THE ISOLATED CILIARY BILAYER IS USEFUL FOR STUDIES OF AQUEOUS HUMOR FORMATION*

BY *Marvin L. Sears,* MD, *Eichi Yamada,* MD, PhD (BY INVITATION), *Dean Cummins,* MD, PhD (BY INVITATION), *Naoki Mori,* MD (BY INVITATION), *Alden Mead* (BY INVITATION), AND (BY INVITATION) *Masakazu Murakami,* MD, PhD

INTRODUCTION

UNCOVERING THE PRECISE MECHANISM OR DRIVING FORCE OF AQUEOUS humor formation has remained a challenge for physiologists interested in this process. Confronting an analysis of the variables influencing aqueous humor formation, virtually all investigators have turned to in vitro measurements.

A good deal of information has been obtained from excised iris ciliary body preparations utilizing either transmural measurements of its bioelectrical properties or measurement of saturation kinetics for different solutes, such as ascorbic acid.[1-3] The complex geometry of this preparation and its failure to support adequate flow of solute[4] and solvent have promoted the use of separately cultured nonpigmented and pigmented epithelial cells and/or corresponding layers.[5,6] In these preparations, uptake studies of solutes have given information additional to that obtained in excised iris ciliary bodies. Also, studies of membrane potentials by intracellular recordings in cultured ciliary epithelial cells have given us important data.[7]

There are still important reasons for developing a more suitable tissue layer for the study of aqueous humor formation comparable to the natural secretory cells. This tissue layer should include both pigmented and nonpigmented epithelium to account for (1) the role of the pigmented

*From the Yale University School of Medicine, Department of Ophthalmology and Visual Science, New Haven, CT. Supported by MERIT Award EY08879 from Research to Prevent Blindness, Inc, New York, and grant INT-8715680 from the National Science Foundation, Washington, DC.

epithelium, a cell that has characteristic features of "active" functions (eg, basolateral surface infoldings, closely packed intramembranous particles, abundant mitochondria, distinct Golgi apparatus, numerous microvilli extending into the "ciliary channels" at its apical surface), (2) the gap junctions between the two cell layers that make the cells of both layers a functional syncytium, and (3) true vectorial or transepithelial transport. For these reasons we have tried to develop a good system in which *transepithelial* transfer of materials can be demonstrated. This study describes how the natural double epithelial cell layer is isolated and discusses some of the preliminary results we have obtained to authenticate its properties.

MATERIALS AND METHODS

PERFUSION TECHNIQUE FOR ISOLATING BILAYER OF CILIARY EPITHELIUM

New Zealand Albino rabbits usually weighing between 2 and 3 kg were anesthetized intravenously with a 1:1 mixture of ketamine hydrochloride and xylazine hydrochloride, then heparinized with an excess of sodium heparin, usually 1000 to 2500 U/kg. The animals were then euthanized in accordance with the rule of the National Institutes of Health as adopted and approved by the Association for Research in Vision and Ophthalmology regulations. The eyes were then enucleated and cleaned of excess connective tissue so that the two posterior ciliary arteries could be identified and isolated (Fig 1). Both arteries were cannulated with fine polyethylene tubing that had been drawn over a hot plate. These were attached by three-way stopcocks to a peristaltic perfusion pump calibrated to deliver about 500 μl/min of perfusion fluid.

The eyes were then incised posteriorly. Six scleroretinouveal flaps were created so that the perfusion of the ciliary process could be visually monitored under a dissecting microscope (Fig 2). The perfusion was begun with Ca^{++} Mg^{++} free Hanks solution, then switched after 1 to 2 minutes to oxygenated Hanks with 0.1% collagenase at 37°C at pH 7.4 to 7.5 for less than 10 minutes. After this interval the perfusion fluid was switched again for the 10 final minutes, this time to a balanced salt solution at 30°C to which a 10% fetal calf serum had been added to inactivate any enzyme activities.

During the perfusion, the vitreous was gently, quickly cut and dissected off the retina and pars plana at its base, sometimes with the aid of 0.1% hyaluronidase, then the zonules chemically stripped with a very few microliters of dilute alpha-chymotrypsin. The chymotrypsin procedure is better than simple mechanical removal. Without such treatment, the lens

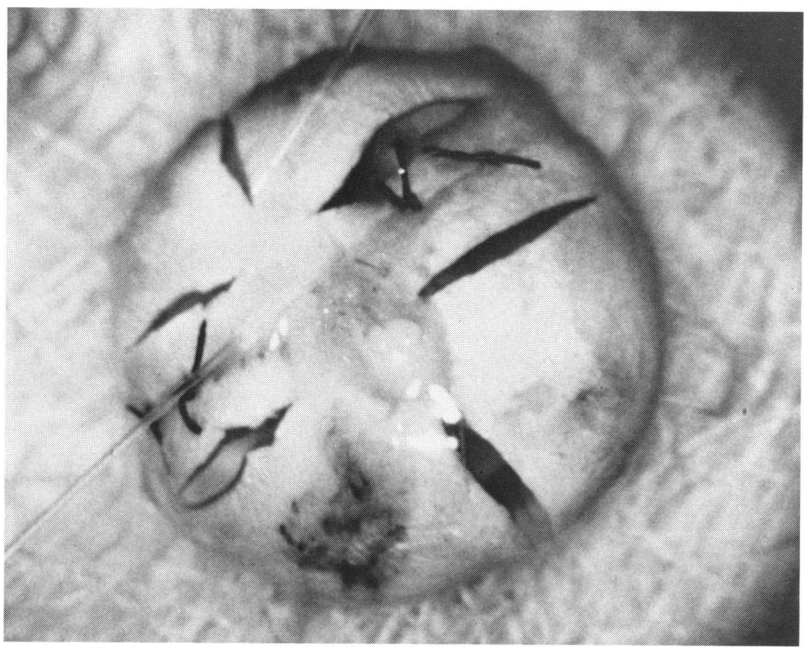

FIGURE 1
Both posterior ciliary arteries cannulated and slits that have been made in posterior portion of enucleated rabbit globe.

always has some nonpigmented epithelium present on its surface. With the treatment as described, the lens was devoid of any ciliary epithelium. These chemical dissections were quick, easy, and atraumatic. At the completion of the perfusion, segments of the iris ciliary body were created and placed in a small Petri dish, containing shallow medium and 10% serum, mounted on a platform stage under a dissecting microscope. The time from euthanasia to completion of the perfusion was always less than 25 minutes.

With the aid of a micromanipulator and microinjector upon which a glass needle micropipette was mounted, either the iris epithelium (posterior surface) or the ciliary epithelium (anterior surface) was pierced and the stroma inflated (injected) with cool Hanks solution. The anterior and posterior aspects of the ciliary segment were incised and lateral incisions created so that the bilayers of ciliary epithelium were floated free from the iris. A "recovery" period of about 10 minutes was permitted, during which the tissue was floated in oxygenated Balanced Salt Solution Plus

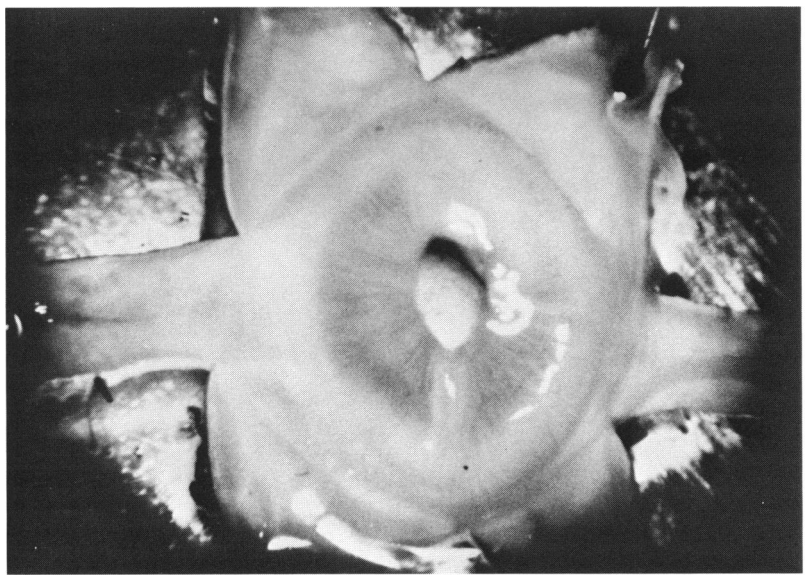

FIGURE 2
Open rabbit eye with vitreous and lens removed. Perfusion was done with eye open once ciliary arteries had been cannulated in order to speed removal of lens and vitreous and to facilitate direct observation of perfused ciliary processes.

(Alcon) fortified with ascorbate in 0.1 mM concentration and thiourea in 1.0 mM concentration.

MOUNTING AND MORPHOLOGY

In the meantime, plastic coverslips machined with beveled apertures of different sizes and shapes and coated with Dow-Corning high-vacuum grease, had Nitex mesh 150 μm mounted on one and a doughnut of filter paper mounted on its mate. The bilayer tissue was captured on the mesh much as a thin section is captured on a grid. The two plastic coverslips were then brought together, with the filter paper serving to reduce slippage of the bilayer during sandwiching. The sandwich was then mounted in a four-port Ussing-type chamber designed and built in our laboratories.

Some preparations were selected for examination under the light and electron microscope in two ways: (1) by looking at adjacent segments and (2) by examining the used preparations at the end of an experiment. Pieces of the isolated, bilayered ciliary epithelium were cut into segments

perpendicular to their long axis. The segments were fixed with modified Karnovsky's fixative containing 2% formaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. Some specimens were fixed for 2 hours or were left overnight. The tissues were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours and were stained en bloc with 2% uranyl acetate solution. They were then dehydrated in graded concentrations of ethanol and embedded in epoxy resin. For light microscopy, semithin sections were made on a Porter Blum MT-2 microtome or on an LKB ultratome and stained with buffered toluidine blue. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H600 electron microscope (at 75 kV). In addition, selected preparations were exposed to horseradish peroxidase on the pigmented epithelium side and examined under the electron microscope after suitable fixation, embedding, and section as previously described. These examinations, taken together with the transepithelial potential and resistance measurements, confirmed the reliability of any single preparation by establishing the integrity of the zona occludens of the nonpigmented epithelium.

BIOELECTRICAL MEASUREMENTS

The tissue was mounted in an Ussing-type chamber with four electrode ports, one port for voltage and one for current in each hemichamber with a volume of 2 ml. The electrodes were Ussing system electrodes from World Precision Instruments. Through a preamplifier electrodes were attached to a World Precision Instrument Company DVC-1000 voltage/current clamp amplifier. The signals from the apparatus were routed to a MacLab Analog Digital Amplifier, which, in turn, sent the signal to a MacIntosh SE 30 computer. There the signal was charted and stored using "MacLab Chart V 3.0" software. Using "chopstick" electrodes, open circuit membrane resistance of the epithelial bilayer was also directly measured with a millivolt and ohmmeter (WPI EVOM) to confirm the (resistance) "viability" of the preparations. Recording electrodes were positioned within 1 mm of the tissue on each side of the bilayer.

Drugs were added to each hemichamber by a simultaneous push-pull syringe arrangement. In the experiments to be reported, the bathing solutions consisted of modified Tyrodes solution 295 mOsm/l maintained at pH 7.4 to 7.5 throughout each experiment by HEPES buffer. Oxygenation was provided for by 95% oxygen and 5% carbon dioxide. All experiments were conducted at 30° to 32°C to prolong the interval for which the bilayer could be studied.

RESULTS

Progressive isolation of the ciliary epithelial bilayer can be seen in stages in Figs 1 through 18.

Figs 3 and 4 show perfused ciliary processes in progressive isolation with removal of iris and collagenous connective tissue within the processes.

Figs 5 and 6 illustrate light microscopy of different ciliary tips with stroma no longer adherent to the basal lamina of the pigmented epithelial cell layer. The nonpigmented epithelium and the pigmented epithelium appear normal at this level with a continuous arrangement, even in the plicated portion of the processes. The stroma appeared to collapse on itself and seemed retracted from the basal lamina of the pigmented epithelium. Of course, the fragile bilayer is easily fractured during sectioning (Fig 6).

Phase contrast microscopy shows flattening ciliary processes (Fig 7) and a view through the double cell layer (Fig 8). At a higher-power view (Fig 9), an occasional pleat appears. Fig 10 shows a thin section of the double cell layer. The overall architecture is well prepared. Ciliary channels are evident in these iridial ciliary processes.

Higher-power views of the isolated double cell layer (Figs 11 and 12) show preservation of intracellular organelles. Fig 11 shows a roughened basal lamina of the pigmented epithelium. Fig 12 shows a completely preserved pigmented epithelium lamina, actually fused to the capillary endothelial lining, incompletely detached.

The intercellular junctions were intact after perfusion (Figs 13 and 14).

Results of experiments done with a marker for the intercellular zonula occludens or tight junctions are shown in Figs 15 and 16. Reaction product from the horseradish peroxidase placed on the pigmented epithelium side of the preparation does not reach the nonpigmented epithelium side, even after 1 to 2 hours of incubation. The product is seen between the pigmented epithelium cells and up to the tight junction of the nonpigmented epithelium cells but does not penetrate this cell layer.

These latter "morphologic" results were confirmed by direct measurements of the resistance of the bilayer, which yielded a total resistance of 350 ohms and for our amount of 0.064 cm^2, a value of 50 ohm cm^2. (The resistance of the bathing solutions and chambers were previously measured and substracted out.) The resistance was susceptible to 200 μM EGTA in a calcium-free solution, dropping to very low levels after its addition to the chamber (Fig 17).

In more than 40 experiments the ciliary epithelial bilayer generated a spontaneous transepithelial potential difference of 650 μV, nonpigmented

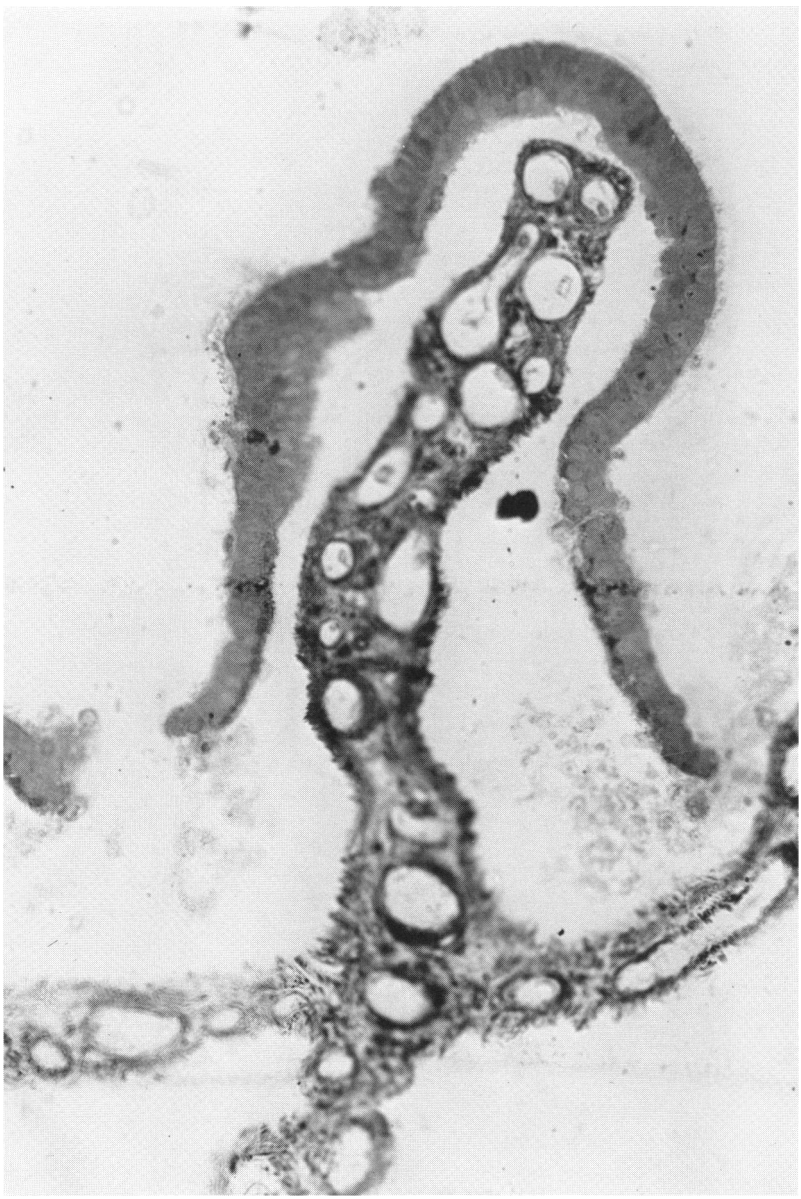

FIGURE 3
Perfused ciliary processes in progressive isolation with removal of iris and collagenous connective tissue within processes. Stroma appeared to collapse on itself and seemed retracted from basal lamina of pigmented epithelium.

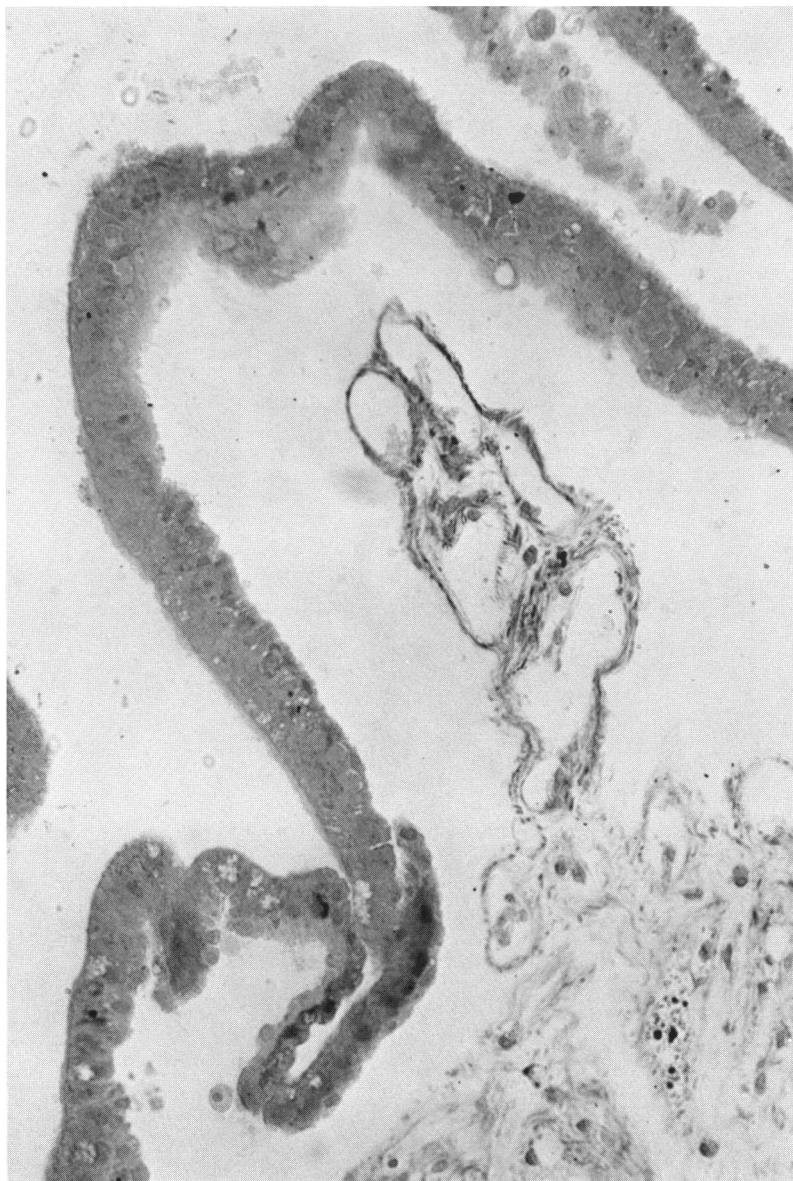

FIGURE 4
A more posterior region of ciliary processes.

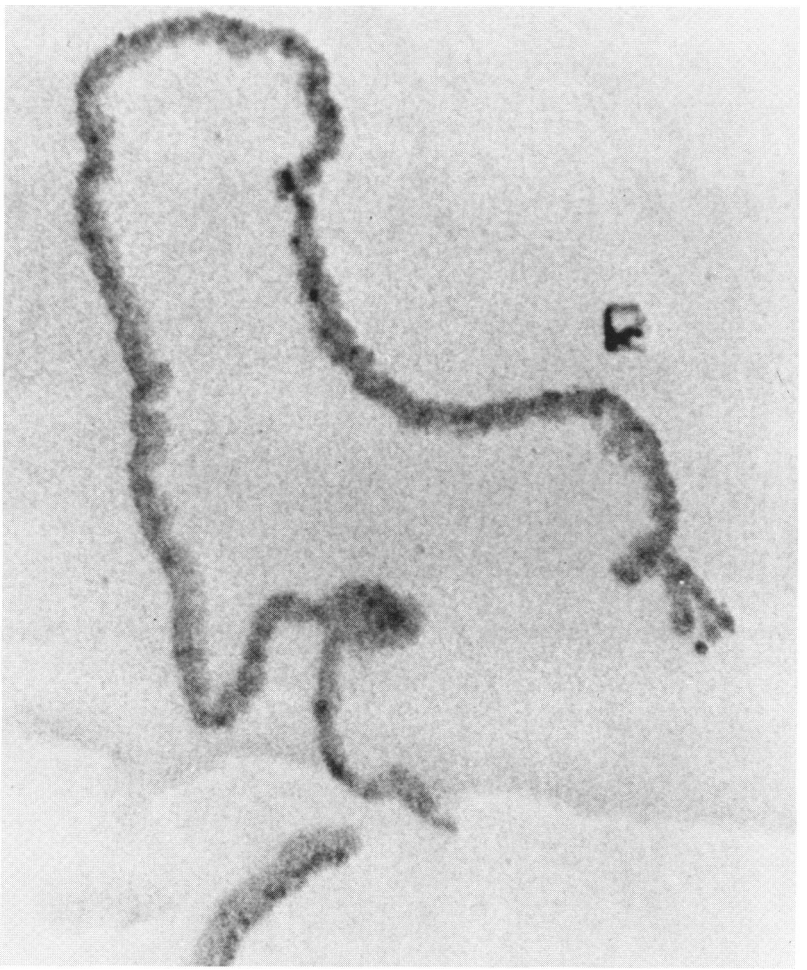

FIGURE 5
Light microscopy of different ciliary tips with stroma no longer adherent to basal lamina of pigmented epithelial cell layer. Nonpigmented epithelium and pigmented epithelium appear normal at this level with a continuous arrangement, even in plicated portion of processes.

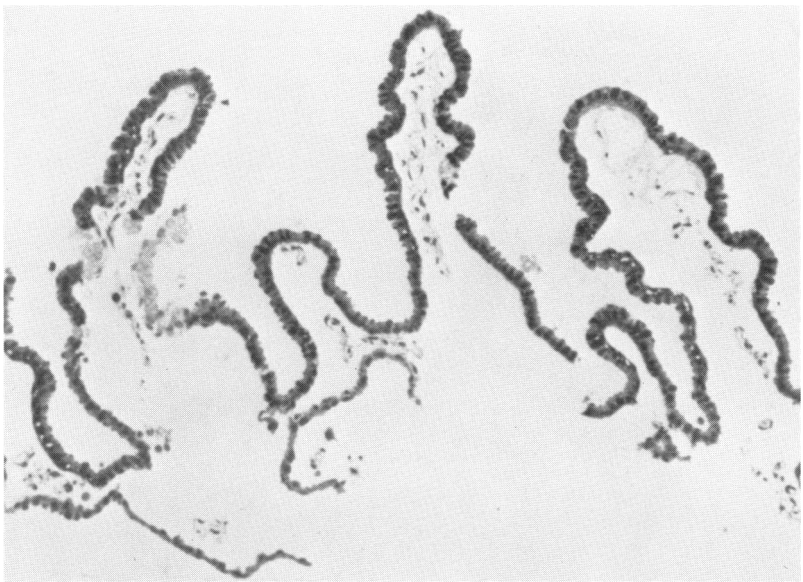

FIGURE 6
A continuous layer of isolated plicated ciliary process in section for light microscope.

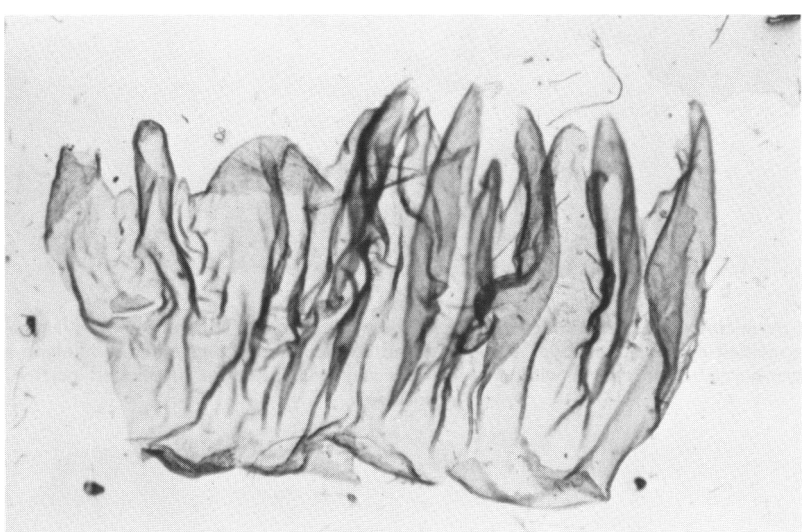

FIGURE 7
8× magnification of flattening ciliary processes.

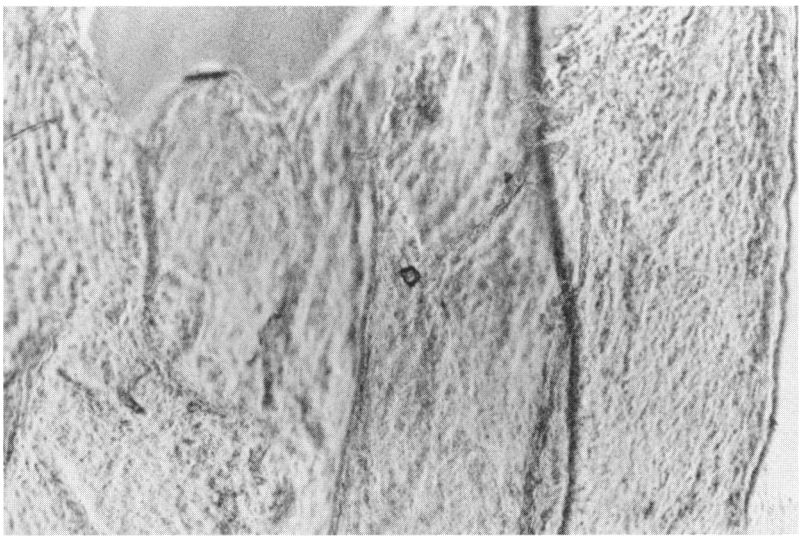

FIGURE 8
A view through double cell layer with phase contrast microscopy (magnification, ×250).

epithelium side negative. It was possible after some practice to develop two preparations in parallel from a single eye which could be mounted in separate Ussing chambers. In this way, each preparation served as its own control (baseline) and a second preparation could be used as an additional paired control. The results could then be expressed in either of two ways, yielding a very small standard error (Fig 18).

Ouabain, 10^{-4} M, added to either side of the chamber, was an effective inhibitor of the transmembrane current, blocking the sodium pump as reflected by a time-dependent decrease in the transmembrane potential (Fig 18).

After bicarbonate depletion (Fig 19) a rapid decline occurred in the spontaneous transepithelial potential difference. During these experiments pH was kept constant by addition of appropriate amounts of HEPES buffer. In separate experiments, propionate was substituted for bicarbonate and also produced a rapid drop in the transepithelial potential difference.

Observations were made after addition of carbonic anhydrase inhibitors to the pigmented epithelial side of the chamber. A decrease of 20% to 30% in the transepithelial potential difference was observed. Certain small differences were seen among the different carbonic anhydrase inhibitors (Fig 20).

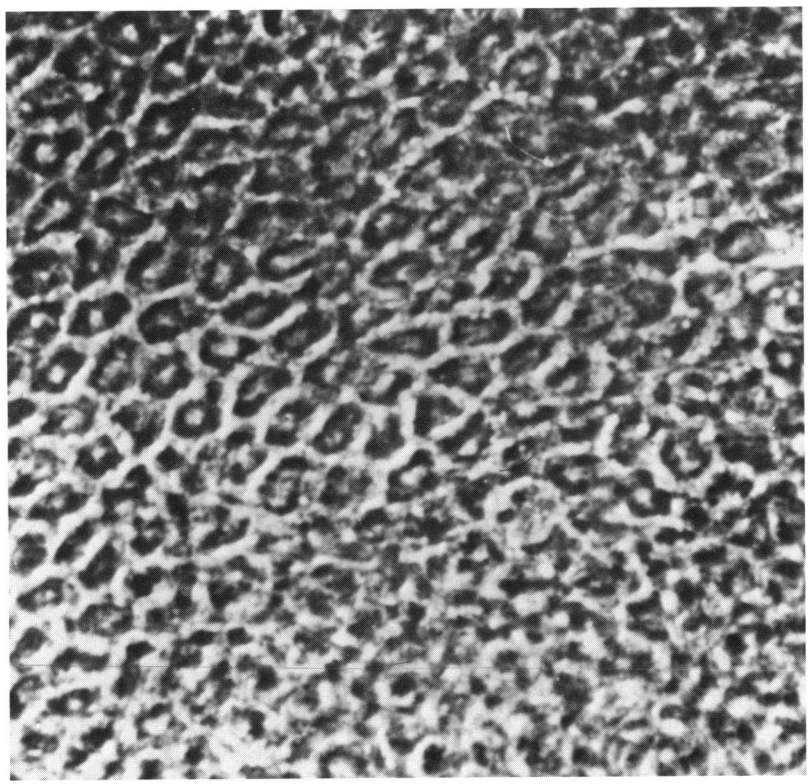

FIGURE 9
400× magnification through a similar double cell layer. An occasional pleat appears.

DISCUSSION

An isolated ciliary epithelial bilayer has been prepared by perfusion, microdissection, and recovery techniques. This preparation has bioelectric properties that indicate its usefulness for the study of the mechanisms of aqueous humor formation. The bilayer holds a steady resistance and generates a spontaneous transepithelial potential. Furthermore, the resistance is rapidly susceptible to EGTA in a calcium-free solution. The level of the resistance, 50 ohms cm^2, indicates that the ciliary epithelium is a "leaky" epithelium, confirming the findings of Wiederholt and associates[8] for the mechanically stripped preparation of shark epithelium. The trans-

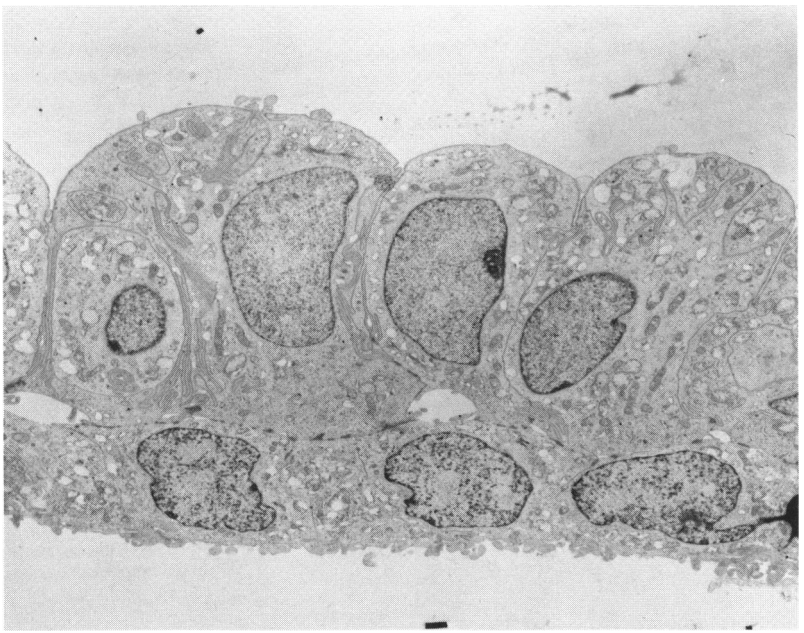

FIGURE 10
A thin section at ×2000 of double cell layer. Overall architecture is well prepared. Ciliary channels are evident in these iridial ciliary processes.

epithelial potential difference is rapidly decreased by 10^{-4} M ouabain, indicating the electrogenic nature of the sodium pump that generates this potential difference. An interesting initial hyperpolarization occurs when ouabain is added to the nonpigmented epithelium side of the preparation, confirming suspicions of a concentration-dependent effect of ouabain; ie, initial stimulus from a low concentration followed by a rapid decline as the binding of ouabain to the sodium potassium ATPase sites increases. The pump is apparently bicarbonate-dependent, because under the conditions of constant pH (7.4) maintained with HEPES buffer, the transepithelial potential difference rapidly falls when sodium bicarbonate is exchanged either with sodium chloride or with sodium propionate. It is further interesting to observe the coincidence between the 20% to 30% drop in potential difference seen after addition of carbonic anhydrase inhibitors to this isolated epithelial preparation, and the 29% drop in the rate of sodium into the posterior chamber of rabbits[9] and dogs[10] observed by others.

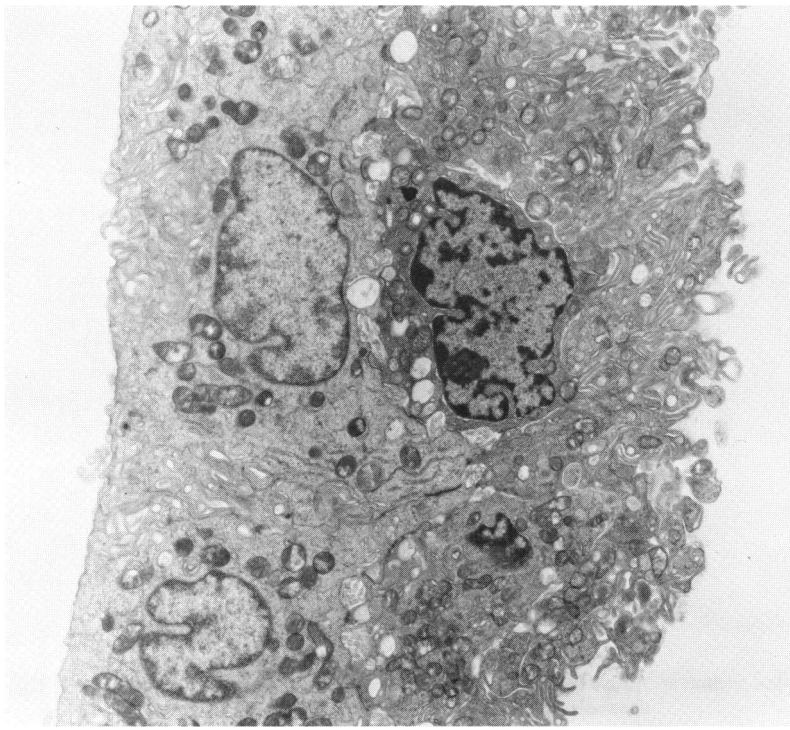

FIGURE 11
Figs 11 and 12 show a higher power view of isolated double cell layer. Both show preservation of intracellular organelles. This figure shows a roughened basal lamina of pigmented epithelium.

All of these findings, taken together with the morphologic integrity of the preparation, indicate that the isolated bilayer can provide physiologists with what is required for a detailed study of the mechanism of aqueous humor formation. Such studies can be done in a preparation that preserves the ciliary epithelial layers in their usual state, avoiding problems with relatively inert iris ciliary body preparations and avoiding the loss of polarity and dedifferentiation that occurs in cultured or transformed cells while providing a preparation in which true vectorial transport can be accomplished and analyzed.

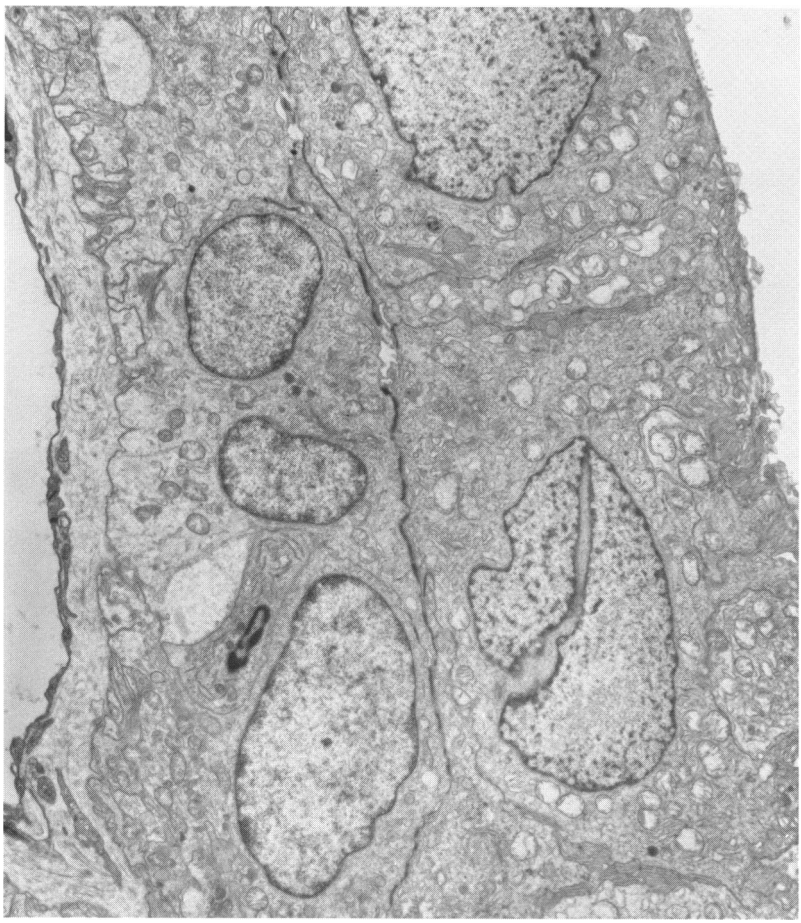

FIGURE 12
A completely preserved pigmented epithelial lamina, actually fused to capillary endothelial lining, incompletely detached. Intercellular junctions were intact after perfusion.

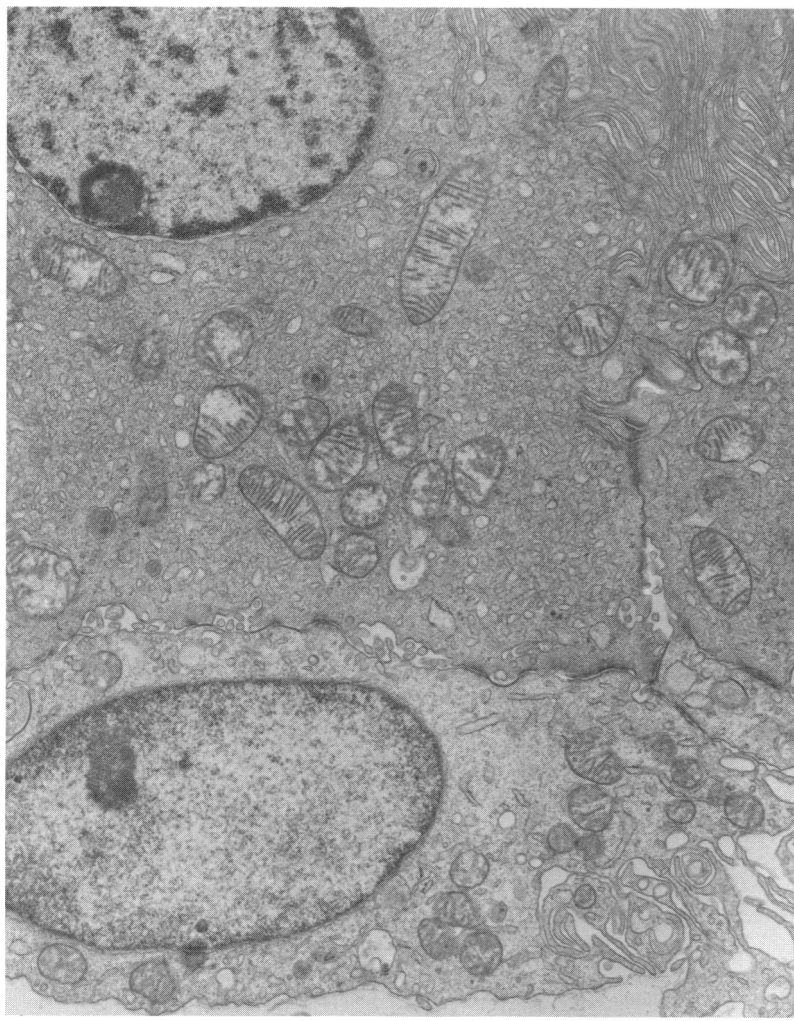

FIGURE 13
Junction complexes and gap junctions between pigmented epithelium and nonpigmented epithelium and between adjacent nonpigmented epithelium.

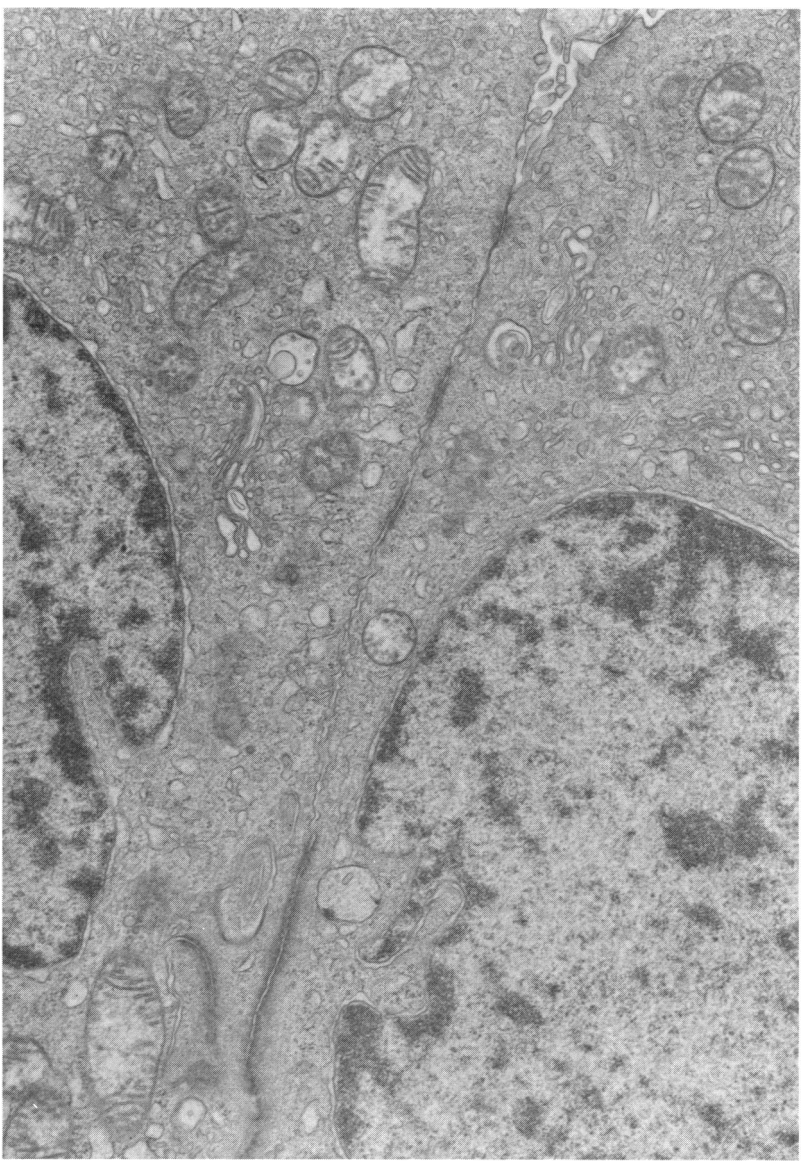

FIGURE 14
Junctional complexes between adjacent nonpigmented epithelium with excellent preservation.

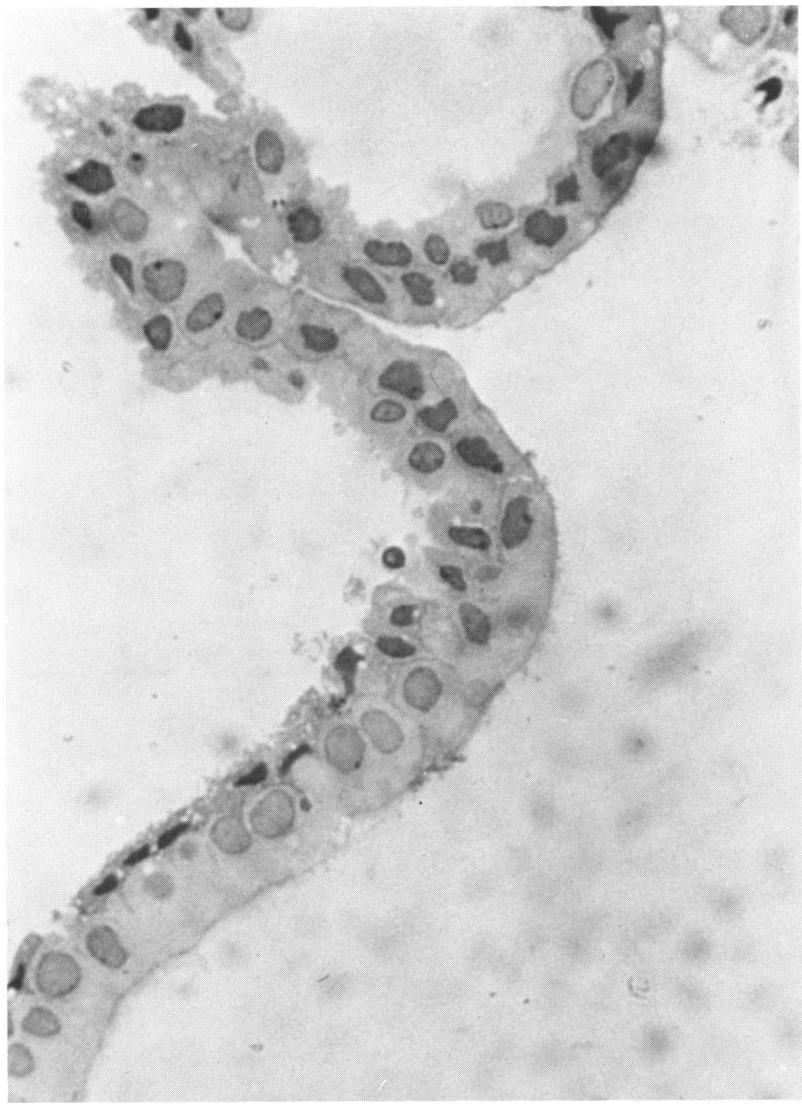

FIGURE 15

Results of experiments done with a marker for intercellular zonula occludens or tight junctions are shown in Figs 15 and 16. Reaction product from horseradish peroxidase placed on the pigmented epithelial side of the preparation does not reach nonpigmented epithelial side even after 1 to 2 hours of incubation. Product is seen between pigmented epithelial cells and up to tight junction of nonpigmented epithelial cells but does not penetrate this cell layer.

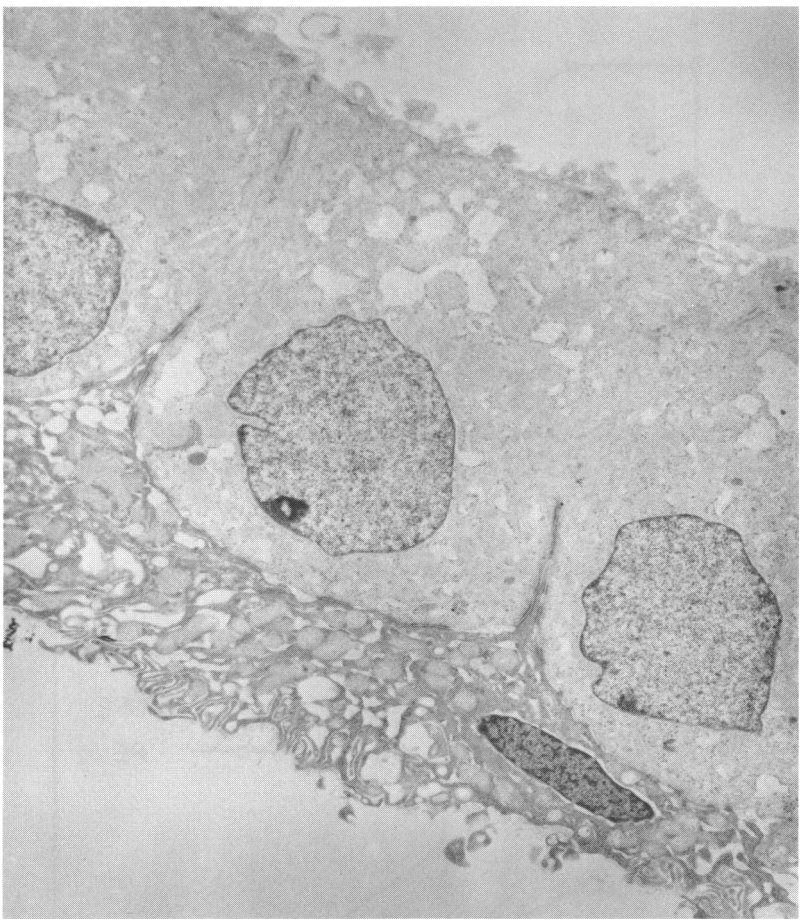

FIGURE 16

Results of experiments done with a marker for intercellular zonula occludens or tight junctions are shown in Figs 15 and 16. Reaction product from horseradish peroxidase placed on the pigmented epithelial side of the preparation does not reach nonpigmented epithelial side even after 1 to 2 hours of incubation. Product is seen between pigmented epithelial cells and up to tight junction of nonpigmented epithelial cells but does not penetrate this cell layer.

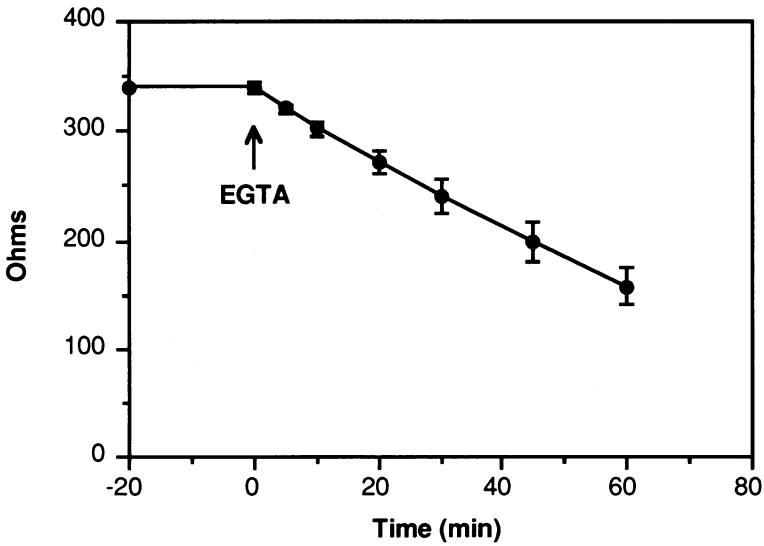

FIGURE 17
Resistance was susceptible to 200 μM EGTA in a calcium free solution, dropping to very low levels after its addition to chamber.

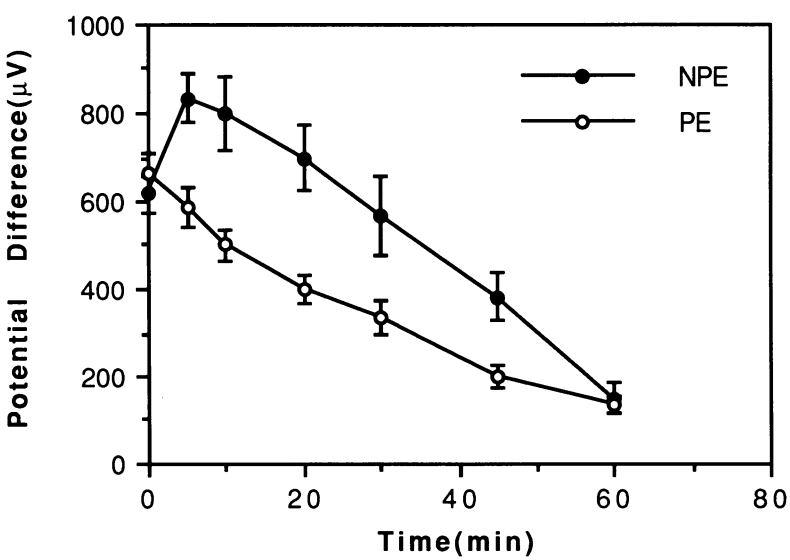

FIGURE 18
In more than 40 experiments, ciliary epithelial bilayer generated a spontaneous transepithelial potential difference of 650 μV, nonpigmented epithelium side negative. Ouabain, 10^{-4} M, added to either side of chamber was an effective inhibitor of transmembrane current, blocking sodium pump as reflected by a time-dependent decrease in transmembrane potential.

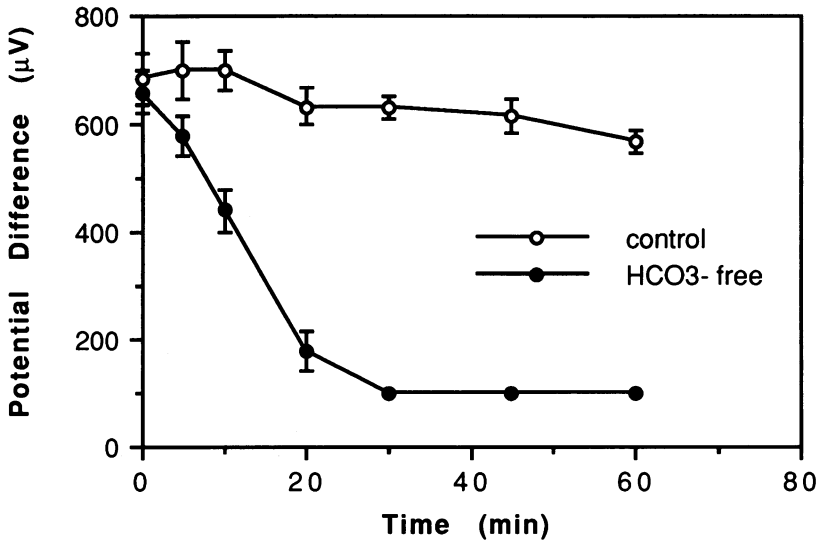

FIGURE 19
After bicarbonate depletion with NaCl a rapid decline occurred in the spontaneous transepithelial potential difference. pH was kept constant by addition of HEPES buffer. Propionate substituted for bicarbonate also produced a rapid drop in transepithelial potential difference.

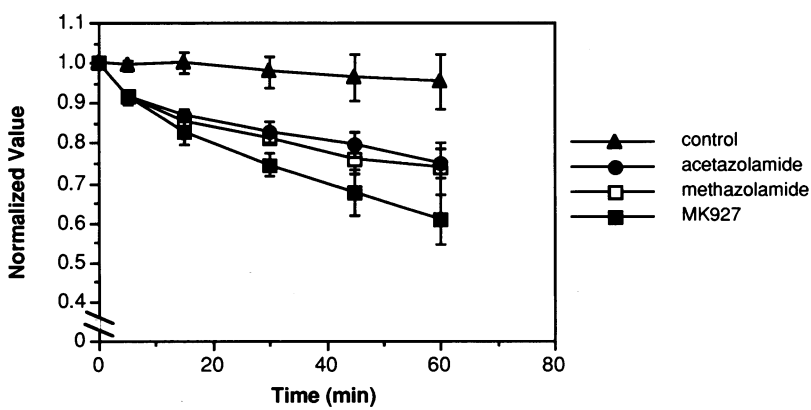

FIGURE 20
Observations were made after addition of carbonic anhydrase inhibitors to pigmented epithelial side of chamber. A decrease in transepithelial potential difference of from 20% to 30% was observed. Certain small differences were observed among different carbonic anhydrase inhibitors.

SUMMARY

An intact ciliary epithelial bilayer has been isolated from the rabbit eye by perfusion, microsurgical dissection, and recovery techniques. Vital subcellular organelles and intercellular junctions of this epithelial bilayer preparation are very well preserved. The total electrical resistance of the epithelial bilayer is 350 ohms, and the transepithelial potential is 650 μV, nonpigmented epithelium side negative. The electrical resistance is reduced by 0.2 mM EGTA and the transepithelial potential reduced by 0.1 mM ouabain. Bicarbonate depletion at a constant pH of 7.4 rapidly and significantly reduces the transepithelial potential. Carbonic anhydrase inhibitors decrease transmembrane potential by as much as 30%. These morphologic and physiologic experiments authenticate the validity of this bilayered epithelial preparation for future use in detailed studies of the mechanism of aqueous humor formation.

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DISCUSSION

DR PAUL R. LICHTER. Doctor Sears and colleagues have provided us with a very important paper describing a new in vitro preparation of ciliary epithelia that promises to yield new information on the mechanism of solute-coupled aqueous secretion. This preparation not only allows for the study of basic ocular physiology

but has significant potential for yielding valuable clinical information.

The transport of water in tissues has been an elusive subject for physiologists. Much of the problem lies in appropriate in vitro tissue preparation and the complexities therein. In the 1950s, physiologists were using frog skin to study water transport because this was a relatively thin, flat tissue that could be placed in a chamber and evaluated under a variety of conditions. Imagine the complexity of studying the highly tortuous ciliary epithelium compared to the flat frog skin.

The major features of the preparation by Sears and colleagues are that it is an isolated bilayer of pigmented and nonpigmented cells, free of the underlying stroma, and with a simple planar geometry that allows it to be mounted as a flat sheet.

Planar geometry facilitates transport measurements in the Ussing chamber. The absence of stroma should permit a better perfusion of the pigmented cellular layer, and this may well enhance oxygen and nutrient availability to these cells. Possible disadvantages include fragility of the tissue as well as mechanical damage, which could compromise the epithelial barrier function.

I would like to pose several questions to Doctor Sears:

First, you have ascribed the rapid decline of the transepithelial potential following the bicarbonate depletion to the inhibition of the electrogenic Na^+/K^+ pump. Recent studies by Wolosin at Mount Sinai in New York indicate that the ciliary epithelium contains an electrogenic $NaHCO_3$-cotransport mechanism. Could the inhibition of this transporter account for your observations? Have you tested the effect of ouabain in HCO_3-free conditions?

Second, in a 1984 paper in *Experimental Eye Research,* Burstein and colleagues showed that when ouabain is added to the nonpigmented epithelial side, there is a small but sustained increase in the electrical potential, as opposed to your finding of an increase and then a drop. Does this mean that the tight junctions in your preparation are not as tight as they were in the preparation by Burstein and colleagues, even though in your preparation, there was a decrease in the electrical potential on the pigmented epithelial side similar to that shown by Burstein?

Third, What is the location of the Na^+/K^+ ATPase in the two cell layers? If, in fact, it is in the intact basal lateral membrane of the nonpigmented epithelium, how can ouabain have any effect at all on the electric potential?

Fourth, Why have ascorbic acid rates in vitro not been demonstrated to be as high as they are in vivo? How might your preparation better explain these differences?

Lastly, What is it about the technique of your preparation that allows the preservation of gap and tight junctions when it is known that these junctions are sensitive to the kind of treatment needed to separate the cellular layers from the stroma?

Again, I commend Doctor Sears and colleagues for their work and look forward to further developments as their studies continue.

DR MARVIN SEARS. I would like to thank Doctor Lichter and Doctor Hughes for the thorough review of this paper and kind compliments. This bilayer preparation is still in its infant stages, you might say. We have not gone on to characterize the very complex number of co-transporters that must exist in these cells. However, consistent with the observation made in the first question, we have not been able to show that ouabain has an effect on the bicarbonate depleted preparation, but, on the other hand, the potential is so low in the bicarbonate depleted preparation to begin with that we couldn't expect it to go much lower. While I recognize that this response does not completely answer Doctor Lichter's question, we have done that experiment.

Doctor Lichter has raised other questions. One of them has to do with the issue of the integrity of the junctions in this bilayer. As you have seen from the morphologic studies, the advantage of this preparation is that the gap junctions are indeed completely preserved as are the tight junctions between the nonpigmented epithelial cells. We don't use the enzymatic procedures and other techniques to disrupt cell junctions as the "monolayer or culture" people do. Further, we feel that in the course of ordinary events during aqueous humor formation *both* cell layers are important. For example, the gap junctions have a permeability that could probably be up-regulated or down-regulated and these in fact may have quite a lot of bearing on the circadian changes in flow demonstrated so nicely by Erickson, Gregory, Brubaker, and others. So we are excited about having a preparation in which these junctions are intact so that we can look at the possibility that the regulation of aqueous humor formation may be done through an adjustment of both the numbers and/or permeability of these gap junctions.

The question about the hyperpolarizing response after ouabain has been a mystery to investigators but most have not found a sustained polarizing response but rather a transient one. So I can't account for the differences between Bernstein and the others who have not found such a sustained response. In Bernstein's paper, ouabain was actually used in a different concentration, 10^{-5} M, and the effects are not statistically significant from baseline. Finally, the sodium pumps are actually located on the basolateral membranes of *both* the pigmented epithelium and the nonpigmented epithelium, but there are subunits to these Na^+/K^+ ATPase proteins. It is thought at the moment that the pigmented epithelium has sort of a clean-up Na^+/K^+ ATPase, whereas, the isoforms of the Na^+/K^+ ATPase that existed in the nonpigmented epithelium are perhaps more directly related to secretion. Of course, under conditions of acidity or under conditions of culture the polarity of the pump in these cells may change. In our bilayer I would expect that ordinary polarity would be maintained. So we are looking forward, having authenticated this preparation, to developing more information about regulatory mechanisms of aqueous humor secretion and hope to be more responsive to your questions the next time around. Thank you for your comments and attention.