

STUDIES ON THE CORNEA

II. The Uptake and Transport of Colloidal Particles

by the Living Rabbit Cornea *in Vitro*

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ABSTRACT

In vitro studies of the transport of colloidal particles by the cornea were carried out on intact corneas of adult rabbits in a chamber described by Donn, Maurice, and Mills (2) in which the epithelial or the endothelial surface of the cornea was exposed to thorium dioxide or saccharated iron oxide under various conditions. These studies confirmed the results of previous work *in vivo* and allowed modification of the experimental conditions. Particles are pinocytosed at the apical surface of the corneal endothelium and carried around the terminal bar in membrane-bounded vesicles. Basal to the terminal bar these vesicles fuse with the lateral cell margin and their contents are released into the intercellular space, in which they appear to be carried by a one-way flow down to Descemet's membrane and the corneal stroma. Indications that the endothelial transport is an active process are presented by the different pathways of transport into or out of the corneal stroma, as well as by the approximately 70 per cent reduction in transport activity at low temperatures.

INTRODUCTION

The literature on corneal fine structure and physiological studies of corneal permeability has been reviewed in a previous communication from this laboratory (1), in which the fine structure of the rabbit cornea and the uptake and transport of colloidal particles by the rabbit cornea *in vivo* were described.

Rabbit corneas may also be studied *in vitro* in a chamber described by Donn, Maurice, and Mills (2). Electron-opaque colloidal particles can be added to the fluid bathing either the epithelial or the endothelial surface of the cornea. The present study describes the pathways of transport of colloidal particles by the living cornea maintained *in vitro* and compares these results with those found in the cornea *in vivo* (1). A comparison of transport

phenomena in mammalian (rabbit) corneas and amphibian (frog) corneas will be presented in another communication (3).

MATERIALS AND METHODS

Methods for the Study of the Uptake and Transport of Colloidal Particles by the Cornea in Vitro

Studies on the intact cornea *in vitro* were carried out in a chamber (Fig. 1), described by Donn, Maurice, and Mills (2), in which the cornea can be maintained for up to 8 hours. Potential and resistance measurements may be made across the cornea to determine its state of health, and various solutions can be added to the artificial aqueous humor (4) bathing either or both sides of the cornea.

Large adult rabbits were killed with an intravenous injection of Nembutal (sodium pentobarbital). Within 1 minute of death, the anterior segment of one eye was excised approximately 2 mm posterior to the limbus. The tissue was held by the sclera and immersed in a bath of the artificial aqueous humor, in which the lens and iris were removed. The cornea was then carefully clamped between two Lucite chambers (Fig. 1). These chambers were each fitted with a

room temperature for some experiments and immersed in an oil bath at 0 to -4°C for others.

To measure the potential difference across the cornea, the outflow beakers containing saturated KCl solution were connected by means of Calomel electrodes to an electrometer. The transcorneal potential difference was measured regularly throughout each experiment and was used as an indication of the state of health of the cornea (Figs. 2, 4, 15, and 17).

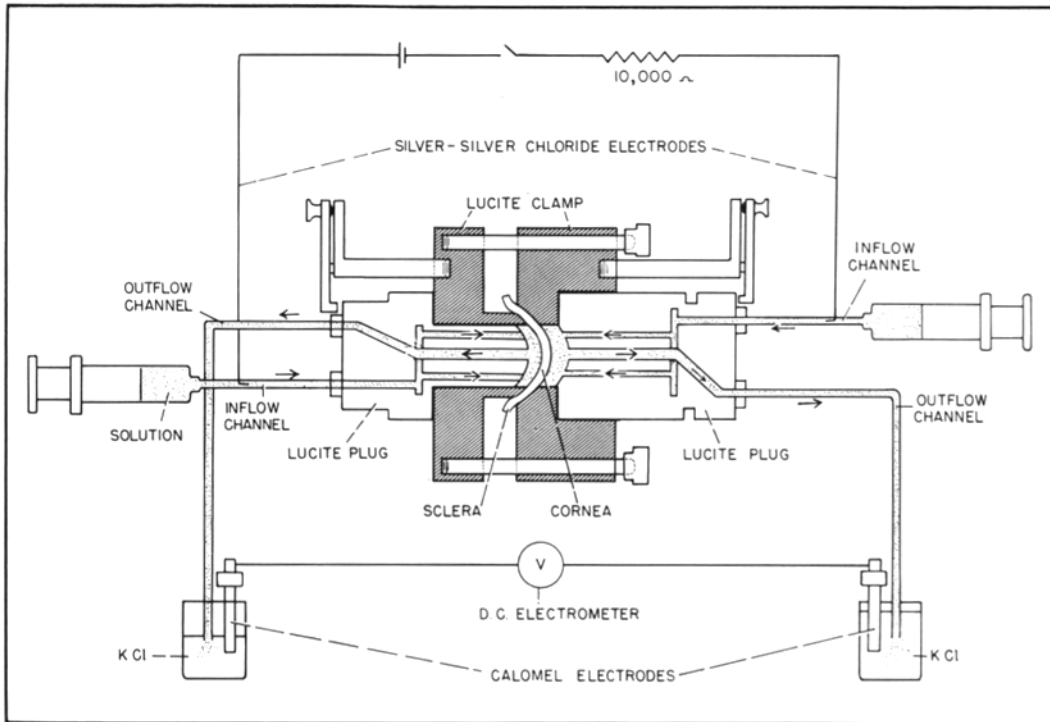


FIGURE 1

This schematic diagram of the perfusion chamber used in these experiments shows the cornea in place, the direction of flow of the perfusing fluid, and the electrical connections necessary to record the transcorneal potential and resistance. Potential difference is measured across the outflow tubes. A known current passes through the cornea via the inflow tubes and the change in potential is a measure of corneal resistance. (Diagram modified from Donn, Maurice, and Mills (2)).

Lucite plug so that the volume of solution could be reduced to approximately 0.5 ml. The plugs were bored with a ring of inflow channels and a single central outflow channel so that fluid could be circulated over both surfaces of the cornea. Polyethylene tubing connected the inflow channels to separate motor-driven syringes which were filled with the artificial aqueous humor. The outflow from each chamber passed through polyethylene tubing, the ends of which dipped into beakers of saturated KCl solution. The clamp holding the cornea was kept at

Initial control experiments were carried out in order to compare corneas which had been fixed immediately upon excision with corneas which had been incubated in the chamber for several hours prior to fixation. Corneas which had been cooled to 0°C in the chamber and corneas in which the potential difference had been neutralized electrically were also compared with normal corneas, and no morphological differences were evident.

Several series of experiments were conducted in which 0.6 to 1.2 ml of thorium dioxide was added to

the inflow to the endothelium, epithelium, or both, under varying conditions as follows: (a) ThO₂ on both epithelial surface and endothelial surface, for 30 to 45 minutes; (b) ThO₂ on endothelial surface only, for 30 minutes; (c) ThO₂ on epithelial surface only, for 30 minutes; (d) Epithelium stripped before mounting the cornea in the chamber; ThO₂ on bare subepithelial stroma for 3 hours; (e) ThO₂ on endothelial surface only, for 3 hours; (f) ThO₂ on endothelial surface only, for 30 minutes; cornea washed vigorously and incubated further for 3 hours; (g) cornea cooled to 0 to -4°C; ThO₂ on endothelial surface only, for 30 minutes.

Corneas were quickly removed from the clamp at the end of a given experiment and immediately dropped into a pool of 1 per cent OsO₄ in M/14 veronal acetate buffer at pH 8.2. The corneas were generally cut in half and transferred to a vial of fresh fixative kept at 0°C in an ice bath. Tissue was fixed for 30 to 60 minutes. As in the normal and *in vivo* experiments described previously (1), the corneas were cut into strips after approximately two-thirds of the fixation period was completed, and the strips were fixed for the remaining time. Dehydration in graded ethanol solutions, orientation of the tissue, and embedding in methacrylate were carried out as in previous work (1).

Thin sections were examined in an RCA EMU 3C or 3F electron microscope.

OBSERVATIONS

The Uptake and Transport of Colloidal Particles by the Cornea in Vitro

When the epithelial surface of a rabbit cornea maintained *in vitro* was exposed to a solution of thorium dioxide by the injection of approximately 2 ml of Thorotrast into the inflow tube leading to the epithelial chamber (Figs. 2 and 3), a small number of particles was found attached to the outer surface of the outermost layer of cells. There was no evidence of vesicle formation on this surface. Occasionally, however, what apparently were large vacuoles containing ThO₂ particles were found at or near the surface of the outermost cells. It is likely that some of the "vacuoles" represent tangential sections through pits or depressions of the irregular cell surface like that shown in Fig. 3 a. However, it is possible that others are enclosed vacuoles formed during the exposure to the test solution.

Small vesicles containing ThO₂ particles are never found in the cytoplasm of the surface cells, nor are free particles ever found there. Particles

are not observed in the intercellular spaces. Desmosomes occur frequently along the interdigitating membranes of the squamous cells (Figs. 3 and 3 a).

The addition of approximately 1.2 ml of thorium dioxide suspension to the fluid bathing

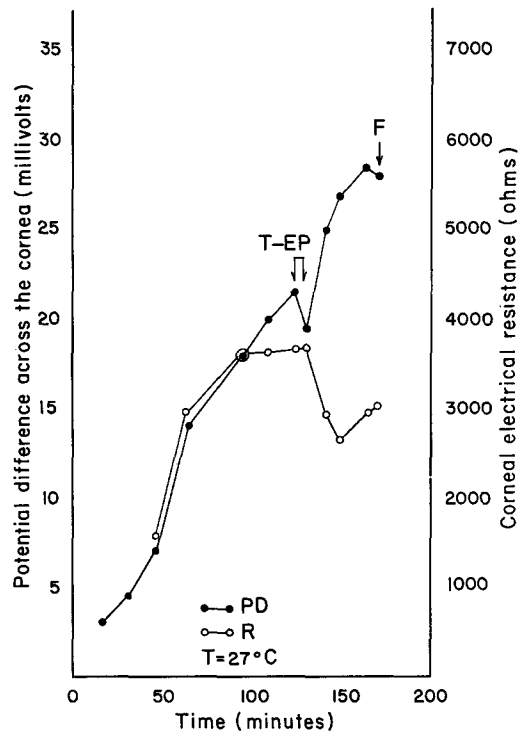


FIGURE 2

A typical record of the changes with respect to time of the transcorneal potential (anterior chamber surface positive) and corneal electrical resistance of a rabbit cornea maintained *in vitro*. A suspension of thorium dioxide was added to the inflow of the epithelium at T-EP and the cornea was fixed at F. A transient reduction in both potential difference and resistance is seen after the addition of the thorium dioxide.

the endothelial surface of a cornea maintained *in vitro* (Fig. 4) produces, after 30 to 45 minutes, an intense uptake of the colloidal particles as shown in Fig. 5. The apical surface of the cell is completely coated with ThO₂ particles, and numerous vesicles containing the particles are seen in the apical cytoplasm. Particles are found in the intercellular space apical to the terminal bar region. Basal to the terminal bar, the intercellular space contains many thorium dioxide

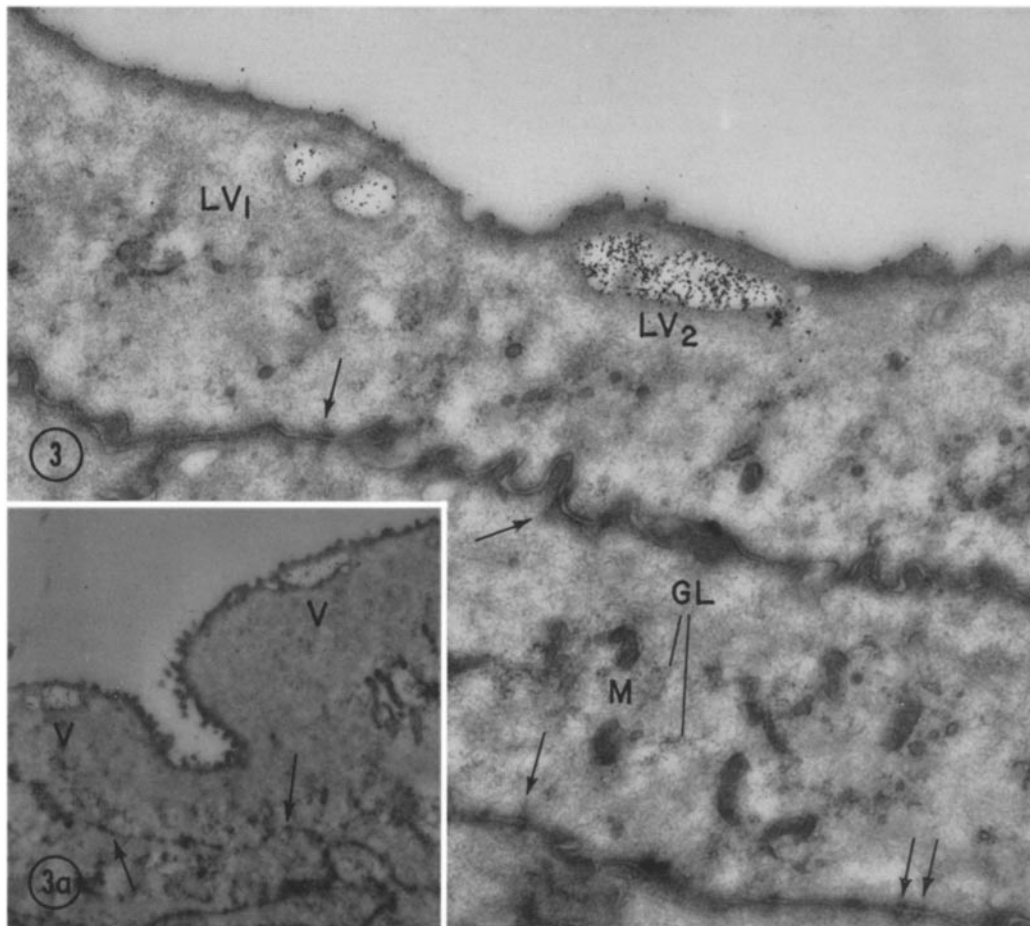


FIGURE 3

An electron micrograph of a part of the surface of the epithelium of a rabbit cornea. This cornea, maintained *in vitro*, had been exposed on its epithelial surface to a thorium dioxide suspension for 30 minutes. Some particles may be seen attached to the outer cell surface, and two large vacuoles filled with particles (LV_1 and LV_2) are found in the cytoplasm near the cell surface. The presence of a process in LV_1 suggests that this vacuole may represent a section through a pit or depression of the cell surface similar to that shown in Fig. 3 *a* (inset). Small mitochondria (M) and glycogen granules (GL) may be distinguished from the fine fibrils which fill most of the cytoplasm. Intercellular desmosomes are also present (arrows). $\times 22,000$.

FIGURE 3 *a*

This electron micrograph shows a part of the surface of the epithelium of a rabbit cornea, maintained *in vitro*, which had been exposed for 30 minutes to a suspension of thorium dioxide on its epithelial surface. Particles of thorium dioxide may be seen attached to the processes of the cell surface. Two large vacuoles (V) containing thorium dioxide are just below the surface. These vacuoles may represent sections through the bottoms of large depressions of the cell surface like that shown in the center of this figure. The fine fibrillar ground substance of the epithelial cell cytoplasm is apparent in this figure. Numerous desmosomes (arrows) are found at the margins of adjacent cells. $\times 9000$.

particles. There is some piling up of particles at Descemet's membrane, and diffusion of the ThO_2 into Descemet's membrane can be seen.

The apical part of an endothelial cell is seen in greater detail in Fig. 6. A vesicle containing thorium dioxide particles can be found (arrow) which appears to be pinching off from the intercellular space apical to the terminal bar, and a

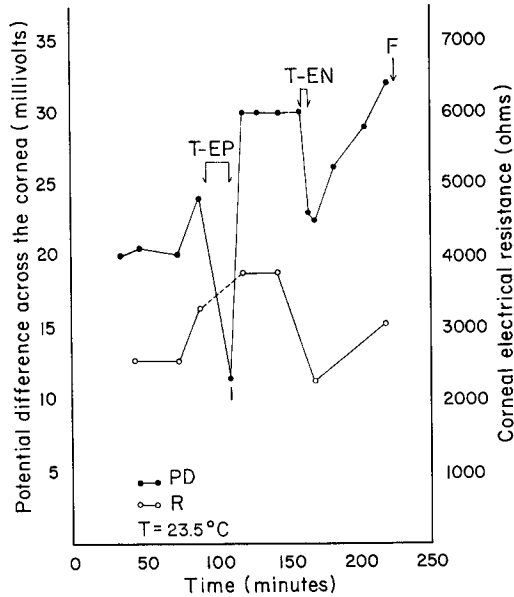


FIGURE 4

A record of the changes with respect to time of the transcorneal potential (anterior chamber surface positive) and corneal electrical resistance of a rabbit cornea maintained *in vitro* and exposed to thorium dioxide on both the epithelial (*T-EP*) and the endothelial surface (*T-EN*). *F* represents the time of fixation. A transient drop in both the potential difference and the resistance may be seen after addition of the colloidal particles to the fluid perfusing either side of the cornea.

row of vesicles seems to be leading to the intercellular space basal to the terminal bar.

Figs. 7 and 8, from oblique sections through the endothelium, demonstrate, respectively, the apparent pinching off of vesicles from the bottom of an invagination of the cell surface and the apparent fusion of a small vesicle containing ThO_2 with the cell membrane basal to the terminal bar.

The diffusion of particles in Descemet's membrane which has been demonstrated in *in vivo* studies (1) is also seen *in vitro*. Fig. 9 shows part of the basal portion of an endothelial cell from a

cornea which had been exposed to ThO_2 on its endothelial surface for 45 minutes. Particles appear to be funneling down the intercellular spaces and spreading out and becoming diluted as they enter Descemet's membrane, suggesting a flow in the intercellular space which would move the particles toward Descemet's membrane. Some particles which appear to be free in the endothelial cell cytoplasm may be seen, on close scrutiny, to be surrounded by a delicate membrane which is not clearly demonstrated in these unstained sections.

Exposure of the endothelial surface of the cornea maintained *in vitro* to ThO_2 for 3 hours preceding fixation produces more intense uptake and transport of the colloidal particles. There is a greater filling of the intercellular space with the thorium dioxide (Fig. 10), as well as more piling up of particles at the apical surface of Descemet's membrane. Large particle-containing vesicles, presumably originating from the fusion of smaller vesicles, and having an internal structure, are found in the apical cytoplasm. Even after a 3 hour exposure to ThO_2 , particles are not found within the terminal bar region.

In the 3 hour ThO_2 preparations, one often finds a thick coating of thorium dioxide particles apparently organized in some manner at the apical surface of the endothelial cell (Fig. 10). This coating may become as much as 5 microns or more thick (as thick as the endothelial cell itself). In these 3 hour experiments it is possible to demonstrate that the ThO_2 particles have passed through Descemet's membrane and have entered the stroma.

A surprisingly different picture is found in electron micrographs of the endothelium of corneas which had been exposed to thorium dioxide on their endothelial surface for 30 minutes, washed vigorously after exposure, and allowed to incubate for 3 hours after the washing (Fig. 11). Very few ThO_2 particles are found either attached to the apical surface of the cell or in the intercellular space. Particles are accumulated at the apical surface of Descemet's membrane and also are found in large and small vesicles in the endothelial cell, particularly in the basal part of the cell. Most of the large vesicles have an internal membranous structure which is presumed to originate from the excess membranes resulting from the fusion of several small vesicles. This picture is essentially identical with that which has been described in the observations on *in vivo*

experiments (1) as resulting from a bleb of colloidal material placed in the stroma and allowed to travel out across the endothelium. This similarity is further discussed in a later section.

In one experiment, it was found after fixation that a considerable portion of the endothelium had been stripped accidentally during the preparation of the cornea for incubation. The bare apical surface of Descemet's membrane was, therefore, exposed to the thorium dioxide for 30 minutes. This particular preparation was then washed and incubated for 3 more hours. Figure 12 is a low power electron micrograph of a portion of the central stroma of this cornea. Thorium dioxide particles fill almost all the interfibrillar and interlamellar spaces, producing what amounts to a negative staining of the fibrous structures of the stroma. That is to say, the light areas in the stroma in this micrograph represent the collagen fibers, while the dark areas show the ThO_2 which had diffused around and between them. Portions of two stromal cells (*S*) are also shown as light areas containing cytoplasmic vesicles densely packed with thorium dioxide.

It was found at the end of the experiment that the leading edge of the diffusing thorium dioxide was just below the basement membrane of the epithelium (Fig. 13). Under these experimental conditions, therefore, the thorium dioxide has an approximate diffusion rate of 100 microns per hour in the stroma. The densest accumulation of particles (Fig. 12) was found approximately 10 to 15 microns behind the leading edge. ThO_2 particles have reached the basement membrane of the epithelium, although they are not found within the basement membrane (Fig. 13). Nevertheless, a few particles may appear in larger vesicles within the cytoplasm of the basal cells of the epithelium. Processes of the basement membrane, normal to

its plane, may be seen entering the stroma in this section. A similar phenomenon has been described by Jakus (5) in the human cornea.

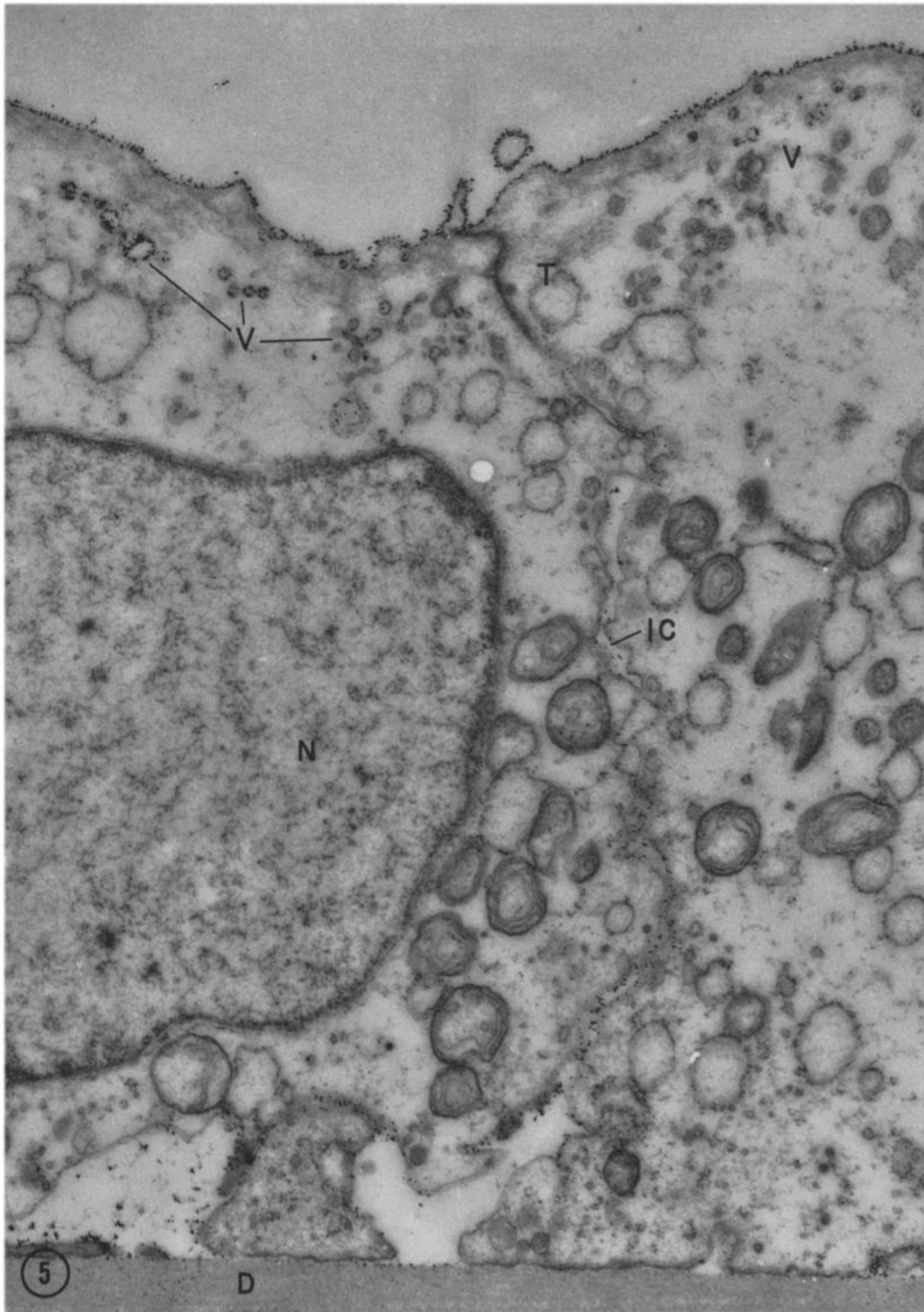
The distribution of marker in the stroma in this experiment strongly resembles that seen in *in vivo* experiments (1) in which a bleb of thorium dioxide was injected into the stroma. In the present instance, the concentration of particles in the stroma is so high that almost the entire anterior third of the stroma resembles the central part of a bleb.

If, on the other hand, the epithelium (and presumably its basement membrane) is deliberately removed before the cornea is mounted in the clamp of the chamber, and the bare stromal surface is exposed to ThO_2 for 3 hours, a different situation is found. Fig. 14 shows a portion of the stromal surface of such a preparation. There is an accumulation of thorium dioxide particles at the surface, and particles which have diffused into the stroma are easily observed for a distance of approximately 15 to 20 microns. Probably because of their extreme dilution, the particles are not recognizable any deeper in the stroma. Stromal cells in this region do not appear to take up the particles. No thorium dioxide is found at the endothelial side of the cornea either in the endothelial cells or at the basal or apical surface of Descemet's membrane. It is possible, and is suggested by the dense fibrillar material seen at the stromal surface in Fig. 14, that all of the basement membrane may not have been removed and that the remnant has prevented the rapid penetration of the ThO_2 into the stroma.

In the last series of experiments, corneas were cooled to 0°C before ThO_2 was added to the inflow of the endothelial side of the chamber. In the first of these experiments, the cornea was allowed to develop a transcorneal potential at room tempera-

FIGURE 5

An electron micrograph showing part of the endothelium of a rabbit cornea, maintained *in vitro*, which had been exposed for 45 minutes to a suspension of thorium dioxide on its endothelial side. The surface of the endothelial cells is entirely covered with ThO_2 particles, and numerous vesicles (*V*) containing particles are found in the apical cytoplasm. Particles are present in the intercellular space apical to the terminal bar (*T*) as well as in the intercellular space (*IC*) basal to the terminal bar. However, no particles are seen in the terminal bar region itself. Particles appear to have been carried down in the intercellular space to the junction with Descemet's membrane (*D*), where their progress is apparently slowed, producing an accumulation of particles at this surface. However, particles do appear to diffuse into Descemet's membrane. *N*, nucleus. $\times 21,000$.



ture before the clamp was immersed in an oil bath at 4°C, and the ThO₂ suspension was added without prior cooling (Fig. 15). In this instance (Fig. 16), some ThO₂ is seen attached to the apical surface of the endothelium and some particles are found in the intercellular space. A single

and Mills (2) before the ThO₂ was added for 30 minutes.

In later work (Fig. 17), the clamp containing the cornea was placed in the oil bath at -4°C immediately after clamping, and the test solution was cooled overnight in the same oil bath before it was

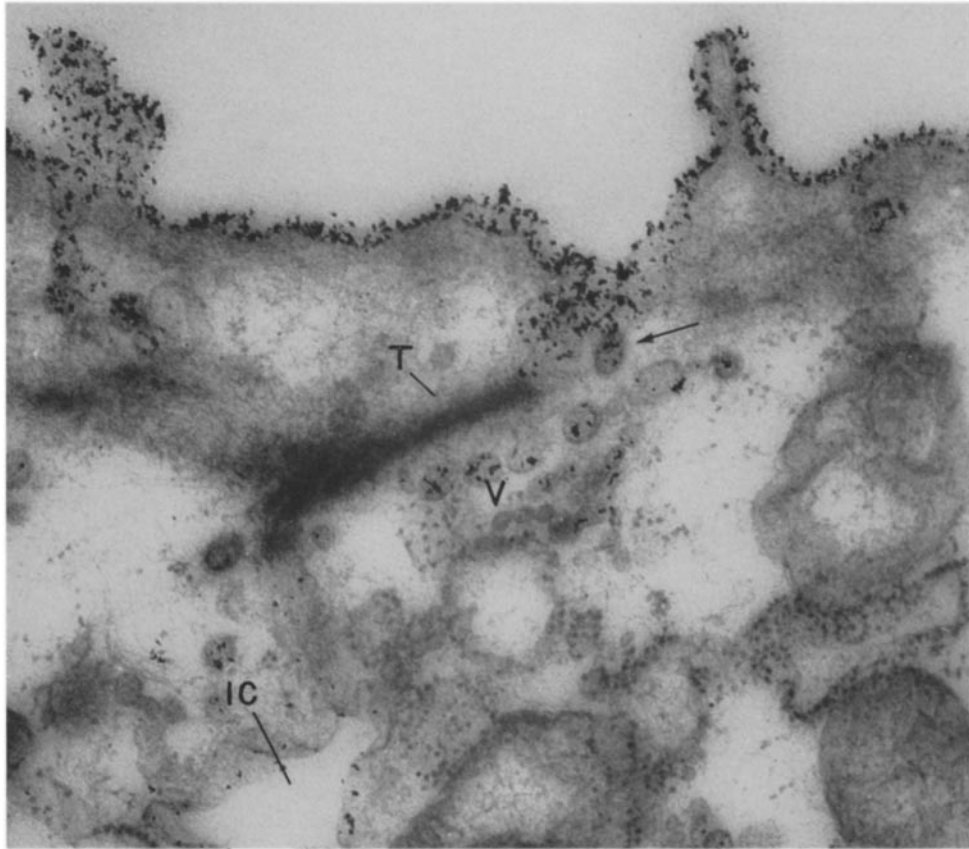


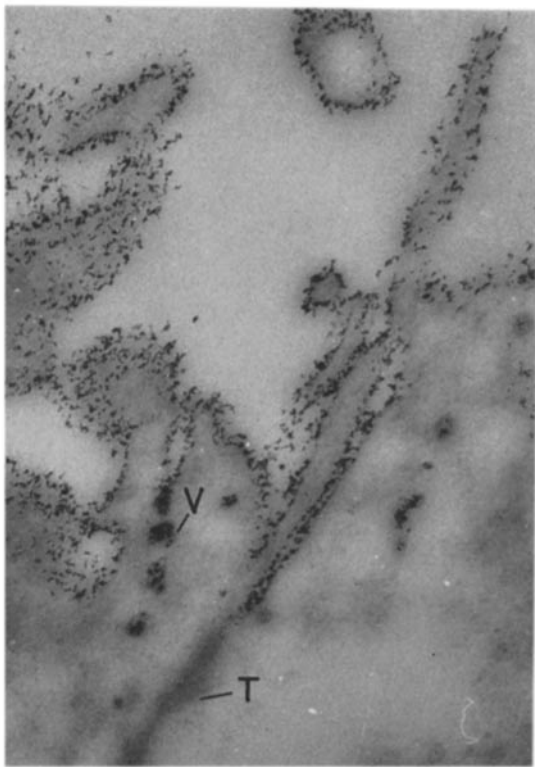
FIGURE 6

An electron micrograph of the apical part of the endothelium of a rabbit cornea, maintained *in vitro*, which had been exposed for 45 minutes to a suspension of thorium dioxide in the fluid bathing its endothelial surface. The surface of the cell is heavily coated with ThO₂ particles. A vesicle containing thorium dioxide particles appears to be pinching off from the cell membrane above the terminal bar (arrow), and a row of vesicles (V) seems to lead to the intercellular space (IC) below the terminal bar (T). $\times 51,000$.

vesicle containing ThO₂ is found in the apical cytoplasm just below the apical surface. Particles of thorium dioxide are not found at the apical surface of Descemet's membrane. This activity may be due to the fact that the transcorneal potential did not drop, after cooling, to the level of 3 to 4 millivolts as reported by Donn, Maurice,

injected into the inflow of the endothelium. This further reduction of the temperature and the placing of the clamp in the oil bath immediately after clamping the cornea reduced the transcorneal potential to less than 1 millivolt (Fig. 17). Some transport is apparently taking place under these conditions, but the extent of this activity is con-

siderably reduced from that seen after 30 minutes' exposure to ThO_2 of a cornea maintained at room temperature (Figs. 5 to 9). The reduction is roughly estimated at 70 per cent, that is, 70 per cent decrease in transport activity. At -4°C one finds some attachment of particles to the surface, the formation of rare vesicles containing ThO_2 , and occasional particles of ThO_2 in the intercellular space below the terminal bar.



(7, 8), it is reasonable to suppose that the cornea will remain relatively normal if these substances are provided in the perfusing solutions.

The use of the *in vitro* apparatus allows a more uniform exposure of the surface being studied to the test solution as well as the use of high concentrations of the tracer. It also makes it possible to modify the corneal environment in a controlled and measurable manner. Electrical measurements

FIGURE 7

An electron micrograph of an oblique section through the endothelium of a rabbit cornea, maintained *in vitro*, which had been exposed for 1 hour to a suspension of thorium dioxide in the fluid bathing its endothelial surface. Particles of ThO_2 coat the surface of the cell. The terminal bar (*T*) appears longer than in sections which are normal to the plane of the endothelium (Figs. 5 and 6). A row of vesicles (*V*) appears to be pinching off from the base of an invagination of the cell surface. $\times 38,000$.

DISCUSSION

The use of the *in vitro* chamber described by Donn, Maurice, and Mills (2) to study the transport of colloidal particles by the cornea has given somewhat fuller measure of control over the experimental conditions. The normal avascularity of the cornea permits maintenance of this structure *in vitro* for periods up to 8 hours, provided the ionic and gaseous balance of the perfusing fluid is maintained in a relatively stable state and there is an adequate supply of substrate (dextrose) for corneal metabolism. Since, *in vivo*, the cornea appears to derive most of its nutrients from the aqueous humor (6) and most of its O_2 from the atmosphere

of the transcorneal potential difference and electrical resistance are also facilitated *in vitro*. While the site of origin and/or the significance of the measured potential difference is not clear in complex systems such as the cornea, frog skin, or intestinal wall, several hypotheses based on the active transport of sodium ions have been proposed to explain this electrical phenomenon (9-12).

Friedman and Kupfer (13) measured the transcorneal potential *in vivo* in the rabbit by means of surgically implanted electrodes. They found that small epithelial abrasions, performed serially, produced a stepwise drop in the potential differ-

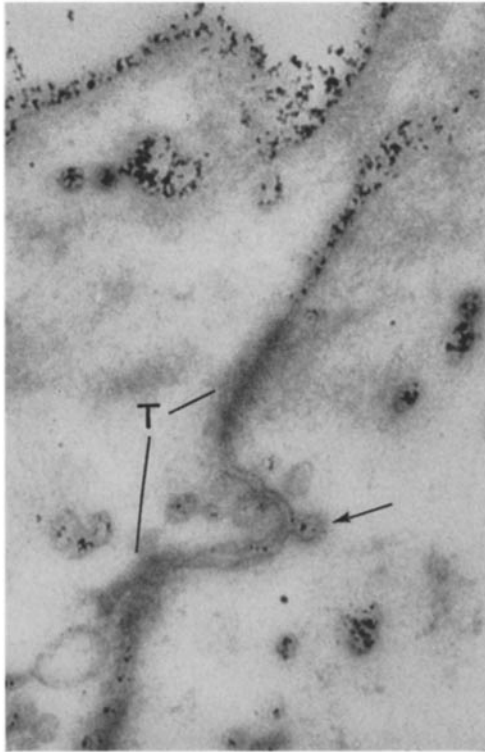


FIGURE 8

This electron micrograph also shows an oblique section through the endothelium of a rabbit corneal preparation identical with that of Fig. 8. The apparent fusion of the ThO_2 -containing vesicles with the lateral cell membrane below the terminal bar (T) is indicated by the arrow. The hook-like convolution of the intercellular space, at which point the vesicle is fusing, probably is actually below the terminal bar. The convolutions of the intercellular space are such that the terminal bar passes into and out of this almost tangential section, creating the illusion of a bipartite attachment zone. $\times 48,000$.

ence across the cornea and that the potential difference dropped to zero when approximately one-third of the epithelium had been abraded. They do not state, however, whether this was a uniform removal of one-third of the thickness of the epithelium or a non-uniform patch-like removal of one-third of the epithelium.

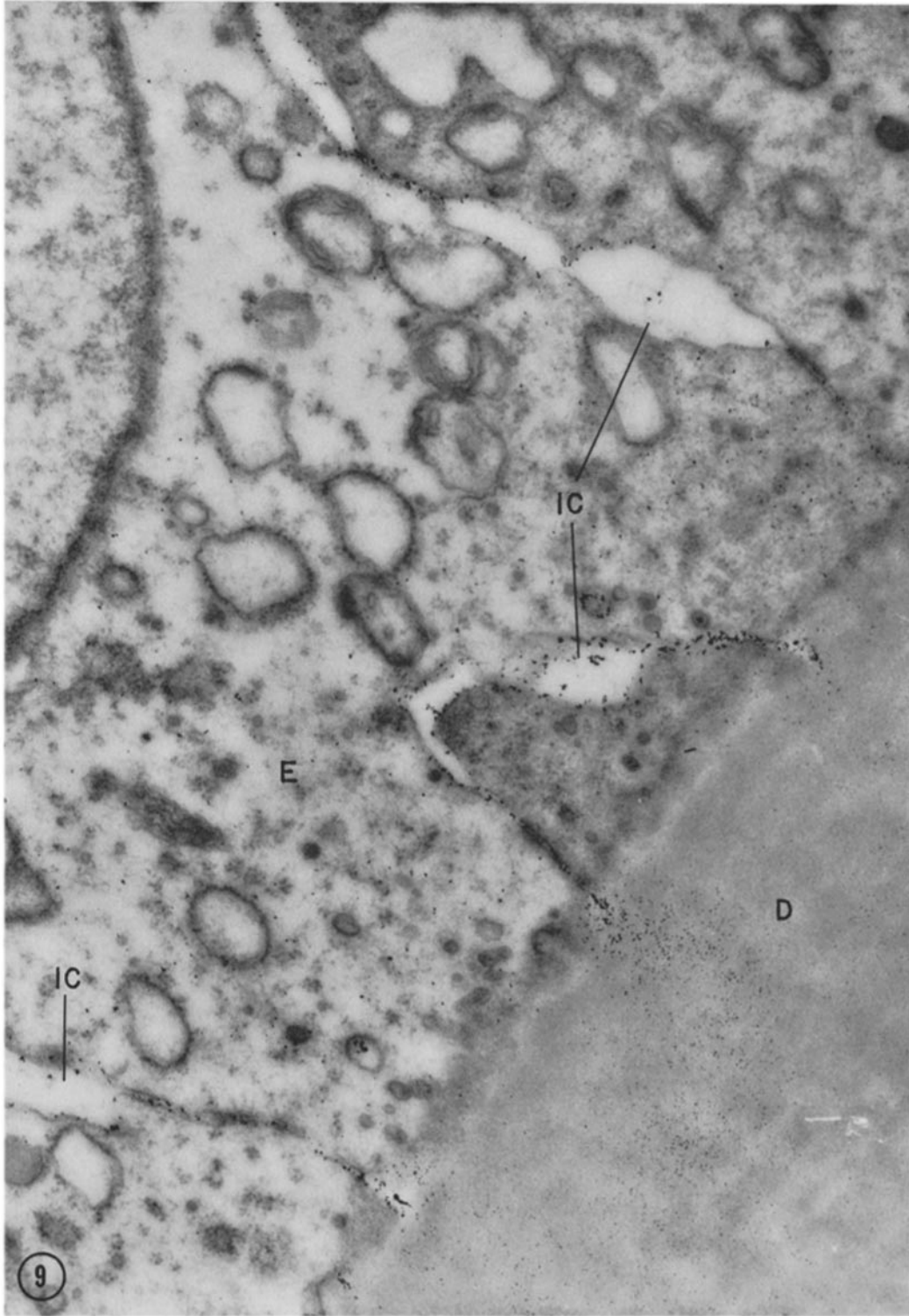
Donn, Maurice, and Mills (2) reported a transcorneal potential *in vitro* of 15 to 42 mv, which disappeared on removal of the epithelium. These workers (14) also measured Na^+ flux across the cornea and reported the active transport of Na^+ inward across the epithelium. Conditions which presumably would greatly inhibit cellular metabolism (0°C) were found to stop the transport of Na^+ as well as to reduce the transcorneal potential to 2 to 3 mv. Though no reliable measurements of the endothelial potential are available, it is a common clinical as well as experimental observation that any damage to the endothelium leads to immediate swelling and loss of transparency in the cornea.

Potential and resistance measurements were used in the present study primarily as an indication of the state of health of the cornea *in vitro* or as an indication of the effectiveness of the cooling to 0°C . The addition of thorium dioxide to the fluid bathing either the epithelial or the endothelial surface usually produced a transitory reduction in both the potential difference and the resistance (Figs. 2, 4, 15, and 17), but the significance of this finding is not clear.

In general, the *in vitro* studies of the uptake and transport of colloidal thorium dioxide particles by the cornea have confirmed and expanded the earlier observations on the transport of ThO_2 and Fe_2O_3 *in vivo* (1). When the epithelium was exposed to the thorium dioxide suspension, only a slight degree of attachment of the particles at the

FIGURE 9

An electron micrograph of the basal part of an endothelial cell (E) of a rabbit cornea, maintained *in vitro*. The cornea had been exposed for 45 minutes to a suspension of thorium dioxide in the fluid bathing the endothelial surface. Thorium dioxide particles are shown funneling down the intercellular space (IC) and diffusing into Descemet's membrane (D). Some of the particles which appear to be free in the cytoplasmic matrix may be seen, on close scrutiny, to be surrounded by a delicate membrane which is not clearly delineated in these unstained preparations. $\times 30,000$.



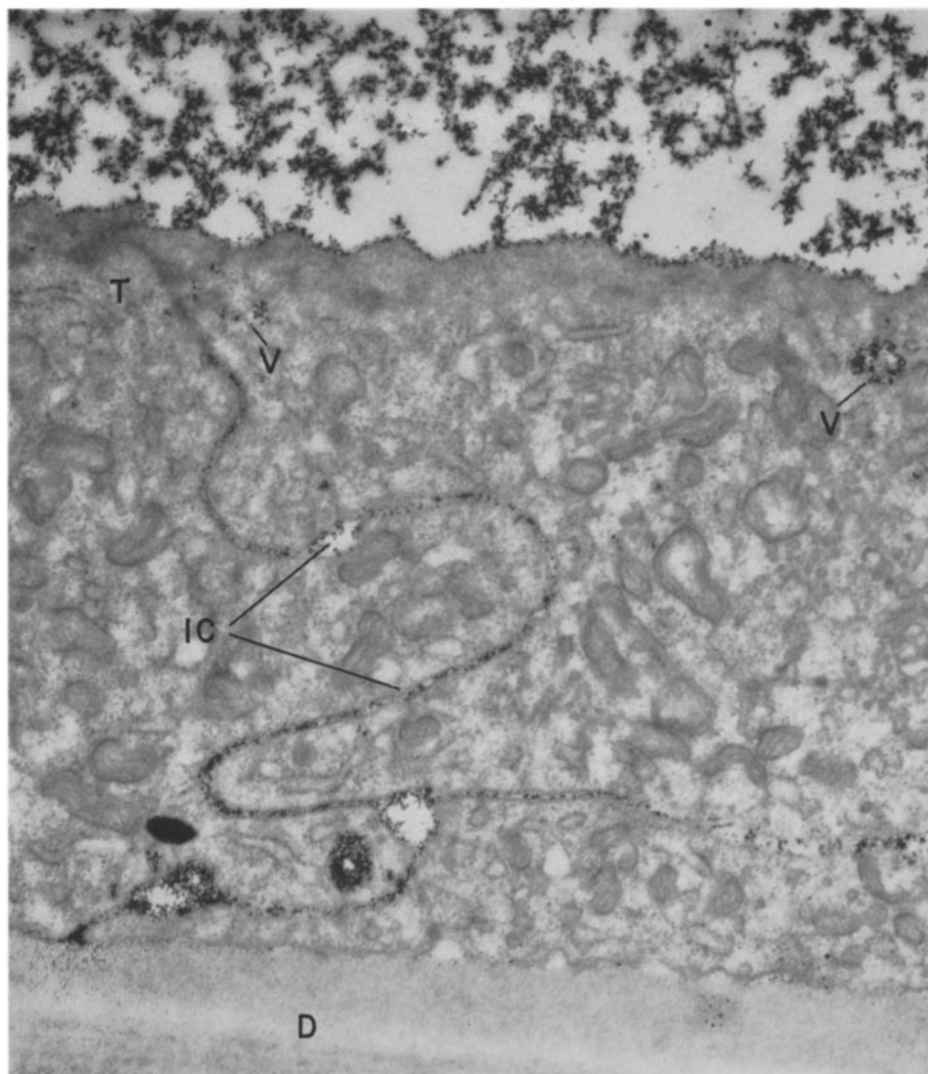


FIGURE 10

In some rabbit corneas exposed *in vitro* for 3 hours to a suspension of thorium dioxide in the fluid bathing the endothelial surface, there is a dense accumulation of ThO_2 particles at the cell surface. The apparently organized coat sometimes becomes as much as 5 microns thick (as thick as the endothelium itself). Large and small vesicles (*V*) containing thorium dioxide are seen primarily in the apical cytoplasm. The entire intercellular space (*IC*) is densely filled with thorium dioxide particles. These particles are found to accumulate at the border of the endothelium next to Descemet's membrane (*D*) and to diffuse into Descemet's membrane. *T*, terminal bar. $\times 29,000$.

surface was observed (Figs. 3 and 3 *a*). Some large vacuoles containing ThO_2 , which presumably formed at the outer surface of the surface cells during the exposure to ThO_2 , were also observed (Fig. 3). There was, however, no apparent transport of particles any farther into the epithelium.

Studies of the permeability of the cornea to sodium ions (14, 15) indicated that this small ion is rapidly and apparently actively transported by or through the epithelium.

It is difficult to conceive that such rapid, active transport occurs across multicellular layers. Dav-

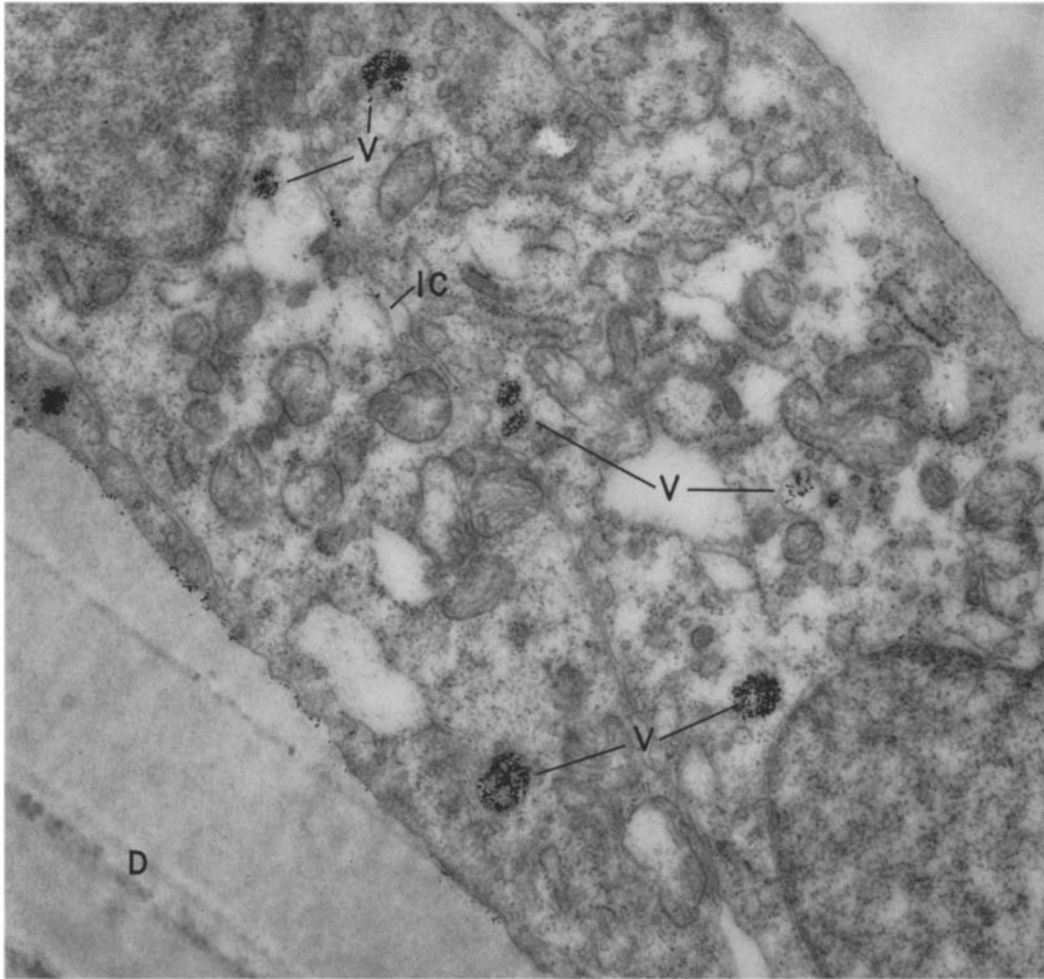


FIGURE 11

An electron micrograph of a part of the endothelium of a rabbit cornea which had been exposed *in vitro* for 30 minutes to a suspension of thorium dioxide in the fluid bathing the endothelial surface. The endothelial surface of the cornea was then vigorously washed and allowed to incubate for 3 hours after the washing. In this instance very few ThO_2 particles are present at the cell surface. Vesicles (V) containing dense accumulations of particles are found primarily in the basal part of the cell. This figure strongly resembles the endothelium of a cornea which had received an intrastromal bleb (1) some time before fixation. It appears likely that this represents a reversal of transport direction after washing. The particles which had reached Descemet's membrane before the washing now act as a reservoir of material which is transported to the anterior chamber side of the cornea. D, Descemet's membrane; IC, intercellular space. $\times 30,000$.

son (16) concluded that successive uptake and secretion by cell layers in epithelia would be most inefficient, and suggested that some type of extracellular transport may exist. Ussing (10), on the other hand, concluded that it must be assumed that sodium transport takes place through the cell membranes, even in multicellular epithelial mem-

branes. The findings in the present study, concerning the transport of colloidal particles by the epithelium, indicate only that some particles may be taken up in the surface cells. There is no evidence of a transport of colloidal tracers into deeper layers of the epithelium.

On the other hand, addition of colloidal

thorium dioxide to the fluid bathing the endothelial surface of the cornea produced evidence of a considerable transport of the colloidal particles into the cornea across the endothelium (Figs. 5 to 11).

The coating of the apical surface of the endothe-

cellular space both apical and basal to the terminal bars but never within these structures. Miller (21) has demonstrated that the terminal bars of the mouse kidney tubular epithelium are barriers to the diffusion of hemoglobin into the intercellular space.



FIGURE 12

A low power electron micrograph of a portion of the stroma of a rabbit cornea which had been exposed *in vitro* for 30 minutes to a suspension of thorium dioxide bathing the bare apical surface of Descemet's membrane. The cornea was subsequently washed and incubated for 3 hours. Thorium particles fill the interfibrillar spaces of the stroma, creating an essentially negative staining of the fibrous component of the stroma. Parts of two stromal cells (*S*) are seen. These contain cytoplasmic vesicles (*V*) with dense accumulations of ThO_2 particles. $\times 4000$.

lial cells with the colloidal particles is similar to the situation during pinocytosis in amebas described in studies by Brandt (17, 18), Brandt and Pappas (19), and Schumaker (20). Vesicles containing ThO_2 particles which are found in the apical cytoplasm appear to form from infoldings of the particle-loaded cell membrane and probably represent pinocytotic vesicles. It is significant that particles may be found in the inter-

The sequence of transport of the particles which may be deduced from static views of this sort is, of course, based largely on the fact that in these experiments only the apical surface of the cell was exposed to the colloidal solutions. Particles found in the intercellular space below the terminal bar must either have by-passed the terminal bar by an inter- or intracellular pathway, or have passed through the terminal bar. This latter possi-

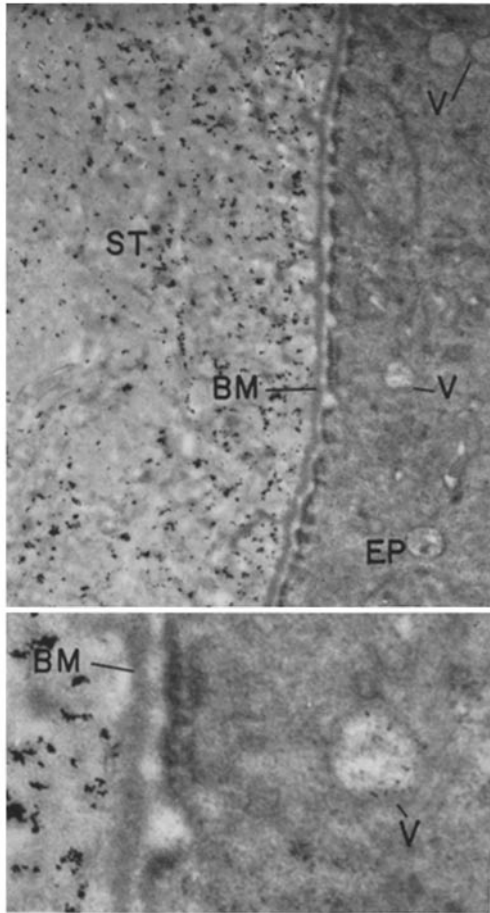


FIGURE 13

This electron micrograph of a section of the same cornea presented in Fig. 12 shows the basal part of the corneal epithelium (*EP*) and the underlying basement membrane (*BM*). ThO_2 particles may be found in the stroma (*ST*) below the basement membrane. No particles are seen in the basement membrane, but vacuoles (*V*) which may contain ThO_2 particles are present in the epithelial cell cytoplasm (see inset). $\times 15,000$; inset, $\times 45,000$.

bility is unlikely because in sections normal to the endothelial surface (Fig. 5) particles are never found in the terminal bar. Studies of normal rabbit cornea (1), and all observations thus far made on experimentally treated rabbit corneas, indicate that a terminal bar is found between all endothelial cells and is continuous in three dimensions. That is to say, the terminal bar forms a continuous ring about any given endothelial cell, just below its apical surface. A similar conclusion was reached

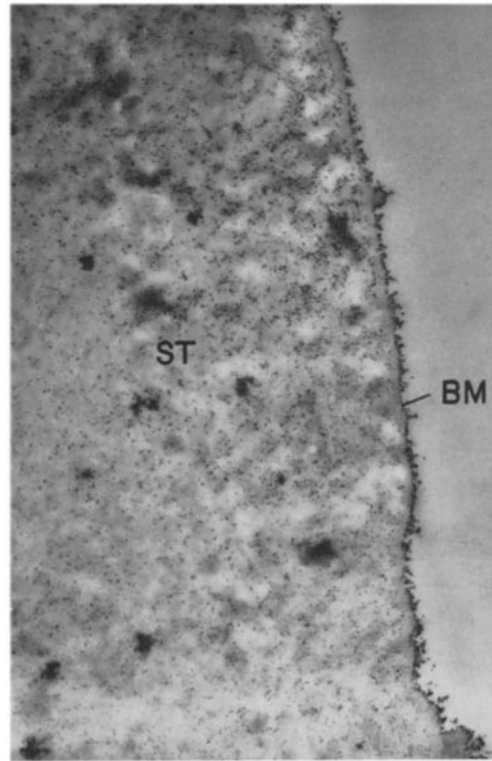


FIGURE 14

This electron micrograph shows the bare stromal surface of a cornea from which the epithelium was scraped before it was exposed *in vitro* for 3 hours to a suspension of thorium dioxide. Particles have attached to the surface and apparently diffused into the stroma (*ST*). Particles are easily recognizable only to a depth of 15 to 20 microns. The dense fibrillar material seen at the surface of this preparation may represent a remnant of the basement membrane (*BM*) which retards the rapid penetration of the ThO_2 particles into the stroma. It is surprising that when ThO_2 is placed on the bare subepithelial surface of the stroma there is less penetration into the stroma than when ThO_2 is placed on the bare apical surface of Descemet's membrane. $\times 17,000$.

by Palay and Karlin (22) in their study of the epithelium of the intestinal villus, in which they likened the terminal bar to the hoop of a cask. If a continuity of the terminal bar is assumed, it is, therefore, unlikely that the particles have bypassed the terminal bar by an intercellular route.

The remaining possibility, that of an intracellular pathway, seems to fit most logically the known morphology of the endothelial cell as well as the

observed way in which the cell apparently handles the colloidal particles.

It is proposed, therefore, that particles are internalized after they have attached to the cell membrane by the pinching off of vesicles from the cell membrane (pinocytosis), and that these particles are carried around the terminal bar in membrane-

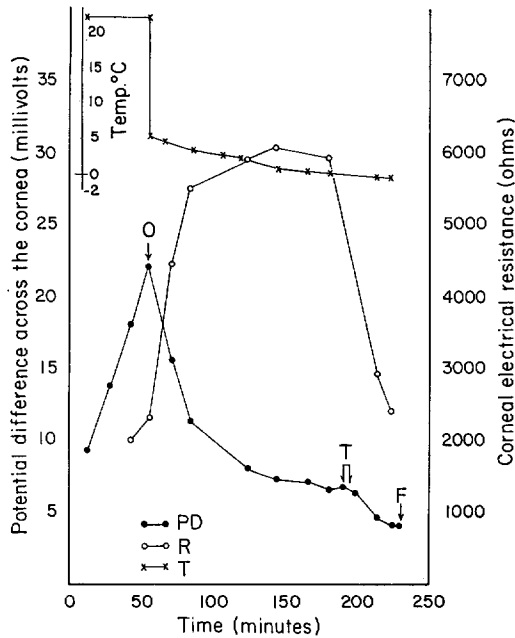


FIGURE 15

In this case, the cornea was allowed to develop a potential difference at room temperature. Subsequently it was cooled to approximately 2°C before thorium dioxide was added to the fluid bathing the endothelial surface. The potential rose until the moment when the cornea was placed in the cold oil bath (O). It then fell precipitously to approximately 7 millivolts, at which point thorium dioxide was added (T). The resistance continued to rise in the cold until the ThO₂ was added, after which it rapidly fell. F, time of fixation.

bounded vesicles. When the vesicles reach the lateral cell membrane basal to the terminal bar, the membrane of the vesicle fuses with the cell membrane and the contained particles are released into the intercellular space, in which they appear to pass freely down to and then into Descemet's membrane.

The attachment of material at the cell surface and its subsequent internalization into vesicles was suggested by Bennett (23) as a means of transport-

ing material across the cell membrane. He also proposed the release of the material into the cytoplasm by the breakdown of the vesicular membrane. Brandt and Pappas (19) demonstrated the attachment and internalization steps of this sequence in amebas but never found the marker particles (ThO₂, ferritin) free in the cytoplasm. Particles were found only in membrane-bounded vesicles in the cytoplasm, although there was some evidence of the fusion of vesicles with the contractile vacuole (18) and the release of particles into this hydrostatic organelle. Fawcett (24) reported the appearance of ThO₂ particles in the intercellular space of the epithelium of the distal convoluted tubule of the frog kidney after the uptake of ThO₂ from the tubular lumen by the tubule cell. Palay and Karlin (25) described the uptake of fat droplets in the intestinal epithelium by a process of pinching-off of vesicles containing fat from the base of the intermicrovillous space. They further described the apparent fusion of the membrane of a vesicle so formed with the membranes of the endoplasmic reticulum, and the subsequent extrusion of the fat into the intercellular space at the lateral margin of the cell by a secondary fusion of the membranes of the endoplasmic reticulum with the cell membrane. They suggest that if the lumen of the endoplasmic reticulum can be considered to be continuous with the extracellular phase, then such a pathway would actually represent an extracellular pathway of fat absorption. The analogy between such a system and the gut of an animal is apparent.

It is interesting that in the frog corneal endothelium, in which no terminal bars are found, particles appear to diffuse freely through the intercellular space (3). Indeed, they are often seen filling this space from its apical to its basal end. However, attachment of particles to the apical surface of the cell and the subsequent pinocytosis of these particles does occur in the frog, although the process probably plays a minor role in trans-endothelial transport in that animal (3).

After a 3 hour exposure of the endothelial surface to thorium dioxide, a dense coating of particles as much as 5 microns thick may be observed at the apical surface of the endothelial cell (Fig. 10). A thick coating of the hair-like structures of the plasmalemma of the ameba after exposure to ThO₂ has been noted by Brandt and Pappas (19). The fact that a coating of the dimensions found in the rabbit corneal endothelium survives the

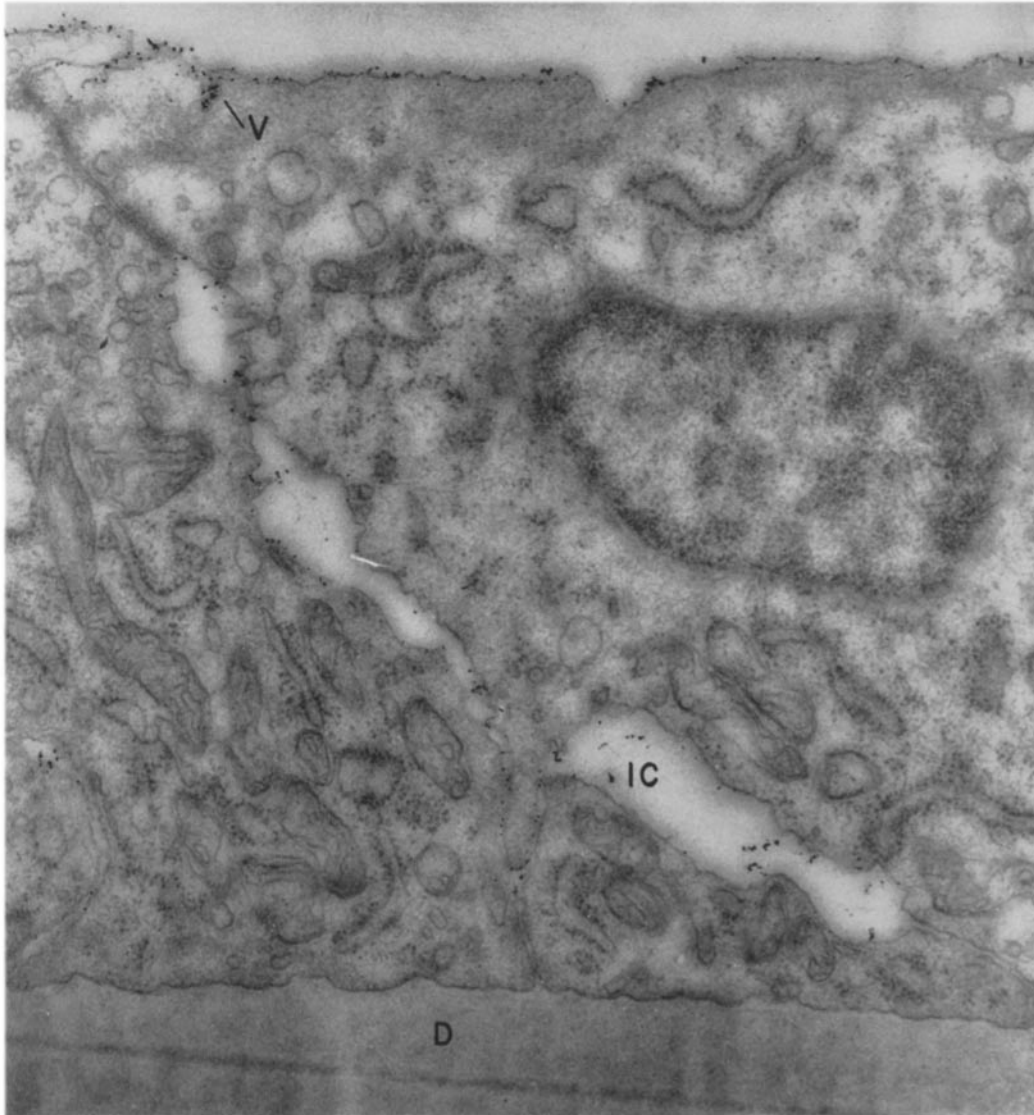


FIGURE 16

An electron micrograph of a part of the endothelium of a rabbit cornea, maintained *in vitro*, which was cooled to 0°C before the addition for 30 minutes of a suspension of thorium dioxide to the fluid bathing the endothelial surface. There is some attachment of thorium dioxide particles to the surface, and a few particles are found in the intercellular space (IC). Only a single vesicle (V) containing ThO₂ particles is found in the apical cytoplasm. No particles are seen at the junction of the endothelium and Descemet's membrane (D). The amount of transport of particles under these conditions appears to be approximately 70 per cent reduced from the amount of transport seen at room temperature (Fig. 5). Cooling of the cornea to -4°C does not appear to modify further the morphological evidence of transport. × 25,000.

preparatory procedures used in electron microscopy is some indication that a degree of structural organization exists in this layer. It is possible that the amorphous coating of the cell surface (1) is responsible for this intense binding of particles. It is also possible that the binding of the first few layers of particles onto the surface alters the binding characteristics of the surface so that it then

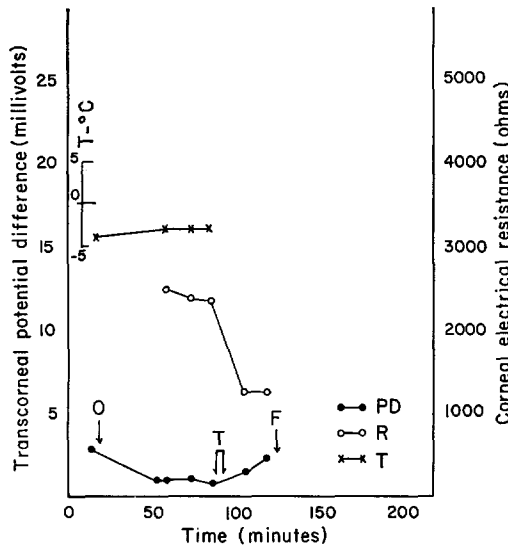


FIGURE 17

In this case the corneal clamp was placed in an oil bath at -4°C immediately after the cornea was mounted in the clamp. The potential never rose above the 3 millivolt level at the start of the experiment, and had fallen to less than 1 mv by the time the thorium dioxide was added to the inflow to the endothelial surface (T). The resistance remained relatively constant until the thorium dioxide was added, at which time it dropped rapidly. O, time of placing the cornea in the -4°C oil bath; F, time of fixation.

attracts more particles. Burgos (26) has reported the binding of dense colloidal particles within the amorphous coats of the epithelial cells of the ductuli efferentes and the epididymis.

The transport of colloidal particles by the corneal endothelium *in vivo* (1) or *in vitro* always appears to be along the concentration gradient under the experimental conditions in this study. This condition appears to obtain whether the transport is into the cornea across the endothelium after the exposure of the endothelial surface to ThO_2 or Fe_2O_3 , or out of the cornea across the endothelium after the injection of a small drop of

ThO_2 or Fe_2O_3 into the stroma. In experiments in which the endothelial surface was exposed to ThO_2 for 30 minutes, washed vigorously, and then allowed to incubate for 3 hours before fixation, a situation was found which more closely resembled that observed in the endothelium of a bleb preparation (1) than that observed in the endothelium of a cornea exposed to colloidal markers on the endothelial surface (compare Fig. 11 with Figs. 12 and 13 of Kaye and Pappas (1)). It is likely that the vigorous washing, which results in the removal of the ThO_2 particles from the endothelial surface of the cornea, has essentially reversed the concentration gradient. The particles which had already been transported into Descemet's membrane before the washing probably now act as a particle source, and the transport which occurs after washing is outward through the endothelium. In *in vivo* experiments in which particles were injected into the stroma, the finding of large as well as small vesicles containing colloidal particles in the basal part of the endothelial cell has been associated with the transport of colloidal particles from the stroma out across the endothelium (1). In the *in vitro* experiments, in which the high concentration was maintained at the aqueous side, vesicles containing the marker are rarely, if ever, found in the basal part of the endothelial cell (Figs. 5 to 10). It is most likely, therefore, that the situation seen in Fig. 11 represents reversal of transport direction after washing, to follow the gradient reversal. The vesicles containing ThO_2 which are found in the basal part of these endothelial cells are probably due to pinocytosis of the particles at the basal cell surface.

Evidence for the free diffusion of the colloidal particles similar to that found in bleb experiments (1) was seen in the experiment in which the bare apical surface of Descemet's membrane was exposed to ThO_2 . ThO_2 was found at all levels of the stroma, the greatest concentration of the marker being in a band only 10 to 20 microns below the basement membrane of the epithelium (Figs. 12 and 13). As in the bleb experiments, the stromal cells take up considerable amounts of the thorium dioxide (Fig. 12).

It was somewhat puzzling, therefore, to find that a 3 hour exposure of the bare stromal surface of the rabbit cornea (following removal of the epithelium) to ThO_2 did not result in a large accumulation of particles in the deep stroma. ThO_2 particles could be recognized to a depth of

only 10 to 15 microns from the bare surface (Fig. 14). Certainly, on the basis of the amount of ThO_2 found to have crossed the stroma when it was placed on a bare Descemet's membrane for only 30 minutes, one would expect to find a great deal of ThO_2 in the stroma when the bare subepithelial surface of the stroma is exposed to ThO_2 for 3 hours.

Several possible explanations for this finding may be advanced. There is some indication (Fig. 14) that the basement membrane is not completely removed by the stripping of the epithelium. The remnant of the basement membrane may act as a barrier to the rapid penetration of the ThO_2 particles into the stroma. It is also possible that the layer of irregularly arranged collagen fibers in the immediate subepithelial stroma (an area which in some vertebrate corneas examined in the light microscope appears as a distinct Bowman's membrane) does not permit so rapid a penetration by the relatively large colloidal markers as do the regular structures of Descemet's membrane and the deep stroma. It is conceivable, too, that there might be some precipitation or coagulation of the stromal ground substance at the bare surface which retards the rapid entry of the colloidal particles.

Cooling the cornea *in vitro* to 0°C has been shown to stop the presumed sodium "pump" in the epithelium, to lower the transcorneal potential difference, and to allow corneal hydration, presumably by interfering with normal metabolic processes (2, 14). In the present study, the cooling of corneas *in vitro* to as low as -4°C reduced the potential difference to less than 1 mv (Fig. 17) and appeared to reduce greatly the quantity of ThO_2 transported (Fig. 16). That it did not completely stop this transport is shown by the attachment of some particles at the apical surface of the endothelial cell, the formation of an occasional vesicle containing ThO_2 in the apical cytoplasm, and the occurrence of some ThO_2 in the intercellular space basal to the terminal bar.

If, as Brandt (17, 18), Schumaker (20), and Brandt and Pappas (19) have suggested, the surface attachment is by a process of adsorption, this attachment would not be reduced by cooling to 0°C . It is also possible that the surface tension changes which accompany the adsorption of particles onto the cell membrane may facilitate the formation of vesicles. The free energy decrease in the cell membrane following adsorption may in

some way be coupled to the mechanism of vesicle formation. Presumably, once a vesicle is formed, even random movement of the vesicle would eventually bring it into contact with the lateral cell membrane, where it may fuse with the cell membrane and discharge its contents into the intercellular space.

It is also possible that transport of particles in the manner proposed may be only indirectly dependent on the cellular metabolism. Future experiments in this area must be designed to test ways in which the transport may be inhibited or enhanced. Preliminary experiments with malonate inhibition indicate that this competitive inhibition may not affect the transport of particles by the corneal endothelium. It would be desirable to test the effect of more potent inhibitors of oxidative processes, such as cyanide and azide.

It would also be of interest to test the effects of substances known to enhance fluid transport in other systems. Pak Poy and Bentley (27) have demonstrated, in electron micrographs, the expansion of the intercellular space of toad bladder epithelium after the administration of posterior pituitary hormone to the isolated bladder. A similar study on the effect of posterior pituitary hormone on the transport of colloidal particles by the corneal endothelium might yield interesting results.

Studies in this laboratory have described the structure of the normal rabbit cornea (1) and have attempted to show how this morphology may be related to the fluid and ion transport phenomena which have been studied in the cornea. The use of electron-opaque particles to study the uptake of colloidal materials by the corneal endothelium *in vivo* and *in vitro* has led to these conclusions: In the rabbit, in which the intercellular space is isolated from the external environment by a terminal bar, a transport mechanism apparently exists to by-pass the terminal bar. This involves the attachment of particles to the apical cell surface, their internalization into vesicles, the movement of the vesicles from the apical to the lateral surface, and the liberation of the particles into the intercellular space at the lateral margin by fusion of the vesicular membrane with the cell membrane. A one-way flow down to Descemet's membrane is proposed to exist in the intercellular spaces of the rabbit endothelium on the basis of a comparison of the transport of particles into and out of the cornea across the endothelium. Observations on the penetration of colloidal particles at the epithelial sur-

face of the rabbit cornea do not demonstrate a transport system for these particles similar to that described by Donn, Maurice, and Mills (14) for the transport of sodium ions across the cornea from the epithelial surface. Nor, for that matter, is there found at the epithelial surface any indication of a two-way transport system similar to that found in the endothelium in the present study.

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of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service. This paper represents part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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