STUDIES ON THE CORNEA

I. The Fine Structure of the Rabbit Cornea and the Uptake and Transport of Colloidal Particles by the Cornea *in Vivo*

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ABSTRACT

Physiological studies have demonstrated that ions, as well as large molecules such as hemoglobin or fluorescein, can diffuse across and within the cornea. Most of the substrates for corneal metabolism are obtained from aqueous humor filling the anterior chamber. In order to receive its nutrients and in order to maintain its normal conditions of hydration, the avascular cornea must transport relatively large amounts of solute and solvent across the cellular layers which cover this structure. It has been suggested in the past that there may be a morphological basis for the transport of large amounts of solvents and solutes by cells by the mechanism of pinocytosis. The use of electron-opaque markers to study fluid movements at the electron microscope magnification level was described by Wissig (29). The present study describes the fine structure of the normal rabbit cornea and the pathways of transport of colloidal particles by the cornea *in vivo*. Rabbit corneas were exposed *in vivo* to suspensions of saccharated iron oxide, thorium dioxide, or ferritin by injection of the material into the anterior chamber. In other experiments thorium dioxide or saccharated iron oxide was injected into the corneal stroma, producing a small bleb. Particles presented at the aqueous humor surface of the rabbit corneal endothelium are first attached to the cell surface and then pinocytosed. It appears that the particles are carried around the terminal bar by an intracellular pathway involving the pinocytosis of the particles and their subsequent transport in vesicles to the lateral cell margin basal to the terminal bar. Particles introduced at the basal surface of the endothelium (via blebs in the corneal stroma) are apparently carried through the endothelial cells in membrane-bounded vesicles without appearing in the intercellular space. There appears to be free diffusion of these particles through Descemet's membrane and the corneal stroma. The stromal cells take up large quantities of the particles when blebs are injected into the stroma.

INTRODUCTION

The earliest electron microscopic studies of the cornea (1–7) were primarily concerned with the characterization of the fibrous components of the corneal stroma and Descemet's membrane. Later work (5, 8–11) dealt with the structure of the corneal epithelium in normal rat, mouse, and human tissue. Sheldon and Zetterqvist (12) described the corneal epithelium of the mouse with a vitamin A deficiency. Only Speakman (13) has described the structure of the corneal endothelium in the rabbit, and attempted to correlate the normal morphology with the physiological activity of the cornea.

Physiological studies by Maurice (14–16),

Davson (17), Langham (18, 19), and Cogan and Kinsey (20, 21) and Kinsey and Cogan (22) have demonstrated that ions, as well as larger molecules such as hemoglobin or fluorescein, can diffuse across and within the cornea. Donn, Maurice, and Mills (23, 24) have measured a transcorneal electrical potential *in vitro* and demonstrated that this potential is related to the "active" transport of Na ions from the epithelial surface across the cornea. Friedman and Kupfer (25) measured a similar potential *in vivo* and found that sodium competitors (Li and other cations) would reduce the potential.

The transparency of the cornea appears to depend upon its degree of hydration; increased hydration leads to swelling and loss of transparency. This may be reversibly demonstrated by cooling corneas to 0°C (17) and subsequently rewarming them. Maurice (15) has postulated that excess hydration causes disruption of the regular packing of the stromal collagen fibers. The loss of transparency would then be due to the creation of excessive scattering of the light rays.

Langham (18, 19) has shown that the cornea derives most of its O_2 supply from the atmosphere. Because the central cornea is avascular, Pirie and Van Heyningen (26) suggested that most of the substrates for corneal metabolism are obtained from the aqueous humor filling the anterior chamber.

In order to maintain its normal conditions of hydration and to receive its nutrients, the cornea must therefore transport relatively large amounts of solute and solvent across the cellular layers which cover this structure.

It has been suggested by Palade (27, 28), Wissig (29), and Brandt (30) that there may be a morphological basis (pinocytosis) for the transport of large amounts of solvents and solute by cells. That is, transport in quantity may be in "quanta" (28) and the morphological basis of this transport may be demonstrable in the electron microscope.

The use of markers to study fluid movements is a standard physiological technique. Evans blue, neutral red, or isotopes have been used for many years to determine fluid volume and exchange in cells and tissues. The use of electron-opaque markers to study similar processes at the electron microscope magnification level was described by Wissig (29), in studies on the transport of colloidal particles across the capillary endothelium.

The cornea provides an ideal system for the study of the manner in which cells transport colloidal particles (as an index of the transport of other solutes or solvents). Electron-opaque particles may be introduced into the anterior chamber of the eye *in vivo*, thereby exposing the apical surface of the corneal endothelium to the marker. Small blebs of marker may be injected intracorneally, and the diffusion of the particles within and across the cornea may be observed in fixed preparations examined with the electron microscope.

MATERIALS AND METHODS

Methods for the Study of the Normal Cornea

Adult albino rabbits were anesthetized with an injection of 60 mg of Nembutal (sodium pentobarbital, in solution) into an ear vein. A fixative solution containing 1 per cent osmium tetroxide buffered to pH 8.2 with M/14 veronal acetate solution and chilled to 0°C was injected into the anterior and posterior chambers of the eye. The eye was then rapidly enucleated, cut in half equatorially, and fixed for 30 to 90 minutes at 0°C in fresh 1 per cent OsO_4 in M/14 veronal acetate buffer at pH 8.2. After approximately two-thirds of the fixation time had elapsed, the lens and iris were removed and the central cornea was cut into strips up to 0.5 mm wide by 1 to 2 mm long. Care was always taken to avoid abrading the delicate endothelial cell layer. The strips were then fixed for the remainder of the particular fixation period used. The material was dehydrated in graded ethanol solutions and subsequently embedded in methacrylate containing 0.075 per cent UnO_3 (31).

In embedding the rabbit corneas, care was taken to orient the block in the capsule so that the face of the block which was seen at the bottom of the capsule contained epithelium, stroma, Descemet's membrane, and endothelium.

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

Adult albino or wild rabbits were anesthetized with an injection of 60 mg Nembutal (sodium pentobarbital, in solution) into an ear vein. Test solutions (0.1 to 0.5 ml) containing thorium dioxide suspensions (approximately 25 per cent ThO_2) were injected into the anterior and posterior chambers of the eye and allowed to remain in the eye for periods ranging from 1 hour to 2 days. These test solutions (Thorotrast, Testagar and Co.) were either dialyzed against ion-free water or used as the material came

from the manufacturer. Other test solutions containing suspensions of saccharated iron oxide (approximately 50 per cent Fe_2O_3) in a solution similar in composition to aqueous humor[1] (32) were also injected into the anterior and posterior chambers of rabbit eyes and allowed to remain for 20 minutes to 2 hours.

In order to determine whether colloidal particles are transported out of the cornea, small blebs were made by injecting approximately 0.05 ml solutions containing thorium dioxide or saccharated iron oxide directly into the stroma with a no. 30 hypodermic needle. These blebs were left in the cornea for 1 hour to 2 days prior to fixation.

At the end of the experimental period, the corneas were fixed, dehydrated, and embedded by the procedure described above for normal specimens.

Sectioning and Examination of Tissue

Blocks showing the best orientation of the epithelium, stroma, and endothelium were selected and trimmed so that sections would include all layers of the cornea.

Thin sections (400 to 800 A) were cut with a Porter-Blum microtome using a diamond knife. Thin sections were mounted on 200 mesh copper grids coated with a thin Formvar film. A film of carbon was then evaporated over the sections in order to minimize sublimation of the methacrylate in the electron beam.

In the cutting of both thick and thin sections, care was always taken to make the sections parallel to the long axis of the tissue block. This was done in order to minimize the possibility of colloidal markers' being moved by the action of the knife. By sectioning in this manner, whatever slight movement of the particles might occur would be at right angles to the direction of transport into or out of the cornea and could not appear as a false transport.

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

OBSERVATIONS

The Structure of the Rabbit Cornea

The intact rabbit cornea measures approximately 0.41 mm in total thickness (23). The cornea embedded in methacrylate varies in thickness from 0.4 mm to as much as 0.6 to 0.7 mm, probably because of swelling or disruption of the stroma during the dehydration and embedding procedures. The external surface of the cornea is covered with a stratified squamous epithelium which is approximately 35 to 40 microns thick. The underlying stroma comprises most of the remaining thickness of the cornea. Descemet's membrane is 8 to 10 microns thick, and the thickness of the endothelium (Descemet's mesothelium) is about 3.5 to 5 microns. In the rabbit, therefore, almost 90 per cent of the corneal thickness is due to the stroma.

Epithelium

The epithelium of the rabbit cornea (Fig. 1) can be divided into three layers: (1) a basal layer (*B*) of columnar cells, one cell thick; (2) an intermediate layer (*I*) of more regular polygonal cells, 1 to 2 cells thick; (3) a layer of flattened cells, 5 to 6 cells thick, which ranges from the "wing" cells adjacent to the intermediate layer to the squamous cells (*S*) of the outer surface.

The nuclei of the basal cells are elongated, as are the cells, in a direction normal to the plane of the corneal surface. Those in the surface cells are elongated parallel to the plane of the cornea, as might be expected in these flattened cells.

Interdigitations of the cell surfaces (Figs. 1 and 2) identical with those described by Jakus (5, 33) in the rat and human corneas, and by Sheldon (10) in the mouse cornea, are found in all layers of the rabbit corneal epithelium but are most frequent in the intermediate and surface layers. The lateral margins of the basal cells have few interdigitations, although complex foldings are sometimes found in the lateral margins near the base of the cell. The free surface of the superficial cells bears processes identical with those forming the interdigitation of cell membranes in other parts of the cornea. Sheldon (10) had described these processes of the mouse cornea as "microvilli," but it is unlikely that they bear any relation to the microvilli composing the brush borders of other specialized epithelia (34, 35).

The cell surface of the outermost layer of cells is covered with an apparently amorphous coating of moderate density. This is probably similar to the extraneous coats described by Chambers (36, 37) in his micromanipulation studies and to the amorphous coats found by Burgos (38) in the epididymal and kidney epithelia.

[1] Composition of artificial aqueous humor: NaCl (0.90 per cent), 100.0 ml; KCl (1.15 per cent), 5.0 ml; $CaCl_2$ (1.22 per cent), 1.0 ml; $MgSO_4/7H_2O$ (3.82 per cent) 0.6 ml; KH_2PO_4 (2.11 per cent), 1.0 ml; $NaHCO_3$ (1.30 per cent), 42.0 ml; dextrose (12.00 per cent), 1.0 ml; total, 150.6 ml. A mixture of 5 per cent CO_2 and 95 per cent O_2 is bubbled through the solution until the pH is 7.38 (32).

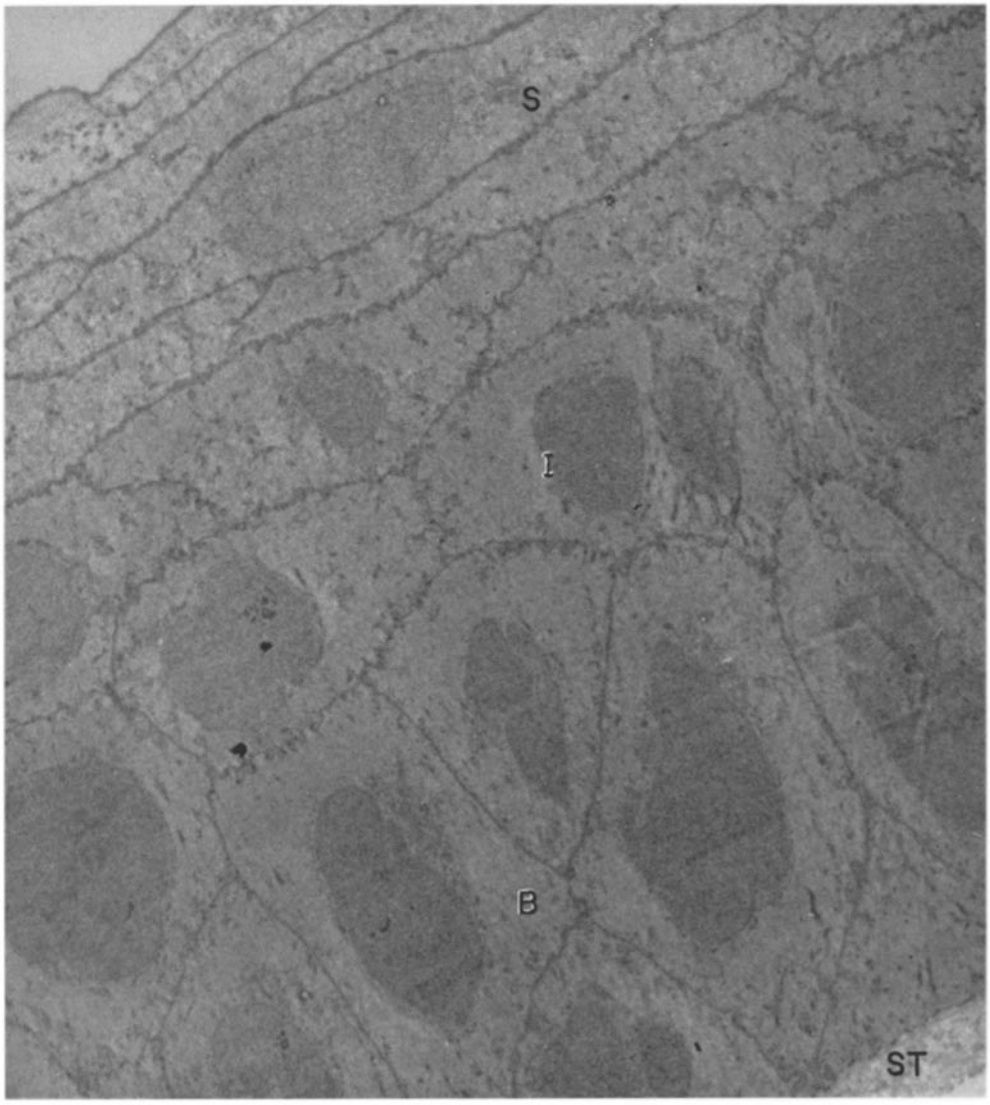


FIGURE 1

A low power electron micrograph of the rabbit corneal epithelium. The columnar basal cells (*B*), the cuboidal cells of the intermediate layer (*I*), and the flattened "wing" and squamous cells (*S*) of the outermost layer may be seen. Numerous interdigitations are visible at adjacent cell borders at all levels except the lateral margins of the basal cells. A part of the stroma is shown at *ST*. × 4000.

Typical desmosomes, similar to those described by Odland (39) in human epidermis and by Jakus (9) in the corneas of various vertebrates, are regularly found in the cell membranes in the intermediate and surface layers and occasionally on the lateral margins of the basal cells (Fig. 2). Desmosomes or, more precisely, half desmosomes are found apparently attaching the basal plasma membrane of the basal cells to the basement membrane which underlies the epithelium (Fig. 3).

The most striking feature of the cytoplasm of the epithelial cells is the paucity of organelles. Very few granular, or even smooth, vesicular components of the endoplasmic reticulum are found in the intermediate and surface cells. Some flattened cisternae of the endoplasmic

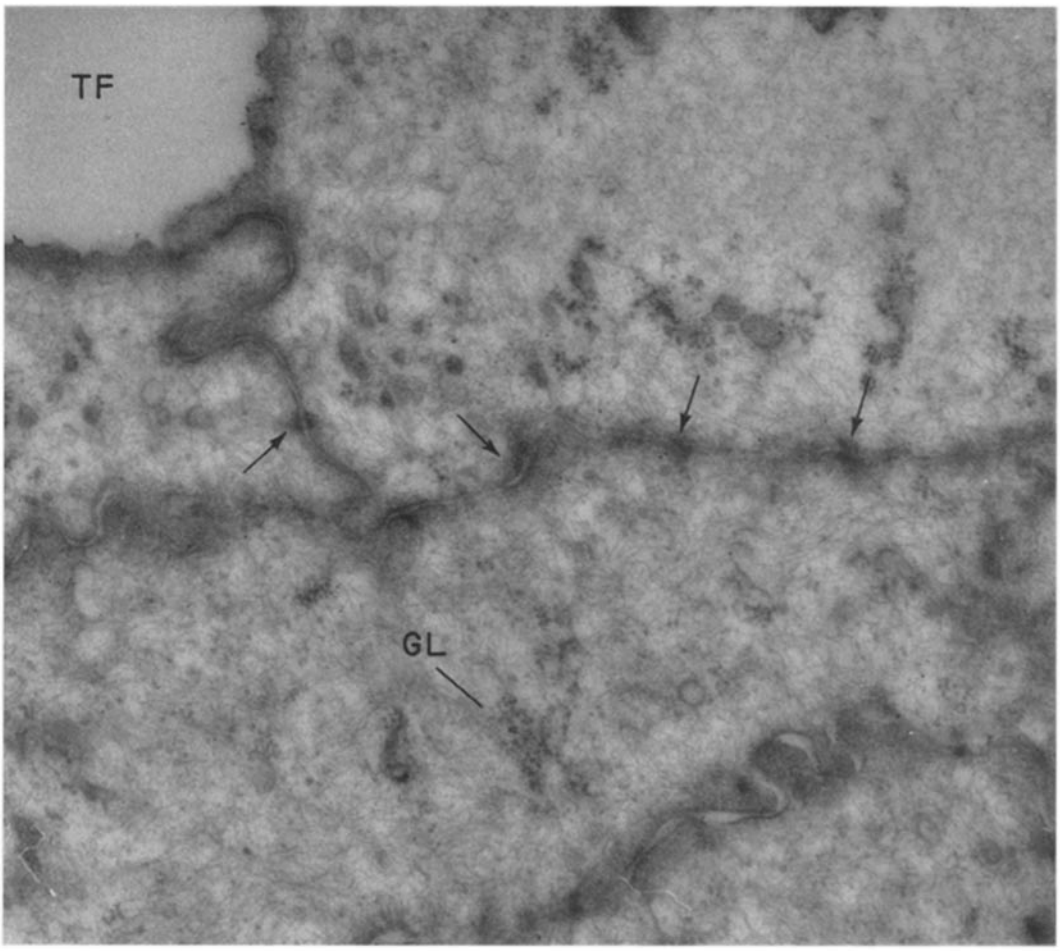


FIGURE 2

An electron micrograph of a part of the epithelium of a rabbit cornea. The tear fluid surface is indicated by *TF*. Numerous desmosomes (arrows) may be seen between or along the interdigitating processes of the cells. The cytoplasm of all the cells is filled with fine fibrils, 50 A or less in diameter. Clusters of small dense particles which probably represent glycogen (*GL*) are seen in several of the cells. Note the paucity of mitochondria, endoplasmic reticulum, and other cell organelles. × 38,000.

reticulum are found in the basal part of the basal cells. Small granules probably containing glycogen are also found in the epithelial cells and are arranged randomly, or occasionally in rosettes (*GL*, Fig. 2). Typical Golgi complexes are, however, often encountered even in the squamous cells. Mitochondria are small (0.2 to 0.3 micron in diameter, 0.5 to 1.0 micron long), simple, and infrequently found in the epithelium. When seen in cross-section, the mitochondria of the rabbit corneal epithelium often show a profile of only a single crista.

Fine fibrils are the most common structures found in the epithelial cells, and these appear similar to those first described by Jakus (5) in phosphotungstic acid–stained sections of the rat cornea. These fibrils measure 50 A or less in diameter, and are of indeterminate length. They fill most of the ground substance of the epithelial cells. They are seen quite clearly even in unstained preparations (Figs. 2 and 3). In the surface cells they generally run in a direction parallel to the long axis of the cells. The same is true in the basal cells. Jakus (5) has ascribed the birefringence of

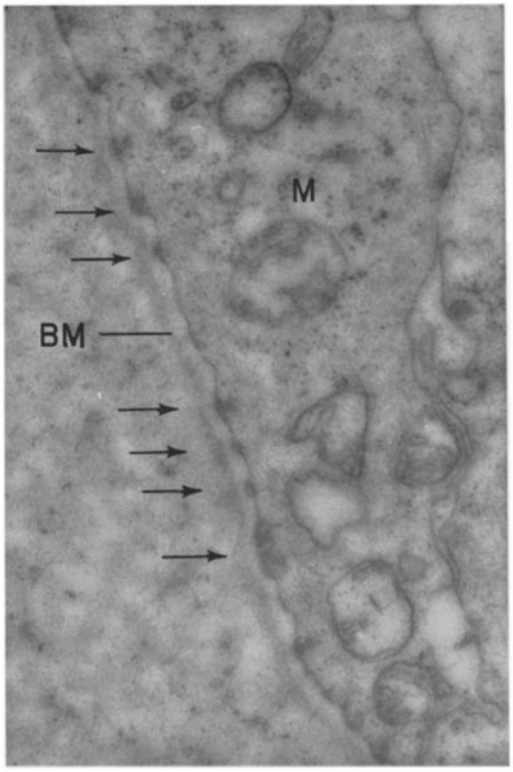


FIGURE 3

An electron micrograph of the basal part of the rabbit corneal epithelium. The half desmosomes which apparently "attach" the basal cell membrane to the underlying basement membrane (*BM*) are well demonstrated in this preparation (arrows). Mitochondria (*M*) may be seen among the fine fibrils which fill the basal cell cytoplasm. × 32,000.

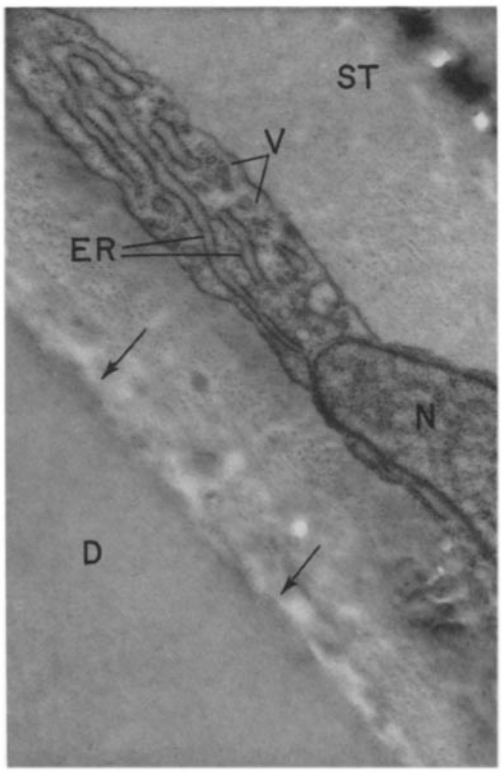


FIGURE 4

An electron micrograph of rabbit cornea which shows part of Descemet's membrane (*D*) and a part of a stromal cell in the adjacent stroma (*ST*). Large flattened cisternae of the rough surfaced (granular) endoplasmic reticulum (*ER*) may be seen in the stromal cell. Numerous large and small vesicles (*V*) are found in the cytoplasm. The collagen fibers of the stromal lamellae are cut transversely in this section. There is some indication of the fibrous nature of Descemet's membrane at its junction with the stroma (arrows). *N*, nucleus. × 17,000.

these cell layers to the orientation of these fine fibrils.

Cross-sections of small unmyelinated nerve fibers are occasionally found in the intercellular spaces just above the basement membrane of the epithelium. The structure of such nerves in the mouse has been described by Whitear (11), and it is possible that the structures lying just above the basement membrane in rat corneal epithelium, which were identified by Jakus (5) as mitochondria, may be cross-sections of nerves.

Underlying the epithelium in the rabbit there is a distinct basement membrane which measures between 100 and 200 A in width (dense zone, Fig. 3). A light zone separates the basal plasma membrane of the basal cell from this basement membrane in some places, while the half desmosomes of the basal plasma membrane appear firmly attached to the basement membrane in others.

Bowman's Membrane

The existence of a distinct Bowman's membrane in the rabbit cornea is dubious. Jakus (5, 33) has suggested that what classically has been called Bowman's membrane may be a somewhat less regularly organized portion of the stroma subjacent to the epithelium. This condition of reduced

regularity appears to exist in the rabbit stroma immediately subjacent to the epithelium (Fig. 3).

Stroma

The stroma, which comprises the major portion of the rabbit cornea, is similar in structure to that of the mouse (11), rat (5), and human corneas (33). It is composed of layers of collagen fibers which appear to run from border to border of the cornea. The orientation of the fibrils in any layer is approximately at right angles to the fibrils in the adjacent layers. The fine structure and periodicity of the collagen in these bundles is not well demonstrated in these unstained preparations.

Stromal cells appear between the layers. These cells are much flattened and elongated and have numerous processes (Fig. 4). The stromal cells contain elongate mitochondria and flattened cisternae of the granular (rough surfaced) endoplasmic reticulum (*ER*), as well as numerous small and large vesicles (*V*). Some of these vesicles are reminiscent of pinocytotic vesicles (27), while others appear to be elements of the agranular endoplasmic reticulum. The cell is widest at the nuclear region. The greatest concentration of cytoplasmic organelles is to be found in the perikaryon, although representative examples of the organelles may be found even in the most attenuated processes of the cell.

Descemet's Membrane

In the unstained preparations examined in this study, Descemet's membrane exhibits a homogeneous fine granularity (Fig. 4). However, in slightly thick sections observed at high magnification an indication of the fine fibrillar nature of this membrane which Jakus (6) described in bovine and other corneas can be found. At high magnification, fine dense fibrils which are closely packed in a matrix of approximately the same density are seen. The fibrils appear to run primarily in the plane parallel to the surface of the cornea.

The basal plasma membrane of the endothelium (which is in contact with Descemet's membrane) appears somewhat denser than the apical plasma membrane (Fig. 5), and frequently shows thickenings which resemble the desmosomal structures at the junction of the epithelium and the basement membrane. No basement membrane similar to that seen under the epithelium is visible beneath the endothelium.

Endothelium

The endothelium (Descemet's endothelium or mesothelium) is a flattened layer of cells attached to Descemet's membrane and lining the side of the cornea exposed to the anterior chamber. This cellular layer most nearly resembles other mesothelia, particularly those of the peritoneum (40–42). The cells are flattened hexagonal blocks (13), the diameter of the hexagon being roughly 3 to 4 times the height of the cell. The nucleus is large and compressed in the direction of the flat plane of the cell. Many ovoid mitochondria are visible, and these often show complex villous or concentric lamellar types of cristae (Fig. 6). There is a large amount of the granular (rough surfaced) component of the endplasmic reticulum. The membranes of the rough surfaced reticulum may be seen to be in direct continuity with those of the agranular vesicular reticulum (Fig. 6, *A*). The preparations used in the present study always show many small vesicles indicative of pinocytosis (27) in the apical and basal cytoplasm (Fig. 6).

The intercellular space of the rabbit corneal endothelium, although very variable in size, is less convoluted than that described in the human (33) or rat (5). The width of this space ranges from approximately 100 A to more than 0.5 micron. This variation in the width of the intercellular space was found in all the preparations in this study and is in agreement with the data of Speakman (13).

A terminal bar is always found a short distance below the apical end of the intercellular space (Figs. 5 and 6, *T*). Since in no case were terminal bars found to be absent in this position, it is reasonable to assume that this structure is continuous in three dimensions. Oblique sections of the endothelium which are approaching the tangential demonstrate a long continuous attachment zone between adjacent cells as far as the section stays superficial. A tongue or flap-like projection of the cell usually covers the apical end of the intercellular space above the terminal bar.

A thin amorphous coating of material of moderate density lies on the apical surface of the endothelial cells (Fig. 6, at arrows), and resembles the previously described coating of the free surface of the epithelium.

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

Injection of suspensions of thorium dioxide or saccharated iron oxide into the anterior chamber of the rabbit eye produced variable results in terms of the uptake of these colloidal particles.

Only a few particles could be found on the surface of the endothelial cells if the cornea was fixed after a 1 hour exposure to 0.1 ml of thorium dioxide in the anterior chamber. No thorium particles have been found within the cytoplasm of the cell either free or in vesicles, in the specimens examined so far.

The situation was quite different in rabbits whose corneas were fixed 2 days after the injection of 0.1 ml of thorium dioxide into the anterior chamber. The eye was slightly cloudy before fixation and the cornea somewhat swollen. The surface of the endothelium was devoid of attached particles, but large membrane-bounded vacuoles containing dense accumulations of the ThO_2 were visible in the cytoplasm (Fig. 7). Some free particles could be seen at the border of the endothelial cell and Descemet's membrane.

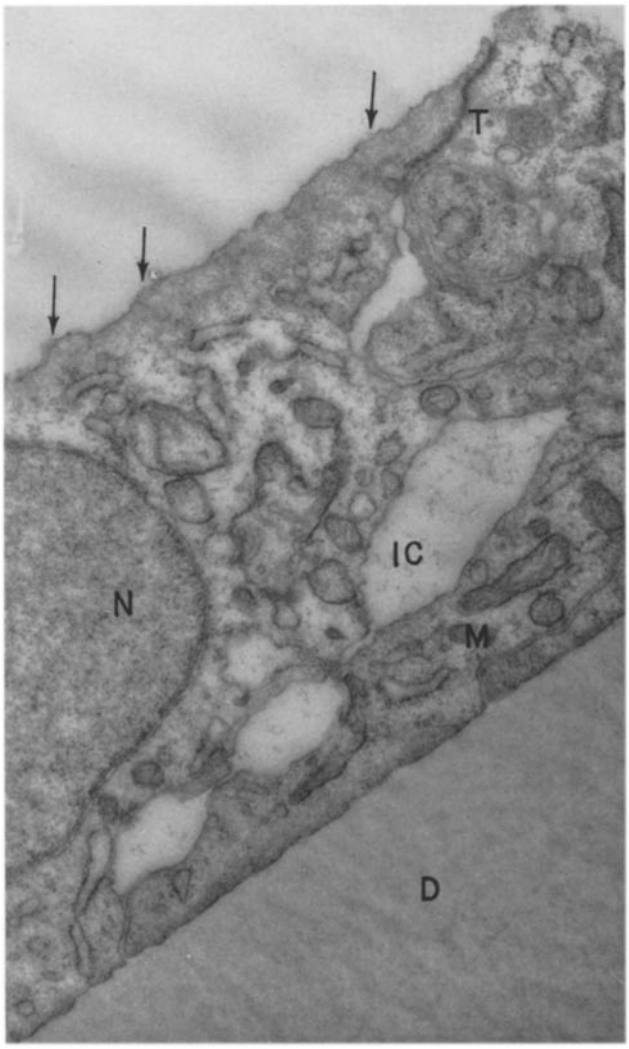


FIGURE 5

An electron micrograph of part of the rabbit corneal endothelium and the underlying Descemet's membrane (*D*). The basal membranes of the endothelial cells are denser than the apical cell membranes. A terminal bar (*T*) is found near the anterior chamber end of the intercellular space (*IC*). This space is very variable in width, ranging from approximately 100 A in the terminal bar region to almost 0.5 micron at its most expanded portion. Cross- and longitudinal sections of numerous mitochondria (*M*) may be found in the cytoplasm. An apparently amorphous material of intermediate density may be seen at the apical surface of the endothelial cell (arrows). *N*, nucleus. × 25,000.

The fact that the cornea was not perfectly healthy after this long exposure to the poisonous and radioactive thorium dioxide particles is also evidenced by the finding of leucocytes within the endothelial layer, usually contained in invagina-

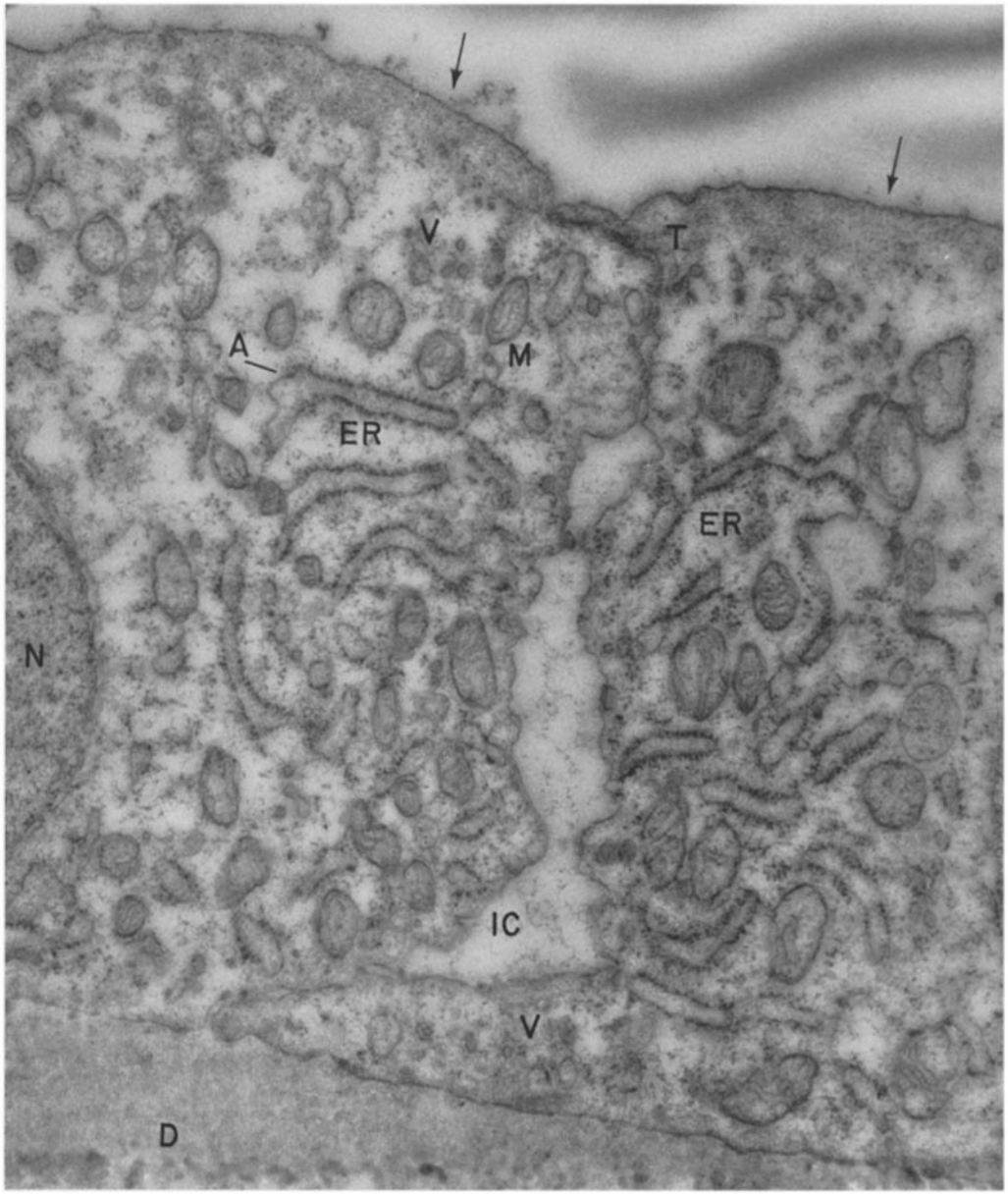


FIGURE 6

This electron micrograph shows parts of two rabbit corneal endothelial cells and of Descemet's membrane (*D*). Numerous mitochondria (*M*), small and large vesicles (*V*), and profiles of the cisternae of the endoplasmic reticulum (*ER*) are clearly visible. Continuity of the membranes of the rough and smooth surfaced elements of the endoplasmic reticulum is shown at *A*. A terminal bar (*T*) is shown at the apical end of the intercellular space (*IC*). Amorphous material of intermediate density is found at the apical surface of the cells (arrows). *N*, nucleus. × 35,000.

tions of the basal surface of the endothelial cells (Fig. 7).

After a 1 hour exposure to 0.2 ml of saccharated iron oxide injected into the anterior chamber, clumps of saccharated iron oxide were found on the apical surface of the endothelium. Both large and small vesicles containing Fe_2O_3 were found in the cytoplasm (Fig. 8). Large numbers of particles, usually in clumps, were found in the intercellular spaces below the terminal bars. Numerous free particles were found accumulated

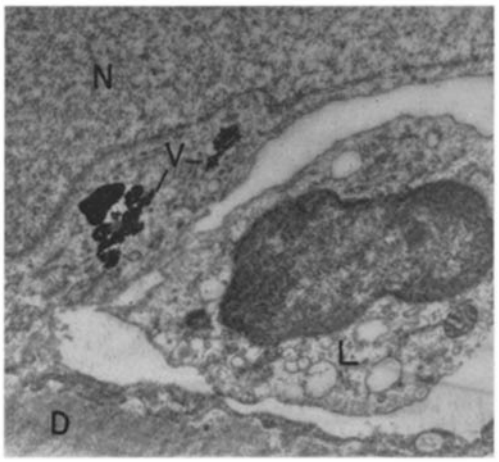


FIGURE 7

An electron micrograph of the basal part of an endothelial cell from a cornea which had been exposed to a suspension of thorium dioxide injected into the anterior chamber and left there for 2 days prior to fixation. Two large vacuoles (*V*) containing dense masses of ThO_2 are found below the nucleus (*N*). The eye was cloudy and the cornea swollen at the time of enucleation. A lymphocyte (*L*) has apparently penetrated between the basal cell surface and Descemet's membrane (*D*). × 24,000.

between Descemet's membrane and the endothelium, and a few particles appeared diffusely in the adjacent part of Descemet's membrane (Fig. 8).

Considerable internal membrane structure is found in the large particle-containing vacuoles in the rabbit corneal endothelium (Fig. 8). This membrane component may take the form of parallel membranes resembling cristae mitochondriales; it may appear as numerous small vesicles within the larger vacuole; or it may appear as complex concentric membranes at the periphery of the vacuole or forming a bull's-eye-like structure within the internum of the vacuole.

In an effort to study the movement of materials within the stroma of the cornea and to determine the possible routes by which materials may leave the cornea, small amounts (1 to 2 mm^3) of either thorium dioxide or saccharated iron oxide suspensions were injected into the stroma, producing a small bleb.

Eyes were fixed either 1 hour or 2 days after the injection. The area of the bleb (the original outlines of which were still clearly visible after 2 days) and its immediately surrounding tissue were fixed and embedded in the usual manner.

In the central part of the bleb, the concentration of opaque particles was so high as to obscure both cellular and extracellular detail. At the periphery of the bleb, where there was still a great concentration of colloidal material, it was possible to study the pattern of the colloidal particles and the reaction of the stromal cells to them (Fig. 9).

Fig. 9 shows a part of the stroma in the periphery of a ThO_2 bleb which had been left in the cornea for 1 hour. It can be seen that the thorium dioxide particles have diffused through the stroma in all directions, and have penetrated the spaces between the collagen fibers. There appears to be a piling up or concentration of the particles at the membrane of the stromal cell, and large as well as small vesicles containing ThO_2 particles may be seen in the cytoplasm of the stromal cell.

Fig. 10, from this same preparation, shows a part of another stromal cell in which small vesicles containing thorium dioxide appear to be formed by pinching off from the bases of deep invaginations of the cell surface (arrows). The large vesicles found in these cells probably represent a fusion of many small vesicles, some of which may appear in cross-section to contain only a single particle, as in Fig. 10.

If a bleb of thorium dioxide is left in the stroma for 2 days, most of the thorium is no longer in the acellular part of the stroma by the time the tissue is fixed (Fig. 11). The stromal cells, however, contain vacuoles with dense concentrations of particles in their interiors. In Fig. 11, the close relationship between the collagen fibers and the stromal cell membrane is particularly well demonstrated.

In Figs. 12 and 13, each of which shows a portion of Descemet's membrane and an overlying endothelial cell taken from a 1 hour bleb and a 2 day

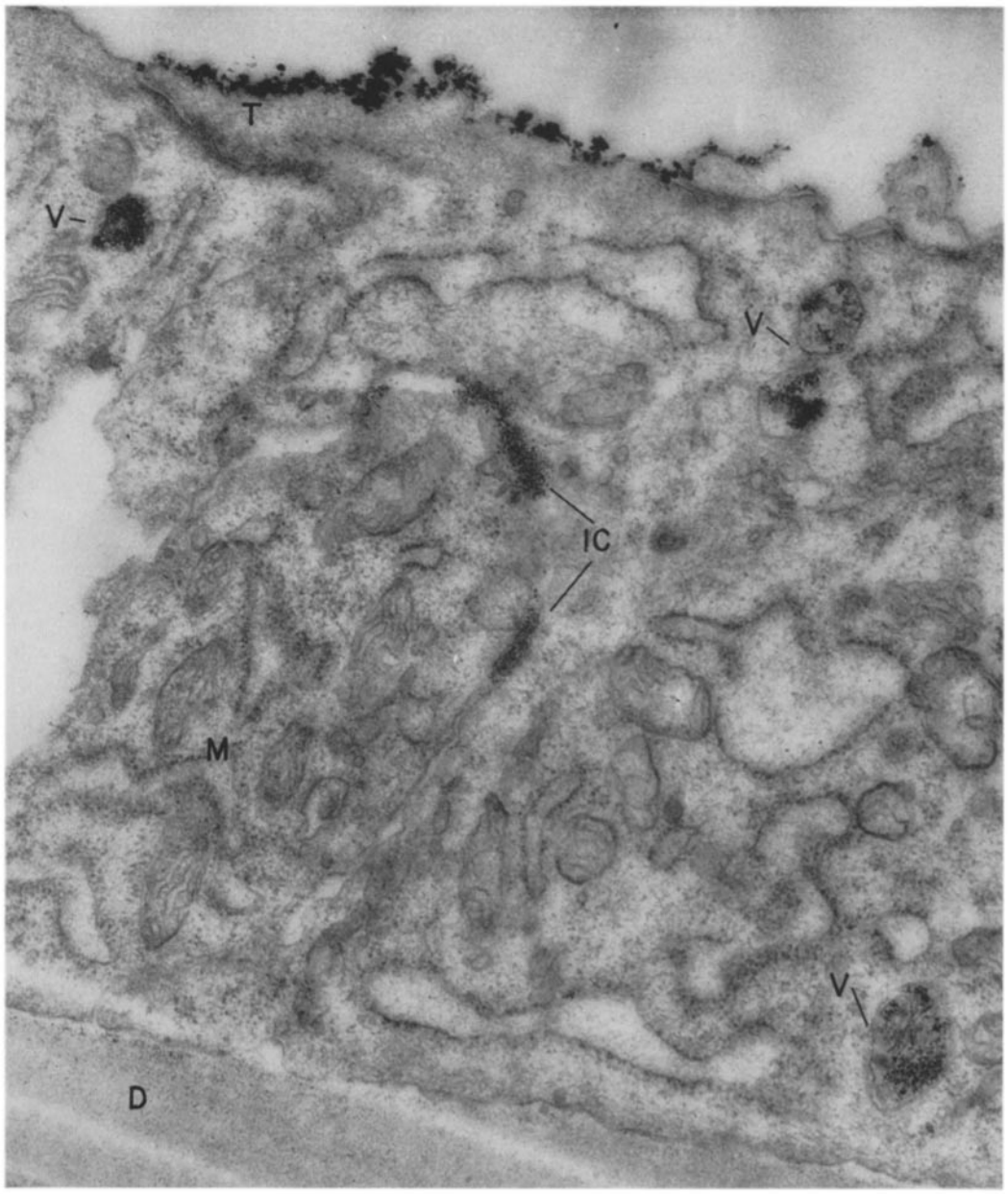


FIGURE 8

An electron micrograph showing a part of the endothelium of a rabbit cornea which had been exposed for 1 hour to a suspension of saccharated iron oxide injected into the anterior chamber. Dense accumulations of the iron oxide particles are found at the cell surface. Several large vacuoles (*V*) contain iron oxide particles. These vacuoles are probably formed by the fusion of smaller vesicles. Iron oxide particles are also found in clumps in the intercellular space (*IC*). A typical terminal bar (*T*) and numerous mitochondria (*M*) are present. *D*, Descemet's membrane. × 40,000.

bleb preparation respectively, it is clearly shown that the thorium dioxide particles can freely penetrate Descemet's membrane traveling in the outward direction. Particles of ThO_2 may be seen at the endothelial surface of Descemet's membrane, and contained in vesicles of various sizes

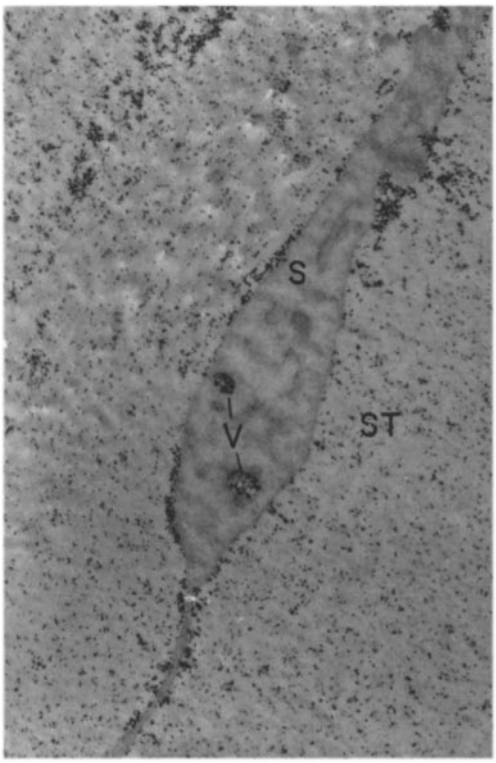


FIGURE 9

This electron micrograph shows a part of the stroma of a rabbit cornea into which an intrastromal bleb of thorium dioxide was injected 1 hour prior to fixation. ThO_2 particles have diffused throughout the stroma (*ST*) and appear accumulated at the membrane of the stromal cell (*S*). Vacuoles (*V*) containing particles of ThO_2 are found in the cytoplasm of the stromal cell. × 16,000.

located at all levels of the endothelial cell cytoplasm. The vesicles are presumably formed by pinocytosis of the particles at the basal surface of of the cell. Some ThO_2 particles may even be seen attached to the free surface of the apical cell membranes, apparently having been transported across the cell. The particles appear to have been liberated at this surface by the fusion of the vesicular membrane with the apical cell membrane and the subsequent release of the contents of the vesicle. This would represent an inversion of the process of pinocytosis in which particles are internalized into vesicles at the apical surface when they have been placed in contact with that surface by injection into the anterior chamber, or by *in vitro* techniques (43).

Fig. 13 shows a view of a bleb prepared 2 days previously. In this instance, the concentration of ThO_2 particles at the border of Descemet's membrane and the endothelial cell is greater than that seen in the 1 hour bleb preparations (Fig. 12). The vesicles containing thorium dioxide in the endothelial cell cytoplasm also appear to be more numerous and to have a more concentrated content of particles.

Although moderately large amounts of ThO_2 are found accumulated at the border of Descemet's membrane and at the basal surface of the endothelial cell, very few or, in most cases, no ThO_2 particles are present in the intercellular space despite the fact that this space may be quite expanded as it approaches Descemet's membrane (Figs. 12 and 13). Large numbers of particles have been noted in the intercellular space (Fig. 8) when the colloidal particles were placed in the anterior chamber and allowed to travel into the cornea.

Similar results occur whether saccharated iron oxide or thorium dioxide particles are used as the markers in bleb experiments. The slight modifications observed with the iron oxide particles apparently are due to their particular clumping behavior (Fig. 14).

Fig. 14 shows a portion of rabbit corneal stroma from a preparation in which a bleb of saccharated iron oxide had been produced 1 hour before fixation. This section was taken near the edge of the dense part of the bleb, *i.e.*, the red-brown color of the bleb was plainly visible in the block. Note the numerous large and small clumps of iron oxide particles, and a part of a stromal cell which exhibits several vesicles containing colloidal particles. There appears to be an accumulation of particles at several places along the membrane of the stromal cell. The distribution of particles in the stroma seems less regular than that found in ThO_2 blebs. Part of this difference may be due to a clumping of the saccharated iron oxide, and part may also be due to the fact that the small iron oxide particles (30 to 50 A) are difficult to

recognize when they occur singly or in very low concentrations.

Fig. 15 shows a part of a stromal cell found some distance from the central part of the bleb. While iron oxide particles are observed infrequently and with some difficulty in the stroma (arrows), a large vacuole containing a dense mass of colloidal particles is found in the cytoplasm.

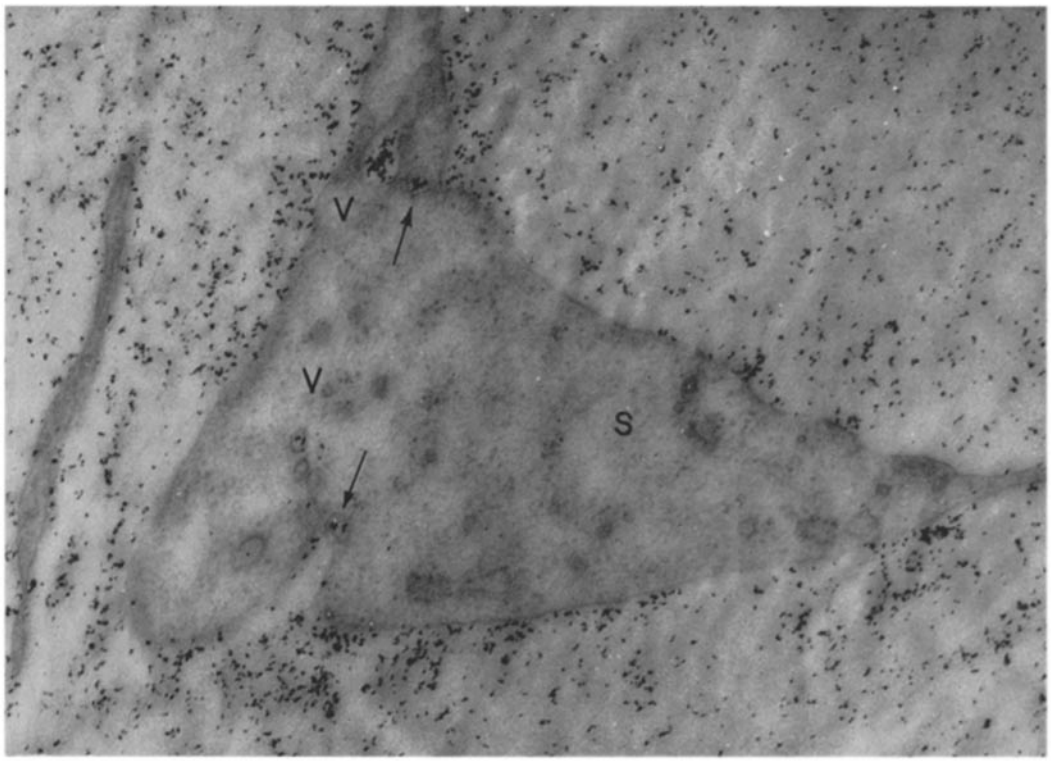


FIGURE 10

This electron micrograph shows another part of the stroma of the cornea described in Fig. 9. ThO_2 particles are present throughout the stroma. Large and small vesicles (*V*) containing the ThO_2 particles are found in the cytoplasm of the stromal cell (*S*). Vesicles containing particles appear to be pinching off from deep invaginations of the cell surface (arrows), apparently demonstrating pinocytosis of the thorium dioxide by the stromal cell. × 40,000.

Parts of two mitochondria (*M*) and several flattened cisternae of the granular endoplasmic reticulum (*ER*) are particularly well demonstrated in this stromal cell.

In contradistinction to the results obtained with thorium dioxide, large numbers of iron oxide particles do not accumulate at the apical surface of Descemet's membrane. This may be due partly to the fact that the clumping reduces the number of freely diffusing particles, or it may be due to the fact that the small iron oxide particles are difficult to identify with certainty when they are in low concentration.

In bleb experiments with both thorium dioxide and saccharated iron oxide, the location of the droplet was usually closer to the endothelium than to the epithelium. Unfortunately, therefore, the concentration of particles which diffused as far as the basement membrane of the epithelium was too low to permit any conclusions as to whether these particles would pass through this structure. *In vitro* observations on the diffusion of particles through the stroma (43) indicate that the basement membrane of the epithelium may be a barrier across which the particles do not pass readily.

DISCUSSION

Early histological studies of the cornea (44) established its basic structure as composed of a stratified squamous epithelial outer surface, a fibrous lamina propria, a glassy membrane deep to the lamina propria (Descemet's membrane), and a

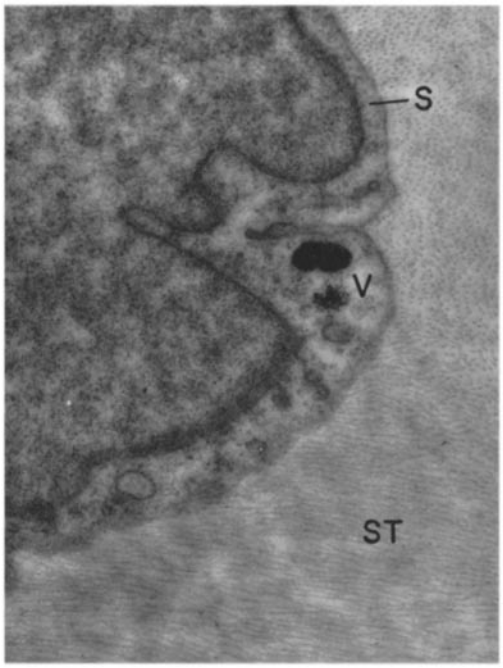


FIGURE 11

An electron micrograph of a part of the stroma of a rabbit cornea in which an intrastromal bleb of thorium dioxide had been produced 2 days prior to fixation. In this instance the ThO_2 particles seem to have disappeared from the acellular part of the stroma (*ST*). However, vacuoles (*V*) containing dense accumulations of thorium dioxide particles are found in the cytoplasm of the stromal cell (*S*). The intimate association of the collagen fibers of the stroma with the cell membrane of the stromal cell is well demonstrated in this figure. × 17,000.

membrane one cell thick lining the surface exposed to the aqueous humor (the endothelium, Descemet's endothelium, etc.). These studies did little, however, to further the understanding of the origin and maintenance of corneal transparency. Later light microscopic studies established the existence of a definite basement membrane under the epithelium (45). Other studies demonstrated the mucopolysaccharide nature of the stromal ground substance (46, 47) and provided some information on the innervation of the cornea (48, 49).

The demonstration of the mucopolysaccharide nature of the ground substance of the stroma and the later chemical demonstration of the collagenous nature of the fibrous component of the stroma permitted speculation on the cause of the excess hydration often seen in corneal pathology. However, these studies did not provide a morphological basis, especially at the cellular level, for the physiological characteristics of the cornea in relation to the transport or diffusion of ions, water, etc., which were under investigation by numerous workers (14–22, 50, 51).

Early electron microscopic studies of the cornea (1–8) were primarily concerned with the characterization of the fibrous components of the stroma and Descemet's membrane and to some degree with the structure of the epithelium (5, 10, 12).

Until now, only one author has been primarily concerned with the structure of the endothelium with respect to its permeability characteristics. Speakman (13) noted lack of penetration of stains into formalin-fixed flat preparations of rabbit, cat, and monkey corneal endothelium, but observed hexagonal outlining of the cells and apparent filling of the intercellular channels by the dye precipitates. In the first published electron micrographs of reasonably well preserved rabbit corneal endothelium he also described "relatively wide S-shaped intercellular spaces . . . broken into compartments by adhesions between adjacent cell membranes." Speakman also noted the occurrence of numerous mitochondria throughout the cytoplasm. Because of the relatively low resolution of his electron micrographs he did not find any intracellular vacuoles and he therefore believed that pinocytosis probably did not occur. He concluded, on the basis of the lack of vacuoles and lack of infolded cell membranes, that the corneal endothelium was not involved in water transport.

Examination of the electron micrographs of the normal rabbit corneal endothelium made during the present study reveals numerous small cytoplasmic vesicles and vacuoles especially in the apical part of the cell (Fig. 6). Studies by Palade (27, 28), Bennett (52), and Brandt and Pappas (53) suggested that pinocytosis was a major component of cellular activity in cells

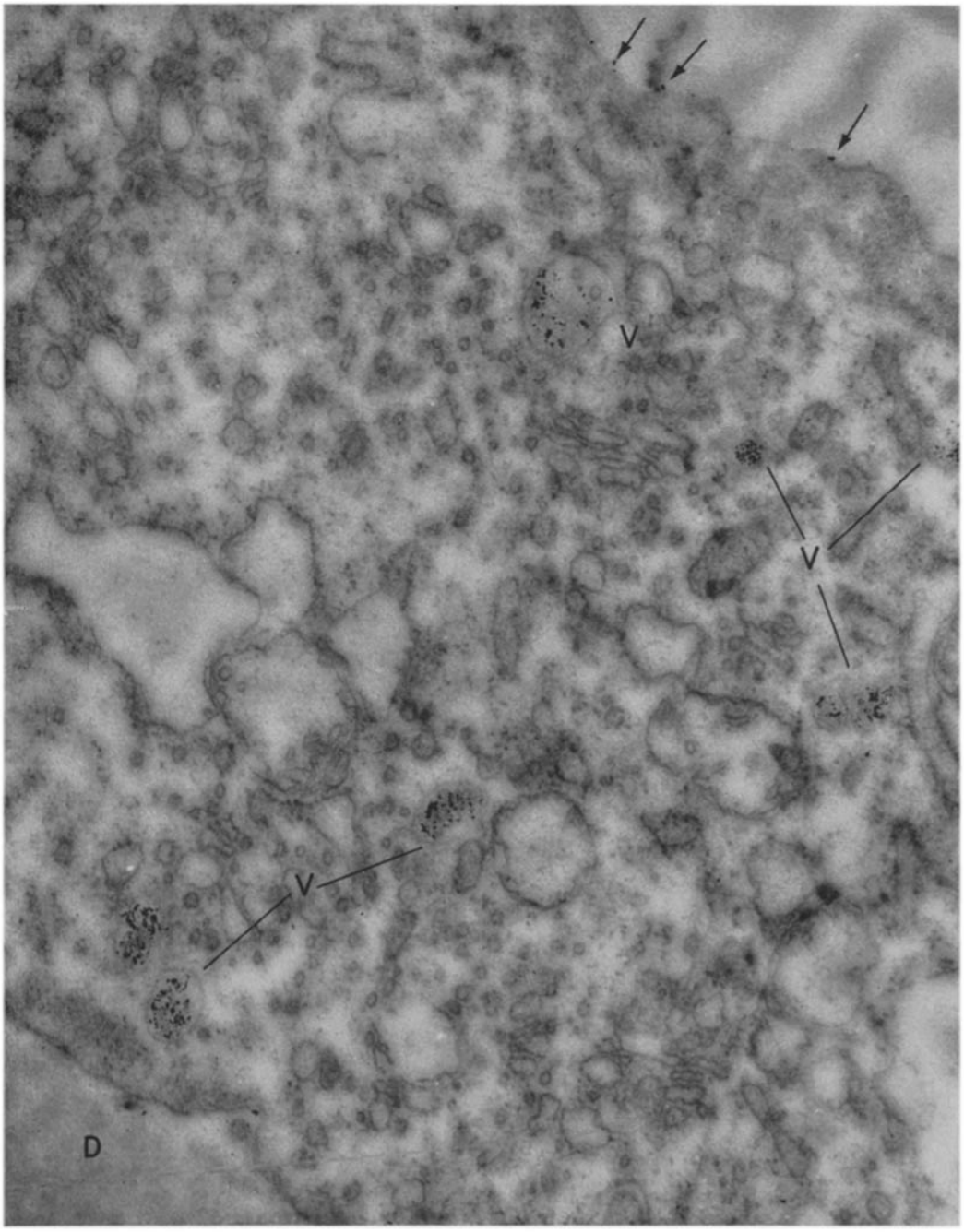


FIGURE 12

An electron micrograph of a part of the endothelium of a rabbit cornea in which an intrastromal bleb of thorium dioxide had been produced 1 hour prior to fixation. ThO_2 particles which are traveling out of the cornea across the endothelium are found at the junction of Descemet's membrane (*D*) and the basal surface of the endothelial cell. Vesicles (*V*) containing thorium dioxide are seen at all levels of the endothelial cell cytoplasm. Particles are also found at the apical surface of the cell (arrows), apparently having been transported across the cell in vesicles. No particles are observed in the intercellular space. × 45,000.

engaged in fluid transport. The finding (Figs. 5 and 6) of terminal bars, or adhesion plates, at the apical end of the intercellular space between rabbit corneal endothelial cells led to the assumption that free diffusion might be hindered in the intercellular space and that a mechanism such as pinocytosis might be necessary for some aspects of transport across the endothelium.

The data of Maurice (15, 54) reveal that small ions (approximately 5 A in diameter) apparently penetrate the endothelium rapidly and at an approximately uniform rate when placed on the stromal side of the cornea. As the size of the ion was increased, the rate of penetration across the endothelium decreased, or, as Maurice puts it, the "obstruction" to penetration increased. From these data Maurice concluded that this diffusion out of the cornea through the endothelium was occurring in channels, probably the intercellular spaces. He derived an approximation of the width of the intercellular space as 80 to 100 A if he assumed minimal tortuosity to exist in these spaces. The electron micrographs of the normal rabbit cornea in this study and that of Speakman (13) suggest that the intercellular space may vary from Maurice's postulated figure of approximately 100 A to as much as 1000 to 5000 A. The tortuous nature of the intercellular space demonstrated by Jakus (33) in the human cornea and noted in the frog cornea in this laboratory (55) is not marked in the rabbit.

On the basis of the finding of regular terminal bars in the rabbit corneal endothelium, and the fact that the intercellular spaces (Figs. 5 and 6) may be 10 to 50 times the width calculated by Maurice from penetration studies on the rabbit cornea, it seems more likely that the barrier to penetration of large molecules may be located in the terminal bar. In an electron microscopic study of hemoglobin proteinuria, Miller (56) found that the terminal bars of the kidney tubular epithelium appear to stop the hemoglobin molecules from penetrating into the intercellular spaces. If this is the case in the cornea, then some mechanism other than simple diffusion must be operating to transport large molecules across the corneal endothelium. Frog corneal endothelium, showing no terminal bars, might be expected to behave differently from rabbit corneal endothelium in penetration experiments, and in fact it does (55).

The fine structure of the epithelium of the rabbit is similar to what has been described by previous workers in the rat (5), mouse (10, 11), and human (33).

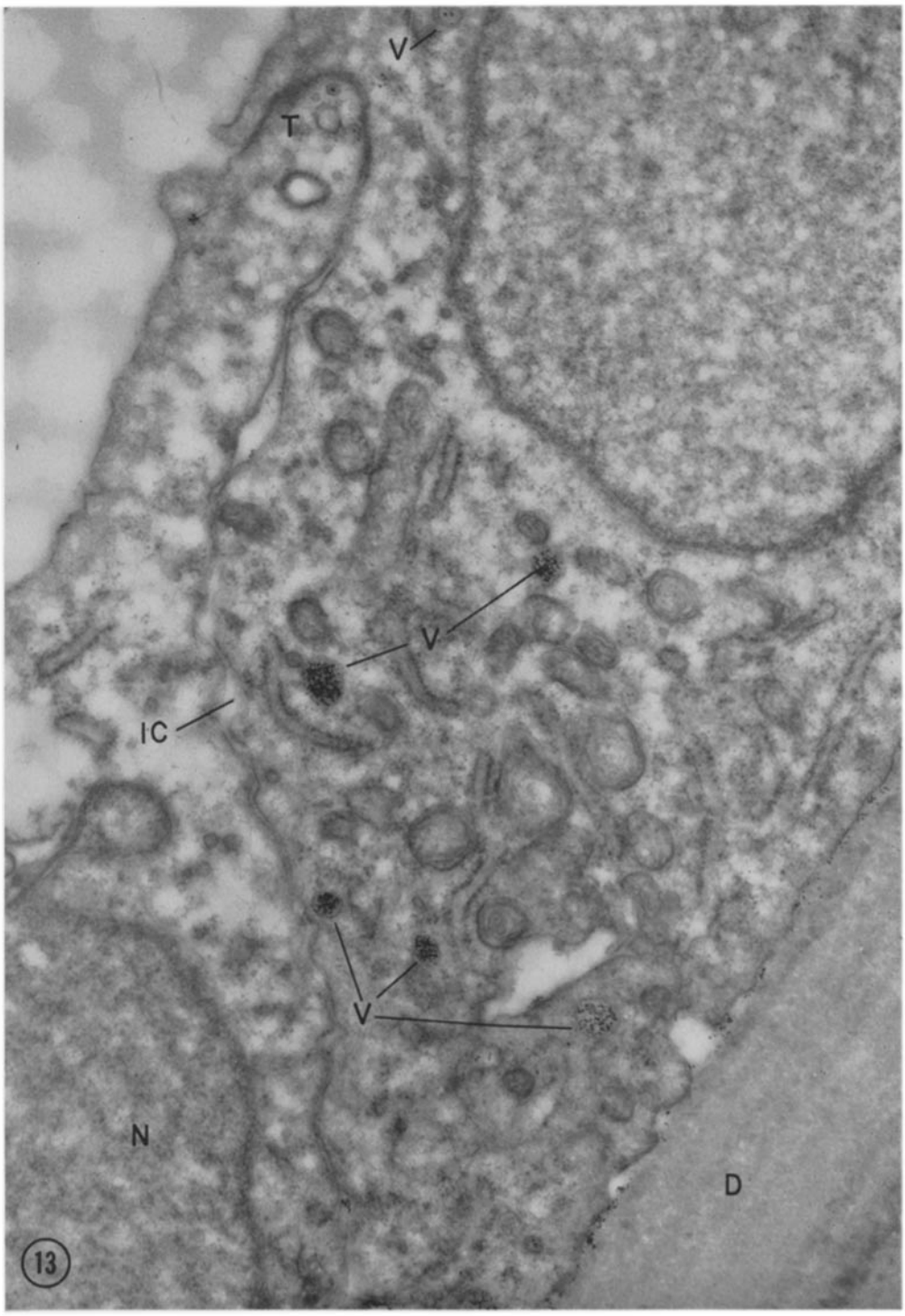
The half desmosomes found at the basal plasma membrane of the basal cells (Fig. 3) are similar, in their relation to the basement membrane, to those described by Jakus (33) in the human cornea and by Whitear (11) in the cornea of the mouse, as well as to structures described by Selby (57), Odland (39), and Charles and Ingram (58) at the dermal-epidermal junction of human skin and by Weiss and Ferris (59) and Porter (60) at the basement membrane of amphibian epidermis.

Teng and Katzin (45) described a basement membrane, typically periodic acid-Schiff–positive, under the corneal epithelium in human, beef, monkey, rabbit, and cat corneas. Jakus (5, 33) has described a fine fibrillar basement membrane in rat and human corneas stained with phosphotungstic acid and examined in the electron microscope. The basement membrane in the rat is approximately 300 A thick, while in the human it is described as being 100 to 300 A thick.

The basement membrane of the rabbit corneal epithelium (Fig. 3), which measures 100 to 200 A in thickness, is considerably thinner than that reported in the mouse by Whitear (11), who described the basement membrane as a fibrillar layer approximately 300 A thick. Sheldon (10)

FIGURE 13

This electron micrograph shows apart of the endothelium of a rabbit cornea in which an intrastromal bleb of thorium dioxide was produced 2 days prior to fixation. A dense accumulation of ThO_2 particles is found at the border of Descemet's membrane (*D*) and the endothelium. Vesicles (*V*) containing large numbers of ThO_2 particles are seen at all levels of the cytoplasm. The vesicles in this preparation have a much denser accumulation of particles than those found in the endothelium of a 1 hour bleb preparation (Fig. 12). Few, if any, thorium dioxide particles are found in the intercellular space (*IC*). *T* indicates a typical terminal bar at the apical end of the intercellular space. *N*, nucleus. × 33,000.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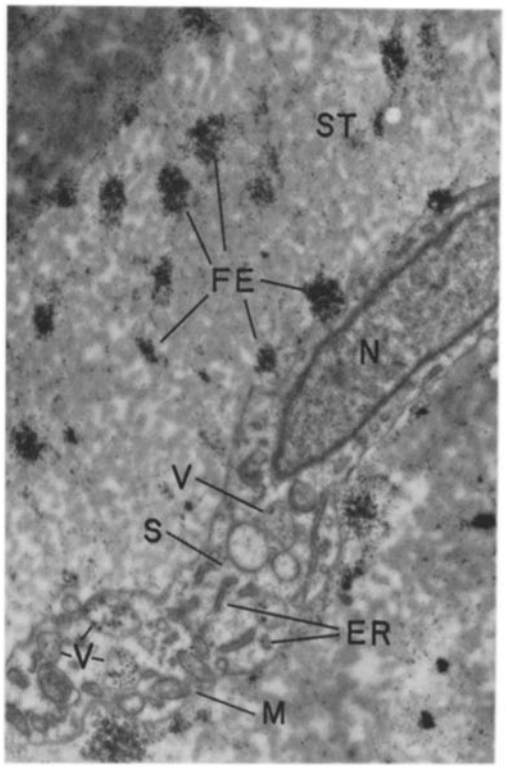

FIGURE 14

This figure shows a part of the stroma of a rabbit cornea in which a bleb of saccharated iron oxide was injected 1 hour before fixation. In this section near the center of the bleb, large and small clumps of iron oxide (*FE*) are present throughout the stroma (*ST*) and at the surface of the stromal cell (*S*). The distribution of Fe_2O_3 in the stroma seems less regular than that of ThO_2, perhaps owing to the clumping of the Fe_2O_3. Large vacuoles (*V*) containing iron oxide are found in the cytoplasm of the stromal cell. Mitochondria (*M*) and elements of the granular (rough surfaced) endoplasmic reticulum (*ER*) are well demonstrated in the stromal cell. *N*, nucleus. × 10,000.

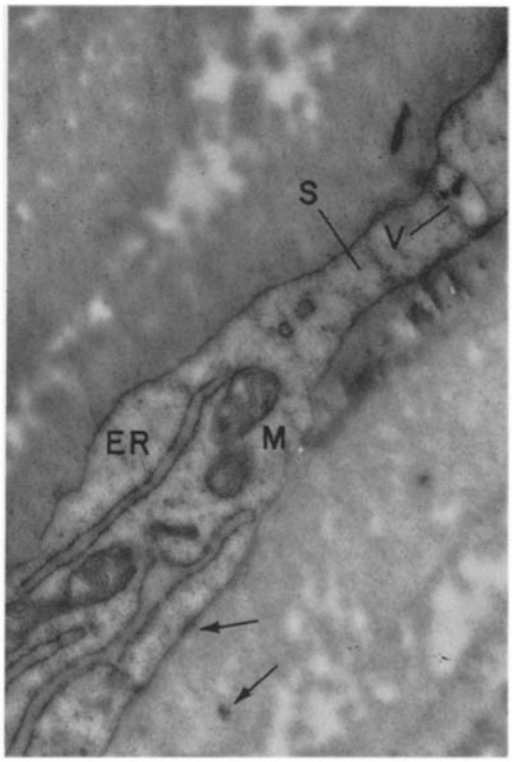

FIGURE 15

An electron micrograph of a part of the stroma of a rabbit cornea in which a saccharated iron oxide bleb was produced 1 hour before fixation. This section is taken some distance from the center of the bleb. While particles of iron oxide are scarce and difficult to identify in the acellular part of the stroma (arrows), a large vacuole (*V*) containing a clump of iron oxide particles is seen in the cytoplasm of the stromal cell (*S*). Mitochondria (*M*) and flattened cisternae of the rough surfaced endoplasmic reticulum (*ER*) of the stromal cell are particularly well shown in this figure. × 17,000.

described the basement membrane of the mouse corneal epithelium as being 600 A thick, but may have included both the clear and dense zones in his measurement. It is also possible that the phosphotungstic acid staining used by other workers to demonstrate the periodicity and fine structure of the fibrous components of the cornea tends to increase the apparent thickness of the basement membrane. The preparations used in the present study were unstained.

The mitochondria of the cells of the outer layers of the rabbit corneal epithelium often show a profile of only a single crista when they are cut in cross-section. It is conceivable that the light center and dense periphery observed in cross-sections of mitochondria in the mouse corneal epithelium (10, 11) may be due to a swelling of the internum of the single crista and a concomitant compression and increase in staining intensity of the ground substance of the mitochondrion. An internal swelling would also tend to place several membranes in closer apposition than normal, thus producing a peripheral structure of increased density.

The simple structure and, above all, the paucity of the mitochondria in the rabbit corneal epithelium are of particular interest in view of numerous physiological and biochemical studies on the metabolism of the cornea. De Roetth (61) reported that the bovine corneal epithelium did not appear to have aerobic glycolysis when lactate and CO_2 production in the whole cornea was compared with that in the cornea denuded of its epithelium. These results were essentially confirmed by Langham (19). More recently, Kinoshita and Masurat (62), using C^{14}-labeled glucose and pyruvate, have compared the relative roles of the tricarboxylic acid cycle and the hexosemonophosphate shunt as aerobic pathways of glucose metabolism in the bovine corneal epithelium. They reported that, from the relative rates of oxidation of the three carbons of pyruvate, it would appear that the tricarboxylic acid cycle is functioning in the corneal epithelium but that the hexosemonophosphate shunt appears to play the most important role. Their results suggest that the majority of the C-2 atoms of glucose are oxidized via the shunt. Pirie (63) concluded that an anaerobic-like glycolysis is the main pathway of glucose breakdown in the cornea. She estimated that the cornea glucolizes 65 per cent of all the glucose metabolized and oxidizes 35 per cent, mostly by direct oxidation through the pentose shunt (hexosemonophosphate shunt).

Mitochondria are now well known to be the site of enzymes facilitating many of the oxidative processes of cells, particularly the tricarboxylic acid cycle and the cytochrome system (64). Siekevitz and Watson (65, 66) have demonstrated, in combined electron microscopic and cytochemical studies of isolated membrane fractions from rat liver mitochondria, that better than 70 per cent recovery of the succinic dehydrogenase and cytochrome *c* oxidase activity of the intact mitochondria is found associated with one of the membrane fractions.

The low level of aerobic glucose metabolism in the corneal epithelium (19, 61, 62), operating primarily via the hexosemonophosphate shunt, correlates well with the scarcity and internal simplicity of the mitochondria of the rabbit corneal epithelium described in the present study.

It has been suggested, on the basis of its staining properties (46, 47), its development in the chick embryo, and its fine fibrillar structure (6), that Descemet's membrane may represent the basement membrane of the corneal endothelium. Though the dimensions of Descemet's membrane (up to 10 microns thick in the rabbit, Fig. 5) are much greater than those of the corneal epithelial basement membranes (100 to 300 A), certain morphological observations in the rabbit cornea may lend further support to the hypothesis that Descemet's membrane is the basement membrane of the endothelium. The increased density of the basal plasma membrane of the endothelial cell, and the frequent thickenings of the membrane which resemble the desmosomal structures at the junction of the epithelium and basement membrane, have been previously described (Fig. 5). Coupled with this observation is the fact that no typical basement membrane is visible beneath the endothelium. Typical basement membranes have been described under the serosal mesothelium (40, 42), which cell layer the corneal endothelium closely resembles.

Studies of the uptake or transport of colloidal particles by the rabbit corneal endothelium *in vivo* have given somewhat equivocal results. Only a few ThO_2 particles appear to attach to the apical surface membrane of the endothelial cell, and none are found in the cytoplasm, either free or in vesicles, after a 1 hour exposure to 0.1 ml of thorium dioxide in the anterior chamber. This is puzzling when one observes the rapid transport by the rabbit corneal endothelium of saccharated iron oxide, or the transport by the frog corneal endothelium of either ThO_2 or Fe_2O_3 (55).

There are several possible explanations for this result. It has been observed in *in vitro* studies (43) carried out in the chamber of Donn, Maurice, and Mills (23) that ThO_2 rapidly settles out of the perfusing fluid after injection. In *in vitro* experiments, therefore, the clamp was always placed so that the surface being exposed to the test solution was essentially horizontal. Whether this settling was due simply to the density of the particles or to a change induced in the suspension by its dilution with the artificial aqueous humor is difficult to determine. It is possible that a similar settling or precipitation occurred when the test solution was injected into the anterior chamber of the rabbit eye. Such a precipitation would greatly decrease the amount of thorium dioxide to which the central cornea was exposed. The aqueous humor was not usually tapped off before injection of the test solution. It is possible, therefore, that some dilution of the ThO_2 in the anterior

chamber, or reaction with the small amount of protein normally occurring in the aqueous humor, may have affected the results. Saccharated iron oxide appeared, by gross examination of the rabbit eye, to be distributed more evenly in the anterior chamber.

In the rabbit, two separate pathways appear to function in the transport of colloidal particles across the endothelium and into the cornea. Particles attach to the surface of the endothelial cell, and are internalized by the formation of vesicles (Fig. 8). Particles also appear to diffuse only toward Descemet's membrane in the intercellular space (Figs. 8, 12, and 13). In the rabbit corneal endothelium, in which a terminal bar is always found near the apical end of the intercellular space, particles appear apical or basal to the terminal bar but never within it (Fig. 8). The pathway of transport from the stroma to the anterior chamber appears to be entirely by way of membrane-bounded vesicles, with no involvement of the intercellular space (Figs. 12 and 13). A proposed mechanism for the circumventing of the terminal bar during the transport of colloidal particles into the cornea by the rabbit corneal endothelium is presented in another communication (43).

In the rabbit, particles accumulate at the border of Descemet's membrane and the endothelium, giving the appearance of a piling up of particles at Descemet's membrane. There is, however, considerable apparently free diffusion of particles into Descemet's membrane (Fig. 8), so that the piling up may simply be due to a drop in rate of movement of particles traveling from the aqueous environment of the intercellular space into the relatively dense semisolid phase represented by Descemet's membrane.

The large vesicles containing particles of saccharated iron oxide which are found in the rabbit corneal endothelial cells after injection of the marker into the anterior chamber (Fig. 8) may be the equivalent of the dense bodies described by Odor (40) in her study of the uptake of colloidal particles by the mesothelium of the visceral serosa of the rat. However, there is one major difference between the structure of the dense large vacuoles in the corneal endothelium and that of the dense bodies of the mesothelium. Whereas Odor (40) states that "no evidence of mitochondrial structure has been noted in the particle-containing bodies," considerable internal membrane structure, in the form of parallel membranes, small vesicles, or concentric lamellae, is found in the large iron oxide–containing vacuoles in the rabbit corneal endothelium. It is hard to conceive, however, that these internal membranes indicate that mitochondria are directly involved in the accumulation and transport of colloidal particles. It is more likely that these internal membranes represent the excess membrane remaining from the fusion of numerous small particle-containing vesicles to form the large vacuole. This was suggested by Clark (67) in his study of the uptake of colloidal particles by the epithelium of the intestinal villus of the immature rat and mouse.

Thorium dioxide and saccharated iron oxide appear to diffuse freely when a small drop of the test solution is injected into the stroma (Figs. 9, 10, and 14). This finding is in agreement with the results of Maurice (15, 16, 54), who found that the diffusion of particles in the stroma was a function of particle size. Maurice, using ions, fluorescein, and proteins of various molecular sizes such as albumin, mammalian hemoglobin, and *Planorbis* hemoglobin, and measuring the spread of the diffusing substance, estimated that particles up to approximately 180 A would diffuse in the stroma. The saccharated iron oxide particles range from 30 to 50 A in diameter, while the ThO_2 particles range from 100 to 150 A in diameter.

Maurice (54) had also concluded from conductivity studies and from measurements of diffusion rates across and along the cornea that large diffusion channels probably did not exist in the stroma. The existence of such large channels had been postulated after examination of early electron micrographs of the cornea which showed spaces between stromal lamellae. These spaces were probably caused by unequal shrinkage of the tissue during embedding (5). Maurice concluded, therefore, that since the collagen is not fully hydrated in the stroma, small interfibrillar spaces up to 200 to 300 A occur, and may represent the only diffusion path.

Observations in the present study on the diffusion of colloidal particles in the stroma support the data of Maurice. Particles are found around and between individual collagen fibers and appear to be stopped or accumulated only at the cell membranes of the stromal cells (Figs. 9, 10, and 14). Particles also appear to diffuse freely out through Descemet's membrane, for they are found accumulated at the border of this membrane

with the endothelium within 1 hour after a bleb is placed in the stroma. Particles are also found in vesicles at all levels of the cytoplasm of the endothelial cell as well as attached at the apical surface in these preparations (Figs. 12 and 13).

Maurice (54) has studied the diffusion through the endothelium out into the aqueous humor of Na^{24}, Br^{82}, and Ce^{134} applied in solution to the epithelial or bare stromal surfaces. On the basis of the rate of accumulation of radioactivity in the aqueous humor, Maurice concluded that these ions diffused through the intercellular space of the endothelium.

In the present study, particles traveling out across Descemet's membrane and the endothelium were rarely, if ever, found in the intercellular spaces. Indeed, particles were found in vesicles within the cytoplasm of the endothelial cell (Figs. 12 and 13). It appears, from observations in the present studies of the transport of colloidal particles in both directions by the endothelium, that there may be a one way flow in the intercellular space. When particles are placed on the apical surface of the endothelium, they rapidly appear in the intercellular space and are transported down to Descemet's membrane, where they accumulate to some extent (Fig. 8). Particles coming out through Descemet's membrane also appear to accumulate below the basal membrane of the endothelial cell but are rarely found in the intercellular space (Figs. 12 and 13). It is possible, however, that the ions studied by Maurice (54) may behave differently from the ThO_2 or Fe_2O_3 particles because of their smaller size (approximately 5 A) and their charge. It would be interesting to observe whether the transport of fluorescein or hemoglobin out through the endothelium, which had been described by Maurice (15, 16), actually occurs through the intercellular space as he proposed, or whether these substances more likely follow the intracellular pathway reported in the present study.

The presence of a high concentration of colloidal particles in the stroma may act as a stimulus to the uptake of the particles by the stromal cells. Large and small vesicles containing thorium dioxide or saccharated iron oxide are found in the stromal cells in the bleb preparations (Figs. 9, 10, 14, and 15). Whether this uptake is another example of the general ability of cells to ingest materials presented at their surface or whether this is a specific activation of the stromal cells to behave like macrophages is still unsettled.

Weimar (68) has reported the apparent transformation of stromal cells to fibroblasts in corneal wound healing. She has further described (69) the activation of corneal stromal cells to take up neutral red after wounding of the epithelium. These two phenomena, coupled with the observations in the present study, suggest that the stromal cells do not represent a specialized cell type, but rather are relatively undifferentiated fibrocytes which may, under the proper stimuli, behave as fibroblast-like or macrophage-like cells.

During the course of this work Dr. Kaye was a Predoctoral Research Fellow of the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service. This paper represents part of a thesis submitted by Dr. Kaye 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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