CELLULAR MECHANISM OF INTESTINAL PERMEABILITY ALTERATIONS PRODUCED BY CHELATION DEPLETION

M. M. CASSIDY and C. S. TIDBALL

From the Armed Forces Institute of Pathology, and the Department of Physiology, The George Washington University School of Medicine, Washington, D. C.

ABSTRACT

The absorption of phenolsulfonphthalein (phenol red) was used as a measure in vivo of intestinal permeability in anesthetized rats. A chelating agent, sodium ethylenediaminetetraacetate (NaEDTA), placed in the lumen evoked a fivefold increase in membrane permeability; at the same time the mucosal content of magnesium and calcium decreased significantly. Making either magnesium or calcium available to the luminal surface of the membrane in isotonic solution restored normal permeability and brought the cation contents above the original levels. Electron micrographs of tissues treated in vivo with NaEDTA revealed (*a*) rounded swellings on the microvilli in the area of the junctional complexes between adjacent epithelial cells, (*b*) widening of intercellular channels particularly in the region of the intermediate junctions (zonulae adhaerentes), and (*c*) loss of architectural detail in the region of the desmosomes (maculae adhaerentes) with separation of their dense borders. All of these alterations in fine structure could be reversed by in vivo cation replacements which reinstated normal permeability. The implications of these findings on mechanisms of fluid transport across epithelial membranes are discussed, and a working hypothesis for the role of divalent cations in membrane permeability regulation is presented.

The ability of the chelating agent sodium ethylenediaminetetraacetate (NaEDTA) to increase the permeability of the intestinal epithelial membrane has now been reported by a number of different laboratories (1–3). The relation of these changes to the magnesium and calcium contents of the membrane was suggested in an earlier report which established the reversibility of the phenomenon (4). The present article utilizes a newly developed method for the simultaneous determination of calcium and magnesium in samples derived from soft tissues,[1] in order to clarify the relationship between the mucosal content of these alkaline-earth cations and the permeability alteration. In addition, electron micrographs are presented which delineate changes in the fine structure of this epithelial tissue during chelation depletion and its attendant increase in permeability. Both permeability and structural alterations could be reversed either partly or completely by treating in vivo with either isotonic $MgCl_2$ or $CaCl_2$. Therefore it seems likely that the phenomena described are of physiological significance and that their further elucidation may contribute to our understanding of fluid transport across epithelial membranes as well as the nature of molecular forces involved in cell adhesion.

METHODS

The Principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed during this study. Male

Sprague-Dawley rats, 200–300 g in weight, were anesthetized with 40 mg/kg of sodium pentobarbital after having been fasted for 24 hr. The small intestine was divided into proximal, middle, and distal segments by counting the vascular arcades betwen the pylorus and the ileocaecal valve and making the divisions such that an equal number of arcades was present in all three portions. Both ends of the segments were fitted with cannulas fashioned of polyethylene and rubber tubing to facilitate washing out, introducing of test solutions, and rinsing procedures. All solutions placed in contact with the intestinal lumen were adjusted to pH 7.0 and 300 mosmoles/liter. Sodium chloride solutions (approximately 0.9%) were utilized in the absorption studies. After cleansing the loop of debris with saline, 3 ml of saline containing 1 μM/ml phenolsulfonphthalein (phenol red) were left in the intestinal loop for 40 min. The loop was then washed out with saline not containing phenol red. The difference between the phenol red introduced and that recovered was taken as the amount absorbed. This phenol red absorption was expressed as a percentage of the total amount placed in the loop. The group of experiments designated as control were subjected to all of the above procedures for one or more absorption periods. Succeeding absorption periods were initiated similarly except that NaEDTA was also present in the test solution. After treatment with NaEDTA the following standardized rinsing procedure was used to study the reversal of the NaEDTA effect: a 30 ml volume of the rinsing solution was flushed through the loop in 3 ml increments; the final 3 ml volume was allowed to remain in contact with the mucosa for 10 min; this was then removed by gravity flow prior to initiating another absorption period. The rinsing solutions consisted of 100 mM/liter $CaCl_2$ or $MgCl_2$ with minor adjustments to bring pH and osmolarity to specified values.

Phenol red was analyzed by determining the optical density of centrifuged intestinal washings diluted and buffered to pH 10. The absorption maximum was at 560 mμ. A Beckman DU spectrophotometer was used for the optical density measurements and a Beckman model 76 pH meter (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) was used for hydrogen ion adjustments. An Advanced Instrument osmometer (Advanced Instruments, Inc., Newton Highland, Mass.) was used to determine the osmolarity of the solutions.

It has been demonstrated clearly in an earlier paper (4) that no significant difference in phenol red absorption exists (*a*) between earlier and later control periods in the same animal, (*b*) between control periods in either half of the small intestine and, (*c*) between experimental periods (NaEDTA) in either half of the same intestine. For purposes of comparison with previous work and on the basis of assumptions drawn from the above determinations, all figures obtained for percentage absorption per 40 min per ⅓ intestine are expressed as percentage absorption per hour per ½ intestine, i.e., 3 μM phenol rec = 100%. Those portions of the intestinal segments between the inflow and outflow cannulas were removed and treated in the following manner. The tissue was weighed and, in some cases, dried, wet-ashed, and analyzed for calcium and magnesium. In the majority of experiments, however, the mucosa was scraped gently from the underlying layers by means of a blunt spatula and glass slide, then ashed, and analyzed separately. The calcium and magnesium

TABLE I

Calcium and Magnesium Content of Rat Tissue

Tissue	Calcium	Magnesium	H_2O content
	mEq/kg	*mEq/kg*	%
Stripped mucosa	2.21 ± 0.18 (39)	5.28 ± 0.44 (39)	84.2 ± 1.9 (10)
Smooth muscle layers and connective tissue	0.95 ± 0.63 (29)	8.61 ± 2.35 (29)	79.5 ± 0.8 (6)
Complete small intestine	3.16 ± 0.45 (30)	13.89 ± 1.91 (30)	81.2 ± 1.3 (11)
Plasma (mEq/liter)	7.85 ± 0.22 (6)	4.31 ± 0.40 (6)	—

The values determined for rat tissue samples are expressed as mEq/kg wet weight total intestine (including muscle layers and connective tissue). Variation is indicated by ±SE of the mean, and the figures in parentheses refer to the number of separate samples used in calculating each mean.

TABLE II

Permeability and Divalent Cation Relationships in Rat Small Intestine with Chelation Depletion

Data: means ± SE for the number of experiments in parentheses

Tissue analyzed	Experiment	Calcium	Magnesium	Permeability (phenol red absorbed)
		mEq/kg	*mEq/kg*	*% /hr/½ intestine*
Complete small intestine	Control	3.16 ± 0.45 (8)	13.89 ± 1.51 (8)	1.85 ± 0.40 (8)
	NaEDTA treated	2.53 ± 0.63 (7)	14.06 ± 2.81 (7)	11.97 ± 2.50* (7)
	$CaCl_2$ rinse after NaEDTA	6.08 ± 1.10*‡ (6)	13.70 ± 2.87 (6)	5.65 ± 1.53‡ (6)
	$MgCl_2$ rinse after NaEDTA	3.58 ± 1.71 (7)	16.58 ± 1.63 (7)	1.64 ± 1.04‡ (7)
Stripped mucosa	Control	2.21 ± 0.18 (39)	5.88 ± 0.44 (39)	1.92 ± 0.38 (39)
	NaEDTA treated	1.20 ± 0.15* (32)	3.88 ± 0.42* (32)	10.51 ± 0.84* (32)
	$CaCl_2$ rinse after NaEDTA	2.72 ± 0.19‡ (16)	7.36 ± 1.92‡ (16)	5.56 ± 1.26‡ (16)
	$MgCl_2$ rinse after NaEDTA	1.74 ± 0.24 (18)	6.82 ± 1.19‡ (18)	3.72 ± 0.86‡ (18)

* Significantly different from control at 95% confidence level.

‡ Significantly different from value for NaEDTA treated at 95% confidence level.

content of ashed samples of tissue was determined by the assay method described in another paper.[1]

Electron Microscopy Methods

For electron microscopy, fixation was started by injecting the fixative into the intestinal lumen as recommended by Palay and Karlin (5). All tissue specimens were fixed for 1 hr at room temperature in Dalton's osmium tetroxide fixative at pH 7.4. The tissue was then washed for 30 min in 10% ethanol, with several changes of solution. The specimens subsequently were dehydrated rapidly in graded ethanols and propylene oxide. Infiltration of the tissue with catalyst-containing resin was done at room temperature and the tissue embedded in Epon 812. Sections 600–900 A thick were cut and mounted on 200-mesh copper grids. Before examination in the electron microscope the sections were doubly stained, first in uranyl acetate (6), and then in lead hydroxide (7). Microscopy was performed with an RCA EMU 3-G electron microscope operated at 50 kv. Micrographs were taken at original magnifications of 12,000–40,000 diameters and enlarged or reduced photographically, as required.

[1] Cassidy, M. M., and C. S. Tidball. Calcium and magnesium content of the gastrointestinal tract of several species using a micro-complexometric method for the determination of calcium and magnesium. Manuscript submitted for publication.

RESULTS

In a series of preliminary observations the normal calcium and magnesium contents of the rat intestinal mucosa, complete intestinal wall, and plasma were determined; the content of the intestinal smooth muscle and connective tissue component was inferred by subtraction. These values are shown in Table I. The calcium and magnesium values for mucosa alone in 39 determinations averaged 2.21 and 5.28 mEq/kg, respectively; whereas the values for complete intestinal wall averaged 3.16 and 13.89 with somewhat more

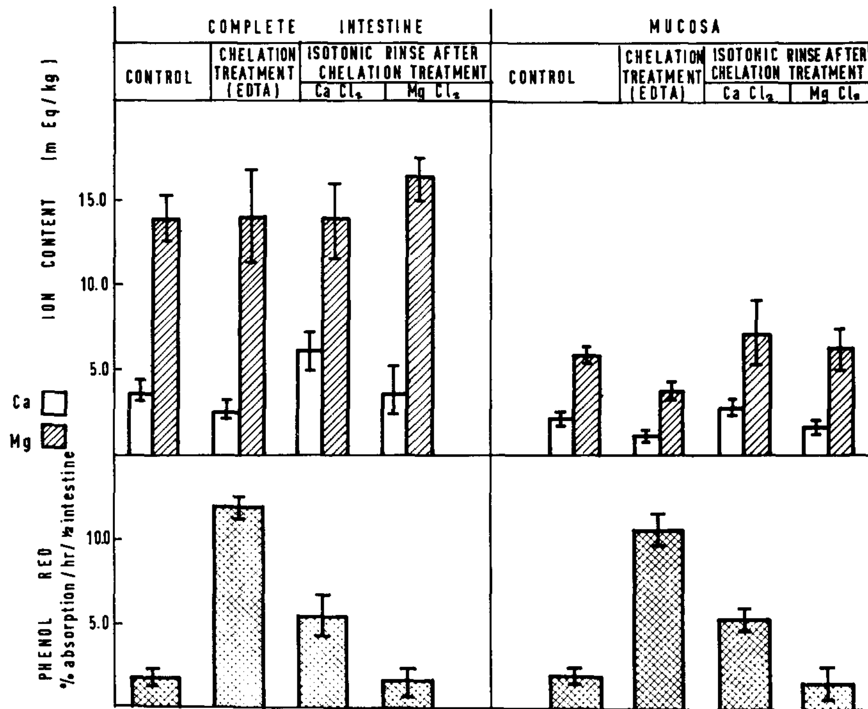


FIGURE 1 The effect of chelation treatment with 25 mM NaEDTA, in loops of rat small intestine in vivo, on the absorption of 1 mM phenolsulfonephthalein and on the calcium and magnesium contents of the tissue. Reversal of the chelation depletion phenomenon with restoration of normal permeability is shown following isotonic rinsing of the loops with the appropriate ionic species. The group of experiments on the left represents analysis of the complete intestinal wall following periods of depletion and replacement; the group on the right is that in which the mucosa alone was subjected to analysis.

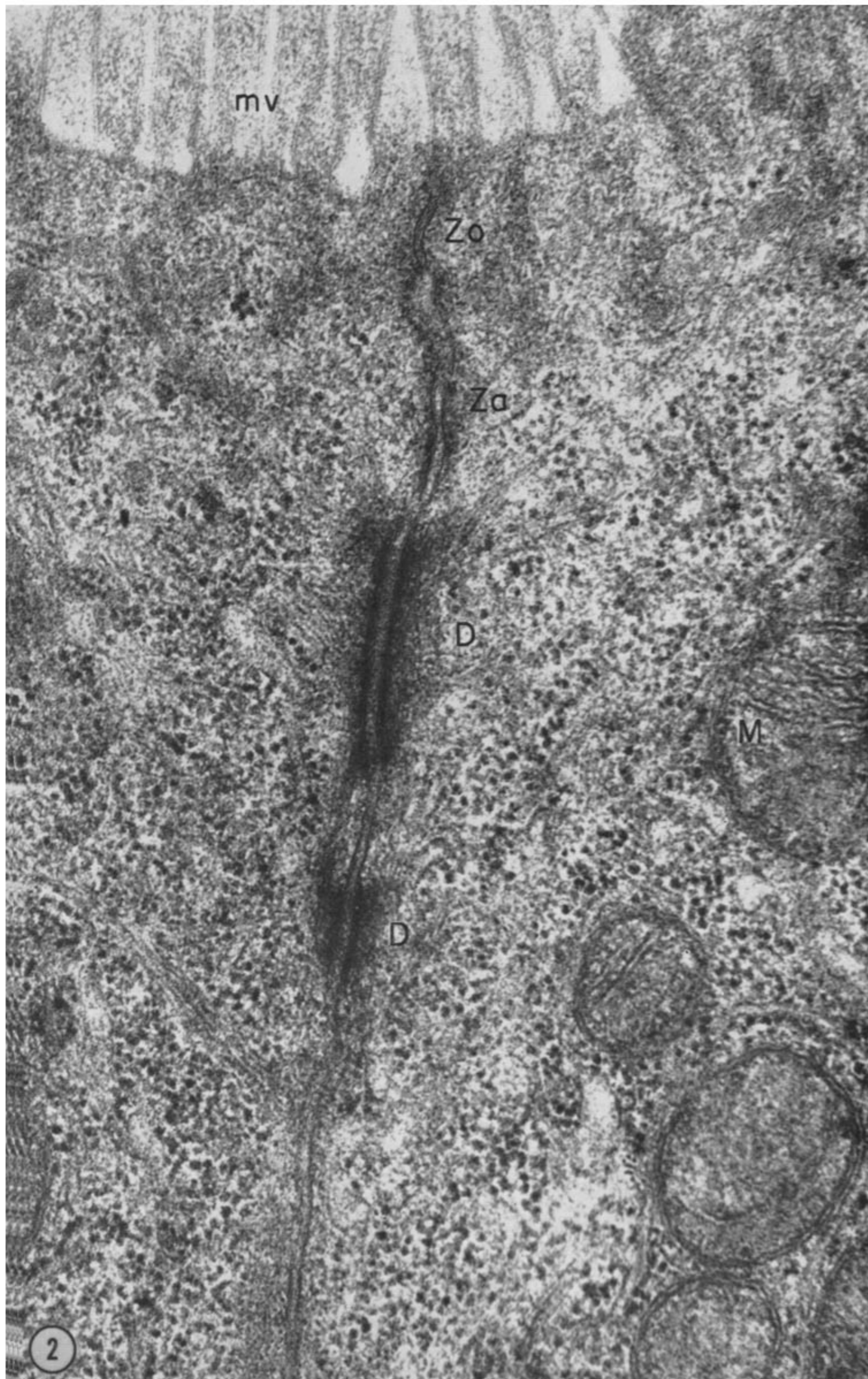
variation. The results are expressed on the basis of small intestine wet weight, but in a number of instances the dry weight of the tissue was also obtained. A summary of the water content of the individual tissues is also included in Table I.

When NaEDTA was placed in the lumen a considerable increase in phenol red absorption occurred, as shown in Table II. In an initial series of experiments in eight animals, the mean difference in phenol red absorption between control and treated segments was 10.12% absorption which is statistically significant at the 99% confidence

Abbreviations

Zo, zonula occludens
Za, zonula adhaerens
D, desmosome
M, mitochondria
L, lumen
fl, fusion line in zonula occludens
is, intercellular space
mv, microvilli

FIGURE 2 Junctional complex between two adjacent cells in the epithelium of the intestinal mucosa from the control group of rats used in these experiments. The tight junction (zonula occludens) is located nearest the lumen and is followed by the intermediate junction (zonula adhaerens) and two desmosome regions (maculae adhaerentes). Bundles of dense cytoplasmic fibrils converge on either side of the desmosomes. Specimen fixed in 1% OsO_4 in Dalton's buffer (pH 7.4) and embedded in Epon. Section stained with uranyl acetate and lead citrate. × 40,000.


mv
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level. The block diagram of Fig. 1 depicts, in the lower half, the changes in phenol red absorption during the experimental conditions described; concomitant values for tissue calcium and magnesium are shown in the upper half of the figure in which vertical bars represent the standard error attached to each mean. Although the calcium content of the small intestine fell from 3.16 to 2.53 mEq/kg during a period of exposure to NaEDTA, this decrement did not constitute a significant depletion. During the same period the level of magnesium within the tissue was not altered. When an isotonic solution of $MgCl_2$ was applied to the lumen following a period of chelation treatment, phenol red absorption returned to control values. The magnesium content of the tissue was slightly elevated to 16.58 mEq/kg, while the calcium values were essentially normal. However, when $CaCl_2$ was employed as the rinsing fluid after chelation treatment, the phenol red absorption merely was returned halfway to normal in spite of a significant increase in calcium content even above the original control values.

These preliminary experiments intimated a trend in cation depletion and replacement which was investigated further by subjecting the mucosal epithelium alone to analysis. In a subsequent series of experiments in 39 animals, the effect of NaEDTA on the mucosal calcium and magnesium was examined. As can be seen from the second half of Fig. 1, phenol red absorption was increased markedly with chelation treatment. Simultaneously the level of tissue calcium was depleted by 47% and that of tissue magnesium by 27% of their normal values, respectively. In 11 experiments the water content of NaEDTA-treated intestinal segments was 82.2% $\pm$ 1.50, which is not significantly different from the value for untreated tissue of 81.2% $\pm$ 1.32.

The effect of isotonic calcium and magnesium solutions on the chelation depletion phenomenon of the epithelial membrane was also studied. The application of 100 mM $CaCl_2$ solution to the lumen following a period of chelation treatment produced a partial reversal of the elevation in phenol red absorption as shown in the lower right half of the diagram in Fig. 1. At the same time, the mucosal calcium content was returned to 2.72 mEq/kg, a value slightly greater than that obtained for the prechelation group of experiments. An unexpected finding was that the magnesium level of the mucosal epithelium also rose during the $CaCl_2$ rinse; the value of 7.36 mEq/kg obtained greatly exceeded that of the NaEDTA-treated tissue and was even greater than that of the control group.

When $MgCl_2$ was used as the rinsing fluid the phenol red absorption returned to control values. The mucosal magnesium was restored to 6.82 mEq/kg but calcium levels were not significantly different from those of the NaEDTA-treated tissue. A complete summary of the numerical values with appropriate statistical analyses for all of these experiments is given in Table II.

Fine Structure of Normal Intestinal Epithelium of the Rat

Fig. 2 shows a portion of the normal rat intestinal epithelial layer obtained from control intestinal segments used in the physiological experiments described. The view shows microvilli at the luminal surface and the boundary between two adjacent epithelial cells. A characteristic tripartite junctional complex is seen which conforms to the generalized scheme advanced by Farquhar and Palade (8). The elements of the complex, in an apical-basal direction from the luminal side, are identified in the Palade nomenclature as follows: the zonula occludens or tight junction characterized by fusion of the adjacent cell membranes with obliteration of the intercellular space; the zonula adhaerens or intermediate junction characterized by the presence of an intercellular space 150–200 A in width; and the macula adhaerens or desmosome characterized by the presence of dense laminar plaques in the cytoplasm parallel and adjacent to the inner leaflet of each unit membrane structure. Bundles of cytoplasmic fibrils are usually found in association with these dense lamellae. Desmosomes often appear singly in other areas of cell-to-cell attachment although two such structures are clearly visible in Fig. 2.

Effect of Chelation Depletion on the Structure of the Rat Intestinal Epithelium

Chelation treatment of the mucosal epithelium with concomitant increase in permeability of the epithelial membrane was associated with certain topographical alterations in the components of the junctional complex. In addition, certain features of these changes wrought by calcium and magnesium withdrawal, such as fenestration of the intercellular space, were clearly visible along the lateral cell borders. By far the most obvious change

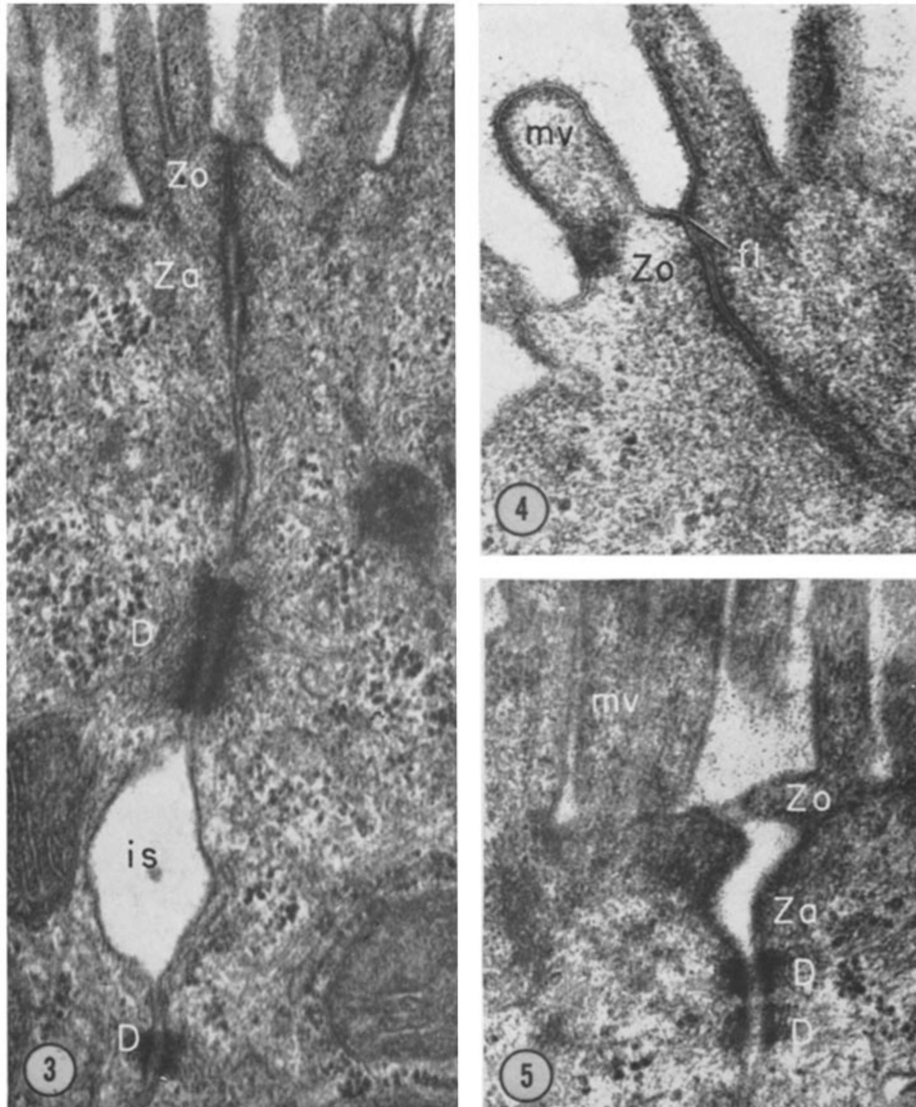

FIGURE 3 Junctional complex between two cells from NaEDTA-treated mucosa. In an apical-basal direction, the zonula occludens, zonula adhaerens and first desmosome all appear perfectly normal. A widening of the intercellular space is clearly visible between the first and second desmosome areas. × 25,800.

FIGURE 4 Tight junction between two epithelial cells from NaEDTA-treated mucosa. The fusion line (*fl*) which represents the fused outer leaflets of the adjoining cell membranes, is clearly visible all along the junction. The short, club-shaped microvilli seen here are typical of the treated preparations, replacing the more filamentous structures observed in control sections. The zonula occludens depicted here is representative of the treated mucosal cells in which disruption of this particular region rarely was noted. × 71,500.

FIGURE 5 Disorganized fine structure within the tight junction between surface epithelial cells of the NaEDTA-treated mucosa. A lateral separation of the walls of the intermediate junction with some disorientation of the desmosomes is also evident. There is also a loss of visible intercellular dense material. × 66,000.

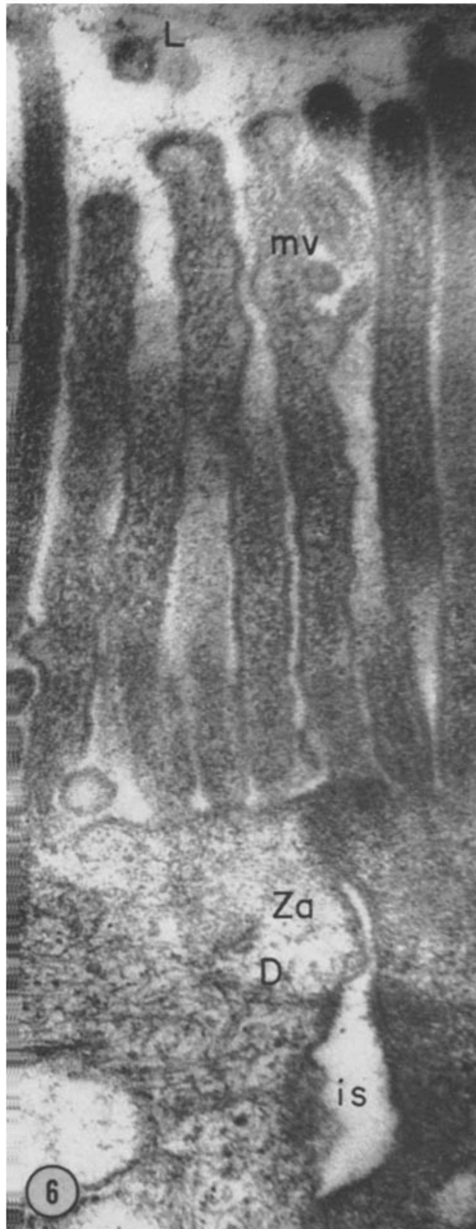

FIGURE 6 The apical portion of a junctional complex between NaEDTA-treated mucosal cells. The more usual club-shaped contour of the microvilli is here replaced by a surface amplification which appears as a series of rounded swellings on the microvillar membrane. This phenomenon was observed in several but not all of the treated preparations. The walls of the zonula adhaerens (*Za*) and the desmosome (*D*) have separated some distance, leading to an enlargement of the intercellular space (*is*). × 83,600.

induced by NaEDTA was a widening of the intercellular space either in the zonula adhaerens region (Fig. 5) or, as appeared more regularly, in the intermediate regions between two desmosome structures (Fig. 3). Distention of the intercellular space in these areas was accompanied by disappearance of the intercellular dense material normally present. Figs. 6 and 7 demonstrate the enlargement of intercellular space which was observed. The extensive nature of this cell-to-cell separation can be seen by referral to Fig. 7 in particular. Unfolding of the interdigitations of the cellular membranes occurred, although in many places close apposition of the individual cell membranes was maintained and the intercellular space was still approximately 200 A in width. This partial separation of cell-to-cell attachment is also visible in Figs. 5 and 6.

Many of the desmosome structures in the NaEDTA-treated tissues showed a marked separation of the dense borders with loss of electron opaque constituent material as in Figs. 4 and 6. The cytoplasmic plaque and associated fibrils were retained although the desmosome itself frequently appeared to be out of register. In many samples of treated tissue a striking fact observed was that many perfectly normal desmosomes were seen in association with fenestration of the interdesmosomal regions of the cell membranes.

No obvious changes were noted in other components of the epithelial cell structure. Although a lateral separation of the membranes of the zonula occludens or tight junction is shown in Fig. 5, it represents the only such alteration observed in this region. More than 200 junctional complex areas from chelated tissue were scrutinized in this study. However, the appearance of the microvilli bordering the apical surface was frequently modified by treatment with NaEDTA as in Fig. 6. The perfectly smooth limiting membrane of these apical projections seen characteristically in control preparations was replaced by a series of rounded swellings over most of the microvillar surface. Conceivably these alterations could be produced by fixation damage, but they never were observed in control tissues and were noted only in treated preparations whose permeability had been altered vastly by chelation depletion. In the same preparations several intracellular electron-transparent vacuoles were encountered and extensive mitochondrial swelling was apparent.

It should be emphasized at this point that the

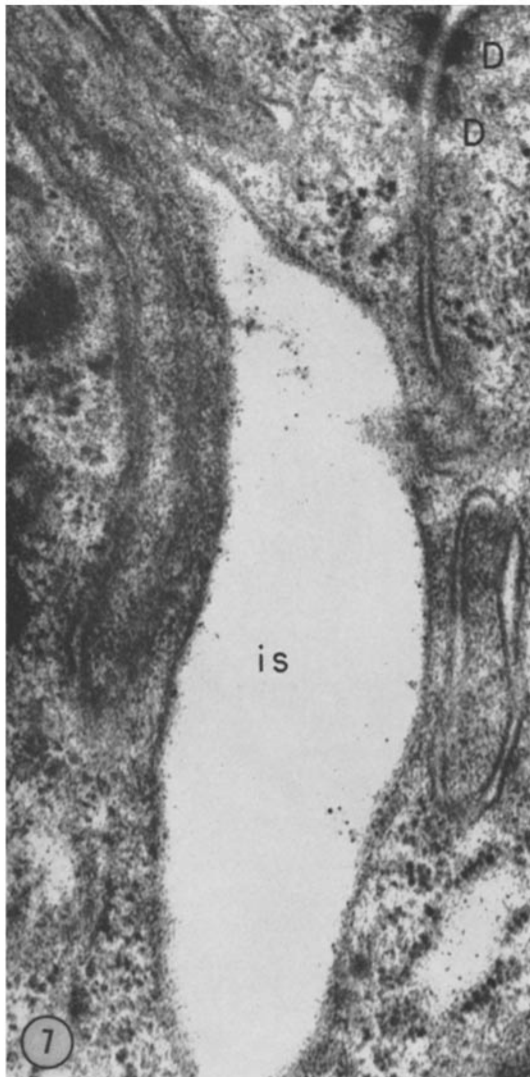

FIGURE 7 Another example of the extensive labyrinthine system of compartments which may be formed from the expanded intercellular spaces (*is*) occurring in mucosal tissue which has undergone chelation depletion. The upper right-hand portion of the micrograph shows two contiguous desmosomes (*D*) with laterally separated walls. × 44,000.

conspicuous alteration in morphological structure outlined above was characteristic of the NaEDTA-treated material compared to control material which had been subjected to similar experimental manipulations in vivo. The examples presented in Figs. 3, 5, and 6 are representative of these alterations. Nonetheless it was equally true that portions of the treated tissues exhibited a completely normal appearance, and that two adjacent junctional complexes could present markedly different morphological appearances varying from normal to severely disorganized.

The Fine Structure of Intestinal Epithelium Exposed to Calcium or Magnesium following a Period of Chelation Depletion

Electron microscope studies of the epithelial tissue structure which had been rinsed with either calcium or magnesium solution in vivo following treatment with NaEDTA showed that the appearance of the cells was similar to that described for the normal cells of the control tissue. In some instances, as in Fig. 8 (calcium-rinsed), the lateral borders of the desmosome still showed a partial estrangement, whereas the lateral cell membranes of the intermediate zone and lower areas appeared normally aligned. In Fig. 9 (magnesium rinsed) the sharp angulations of the lateral border membranes were more prevalent than in control tissue. The microvillar projections were indistinguishable from those of control preparations, and the over-all appearance of depleted intestinal segments replaced by either the calcium or magnesium ion was normal.

DISCUSSION

A previous publication delineated the criteria which justify the use of phenol red absorption as an indicator of passive permeability and established the reversible nature of permeability alterations produced by placing a chelating agent in the lumen of the small intestine. Magnesium and calcium were implicated because of their ability to restore normal permeability after chelation treatment (4). Owing to the small number of these divalent cations involved, analysis of the chelation effluent in the presence of excess NaEDTA was not feasible. Therefore, in order to understand the role of these ions in permeability regulation, it was necessary to develop a quantitative picture of their presence in the tissue under differing permeability conditions.

In experiments involving analysis of the complete intestinal segment, although the permeability alterations attending chelation treatment were prominent, no significant depletion of either ion

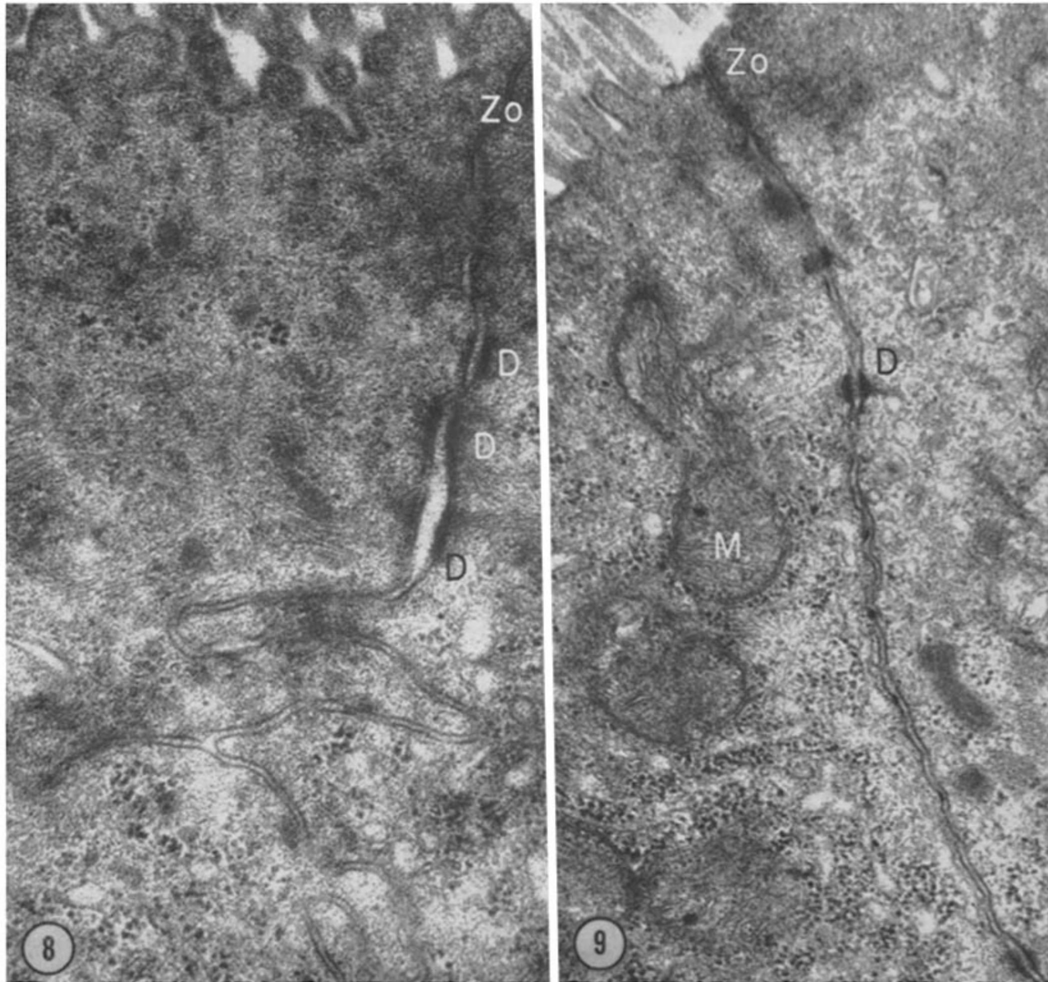

FIGURE 8 Junctional complex between mucosal cells of a loop which was rinsed in isotonic $CaCl_2$ solution after exposure to NaEDTA. In their fine structure the elements of the complex resemble generally those of normal cells, but the side walls of the desmosomes still appear separated and there is a loss of intercellular matrix compared to normal. The particular experiment from which tissue for Fig. 8 was derived showed an increase of phenol red absorption from 1.7% to 10.7% following treatment with NaEDTA. Rinsing with $CaCl_2$ returned the phenol red absorption to 2.6%, while the divalent cation content of the tissue reverted to normal levels. × 42,500.

FIGURE 9 Junctional complex between mucosal cells exposed to NaEDTA and then rinsed with isotonic $MgCl_2$ solution. The intestinal loop used for the preparation of this tissue exhibited an increase in phenol red absorption from 1.5% to 8.7%. Absorption returned to 1.3% with complete restoration of magnesium and partial restoration of calcium following the $MgCl_2$ rinse. × 18,400.

was detected. When the chelation period was followed with an isotonic $CaCl_2$ rinse, a partial restoration of permeability occurred and was accompanied by a significant increase in calcium content. In contrast, rinsing with isotonic $MgCl_2$ after chelation reinstated normal permeability, although the postrinse magnesium content was not significantly greater.

Since the locus of absorptive function in the intestine could be presumed to exist in the mucosal epithelium, a study of the cation content of this layer alone was undertaken. On this basis a rela-

tionship between the content of the two divalent cations in question and the permeability of the membrane emerged.

In the rat, although the concentration of magnesium in plasma is approximately half that of calcium, considerably more magnesium than calcium is present in the complete wall of the small intestine. The partition of these ions in the mucosal epithelial layer approximates 2:1 in favor of magnesium, whereas a ratio of 8:1 is found in the submucosal layers comprised of smooth muscle and connective tissue. These observations on divalent cation content are those for a common laboratory animal not characterized previously. Furthermore, they represent greater precision than values already recorded, which are largely in ruminants (9).

The chelating agent sodium ethylenediaminetetraacetate has a greater affinity for calcium than for magnesium, as revealed by the stability of the chelate complexes. The stability constant (the logarithm of the dissociation constant for the complex) is 10.59 for calcium and 8.69 for magnesium (10). The preferential affinity is reflected in the chelation experiments when mucosa alone was analyzed: the normal calcium content was depleted by 47%, whereas magnesium values fell by only 27%. The alterations in permeability for the second series were no different than those previously discussed. The application of an isotonic calcium solution to the membrane following depletion completely reinstated normal calcium levels in this tissue. Surprisingly, magnesium content in the mucosa of these calcium-rinsed segments also rose significantly and even exceeded values determined under control conditions. A possible interpretation of this finding may involve the time interval between chelation treatment of the mucosal surface and its excision for analysis. The close proximity of magnesium in submucous layers and the known lability of magnesium lend credence to the concept that replacement of mucosal magnesium occurred from deeper layers which in turn could be replenished from the intact blood supply. That the blood supply is not the entire source of replacement can be adduced from experiments in which an isotonic magnesium solution was rinsed through the lumen following chelation treatment. Magnesium was replaced as would be expected but the calcium content, though elevated above the depleted state, remained below control values.

Correlation of Membrane Phenomena with Epithelial Fine Structure

It is worthy of emphasis that in this presentation only tissues with known permeability characteristics as well as known contents of calcium and magnesium were subjected to electron microscopic scrutiny. It is significant that procedures which produced alterations in permeability consistently reduced the content of these divalent cations and brought about characteristic changes in fine structure. Equally significant should be the fact that reversal of these procedures returned both the cation content and the epithelial structure to normal.

In an extensive survey of epithelial tissues, Farquhar and Palade have provided a background and a terminology for the characterization of cellular attachments (8, 11). Tissues from our control experiments appear identical with those published by these authors. The tripartite junctional complex is present and characteristically the zonula occludens consists of fusion of the outer leaflets of the adjacent cell membranes. The zonula adhaerens may be quite straight or tortuous and maculae adhaerentes or desmosomes occur intermittently with generally more than one such in a given plane of sectioning. These desmosomes contain electron-opaque material between their leaflets although it is not always possible to distinguish a median stratum.

According to contemporary viewpoint, the zonula occludens, which girdles the entire cell, provides a barrier restricting intercellular diffusion. It is considered by some that primary changes in membrane potential occur at this level (12). By contrast, the desmosomes and related structures are believed to be involved mainly in cellular adhesion; however, the precise nature of the functional aspects of these membrane specializations has not been explored fully.

Marked changes in appearance of these elements during NaEDTA treatment has been noted previously. Sedar and Forte (12) described enlargements of the intercellular space in the zonula adhaerens, with some slight opening of the membranes in the zonula occludens. Their experiments on the gastric mucosae of *Rana pipiens* included the monitoring of changes in transmucosal potential difference when NaEDTA was applied to the membrane in vitro. These investigators suggest that the primary locus of the chelation depletion

phenomenon is in the junctional complex region, where they envision the divalent cation maintaining structural and physiological integrity by electrostatic binding from cell-to-cell surface. It follows from this interpretation that increases in permeability produced by calcium and magnesium removal result from the creation of extracellular channels between the epithelial cells. Sedar and Forte, however, found that it was difficult to detect consistent changes in the zonula occludens although in some instances disorganization within the tight junction was seen.

In our in vivo studies changes induced by NaEDTA in the zonula occludens were virtually absent; we believe the single instance noted in Fig. 5 to represent a permanent distortion of the tissue as opposed to the reversible phenomena which are the subject of this paper. In addition, it was noted that the areas of intercellular distention became more prominent at sites considerably more basal than the zonula occludens (see Fig. 3). In general the changes induced by NaEDTA in the zonula adhaerens were more striking than those observed in the desmosome area. Frequently several intact complexes could be seen in tissue known to have exhibited a reasonably large increase in permeability to phenol red. It is tempting to suggest that functional modification of this absorptive epithelium may be related to the number of intercellular sites affected rather than the degree of distention produced at each particular zone of cell attachment. In a study of calcium withdrawal on the structure and function of toad bladder in vitro, Hays et al. observed complete unfolding of adjacent plasma membranes with considerable sloughing of the epithelial cells (13). The importance of calcium in cell adhesion mechanisms has long been recognized (14). Investigations range from the development of adhesion sites in amphibian epidermis (15) to the similar processes in slime molds (16). However, the parallel efficacy of magnesium in promoting the reinstatement of normal permeability and restoration of morphological identity allows two possible interpretations regarding the physiological specificity of these two divalent cations. Either calcium exerts a nonspecific role in cell adhesiveness (17) or the membrane phenomena under discussion are a reflection of the withdrawal of magnesium as a specific enzymatic cofactor in the maintenance of normal cell membrane permeability.

The possible mode of penetration of phenol red as a test molecule in these experiments is worthy of consideration. The phenol red molecule is an irregular tetrahedron whose greatest dimension in any single plane approximates 15 A. The substance is ionized completely at physiological pH and virtually nonlipid soluble. The extra hydration shell may be assumed to contribute at least an additional 5 A to the over-all size, which means that a roughly spherical particle of 20 A diameter is presented to the membrane. The three most likely paths of entry are pinocytic uptake, passage through intercellular channels, and sieving through aqueous pores in the plasma membrane of the luminal surface. Pinocytic vesicles were seen only rarely in tissue from control segments; no increase in vesiculation could be observed in chelated tissue. The current view of the zonula occludens, as an area where fusion of the outermost surfaces of adjacent membranes occurs, is not consistent with passage of a 20 A particle. Unfortunately the extensive circumference of this zone does not make it easy to monitor all portions of it. Neither the present work nor that of Sedar and Forte resolves the question of possible modifications of the zonula occludens to permit free passage of large ions by this route. An accepted estimate of intestinal epithelial equivalent pore radius in the rat is 4.0 A (18). The minimal absorption of a 20 A ion is in accord with the general concept of restriction by pore size. The fivefold increase in phenol red absorption observed with cation depletion could be attributed to an increase in pore size but would require an increase on the order of three times the accepted equivalent pore radius for even single-file passage of this particular ion. Such restrictive movement would necessitate an accompanying unidirectional transfer of fluid; however, fluid movements across tissue subjected to chelation depletion were not evaluated. That calcium and magnesium may be responsible for the effective patency of aqueous pores in the plasma membrane has been suggested by Whittembury, et al. (19). It, therefore, seems reasonable to assign the primary locus of the NaEDTA effect to those membranes of the epithelial cells which border on the lumen. Preliminary experiments in a canine intestinal mucosal preparation in vitro lend credence to this hypothesis. As a result of tissue exposure to NaEDTA, a marked increase in intracellular sodium and a concomitant decrease in intracellular potassium occur (20).

Lack of knowledge as to the intimate cellular

events accompanying fluid transport across epithelial membranes has impeded the development of a coherent hypothesis to explain both intestinal absorption and secretion of water. Earlier in vivo experiments from this laboratory revealed NaEDTA-induced, marked increases in net water movement from blood to gut lumen in canine intestinal loops in situ (21). Water is now believed to be transported across biological membranes in response to the active transport of solutes which create a micro-climate of osmolarity difference. The relation between ion transport and solute movement has been demonstrated under certain circumstances (22), but considerable controversy exists in the literature (23). This has been due in part to reliance on data derived from measurement of bulk compartment fluid transfer. Very recently two groups of workers have proposed hypotheses for the route of epithelial water transport which intimately involve the intercellular channels between epithelial cells (24, 25). These lateral border spaces are viewed as a structural device which prevents actively transported solute from diffusing away before sufficient water has followed to achieve osmotic equilibrium. Sodium pumping activity has been indicated by Farquhar and Palade to be localized along the lateral borders of the epithelial cells (26). Diamond and Tormey provide evidence of enlargement of the intercellular channels under conditions of graded water transport activity in the gall bladder (25). Gross pathological disturbances in fluid transport which occur in ulcerative colitis have been shown to be accompanied by alterations in ultrastructure very similar to those described in this study (27). Dilation of these spaces would therefore appear to be related to fluid transport in general.

In summary, the most attractive explanation of chelation depletion effects as we have described them is as follows: removal of calcium and magnesium causes an increase in equivalent pore size sufficient to allow a significant inward movement of sodium into the cell; in turn, considerably more sodium is pumped across the lateral cell surface into the intercellular channels creating an osmotic gradient by which water moves to dilate these lateral border spaces. Not only does this interpretation provide a rationale for the action of NaEDTA, but it offers a working hypothesis which readily may be evaluated in subsequent studies relating to divalent cations and water movement across epithelial membranes.

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