

# LOCALIZATION OF TRANSPORT ADENOSINE TRIPHOSPHATASE IN RAT CORNEA

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## ABSTRACT

Increasingly, physiological and biochemical data on the mammalian cornea have emphasized the importance of the endothelial rather than the epithelial layer in regulating the state of hydration of the cornea, via a transport ATPase. This view is supported by the observations reported here. With the Ernst method, the first cytochemical procedure to be readily responsive to ions and ouabain, the major sites of enzyme reaction product are the intercellular spaces of the endothelial layer. This localization was not found by us with the so-called ATPase method of Wachstein and Meisel.

## INTRODUCTION

It has been suggested, from biochemical and physiological findings (1-3), that among the fluid pump mechanisms for removing the water which constantly enters the cornea, at least one is a cation-dependent ATPase (transport ATPase). This  $\text{Na}^+$ - $\text{K}^+$ -stimulated, ouabain-inhibited ATPase is therefore considered to play a role in regulating the hydration and thickness of the cornea. Both outer (epithelial) and inner (endothelial) layers of the cornea have been considered sites of transport ATPase, and there is still disagreement concerning their relative importance. The cytochemical observations reported are consistent with the assumption that the endothelial layer is the site of greater activity and also that some transport ATPase is also present in the epithelial layer.

It is generally agreed that interpretation of localizations obtained with the Gomori-type lead procedure of Wachstein and Meisel (4) is difficult, particularly with regard to the transport ATPase. In 1972 Ernst (5, 6) introduced a major departure from this procedure: lead was replaced by strontium as the trapping agent for the enzymically

liberated orthophosphate ions, and *p*-nitrophenyl phosphate (NPP) was substituted for ATP. This procedure reveals the second of the two steps postulated for the action of transport ATPase (reviewed by Ernst [5]). The first step is a  $\text{Mg}^{2+}$ - $\text{Na}^+$ -dependent phosphorylation of the enzyme by ATP. The second step is a  $\text{Mg}^{2+}$ - $\text{K}^+$ -dependent dephosphorylation of the phosphorylated intermediate. The enzyme catalyzing this dephosphorylation appears to be sensitive to ouabain and to fixation. It has a fairly wide substrate specificity that includes NPP.

Our observations with *in situ* cytochemistry utilizing the Ernst procedure lend support to the conclusions derived from the physiological data which increasingly emphasize the endothelium's importance in regulating corneal hydration via a transport ATPase. With the Wachstein-Meisel cytochemical method, which does not respond either to  $\text{Na}^+$  and  $\text{K}^+$  or to ouabain, it is not the endothelium but rather the epithelium which shows activity (but cf. reference 7).

## MATERIALS AND METHODS

Corneas of adult albino rats (160–200 g) were excised under ether or pentobarbital anesthesia and were immediately fixed in either: (a) cold 3% formaldehyde (freshly prepared from paraformaldehyde) –0.1 M cacodylate buffer, pH 7.2 (6) for 20 min, 60 min, and overnight, or (b) cold 3% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) –0.1 M cacodylate buffer, pH 7.2 (8) for 20 min, 60 min, and 3 h.

After washing in cold 0.1 M cacodylate buffer, pH 7.2, with 5% sucrose, the corneas were processed in the following manner for light and electron microscopy.

### Light Microscopy

Frozen sections, 10–20- $\mu\text{m}$  thick, were cut on a Sartorius freezing microtome and incubated in the  $\text{Sr}^{2+}\text{-K}^+\text{-NPP}$  medium of Ernst (6), henceforth referred to as the NPPase procedure,<sup>1</sup> or in the  $\text{Pb}^{2+}\text{-ATP}$  medium of Wachstein and Meisel (4), henceforth referred to as the “ATPase” procedure.<sup>2</sup> Incubations were done at room temperature and 37°C, for 15, 20, 30, and 60 min. Visualization of reaction product by conversion to lead sulfide by ammonium sulfide, in the “ATPase” procedure, was done immediately after rinsing sections (in water) after incubation. In the NPPase method the strontium phosphate was first converted to lead phosphate by treating sections twice, for 5 min each, in a 2% lead nitrate solution (6). The sections were rinsed in 0.1 M Tris-HCl buffer, pH 7.2, and then visualized.

### Electron Microscopy

Nonfrozen sections (30–50  $\mu\text{m}$ , cut on a Smith-Farquhar sectioner [Ivan Sorvall, Inc., Newton, Conn.] [9]) or blocks (50–100  $\mu\text{m}$ , hand-cut with a razor blade under a dissecting microscope) were incubated for 30 and 45 min at room temperature in the NPPase and “ATPase” media with added 5% sucrose. After incubation in the NPPase medium the tissues were washed in 0.1 M Tris-HCl buffer, pH 9.0, rinsed in 2%  $\text{Pb}(\text{NO}_3)_2$  (twice, 5 min each), washed in 5% sucrose, and postfixed for 60 min in

1%  $\text{OsO}_4$ -0.1 M cacodylate buffer, pH 7.5, with 5% sucrose. Some of the tissues were stained en bloc with uranyl acetate, pH 5.0, for 1 h at room temperature (10, 11); all were dehydrated in an ascending alcohol series and propylene oxide. Tissues were flat embedded in Epon 812 (12). Thin sections were cut with a diamond knife using an LKB (LKB Instruments, Rockville, Md.) or a Sorvall MT2 microtome. Thin sections were observed, either stained for 10 min in lead citrate (13) or unstained, in a Philips EM 300, using a 30- $\mu\text{m}$  objective lens aperture.

### Controls

The possibility that nonspecific enzyme activity or  $\text{Sr}^{2+}$ - or  $\text{Pb}^{2+}$ -induced substrate hydrolysis (of NPP and ATP, respectively) accounted for or contributed to the reaction product seen in sections incubated in the full NPPase or “ATPase” media was tested by: (a) omitting the substrates, (b) substituting equimolar amounts of Na- $\beta$ -glycerophosphate for the substrates, and (c) substituting ATP for the NPP in the NPPase procedure. The light microscope findings were verified by electron microscopy. Another control, at the light microscope level only, was the incubation in the full NPPase or “ATPase” media of sections heated for 30 min at 65°C before incubation.

The need for  $\text{K}^+$  in the NPPase procedure was tested by substituting choline chloride for KCl. This was studied by both light and electron microscopy.

The effect of ouabain addition to the NPPase and “ATPase” media was also tested by light and electron microscopy. For light microscopy the ouabain concentrations ranged from  $1 \times 10^{-6}$  M to  $5 \times 10^{-3}$  M; for electron microscopy the concentration was  $1 \times 10^{-3}$  M. In some cases sections were presoaked for 30 min at 4°C before incubation.

Finally, magnesium ions were omitted from both NPPase and “ATPase” media. These sections were studied by light microscopy only.

The inhibiting effects of concentrations of ouabain ranging from  $1 \times 10^{-6}$  M to  $1 \times 10^{-3}$  M on NPPase activity were studied on frozen sections fixed for 20 min in cold 3% formaldehyde-0.1 M cacodylate buffer. To obtain sufficient NPP release, the sections were 40- $\mu\text{m}$  thick. 10 sections were incubated in 3 ml of NPPase medium, with continuous shaking, for 30 min at room temperature. The medium was filtered and the amount of nitrophenol released to the medium was read with a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 400 nm, against the complete medium as a blank, according to Ernst (5).

<sup>1</sup> The ingredients of the medium, in final concentrations, are: NPP,  $5 \times 10^{-3}$  M;  $1 \times 10^{-1}$  M Tris-HCl buffer, pH 9;  $1 \times 10^{-2}$  M magnesium chloride;  $2 \times 10^{-2}$  M strontium chloride; and  $1 \times 10^{-2}$  M potassium chloride.

<sup>2</sup> The ingredients of the medium, in final concentrations, are:  $8.3 \times 10^{-4}$  M ATP;  $8 \times 10^{-2}$  M Tris-maleate buffer, pH 7.2;  $3.6 \times 10^{-3}$  M lead nitrate; and  $1 \times 10^{-2}$  M magnesium chloride.

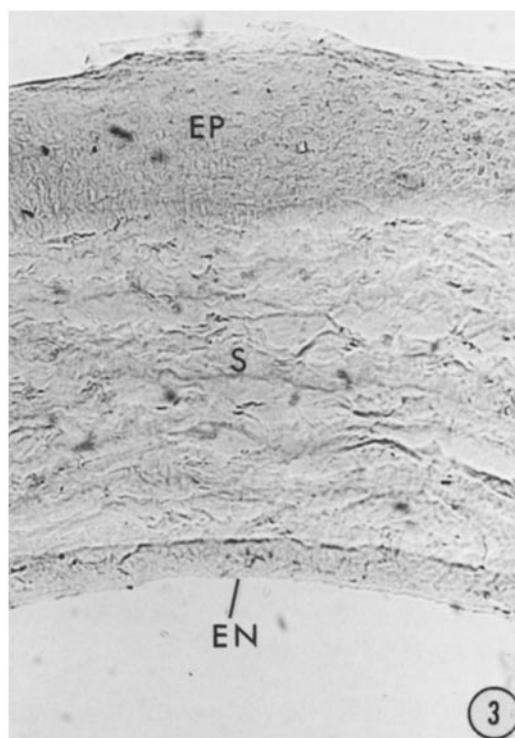
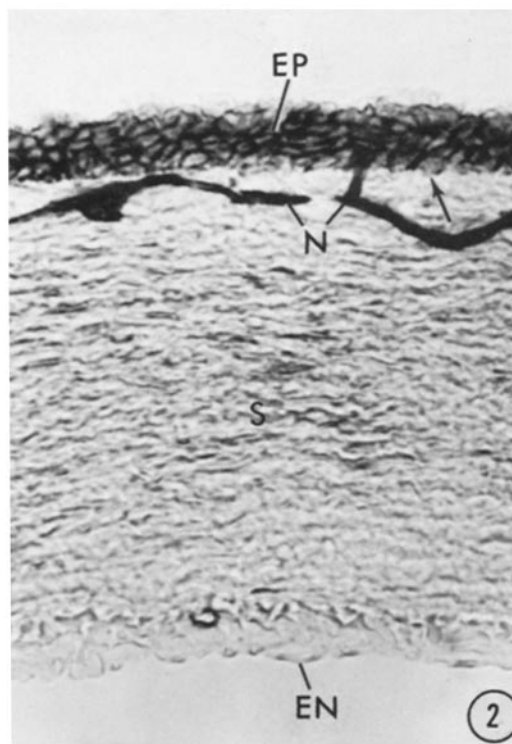


FIGURE 1 Rat cornea. Fixation: 20 min, 3% formaldehyde. Frozen ca. 20- $\mu$ m section. Incubation, NPPase, 40 min room temperature. Reaction product is present in the endothelial cell layer (EN). This region was chosen because the endothelium has folded so that it is viewed face on. The arrows indicate an area where the cells appear separate and the staining at the cell boundaries is more evident. Reaction product is not seen in the stroma (S) or epithelium (EP).  $\times 230$ .

FIGURE 2 Rat cornea. Fixation: 20 min, 3% formaldehyde. Frozen ca. 20- $\mu$ m section. Incubation, "ATPase", 20 min room temperature. No reaction product is present in the endothelium (EN), or stroma (S), but the epithelial layer (EP) is highly reactive. The localization of reaction product at the cell borders is evident. Note the absence of reaction product from the cell membranes at both the basal (arrow) and surface borders. Note also the intense staining of the corneal nerves (N) (cf. reference 37).  $\times 230$ .

FIGURE 3 Rat cornea. Fixation: 20 min, 3% formaldehyde. Frozen ca. 20- $\mu$ m section. Incubation, NPPase, in the presence of  $1 \times 10^{-3}$  M ouabain, 40 min room temperature. No reaction product is seen in endothelium (EN), stroma (S), or epithelium (EP); cf. Fig. 1. To make the section visible in the photograph the iris diaphragm was shut down to the point that cell outlines and bits of debris and dust become evident.  $\times 230$ .

## RESULTS

### Light Microscopy

Dramatically different localizations are obtained by the NPPase and "ATPase" procedures. With the NPPase method the endothelium is deeply stained (Fig. 1). The cell borders are clearly outlined by reaction product. The epithelial layer appears unstained; i.e., it lacks reaction product and is spoken of as "negative" (Fig. 1). However, when 1- $\mu$ m Epon sections of incubated material are examined by phase-contrast microscopy, faint deposits of reaction product are seen at the lateral borders of the cells in the basal layer of the epithelium. With the "ATPase" procedure the endothelium is negative but the epithelial layer cell boundaries are strongly stained (Fig. 2). This procedure visualizes the corneal nerves very well (Fig. 2). The surfaces of some stromal cells also stain lightly. Staining is absent from the negative surfaces, even when they are incubated at 37°C for the longest time tried. Addition of  $1 \times 10^{-3}$  M ouabain to the NPPase medium completely inhibits the staining of the endothelial layer (Fig. 3). A lower concentration ( $1 \times 10^{-4}$  M) is required for such inhibition if sections were presoaked in the  $1 \times 10^{-4}$  M ouabain before incubation.

Spectrophotometric assay of the complete NPPase medium in which 40- $\mu$ m sections are incubated shows the following inhibitions of NPP release by ouabain:  $1 \times 10^{-6}$  M, none;  $1 \times 10^{-5}$

M, 12%;  $1 \times 10^{-4}$  M, 27%;  $1 \times 10^{-3}$  M, 68%;  $5 \times 10^{-3}$  M, 70%.

The staining of the epithelial layer in the "ATPase" procedure appears to be unaffected by the highest ouabain concentration tested,  $5 \times 10^{-3}$  M.

Replacement of  $K^+$  by choline ions in the NPPase procedure results in loss of staining of the endothelial layer. Omission of  $Mg^{++}$  ions greatly diminishes the staining with both procedures.

Negative results are obtained, in both NPPase and "ATPase" procedures, with preheated (30 min, 65°C) tissues, or when substrate is either omitted from the medium or replaced by  $\beta$ -glycerophosphate. Substitution of NPP by ATP in the NPPase medium gives negative results in the endothelium. In the epithelium the findings become similar to those with the "ATPase" method. Tissues fixed overnight in 3% formaldehyde or for 20 min to 3 h in 3% glutaraldehyde are negative in the NPPase procedure; they are slightly positive after 60 min in 3% formaldehyde. With the "ATPase" method positive results are given irrespective of the type or time of fixation employed.

### Electron Microscopy

**NPPase:** Reaction product in the endothelium is most abundant when the cornea is fixed in 3% formaldehyde for 20 min. Because of the brief fixation the preservation of the cytoplasmic structure is inadequate. However, the plasma mem-

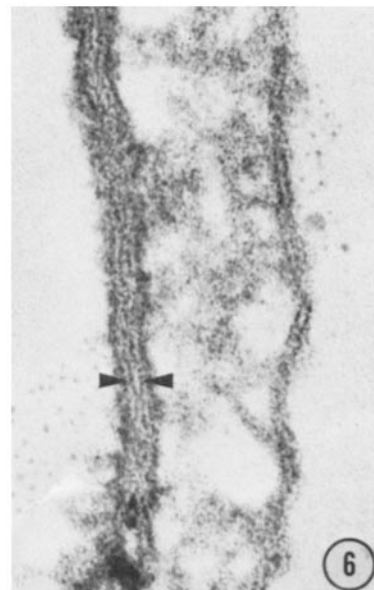
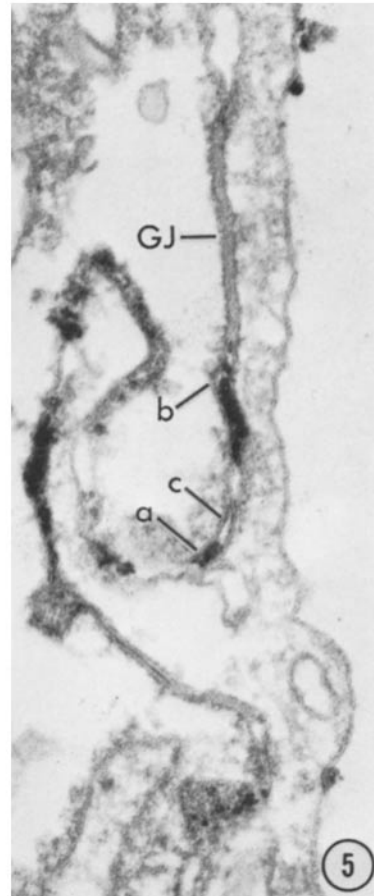
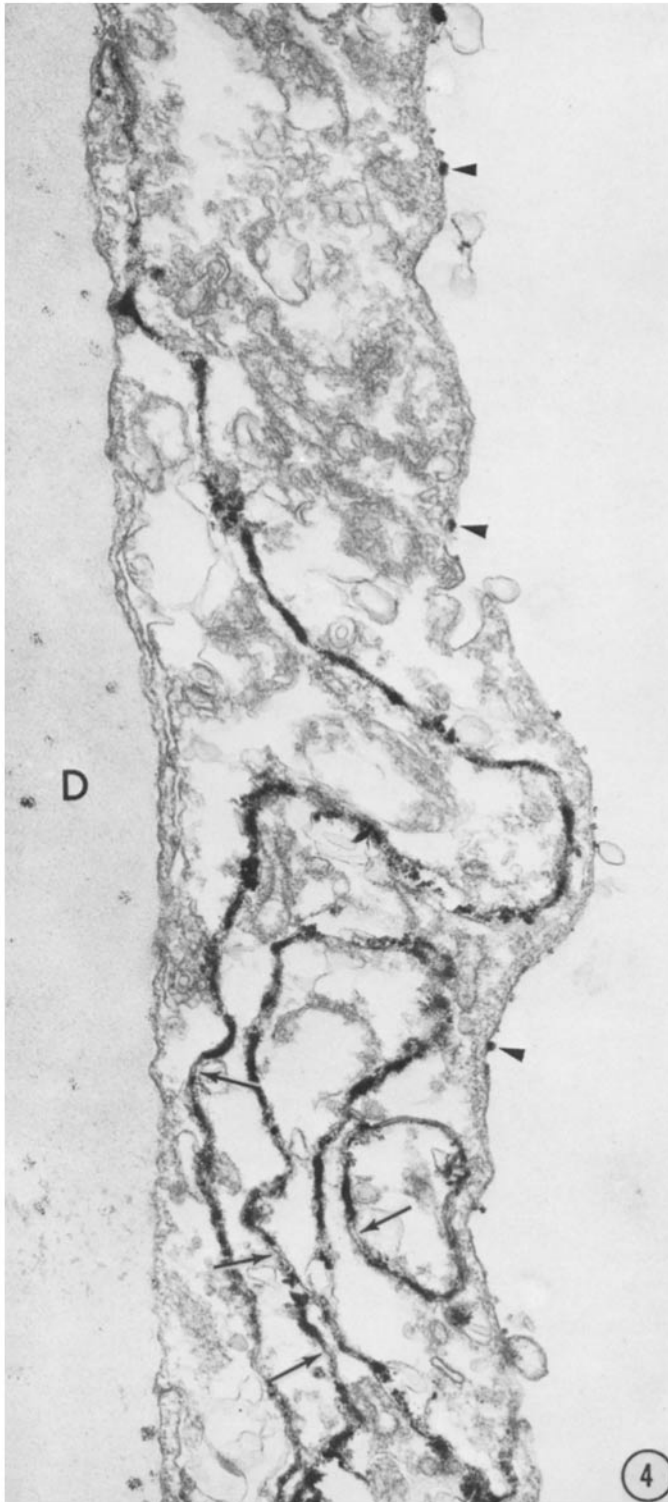
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FIGURES 4-6 Rat cornea. Fixation 20 min, 3% formaldehyde. Ca. 30- $\mu$ m nonfrozen section. Incubation. NPPase, 45 min room temperature. A portion of the endothelium and underlying Descemet's membrane.

FIGURE 4 In most areas dense deposits of reaction product obscure the plasma membranes. However, in regions indicated by arrows the plasma membranes may be seen. Arrowheads indicate some small areas with reaction product on the outer surface of the endothelium. Whether these are artifacts or not is unknown. Note, however, that such deposits are not present on the surface adjacent to Descemet's membrane (D).  $\times 30,000$ .

FIGURE 5 A region that includes a gap junction (GJ). The junction is devoid of reaction product. Below the junction the apposed plasma membranes are seen. Their relations to the deposit of reaction product are interpreted in the following manner: region *a* shows reaction product in the intercellular space at the outer surfaces of the membranes; in area *b* a space is present between one of the plasma membranes and the reaction product, with the other plasma membrane oriented so that reaction product covers it; in area *c* most of the reaction product is missing, and here the tripartite nature of the two plasma membranes is most evident.  $\times 101,000$ .

FIGURE 6 A higher magnification of the gap junction. The typical seven-layered aspect of the nexus with its gap of about 30 Å (between arrowheads) is clearly visible.  $\times 262,000$ .



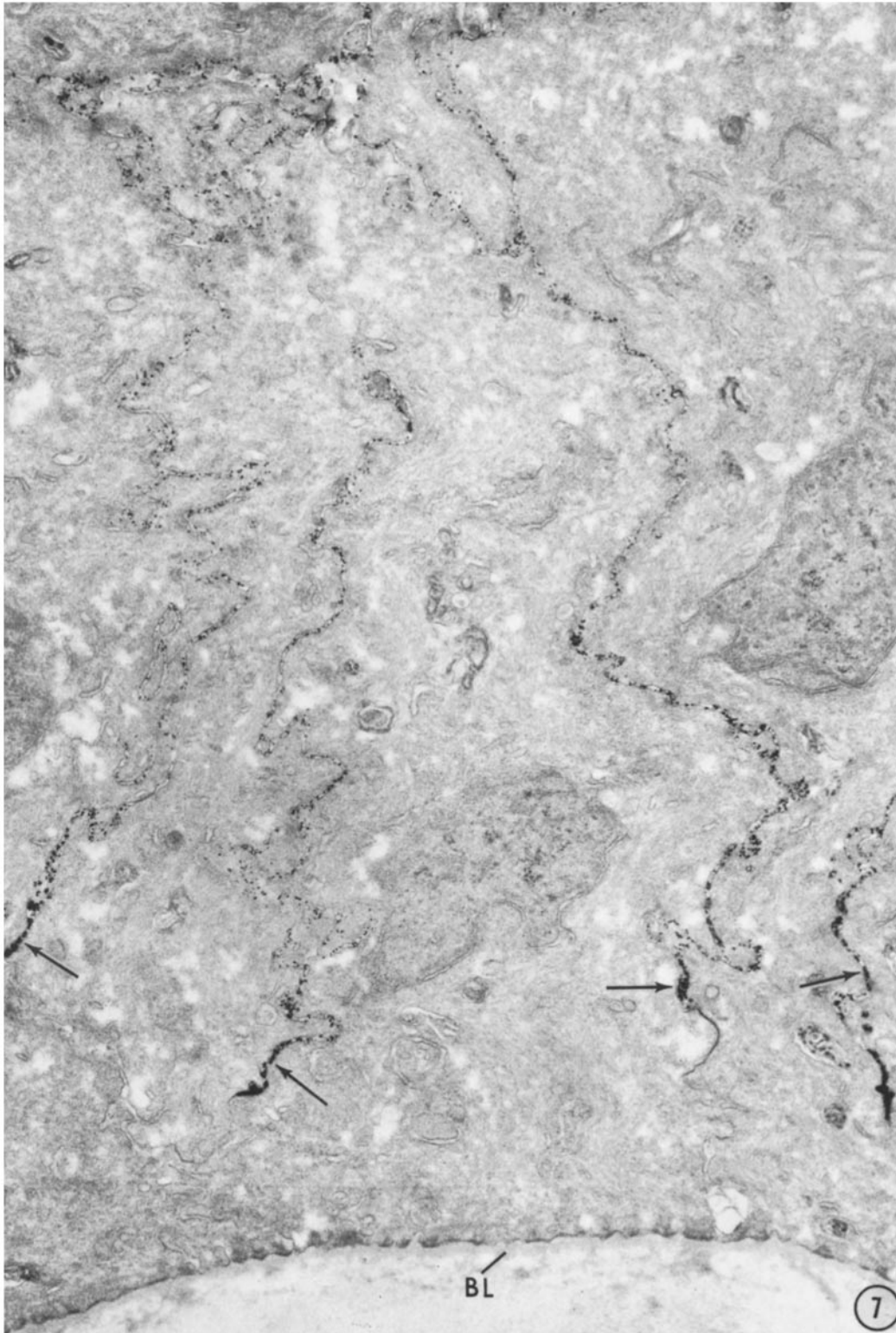


FIGURE 7 Rat cornea. Fixation: 20 min, 3% formaldehyde. Ca. 30- $\mu$ m nonfrozen section. Incubation: NPPase, 45 min room temperature at 37°C. The basal layer of the epithelium is seen. In the intercellular spaces at its upper surface reaction product is scant. This is also true of the lateral surfaces except in small regions, indicated by arrows; cf. Fig. 8. No reaction product is present in the upper layers of the epithelium. Similarly, none is present at the cell base including its contact with the basal lamina (*BL*).  $\times 16,000$ .

branes are well preserved. Reaction product generally fills the intercellular space. In places, reaction product is missing, thus permitting visualization of the two plasma membranes bordering the intercellular space (arrows, Fig. 4), and at higher magnification the tripartite nature of the plasma membranes is evident (Fig. 5). The absence of reaction product may reflect absence of enzyme activity in these areas. However, it is also possible that during sectioning, or at other steps in processing, portions of reaction product are lost. When reaction product fills the intercellular space it generally obscures the plasma membranes (Fig. 4). Reaction product is never seen inside the gap junctions (Fig. 6). The basal portions of endothelial cell membranes, adjacent to Descemet's membrane, are consistently devoid of reaction product.

Electron microscopy reveals some reaction product in the epithelial layer (Fig. 7) although this is insufficient to be seen by light microscope examination of frozen sections (Fig. 1). Reaction product is consistently seen in the intercellular spaces at the lateral borders of the basal and parabasal cell layers (Fig. 7). None is seen at the base of the cells, adjacent to Bowman's membrane, and only scanty deposits are seen in the intercellular spaces between basal and intermediate cell layers.

"ATPase": Irrespective of the fixative employed, the endothelial layer shows no reaction product when examined (not illustrated); i.e., it is negative by electron microscopy as it is by light microscopy (Fig. 2).

The epithelial layer shows dense deposits of reaction product in the intercellular spaces between adjacent cells in the basal and intermediate cell layers (Fig. 8). There is no reaction product at the cell base, adjacent to Bowman's membrane (Fig. 2). No reaction product is present in the most superficial cell layers, including the desquamating cells (Fig. 8). These results are in agreement with those of Kaye and Tice (7).

#### *Controls, Electron Microscopy*

NPPase: The abundant reaction product at the endothelial cell membranes, and also the scant reaction product at the lateral aspects of the epithelial basal and parabasal cell layers, do not form when substrate is omitted from the incubation medium or when the NPP is replaced by Na- $\beta$ -glycerophosphate. Incubation in the NPPase medium, with the NPP replaced by ATP, yields

abundant reaction in the epithelium as in Fig. 8; i.e., the results are like those seen with the "ATPase" procedure. Reaction product is not seen in the endothelial layer. When potassium chloride is replaced by choline chloride or when  $1 \times 10^{-3}$  M ouabain is added to the medium, reaction product is not seen in either endothelium or epithelium.

"ATPase": Omission of substrate from the medium or replacement of ATP by Na- $\beta$ -glycerophosphate yields negative results. However, addition of  $1 \times 10^{-3}$  M ouabain to the "ATPase" medium changes neither the distribution nor the apparent amount of reaction product seen in its absence.

#### DISCUSSION

Dickstein and Maurice (14) have reported elegant experiments demonstrating that the endothelial layer of the rabbit cornea possesses a fluid pump requiring a sodium-dependent, ouabain-inhibited ATPase. This pumping mechanism is capable of removing from the stroma, within 5 min, a volume of fluid equal to the total endothelial volume. Maurice (2) considers that his observations do not support the hypothesis that corneal hydration is regulated by the sodium pump in the epithelial layer. This correlates with the studies of Rogers (15) which showed high levels of Na<sup>+</sup>-K<sup>+</sup>-stimulated, ouabain-inhibited ATPase to be present in the endothelial layers of bovine and feline corneas.

Fischbarg (16) has recently demonstrated a difference of potential across the endothelium. This difference is abolished by  $5 \times 10^{-5}$  M ouabain. A variety of agents which affect the potential have parallel effects upon transendothelial fluid transport. Fischbarg and Lim (17) found that ouabain does not change the endothelium impedance locus; they proposed a model to account for their findings on impedance locus and values of electrical resistance and capacitance across the endothelial layer. They consider the effect of ouabain on the potential difference to be exerted directly on an electrogenic pump.

Although the dominance of the endothelial layer in regulating corneal hydration and thickness has thus gained strong support in recent years (see Rogers [15] and Donn [18] for earlier references), the role of the epithelial layer is less well understood. In a series of publications Green and co-workers demonstrated the presence of a sodium pump in the epithelial layer, and an electric poten-

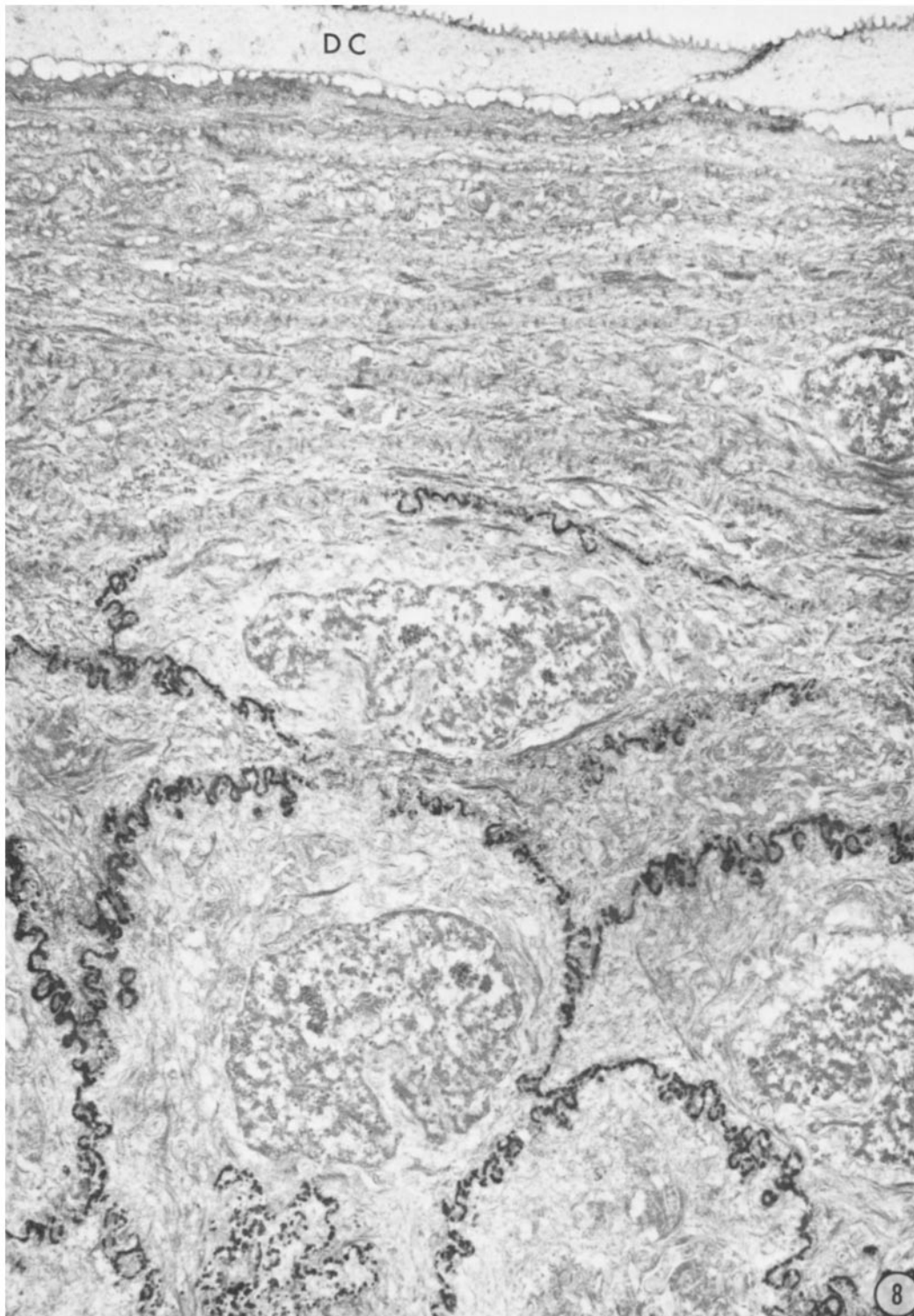


FIGURE 8 Rat cornea. Fixation: 20 min, 3% formaldehyde. Ca. 30- $\mu$ m nonfrozen section. Incubation: "ATPase," 30 min, room temperature. Abundant reaction product (compared to the scant amount in Fig. 7) is seen in the intercellular spaces between cells of the intermediate layers of the epithelium. The basal layer is also reactive except at the surface in contact with the basement lamina (see Fig. 2). Note the absence of reaction product at the level of the superficial and desquamating flat cells (*DC*).  $\times 7,000$ .



tial, negative on the tear side, which is correlated with corneal thickness (3, 19). In 1970 Green (20) reported that ouabain affects the short circuit current of both inner and outer layers of the cornea. He concluded that the data "suggest that the epithelium is the prime target of the ouabain." Rogers (15), while finding a much higher level of transport ATPase in the endothelial layer, was also able to measure a transport ATPase in the epithelial layer. However, the ratio of this activity to the  $Mg^{2+}$ -activated ATPase was far lower in the epithelial layer than in the endothelium. Riley (21) has recently reported results which, while confirming that the epithelial layer acts as a semi-permeable membrane, "provide no support for the postulate that the control of corneal thickness is dependent upon active transport of sodium ions by the epithelium from the tear film to the stroma." It is concluded that the primary role of the epithelium in regard to corneal hydration is as a barrier preventing access of the tear fluid to the stroma." Klyce et al. (22) have recently described a chloride pump, activated by cyclic AMP and driving fluid from the cornea toward the tears, which could be an additional mechanism of corneal dehydration.

Our observations constitute additional evidence for the primacy of the endothelium in the control of corneal hydration via a sodium pump. Heavy amounts of reaction product result from a  $K^{+}$ -dependent, ouabain-sensitive enzyme, presumably ATPase but hydrolyzing NPP, in the endothelium, but only a small amount of such reaction product is present in the epithelium. The latter is insufficient to be visible by light microscopy. By electron microscopy the reaction product is restricted to the lateral borders of basal and parabasal cells.

At the same time our observations strengthen Ernst's view that the NPPase procedure demonstrates the sites of transport ATPase. The NPPase activity does not survive 20 min of glutaraldehyde fixation or overnight fixation in formaldehyde; even 60 min of fixation in formaldehyde markedly reduces the activity. NPPase activity is lost if  $K^{+}$  ions are omitted from the medium or if ouabain is added to it. Although ouabain inhibits its activity, the ouabain levels required are 10–100 times higher than those generally needed for transport ATPase inhibition in biochemical or physiological studies. Ernst (5) suggests that the high concentration required in the cytochemical procedure,  $1 \times 10^{-8}$

and higher, results from the inhibition of  $K^{+}$ -stimulated NPPase by both the  $Sr^{2+}$  ions of the medium and the *p*-nitrophenol liberated into the medium during incubation. In addition, in the avian salt gland studied by Ernst about 20% of the NPPase activity is not  $K^{+}$  dependent. This may be due to a different enzyme but this remains to be established. Our spectrophotometric assay of the nitrophenyl released into the medium demonstrates that some inhibition of corneal NPPase may be obtained with frozen sections at ouabain concentrations as low as  $1 \times 10^{-6}$  M.

The results we have obtained with the Wachstein and Meisel "ATPase" procedure are strikingly different from those of the Ernst NPPase procedure. The endothelial cell membranes are negative and all the epithelial cell membranes, except those at the upper and lower surfaces, are positive. The activity survives the longest fixation times tested, 3 h in glutaraldehyde and overnight in formaldehyde. It is quite possible that the Wachstein-Meisel procedure demonstrates the  $Mg^{2+}$ -ATPase shown by Rogers (15) to be present in the epithelial layer. That the activity is enzymatic and not nonspecific is indicated by the absence of reaction product when the substrate (ATP) is omitted or replaced by  $\beta$ -glycerophosphate, or when heated tissue is incubated in complete medium. Uncertainties regarding the nature of the  $Mg^{2+}$ -stimulated enzyme or enzymes are discussed by numerous authors; see, e.g., Farquhar and Palade (23), Tormey (24), and Marchesi and Palade (25).

Another uncertainty requires discussion. This is the "sidedness" of NPPase sites in the plasma membrane. In the secretory cells of the avian salt gland Ernst (6) found the reaction product on the intracellular side of the membrane. In the cornea we find the product on the extracellular surface (Fig. 5). Comparable differences have been found with modified "ATPase" media. For example, in amphibian epidermis it was found on the extracellular surface (23) while in erythrocyte ghosts it was on the cytoplasmic surface (25).

These findings may reflect differences in enzyme sites among different cell types. On the other hand, this conclusion should probably best be regarded as unsettled. Cornelisse and van Duijn (26) conclude their recent review of theoretical aspects of the local formation of crystalline precipitates in heavy metal cytochemistry with the view that such formation "is essentially a very delicate

process that can easily be disturbed by relatively small changes in the prevailing conditions." Although much work has been done on the nature of possible precipitates when orthophosphate is liberated from ATP under various conditions of incubations (e.g., references 27-29) and preferential adsorption of phosphate complexes in cytochemical controls of different kinds has been demonstrated (e.g., references 30-32), little is known concerning the possible effects of "seeding sites" in the actual conditions which prevail when tissue sections are incubated for "ATPase" activity (cf. reference 33) or for NPPase activity. Decisive seeding sites may be located on the outer surface of the plasma membrane in some cell types and on the inner surface in other cell types.

Previous cytochemical studies of cornea, two at the light microscope level (34, 35) and one at the level of electron microscopy (7), have used the Wachstein and Meisel "ATPase" procedure. Our results are in agreement with theirs concerning the epithelial layer. However, in one of the light microscope studies (35) and in the electron microscope study, some reaction product was also found in the endothelial layer. It is difficult for us to explain these findings. They probably reflect technical differences rather than the different species used. An inhibitory effect of ouabain was either not found (35) or not studied (7, 34); nor was dependence of the cytochemical reaction upon  $\text{Na}^+$  and  $\text{K}^+$  ions reported. Since the physiological significance of the  $\text{Mg}^{2+}$ -ATPase is unknown this discrepancy in the cytochemical studies is not too serious.

Electron microscopy (36) has demonstrated the presence of gap junctions in the corneal endothelium of the rat. These junctions permit penetration of lanthanum hydroxide. In contrast, the epithelial layer is not penetrated by this molecule, thus indicating that the sites of close cell contact are narrowed to 20 Å or less. The absence of reaction product from the gap junctions might suggest that these are not sites of electrogenic pumps, but other explanations are not excluded.

Although it is early to be overly assertive regarding the Ernst NPPase procedure, it appears capable of showing the transport ATPase *in situ*. Its more extensive use is clearly warranted. Even in cells where the short fixation results in inadequate preservation of the cytoplasm, this is more than offset by the visualization of the enzyme sites at the plasma membranes. Direct cytochemical

observations would be particularly valuable where biochemical and physiological data are equivocal.

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