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Opening of Tight Junctions in Frog Skin by Hypertonic Urea Solutions

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Summary. The movements and distribution of lanthanum have been studied in frog skins treated on the outside with Ringer's solution to which 400 mM urea had been added. Under these conditions, ^{140}La moved across the skin. This finding is in contrast with what is observed in control skins, which are impermeable to lanthanum. Examination with the electron-microscope of skins fixed during equilibration with outside hypertonic solutions plus lanthanum, showed penetration of the tracer from the outside into the intercellular spaces of all the inner layers of the epidermis. The occluding zonules (tight junctions) that normally prevent the inward movement of lanthanum beyond the outer border of the stratum granulosum (SG) are open. As a consequence, most intercellular spaces along the lateral cell surfaces of the SG were permeated by lanthanum throughout their extension. In skins treated with hypertonic solutions and then allowed to recover in normal Ringer's, the occluding zonules of the SG were impermeable to lanthanum, and ^{140}La did not move across the epidermis. These observations show that the increased skin permeability caused by outside hypertonic solutions results mainly from a reversible opening of tight junctions.

In 1956, Ussing and Andersen found that the frog skin potential was reversibly and markedly reduced when the solution bathing the outside of the frog skin was made hypertonic by the addition of urea or other hydrophilic solutes. Associated with the drop in potential was a large increase in the passive permeability to anions and cations (Ussing & Windhager, 1964). Furthermore, the movement of substances that normally do not penetrate cell membranes and whose rate of transfer across the frog skin is negligible, markedly increased during the action of the hypertonic solutions (Ussing, 1965; 1966). Based on these findings, Ussing (1965) suggested that outside hyperosmotic solutions caused an opening of the tight junctions sealing together the cells of the outer layers of the epidermis. In an attempt to visualize more directly the high permeability path induced by the hypertonic solutions, skins were equilibrated with a 10-mM Ba^{++} Ringer's solu-

tion on the outside and with sulfate Ringer's on the inside and then examined with the electron-microscope for electron dense $BaSO_4$ precipitates, (Ussing, 1971). No $BaSO_4$ was found in control skins while in the skins treated with hypertonic urea solutions electron dense precipitates appeared in the extracellular spaces of the epidermis. These observations show that a substantial part of the path taken by barium moving inward in skins treated with hypertonic solutions is through the intercellular spaces of the epithelium. No description was made, however, of the distribution of $BaSO_4$ in the outer layer of the *stratum granulosum* (SG), where the main permeability barrier to solute diffusion of the skin seems to be localized (Martínez-Palomo, Erlij & Bracho, 1971).

In the present investigation, we have studied the ultrastructural distribution of lanthanum in skins treated with hypertonic urea solutions, with particular emphasis on the changes in the outer layer of the SG [1]. We have used lanthanum as a tracer because: (1) The distribution of lanthanum can be determined with the electron-microscope after exposing the epithelium to concentrations as low as 0.5 mM added to 'physiological' saline solutions (Martínez-Palomo *et al.*, 1971). (2) In the solutions used in our experiments all the lanthanum is in ionic form. According to the dissociation constants given by Sillen and Martell (1964) approximately 90% of the lanthanum is in the La^{3+} form and 10% in the forms $LaOH^{++}$ and $La(OH)_2^+$. None of the lanthanum is precipitated as $La(OH)_3$. The ionic radius of the lanthanum is 1.061 Å (Moeller, 1963). (3) The tracer is equilibrated with the living unfixed tissue on which it is possible to determine whether the concentrations of lanthanum used had any adverse effects. (4) ^{140}La is an isotope of easy use. The study of its movements can provide a check on the validity of the conclusions reached with the electron-microscope.

Materials and Methods

The technique for mounting the skin to study the effects and movements of lanthanum, and the fixation procedure were the same as described previously (Martínez-Palomo *et al.*, 1971). Briefly, skins taken from the abdomen of adult frogs (*Rana temporaria* and *Rana pipiens*) were mounted either separating two half-chambers of Ussing-Zerahn type, or in the holder designed for isotope uptake measurements. Skin resistance was calculated from the potential changes caused by pulses (10 μamps/cm^{-2}; 10-sec duration). The skins were fixed in the chambers after incubating with selected solutions at either surface. Normal Ringer's solution had the following composition (in mmoles/liter): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.0; Tris chloride buffer, 3.0, pH 7.3 to 7.5. The Ringer's + 400 mM urea had the same amounts of electrolytes and, in addition, 400 mmoles/liter urea were

1 A preliminary communication on these experiments was given to the International Conference on Biological Membranes, Gargnano, Italy, 1971.

included in the solution. When ^{140}La movements were studied, enough isotope to obtain activities between 2 and 5 μC/ml was added to a solution containing adequate amounts of unlabelled lanthanum. The skins were fixed with 2.5% glutaraldehyde in cacodylate buffer for 1 hr; stored for variable periods in cacodylate buffer and dehydrated without postfixation in osmium tetroxide. After flat embedding in Epon and sectioning, they were examined with or without additional counterstaining in a Zeiss EM9-S electron-microscope.

Results

Action of Hypertonic Solutions

Fig. 1*A* illustrates one of four experiments in which the effects of the hypertonicity of the outside solution were measured. The addition of Ringer's +400 mM urea caused a sharp drop in potential difference and a marked decrease in resistance. Replacement of the hypertonic solution by

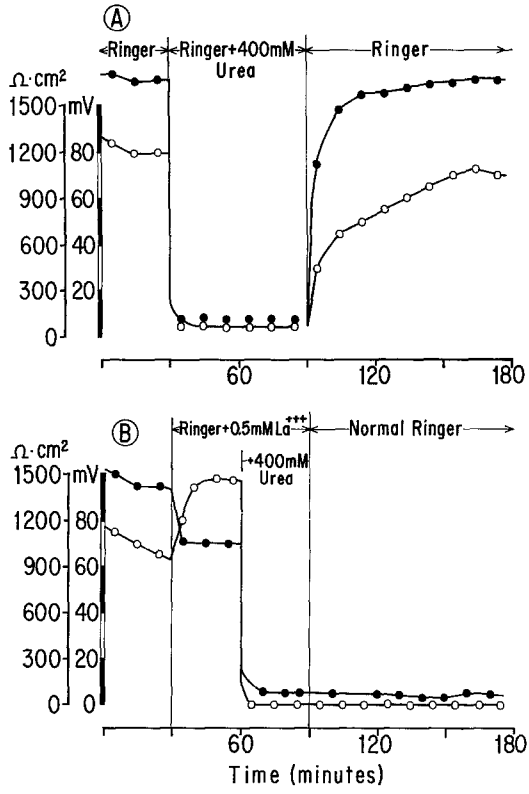


Fig. 1. The effects of outside hypertonic solutions (Ringer's +400 mM urea) on the potential difference and resistance of the frog skin. Filled circles are resistance values (Ω cm^2); open circles are potential difference values (mV). The ordinates are the resistance and potential difference scales. The skin in graph *A* was exposed to the hypertonic solution without any lanthanum. The skin in graph *B* was treated with lanthanum during exposure to the hypertonic solutions

normal Ringer's solution was followed by a recovery of the electrical parameters to values similar to those observed during control periods. The results are in agreement with the findings of Ussing (1966) who added only 200 mM urea to Ringer's solution. We selected a higher concentration of urea to obtain maximum fluxes of tracers across the skin. Biber and Curran (1968) studied the effects of increasing the concentration of the outside solution to levels up to 400 mM above the normal Ringer's concentration, and found an increase in the movement of large nonelectrolytes directly proportional to the concentration of solute added to increase osmolarity. This observation and the reversibility found in the experiments described here suggest that the changes observed with the addition of either 200 or 400 mM urea respond to a common mechanism.

The effects of adding Ringer's +400 mM urea are not modified by including lanthanum (0.5 to 1.0 mM) in the solutions. However, the recovery of the skin potential and resistance after returning to normal Ringer's was blocked when lanthanum was added to the hypertonic solutions. Fig. 1*B* illustrates a typical experiment in which the effects of 0.5 mM La^{3+} on the potential difference and resistance of the skin were first determined. As in previous experiments (Martínez-Palomo *et al.*, 1971; Bracho, Erlij & Garcia, *unpublished experiments*), the addition of lanthanum to the outside solution induced an increase in the potential difference and a reduction in skin resistance. The increase in potential and the reduction in resistance were reversible and result exclusively from an increase in the sodium conductance of the skin (Bracho, Erlij & García, *unpublished observations*). Subsequent treatment of skins with Ringer's +400 mM urea +0.5 mM La^{3+}, was followed by a response (Fig. 1*B*) identical to that observed in the control experiments (Fig. 1*A*). However, in contrast with control experiments, when both sides of the skin were repeatedly washed with normal Ringer's solution without lanthanum, no recovery was observed even after periods as long as 90 min. This blockade was also observed in experiments in which lanthanum was included only in the hypertonic solution.

The effects of Ringer's +400 mM urea on the movements of ^{140}La from the outside solution into the skin are illustrated in Fig. 2. The first part of the experiment shows the typical pattern of lanthanum uptake by the outside surface of the frog skin (Martínez-Palomo *et al.*, 1971). After an initial period of rapid uptake that lasts for about 10 min, there is little or no further increase in the amount of lanthanum in the skin. Urea (400 mM) was added to the outside solution after a constant lanthanum content had been reached. The hypertonicity caused a marked increase in the rate of lanthanum uptake. Similar results were obtained in three additional experi-

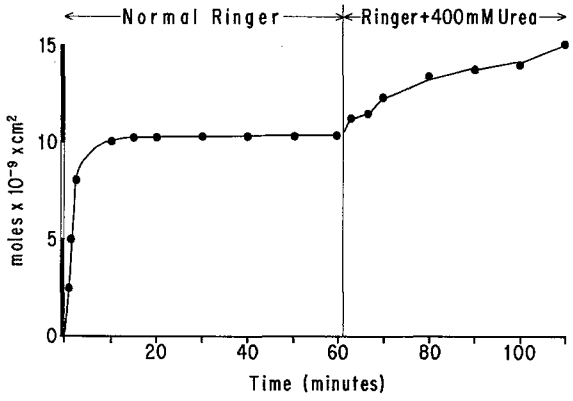


Fig. 2. The effects of outside hypertonic solutions (Ringer's +400 mM urea) on the uptake of lanthanum by the outside surface of the skin. The ordinate is the amount of lanthanum per cm^2 of skin. The vertical line indicates the addition of 400 mM urea to the outside Ringer's solution

ments. In line with these observations is the finding that, after exposing skins to Ringer's +400 mM urea, a movement of ^{140}La from the outside into the inside solution can be measured. In four experiments, the values for the lanthanum permeability coefficient ranged between 0.8×10^{-7} and 2.3×10^{-7} cm sec^{-1}. This small, but finite movement of lanthanum, is in contrast with the failure to detect any ^{140}La moving across the control skins, even after the outside surface had been exposed to the isotope for as long as 5 hr.

Morphological Findings

The general organization of the frog skin fixed 1 hr after exposure to hypertonic solutions is shown in Fig. 3. At the optical microscope level, the most striking modification caused by the addition of 400 mM urea to the Ringer's solution was the formation of vacuoles within the cells of the outer layer of the SG. In addition, the cells in the stratum corneum (SC) and those in the outer layer of the SG appeared to have shrunk under the action of the hypertonic solution.

The intracellular location of the vacuoles seen in the cells of the SG of skins exposed to hypertonic solutions was confirmed with the electron-microscope. The vacuoles were very frequently found near the plasma membrane, but they were separated from it by a narrow rim of cytoplasm. Lanthanum was not found in the interior of the vacuoles (Fig. 6).

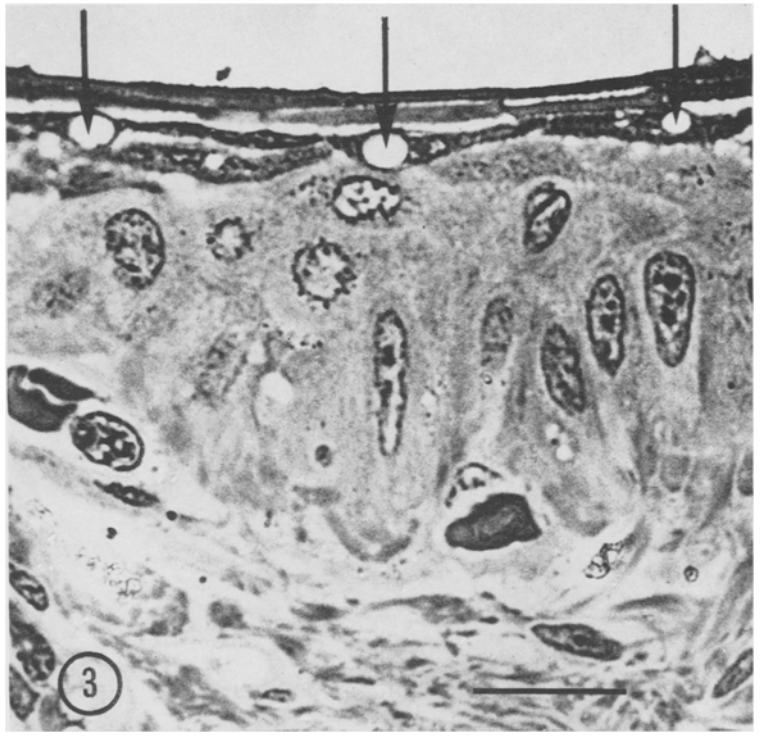


Fig. 3. Photomicrograph of a portion of a frog skin equilibrated on the outside with Ringer's +400 mM urea during 1 hr. The most striking modification is the appearance of large cytoplasmic vacuoles in the external layer of the stratum granulosum (arrows). The specimen was fixed in 2.5% glutaraldehyde and embedded in Epon. 1-μ section stained with toluidine blue. ×2,000. Scale = 10 μ

The electron-microscopical examination of frog skins fixed after equilibration during 1 hr with Ringer's +400 mM urea + lanthanum, demonstrated in addition to vacuole formation, an important modification of the permeability barriers. As in frog skins incubated in normal Ringer's, lanthanum precipitates were observed within the cells of the SC (Fig. 4), and in the space between the SC and the outer layer of the SG. However, in the skins equilibrated with hypertonic solutions, lanthanum precipitates penetrated along the lateral spaces of the SG cells into the intercellular spaces of all the inner layers of the epidermis (Figs. 4, 5 and 6). This observation is in marked contrast with the findings on skins fixed after incubation in normal Ringer's solution, in which penetration of lanthanum is never observed beyond the outer layer of the SG, where movement along the intercellular spaces is barred by zonulae occludentes.

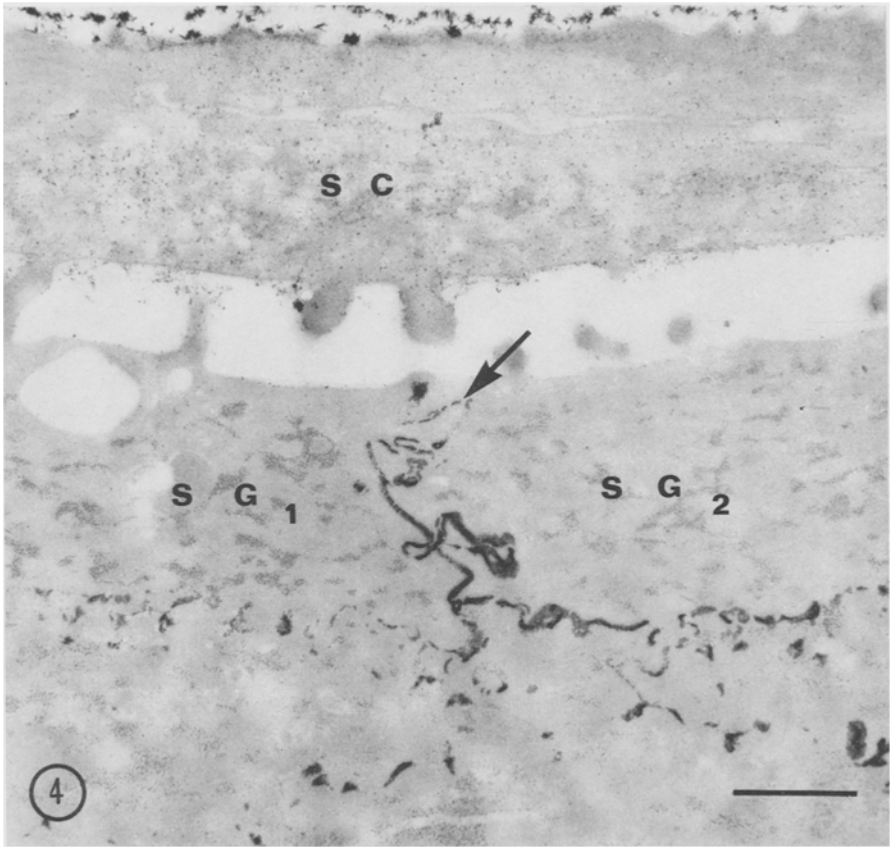
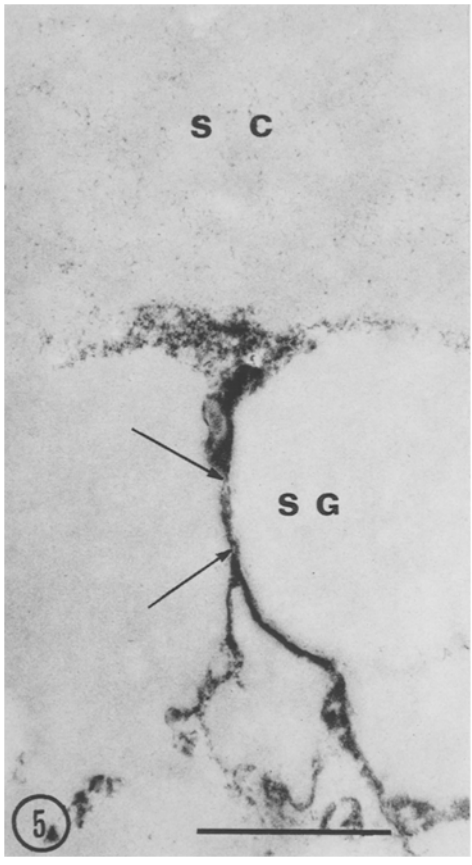
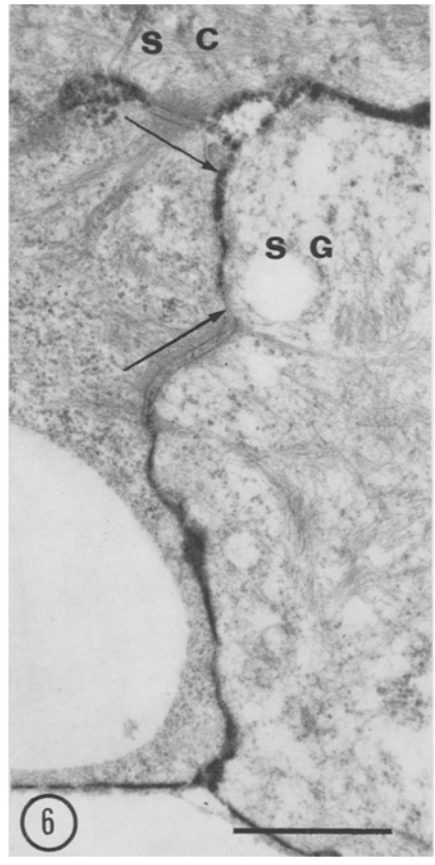


Fig. 4. Outer region of a frog epidermis treated during 1 hr with Ringer's +400 mM urea + lanthanum. Lanthanum precipitate is seen at the surface, and within the cytoplasm of a stratum corneum cell (SC), in the intercellular space that separates the stratum corneum from the stratum granulosum and along the lateral intercellular space between two stratum granulosum cells (SG_1 and SG_2). Lanthanum is also present at the sites where zonulae occludentes restricts, under normal conditions, the passage of tracers (arrow). The specimen was fixed in 2.5% glutaraldehyde and embedded in Epon. No counterstaining was used. × 18,000. Scale = 1 μ. Reduced to 9/10

Examination of a considerable number of specimens revealed that most zonulae occludentes became permeable to lanthanum under the action of the hypertonic external solutions; only in a few instances were intact zonulae occludentes observed. The width of the lanthanum channel at its narrowest point along the continuous lateral intercellular spaces of the outer layer of the SG ranged between 150 to 200 Å. The relationship between these values and the width of the channel through the open tight junction in living skins is not yet determined. An estimate of channel radius could





Figs. 5 and 6. Outer region of frog epidermis treated from the outside with Ringer's +400 mM urea + lanthanum. A dense precipitate is seen in the cytoplasm of cornified cells (SC), at the intercellular space between the stratum corneum and the stratum granulosum (SG) and along the lateral intercellular space between adjacent stratum granulosum cells. The sites where tight junctions are normally present (arrows) are permeated with lanthanum. Specimes fixation as for Fig. 4. The section shown in Fig. 5 was not counterstained, while that shown in Fig. 6 was stained with uranyl acetate and lead citrate. Fig. 5, × 30,000. Fig. 6, × 25,000. Scales = 1 μ. Reduced to 9/10

be obtained by the determination of the permeabilities to different large hydrophylic solutes in the living skin with open tight junctions.

Altered tight junctions were identified mainly on a topographical basis; in normal skins they form a complete belt that girdles the entire cell perimeter at the external region of the outer SG cells (Farquhar & Palade, 1965). Other morphological criteria cannot be used to locate modified zonulae occludentes, since they lack the conspicuous cytoplasmic condensations seen in other types of intercellular junctions (Martínez-Palomo,

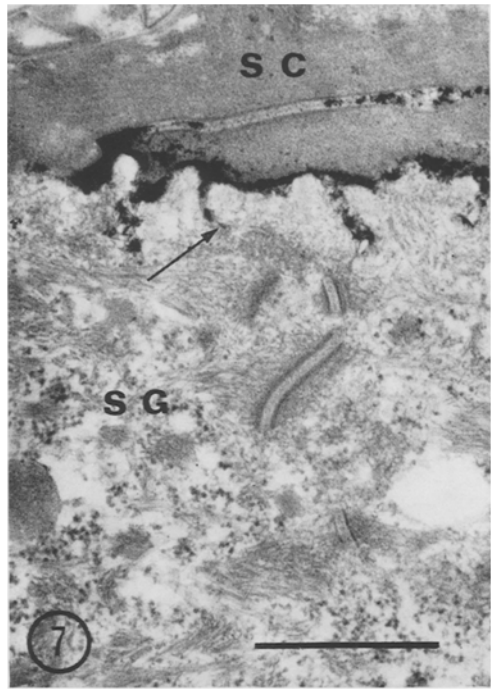


Fig. 7. Frog skin treated with hypertonic solutions and subsequently washed with normal Ringer's until electrical parameters recovered. Lanthanum is prevented from entering into the lateral intercellular space between two cells of the stratum granulosum (SG) by a tight junction (arrow). Specimen fixation and staining as for Fig. 6. × 25,000. Scale = 1 μ

1971). Lanthanum precipitates were never observed within the cytoplasm of the SG cells; therefore, the tracer found along the inner spaces of the epidermis penetrated through opened tight junctions.

The permeability barriers in frog skins subjected to hypertonic solutions and subsequently washed with normal Ringer's, until the potential difference and resistance recovered completely, were found to be intact. In these specimens the lanthanum tracer did not penetrate beyond the space that separates the SC and the SG (Fig. 7), as observed in control skins. Very few intracellular vacuoles remained in the SG of skins fixed after recovery from hypertonicity.

Discussion

The results of this investigation show that the effects of outside hypertonic urea solutions on the permeability of frog skin derive mainly from the opening of the zonulae occludentes (tight junctions). This conclusion is in

agreement with the suggestions made by Ussing in 1965. Furthermore, our findings localize the site where either the local volume circulation (Ussing, 1969; Patlak & Rapoport, 1971) or the solute-solute interactions in free solution (Franz & van Bruggen, 1967; Biber & Curran, 1968) that lead to asymmetrical solute fluxes may take place.

The hypertonic solution treatment provides the first instance in which an opening of tight junctions can be detected. It has been suggested that the increases in permeability caused by treating epithelia with Ca^{++}-free solutions to which EDTA has been added, also result from the opening of tight junctions. However, the available anatomical evidence is not completely convincing. Hays, Singer and Malamed (1965) found that after treatment of the toad bladder with Ca^{++}-free solutions containing EDTA, the epithelial cells could be mechanically detached with great ease. Unfortunately, no study of the appearance of the junctional complexes before mechanical separation was published. Sedar and Forte (1964) working with frog gastric oxyntic glands found consistent changes only in the intermediate junctions. The changes in the tight junctions of EDTA-treated preparations were restricted to focal regions of membrane separation. These authors were unable to establish whether these openings in the zonulae occludens were continuous with the luminal surface. Cassidy and Tidball (1967) working with EDTA-treated rat intestine found an opening of the tight junctions on only one occasion. They concluded that this alteration was due probably to a distortion of the preparation and not a result of Ca^{++} depletion. The changes in epithelial permeability were interpreted as resulting from alterations in cell membrane permeability, and not from the opening of intercellular junctions.

A particularly interesting feature of the present demonstration of opening of tight junctions by hypertonic solutions is the reversibility of the effect, as indicated by the recovery of the electrical parameters, and the restriction of the movements of lanthanum to the outer border of the SG after substituting the hypertonic solution for normal Ringer's. These findings suggest that, in spite of the presence of vacuoles, the cells are not profoundly altered by the treatment; furthermore, the fact that lanthanum did not penetrate into the inner cells fixed during incubation in hypertonic solutions and lanthanum indicates that hypertonicity does not damage the cell membrane.

The lack of recovery after adding lanthanum to urea-treated skins probably results from the action of lanthanum on a component of the junction made accessible by the hypertonic treatment. Lanthanum added to the inside of control skins moves through the extracellular space up to

the inner end of the tight junctions in the outer layer of the SG without affecting the potential difference or the resistance of the skin. When added to the outside solution, lanthanum moves across the cells of the SC and stops at the outer end of the tight junctions in the external layer of the SG and produces only a reversible increase in sodium conductance and transport (Martínez-Palomo *et al.*, 1971; Bracho, Erlij & García, *unpublished observations*). Only when lanthanum penetrated the tight junctions of urea-treated skins was an irreversible effect observed.

Although our knowledge of zonulae occludentes is still in a rather rudimentary stage, it is clear that any scheme of their organization has to take into account that the forces holding together the membranes of neighboring cells are generated in such a manner, that they may be overcome by a process initiated with the addition of outside hypertonic solutions, and that these conditions do not cause a permanent alteration of the cell. It is also necessary to note that the increased permeability disappears when the osmotic gradient across the skin is abolished by increasing the osmolarity of the inside solution (Ussing, 1965). This finding indicates that the opening of the tight junctions does not result only from cell shrinking, but that the osmotic gradient across the epithelium plays an important role in the process.

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