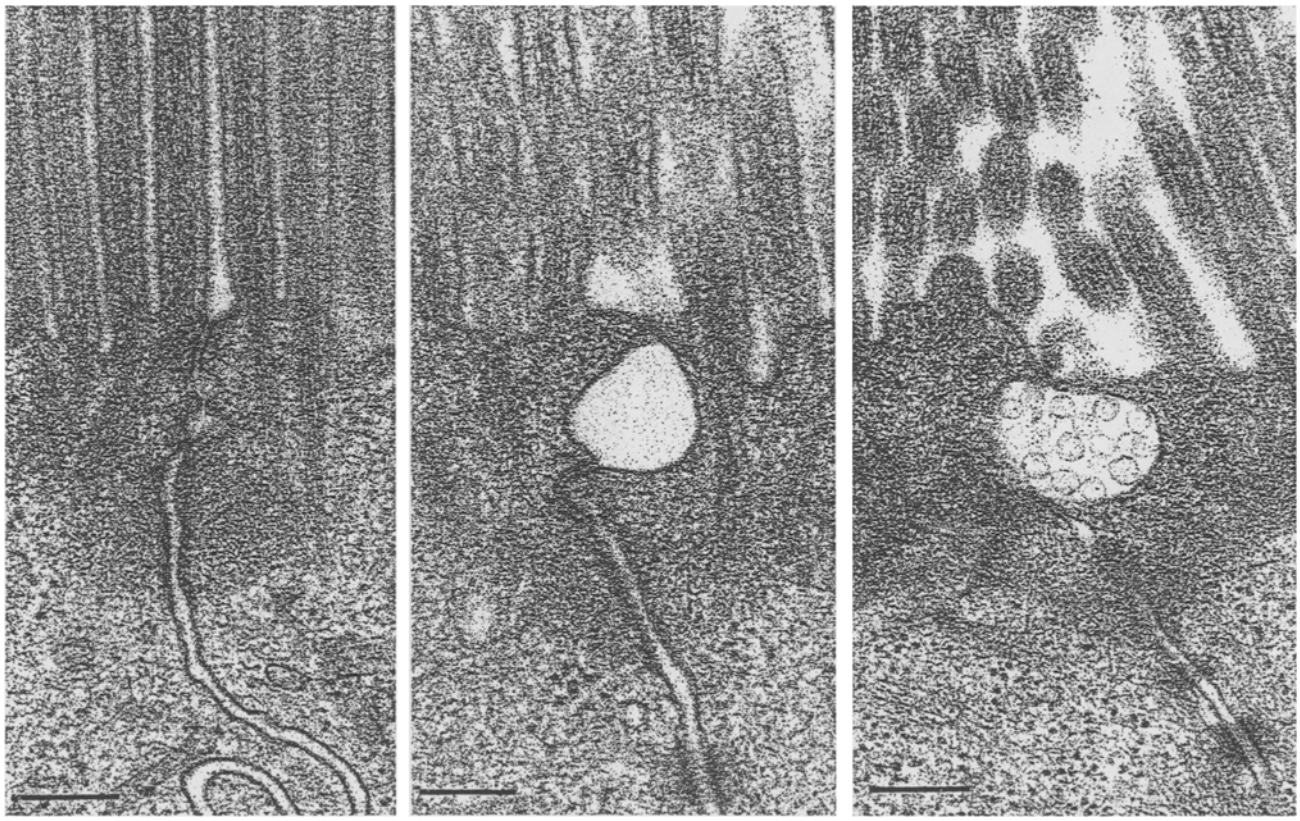


Fig. 7. Variation in appearance of junctional dilatations. Dilatations could appear in series (left), or as extensions over the major portion of the occluding junction (center). Occasional dilitations contain membrane vesicles (right). Bars = 0.2 μm

Table 1. Effects of glucose on junctional interstrand compartments

	No glucose	Glucose	Glucose + ferrocyanide
Frequency of dilated compartments per μm junctional length	0.10	0.89	0.77

Table 2. Effects of glucose on perijunctional actomyosin ring optical transmission ratios[a]

	No glucose	25 mM glucose
Perijunctional ring/ mid-terminal web	1.00 ± 0.02	1.27 ± .09

[a] (Means ± SE); n = 10.

perfused with glucose or amino acids at the time of fixation (either in vitro or in vivo). Condensation of cytoskeletal elements occurred at the level of zonula adherens (Fig. 9) which is immediately subjacent to the occluding junction and represents the major insertion site for the perijunctional actomyosin ring [6]. A rough quantitative measure of this phenomenon was obtained from the optical transmission through electron micrograph negatives of the terminal web in 10 specimens that had been perfused without glucose and 10 specimens perfused with glucose. As shown in Table 2 the optical transmission through negatives of the perijunctional region was 27% greater in epithelia transporting glucose than in epithelial which were glucose-free. Since negatives were analyzed, this indicates that perijunctional actomysin ring density was substantially enhanced by addition of glucose. Although sites showing junctional dilatations typically showed perijunctional condensations (Figs. 6 and 7), such condensations were not invariably accompanied by dilatations (Fig. 9). Since the perijunctional actomyosin ring wraps the apices of absorptive cells, sections cut close to the cell periphery can show ring condensation extending large distances from the junction.

Condensation of microfilaments in the perijunctional region, indicative of the development of tensile forces [8], was the most prominent effect of

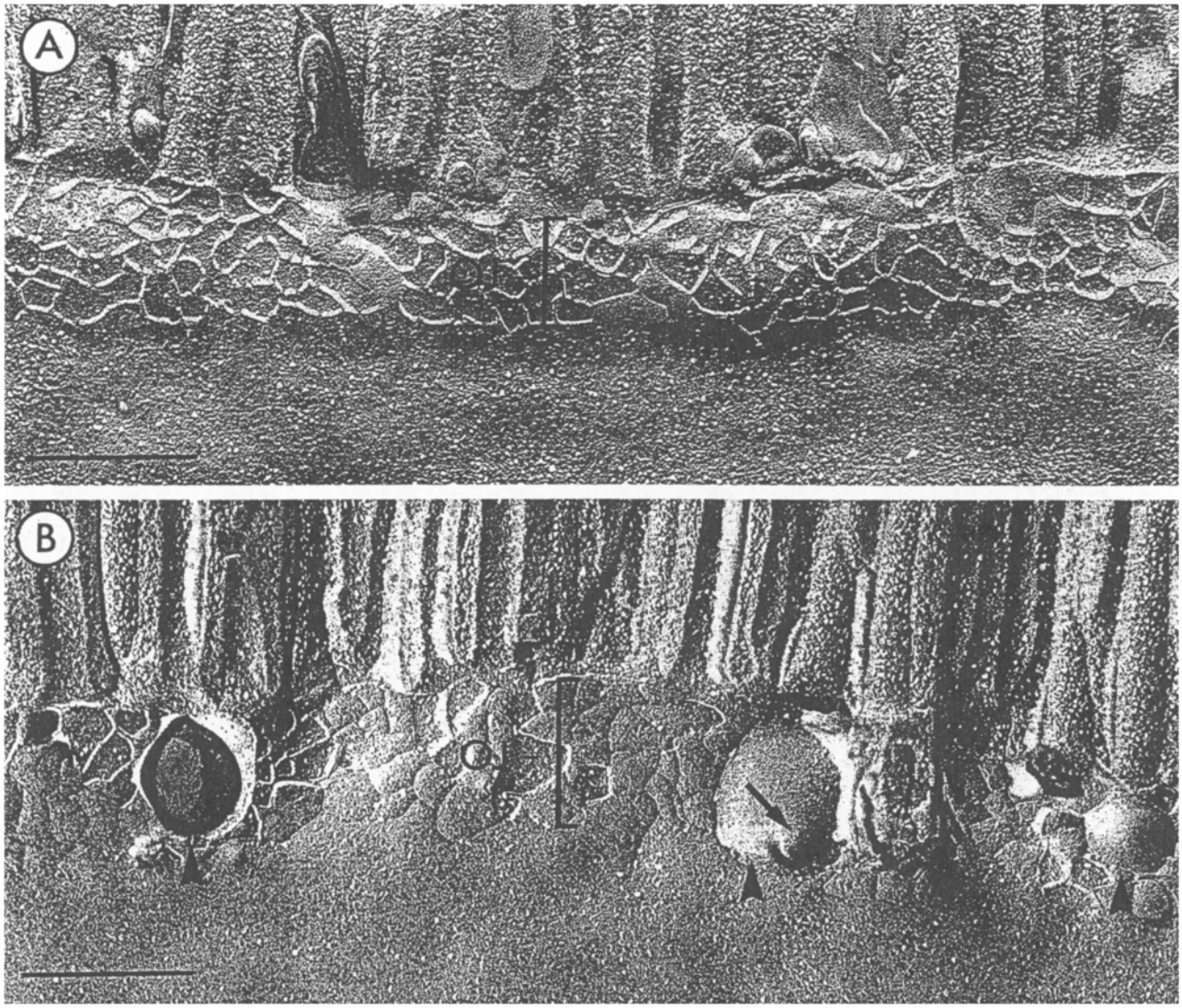


Fig. 8. Freeze-fracture electron micrographs of absorptive cell occluding junctions (*OJ*). (*A*) After perfusion in the absence of substrate junctions consists of a net-like array of strands and/or grooves. (*B*) In contrast, perfusion with glucose elicits focal dilatations of interstrand compartments (arrowheads) which often have concave surfaces and which correspond to the intrajunctional dilatations seen in thin sections (*see* Fig. 6). Some dilatations display a secondary prominent protuberance on their fracture faces (arrow). Such dilated interstrand compartments also distort the anatomy of the junction. For example, at sites where large dilatations exist only two junctional strands separate the luminal from the paracellular space, whereas in glucose-free preparation several junctional strands are always encountered separating these two compartments. Presumably this is the structural manifestation of the increased permeability to hydrophilic solutes and the decreased junctional resistance induced by glucose. *See* Table 1 for quantitative comparison. Bars = 0.5 μm

glucose on the terminal web, but other effects were noted. Condensations of cytoskeletal elements between the rootlets of microvillus actin bundles were often found (*see below*), and occasionally condensation of cytoskeletal elements at this level of the terminal web became confluent. These latter changes suggest that condensation of terminal web microfilaments in response to glucose may occur in central zones of the terminal web as well as in perijunctional regions. To better define the ultrastructural loci of glucose-elicited microfilament condensation, the distribution of such condensations was examined further in transverse thin sections through the apices of absorptive cells at the level of the junctional complex. As shown in Fig. 10, condensation of perijunctional microfilaments occurs around the entire cell perimeter following glucose exposure; it is most marked at the level of the zonula adherens where there is a gap between plasma membranes of adjacent cells, but it is also

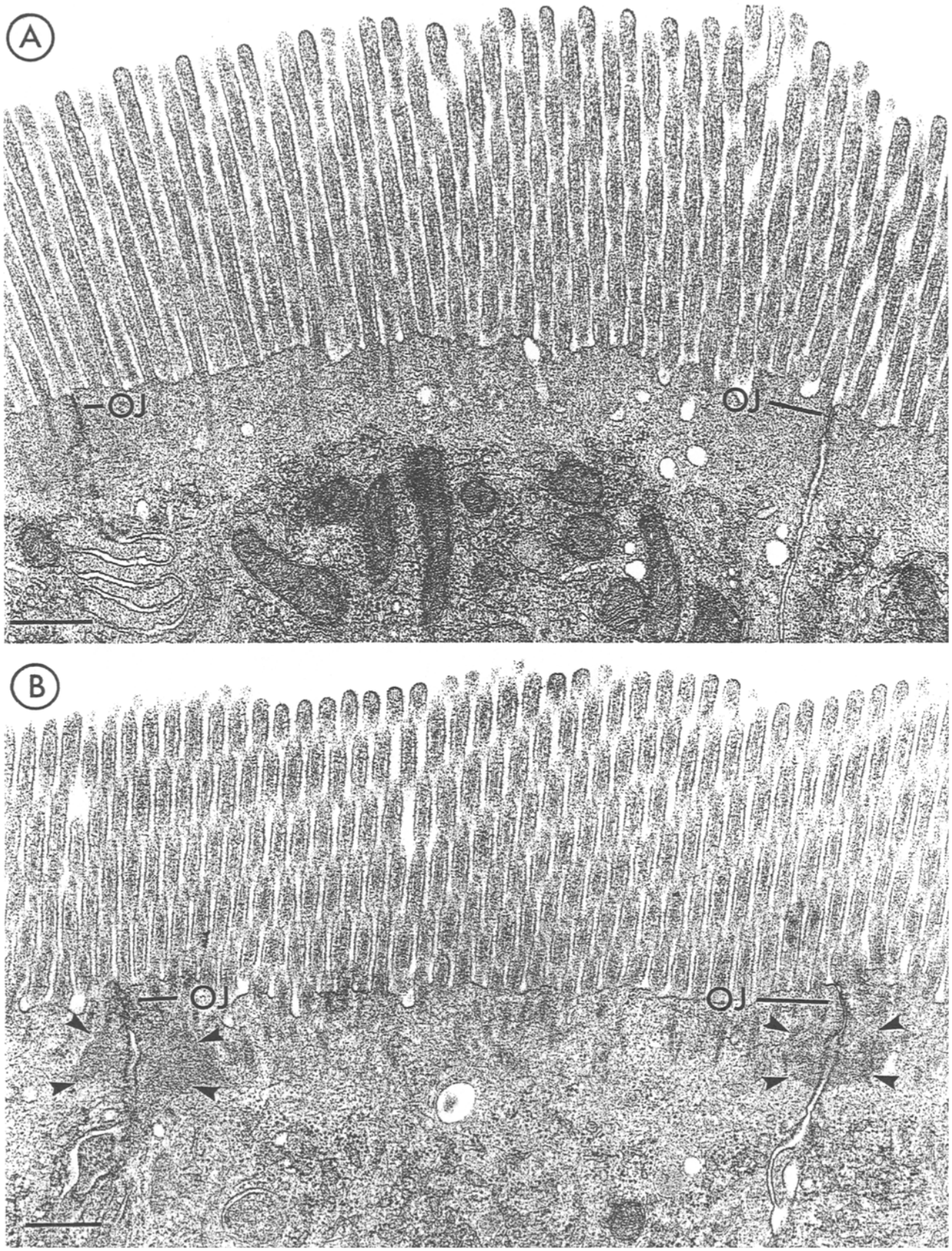


Fig. 9. Effects of glucose on perijunctional actomyosin ring. (*B*) Condensation of microfilaments is evident in absorptive cells perfused with glucose (arrowheads), but is absent in preparations perfused without transportable substrates (*A*). (*OJ* = occluding junction) *See* Table 2 for quantitative comparison. Note that the portions of the perijunctional rings seen in glucose-activated tissues in Figs. 5 and 6 are also condensed. Bars = 0.5 μm

clearly visible at the level of the occluding junctions where membranes between adjacent cells are apposed. Transverse sections also confirm the data shown in Table 2, indicating that many junctional dilatations are formed along the length (cell perimeter) of the junctions as shown in Fig. 10 (right lower inset). Glucose-induced focal condensation of cytoskeletal elements in the inter-rootlet area of the central portion of the terminal web is confirmed in transverse sections (Fig. 10).

Lastly, as shown in Fig. 11, condensation of perijunctional ring microfilaments also was elicited in vivo by glucose perfusion.

4. Effects of Alanine on Epithelial Structure

The sequellae of glucose perfusion described above also followed perfusion with alanine. Not only did this solute result in lateral space dilatation but, as shown in Fig. 12, condensation of perijunctional actomyosin ring elements and junctional dilatations were also elicited.

5. Effects of Fixation by Conventional Procedures

Junctional dilatations or perijunctional condensation of microfilaments were not found in tissues that were removed from in vitro or in vivo perfusions and rapidly dissected under cacodylate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde. Although this conventional fixation protocol preserved ultrastructural details of plasma membranes and intracellular organelles, it evidently reversed the structural responses to Na-coupled transport.

6. Role of Fluid Absorption in Expansion of Lateral Spaces

The lateral spaces in epithelia of renal proximal tubules [1, 16] or gall bladder [17] become distended during high rates of fluid absorption, and it is inferred that this is a mechanical effect brought about by increased hydrostatic pressure associated with increased flow of fluid. Similarly, it might be supposed that the expansion of lateral space shown in Figs. 2*A*,*D*, 4 and 5 of the present paper might be attributed to increased fluid absorption induced by glucose. However, in the accompanying paper on impedance [13] it was shown that the epithelial (impedance) response to glucose was unchanged when fluid absorption was reduced by a luminal osmotic load of ferrocyanide to which the epithelium is impermeable. Figure 2*E*, taken from one of two similar experiments, shows that addition of 10 mM ferrocyanide, a concentration sufficient to greatly reduce fluid absorption [14], did not prevent distention of lateral spaces induced by glucose. Moreover, glucose induced the same number of junctional dilatations whether of not ferrocyanide was present as shown in Table 1. These anatomical findings support the conclusions based on impedance analysis [13], namely that expansion of lateral spaces and widening of tight junctions induced by glucose or amino acids are not direct mechanical effects of increased fluid absorption.

IV. Discussion

A. Correlation of Structure with Function

1. Effects of Glucose or Amino Acids on Permeability and Structure of Occluding Junctions

From a qualitative point of view, the junctional dilatations and distortion of interstrand compartments induced by relatively small concentrations of glucose or amino acids correlate well with changes of fluid absorption, clearances of hydrophilic solutes and junctional resistances that occur during Na-coupled solute transport. Results described in the present paper also shed light on the mechanism of increased permeability. Condensation of perijunctional microfilaments, which largely consist of actin and myosin, and deformation of junctional interstrand compartments indicate that junctional dilatation is an active process involving contractile proteins of the terminal web. The dependance of the contractile response on oxygen [13] bears out this view. Contraction of the perijunctional actomyosin ring with associated alterations of occluding junction structure, decrease of transepithelial resistance, and enhanced epithelial permeability to hydrophilic solutes, can also be induced pharmacologically by Cytocholasin D [8]. Contraction of this ring was first described in isolated brush border preparations [3, 6, 15]. We suggest that a major, and perhaps primary, function of this elaborate contractile system in the terminal web is to regulate the permeability of occluding junctions in response to the presence of luminal nutrients presented for absorption.

From a quantitative point of view it is not yet possible to correlate junctional dilatation with measured changes of permeability or electrical resistance. The functional measurements lead to a well-defined equivalent membrane having the same coefficients of osmotic flow and solvent drag as the glucose-activated intestinal epithelium. Such an equivalent membrane contains cylindrical pores of radius 50Å (or slits 62Å wide) [14]. The diameters of the junctional dilatations described in the present

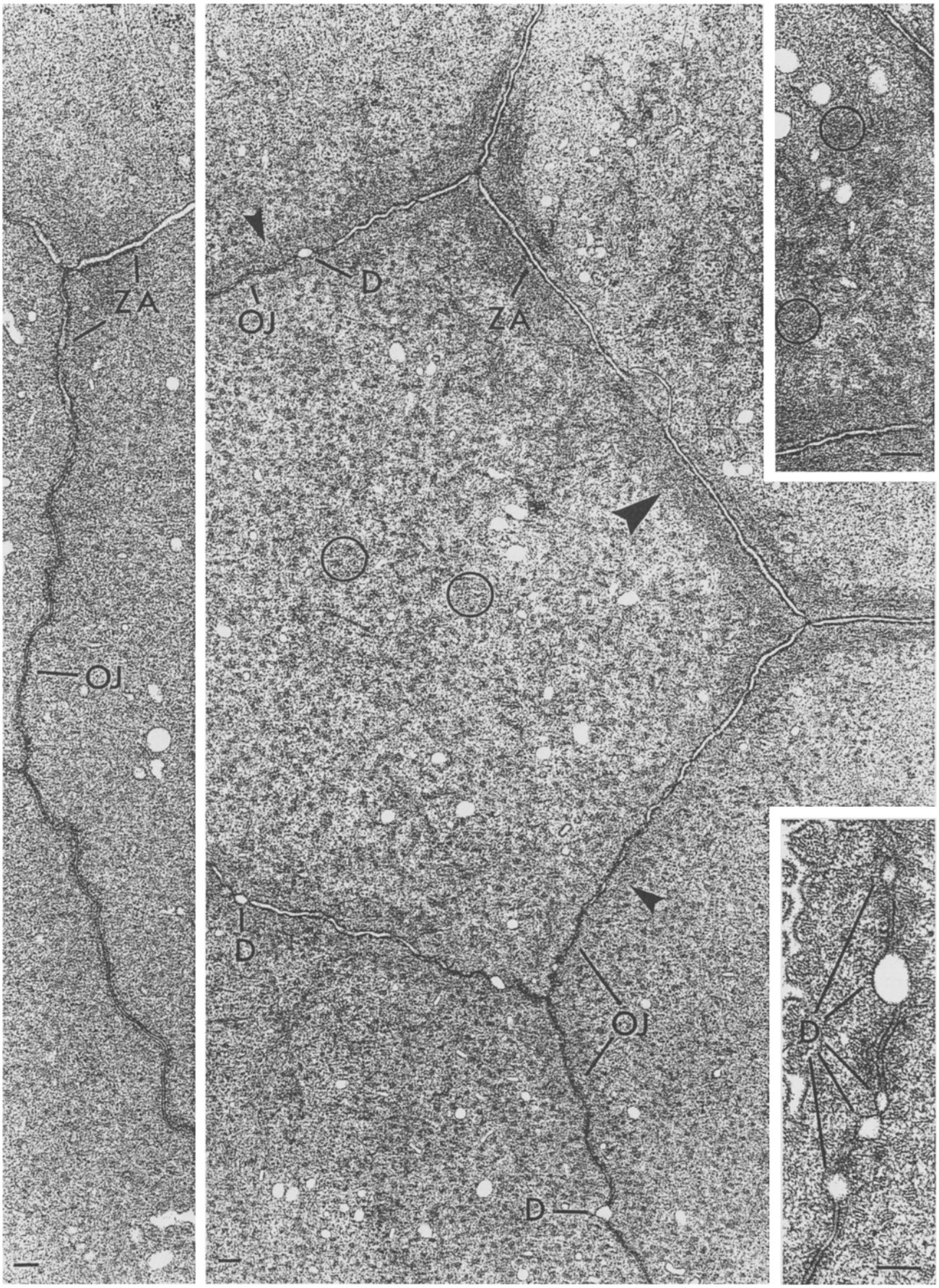


Fig. 10. Effects of glucose on perijunctional actomyosin ring as seen in transverse sections. *Left panel:* No glucose. Junctional dilatations are absent, perijunctional condensation of microfilaments is not seen, and rootlets appear as discrete subtle densities not associated with condensations. *Right panel:* Transverse section of glucose-activated absorptive cells sectioned at the zone of the

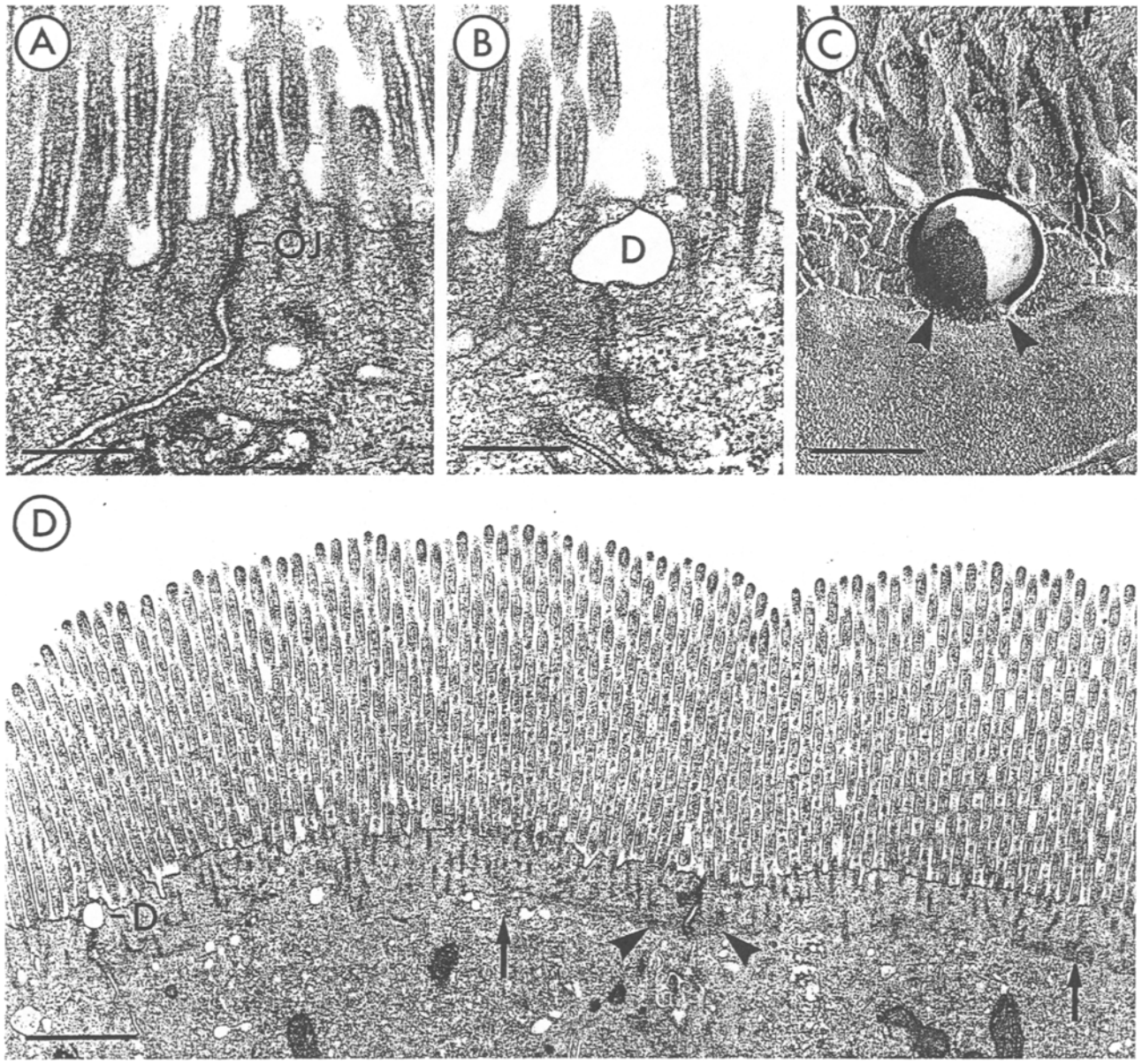


Fig. 11. Effects of glucose on junctions in vivo. (*A*) In the absence of glucose, occluding junctions (*OJ*) contain no prominent dilatations and the perijunctional actomyosin ring is difficult to discern. (*B*) With glucose, obvious junctional dilatations (*D*) are elicited. (*C*) Glucose-associated junctional dilatations represent expansion of the interstrand compartments of the junction as assessed by freeze fracture (arrowheads). (*D*) Glucose perfusion in vivo elicits condensation of perijunctional actomyosin ring elements (arrowheads) as well as focal condensation of microfilaments at other sites within the terminal web (arrows). An associated junctional dilatation is seen at the left (*D*). Bars = 0.5 μm

paper are two orders of magnitude greater than the width of channels having equivalent permeability. However, the dilatations do not span the entire depth of the junctions and may only serve to reduce the effective path-length of the actual channels which determine the coefficients of osmotic flow and solvent drag. For example, such reduction in path-length might be accomplished by a reduction in

junctional complex (occluding junction, *OJ*, or zonnulae adherens, *ZA*, sectioned as shown). The large micrograph shows portions of five neighboring absorptive cells. Although condensation of perijunctional actomysin ring elements is best seen at the level of the zonula adherens (large arrowhead), the condensation does extend to the zone of the occluding junction (small arrowheads). Junctional dilatations are present at this latter site (*D*). In addition to these findings, patchy condensation of microfilaments in the interrootlets zones also occurs (circles). The bottom insert shows that such sections at the level of the tight junctions may show many dilatations (*D*) associated with a single occluding junction. The top insert shows that the condensation of microfilaments in the interrootlet zone of the terminal web may occasionally approach confluence. Bars = 0.2 μm

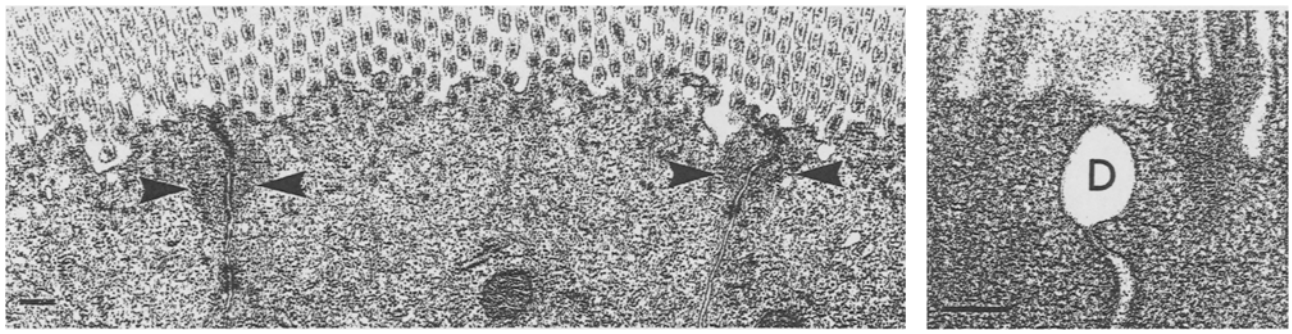


Fig. 12. Effects of 10 mM alanine on absorptive cell junctions. Alterations including condensation of the perijunctional actomyosin ring (left, arrowheads) and intrajunctional dilatations (right, *D*) are indistinguishable from alterations induced by glucose. Bars = 0.2 μm

the number of strands which occur at the site of the junctional dilatations. Since strands represent the interjunctional sites at which the effective barriers to molecular flow reside [4, 9], a reduction in strand number would lead to a reduction of the summated effective channel length at that site. A further complication in relating strand numbers to resistance changes arises from the fact that permeability predicted from structural analysis of crypt cells differs from that of absorptive cells [11].

2. *Effects of Glucose on Lateral Spaces*

Expansion of lateral spaces during perfusion of segments with glucose in vitro was expected because of the large decrease in electrical resistance of the lateral spaces as estimated from our resistance-capacity analog model of the epithelium [13]. The magnitude of the anatomical changes actually found (Figs. 2, 4 and 5) provides strong support for the validity of the model. The mechanism of expansion of lateral spaces during glucose transport is unclear. The expansion occurs even in the presence of sufficient impermeant solute (ferrocyanide) to block most of the fluid absorption as described above and in the accompanying papers [13, 14]. This suggests that expansion of lateral spaces is not secondary to increased fluid absorption but may have an active contractile component related to the concomitant contraction of the apical perijunctional actomyosin ring. The argument that force for lateral space distention stems from increased hydrostatic pressure resulting from increased fluid flow appears unsound to us. The pressure drop for increased flow takes place across occluding junctions, and hydrostatic pressure between cells is unlikely to exceed a few mm of water; whereas, relatively large pressures are required to overcome surface forces between closely apposed lipid membranes unless surfactant is present as in lung alveoli. *A priori*, it therefore seems more likely to us that expansion of lateral spaces induced by Na-coupled solute transport results from generalized activation of contractile elements in the cytoskeleton of epithelial cells. Such cytoskeletal contraction might only be detectable morphologically in regions where actomyosin is most concentrated, such as the perijunctional ring.

3. *Effects of Glucose on Membrane Capacitance*

The lumped or distributed capacitance, C_M, of the electrical model shown in Fig. 1 cannot be related directly to surface area of plasma membranes because it comprises the capacitance of the apical microvilli in series with basolateral membranes and the lateral membrane capacitance is in series with the resistance of the lateral spaces as shown in Fig. 6 of the accompanying paper on impedance [13]. However, the present analysis of structure and *changes* of structure induced by glucose provides a rational basis for interpreting capacitance data in terms of membrane surface area.

In the absence of Na-coupled solute transport, the capacitance of the epithelium is about 8 μF per cm length of intestine (Fig. 1). The measured villus surface area (including crypts) is approximately 8 cm^2 per cm length as noted above (paragraph C, Materials and Methods). Given the usual value of 1 μF per cm^2 plasma membrane, it follows that the capacitance measured in the substrate-free tissue is determined primarily by the basal portion of the basolateral membranes. This implies (i) that the capacitative reactance of the apical membrane (microvilli) is negligible because of its large surface area and (ii) that the closely apposed lateral membranes do not contribute to the capacitance compo-

nent of impedance. The increase of capacitance from 8 to 28 μF per cm induced by glucose (Fig. 1) may be interpreted as an unmasking of the lateral surfaces during expansion of the lateral spaces.

B. Effects of Fixation Procedures on Impedance and on Structure

Correlations between structure and function were strengthened in this investigation because of a fixation procedure which preserved the relevant functional properties of the tissue. Thus impedance of occluding junctions and plasma membranes were comparable in both live and fixed tissues of segments perfused in vitro, and the large changes of impedance induced by Na-coupled solute transport were preserved in the fixed specimens. When live segments were removed from perfusion (either in vitro or in vivo) and subjected to conventional fixation procedures the structural changes induced by glucose were not found. It is possible that the structural changes induced by glucose are reversed by hypoxia during excision of the live tissue and its removal for further dissection under cold fixative. It was shown in the accompanying paper [13] that exposure of glucose-activated segments to low oxygen pressures caused a rapid increase of both junctional and lateral space resistances together with decreased membrane capacitance; hypoxia inhibited the normal responses to glucose. In two experiments of the present series (not described above) we found that in glucose-activated segments perfused in vitro with low oxygen the lateral spaces were collapsed.

The observation that impedances are greatly altered in epithelia fixed at 38°C during perfusion in vivo (in contrast to the lack of change in vitro) calls for further discussion. The impedances of epithelia fixed in vivo were so low at all frequencies that they were not significantly greater than impedance of subepithelial tissue. Evidently, the resistance of the plasma membranes had broken down. Structurally, however, there were no signs of damage to the lipid membranes and the general structure of specimens fixed during in vivo perfusions differed from the structure of specimens fixed during in vitro perfusions only in minor details. The structural changes induced by glucose in vivo were similar to those found in vitro despite the differences in impedance. We emphasize this phenomenon because it suggests that some substance capable of disrupting the resistance barrier of the plasma membrane is liberated by fixatives in vivo but not in vitro, and this may account for certain discrepancies noted in the literature [2].

C. Mechanisms of Intestinal Absorption of Sugars, Peptides and Amino Acids under Physiological Conditions

The three papers of this series have led to a concept of intestinal absorption in which active transport of nutrients plays a necessary, but nevertheless secondary and indirect role in transporting sugars, amino acids or peptides from lumen to blood under physiological conditions. The following resume is based on results from all three papers and the concepts are presented with more clarity than caution in order that they may be challenged, disproved or verified.

We propose that a primary function of active transport of glucose and amino acids is to transport these substances (together with sodium) at high concentration into the intercellular lateral spaces below the occluding junction, thus providing the osmotic force for absorption of fluid. Na-coupled solute transport also triggers contraction of the perijunctional actomyosin ring, resulting in increased permeability of occluding junctions and expansion of lateral spaces, thus providing optimal conditions for transport of luminal nutrients in bulk by solvent drag. When luminal glucose concentration exceeds that required to saturate active transport, the concentration of glucose in basolateral absorbate is some 80 mM greater than that in luminal fluid [14] given our estimated coefficients for transepithelial osmotic flow and reflection; this concentration difference is sufficient to account for the observed rate of fluid absorption [14]. Approximately 50% of the fluid absorption takes place paracellularly through junctions of enhanced permeability. Active transport reaches its maximum capacity at luminal concentrations in the range 10–20 mM, whereas transport by solvent drag increases in proportion to luminal concentrations. At solute concentrations normally found in the duodenum or upper jejunum after a meal (100–300 mM), the greater part of absorption of hydrophilic nutrients takes place by solvent drag and this may include small peptides as well as monomeric sugars or amino acids. As nutrients are removed from the upper intestine by the above mechanisms, their concentrations decrease and the traditional role of active transport becomes a greater fraction of total transport. Thus an additional function of active transport is to provide for complete removal of nutrients from the lower por-

tions of the small intestine where the concentrations are so low that transport by solvent drag becomes negligible.

An unknown step in the mechanism described above is the link between Na-coupled solute transport and the cytoskeletal contractile apparatus. How does activation of the transport system in the apical microvilli trigger contraction of the perijunctional actomyosin ring? Is the ATP-ase associated with the intracellular sodium pump connected with phosphorylation of contractile proteins?

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