Charge and Ion-Binding Differences Between Corneal Epithelial and Endothelial Antigens (37094)

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The ion-exchange properties of protoplasmic polyelectrolyte proteins are now recognized to contribute significantly to the overall ionic composition of a cell (1). This concept of biological ion-exchanger resin has been recently introduced by Damadian (1–3) and experimentally proven in intact cells and in the cell-free system of *Escherichia coli*. It was of interest to test if this concept of the biological ion-exchanger resin applies to protoplasmic molecules derived from mammalian cells.

Our earlier investigation of the tissue-specific antigens from different corneal layers of cattle resulted in the observation of differences in the electrophoretic mobility between the antigens extracted from corneal epithelium and endothelium, the majority of the former being weakly and of the latter strongly anodic (4). One of the aims of this investigation was to see if the proteins from separate corneal layers of other mammalian species exhibit similar characteristic behavior in an electrical field. The main objective, however, was to test if the observed differences in the electrophoretic mobility of extractable corneal macromolecules can be related to the ion-exchange properties of the protoplasmic polyelectrolytes of corneal epithelial and endothelial cells.

Materials and Methods. Rabbit corneas were dissected from fresh rabbit eyes (Rockland, Pa.), carefully rinsed in cold physiological saline, and blotted with filter paper. Different corneal layers were separated and collected as described earlier (4). Special care was exercised to obtain uncontaminated, clean corneal layers. Separated corneal epithelium, stroma, and endothelium were stored frozen at —20° or in liquid nitrogen.

The cell structure of the rabbit corneal layers was broken with the aid of a frozen-tissue pulverizer (Scientific Industries, N.Y.) at —75°. The pulverized powders were extracted with cold saline-phosphate buffer (0.15 *M* sodium chloride, 0.01 *M* sodium phosphate, pH 7.2) in a glass Ten-Broecke tissue grinder. The homogenates were then centrifuged at high speed to sediment all the insoluble residue. The protein concentrations in the supernatants were determined as described by Lowry *et al.* (5).

Muscovy ducks were immunized with homogenized whole rabbit corneas by intramuscular injections in the breast. At every immunization a total of 1 ml of a mixture containing complete Freund's adjuvant (*M. butyricum*) and corneal antigens at the concentration of 100 mg wet weight/ml was injected at five different points. The first three injections were given weekly, all others at monthly intervals. The ducks were bled from the heart 10–12 days after the last immunization.

Duck antisera were absorbed with lyophilized rabbit serum proteins at the concentration of 10 mg/ml in a manner described previously (4).

Scheidegger's (6) microimmunoelectrophoresis technique in 1.5% agar (Noble, Difco Laboratories) and 0.05 *M* veronal-acetate buffer at pH 8.2, 0.05 μm was used. The conditions and details for immunoelectrophoresis were described earlier (4).

Polyacrylamide disc-gel electrophoresis was performed in the standard Canalco 7% alkaline gels. Migration was toward the anode at 5 mA/gel. The gels were stained for protein

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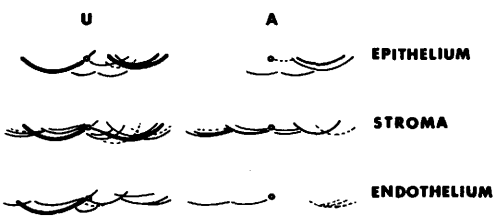

FIG. 1. Immunoelectrophoresis of soluble protein fractions extracted from different layers of a rabbit cornea. The precipitin patterns in the left column were developed with the unabsorbed antiserum (U), while those in the right column with the antiserum absorbed with lyophilized rabbit serum proteins (A). Anode is on the left.

with Coomasie blue (7) and scanned in a Joyce–Loebl Chromoscan at 700 nm.

The ion-exchange properties were determined by equilibrium dialysis of corneal soluble proteins against 0.1 m*M* KCl under conditions similar to those described by Damadian (1). Potassium bound by any given protein fraction was determined by measuring the difference in concentrations of K^+ in the medium and inside the sac before and after equilibrium dialysis for 5 hr at room temperature. The K^+ content of epithelial, stromal, and endothelial proteins was measured in an atomic absorption spectrophotometer (Perkins–Elmer). In all experiments, the protein concentrations inside the sacs were adjusted to be 1.0 mg for epithelial and stromal proteins, and 0.2 mg for endothelial proteins. The protein fractions were freshly extracted and dialyzed against distilled water before equilibrium dialysis.

Results. Figure 1 compares the antigenic compositions of soluble macromolecules from corneal epithelium, stroma, and endothelium. The left column presents immunoelectrophoretic precipitin patterns developed with unabsorbed antiserum. In the right column, where only tissue antigens are visualized by means of anticornea antiserum absorbed with lyophilized rabbit serum proteins, characteristic differences in the electrophoretic mobilities of corneal epithelial and endothelial antigens can be clearly seen. Rabbit epithelial antigens are grouped nearer to the cathode. By comparison, rabbit endothelial antigens moved farther to the anode. The stromal antigens were about evenly distributed between the two electrical poles. When unabsorbed antiserum was used for the development (Fig. 1, left column) such differences in the electrophoretic mobilities could no longer be detected because of the reactions of antibodies to strongly antigenic serum proteins in the tissue extracts.

Since proteins separate in an electrical field on the basis of their net charges as well as molecular sizes, it was of interest to check how the size factor influences the electrophoretic distribution of epithelial and endothelial proteins. With this purpose in mind, freshly extracted corneal proteins were subjected to disc-gel electrophoresis in 7% alkaline gels. As seen in Fig. 2, the differences in mobility distributions between epithelial and endothelial proteins, first observed in agar electrophoresis, were not obscured by the sieving effect of the acrylamide gels. From a cursory examination of the "clustering" of the bands in both gels in Fig. 2, it is obvious that a majority of epithelial protein bands was closer to the cathode, while a majority of endothelial bands moved closer to the anode. Using the spectrophotometric scans, it was possible to draw lines at the mid-mobility points for each gel (starting from the fastest-moving protein band) and calculate the total areas under the scans on each side of the line (Fig. 2). In our experiments, 71.4% of all epithelial proteins versus 43.5% of all endothelial proteins was on the cathodic side (Fig. 2) of the mid-mobility points.

In order to see if these "average" net-charge differences between the mixtures of extractable epithelial and endothelial proteins

TABLE I. Binding of Potassium by the Soluble Protein Fractions from Different Corneal Layers.

Corneal layer (rabbit)	Potassium bound[a] (μmole K/g protein)
Epithelium	268.4 ± 30.5
Stroma	436.8 ± 94.2
Endothelium	2,159.5 ± 389.1

[a] Results of equilibrium dialysis against aqueous KCl (0.1 m*M*) performed under conditions described in "Materials and Methods." Each value is the mean of nine measurements from three different equilibrium dialysis experiments, ± the standard error of the mean.

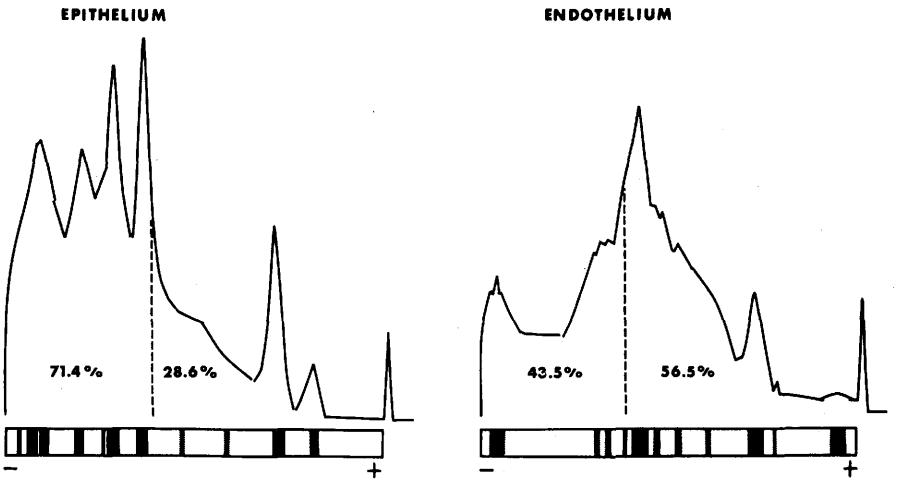

FIG. 2. Polyacrylamide gel electrophoresis of the proteins freshly extracted from corneal epithelium and endothelial (60 μg/gel), performed as described in Methods. Gels were stained with Coomasie blue. The diagrams of stained gels are at the bottom. Gel scans of stained polyacrylamide gels were used to calculate the percentages of relative electrophoretic mobilities of epithelial and endothelial proteins, as explained in the text.

may be reflected in their relative ability to bind cations, equilibrium dialysis experiments against 0.1 m*M* KCl were performed (Table I). Each mean value for potassium binding reported in Table I represents the results from three different experiments, each done in triplicate. Standard errors in Table I were calculated from all combined experimental data. When the mean values for potassium binding from any individual triplicate experiment were calculated, the standard error was significantly smaller: less than 10% for epithelium, 5% for stroma, and less than 10% for endothelium. The larger standard deviations calculated from all combined experiments are an indication of the dependence of the ion binding by extracted macromolecules on conformational changes occurring during the preparation even under carefully controlled conditions. The results clearly indicate that endothelial proteins, even outside of their cellular environment, act as a much stronger cation exchanger, binding nearly 10× more potassium ions than epithelial proteins. The reported difference significantly exceeds the calculated standard deviation of the procedure.

Discussion. The cornea of an eye has a very unique tripartite structure in which a lamellar stroma is limited by several layers of epithelial cells anteriorly, and by a single layer of endothelial cells on the posterior side (8). In addition to its special anatomical features, the avascular cornea has the unusual ability to maintain detergescence under hyper- and hypotonic conditions in different species. For these reasons, the cornea has been a favorite model for the study of ion transport across cellular barriers (9).

Our observation that the differences in electrophoretic mobilities between epithelial and endothelial protoplastic macromolecules were not changed by the sieving effect of polyacrylamide gels, indicates that they were due to net charge rather than to molecular size differences. In polyacrylamide gels, the amount of endothelial proteins that moved further than the electrophoretic mid-mobility point was about twice that of epithelial proteins (Fig. 2). This value is directly related to the relative content of proteins with higher electronegative net charge in the soluble extracts from these corneal cells.

The potential physiological significance of these net-charge differences was demonstrated by the fact that in this report the binding of potassium ions by endothelial extractable macromolecules was ten times greater than that by epithelial macromolecules. Thus, it appears that the cation binding expresses

the net charge differences between corneal endothelial and epithelial protoplasmic macromolecules in a much more pronounced way than the electrophoretic mobility does.

The observation that the extractable proteins from corneal tissues retained their ability to bind potassium ions even in the cell-free environment is significant in terms of a biological ion-exchanger resin concept (1–3). According to this concept, the binding occurs through the nuetralization of fixed charges on intracellular proteins in the absence of any energy source, of a suitable substrate, and of cellular membranes (2). Such an interpretation is supported by the evidence from recent NMR experiments (10), which indicated that potassium ions inside a cell are complexed to fixed charges and/or are solvated in a semi-crystalline state.

Damadian reported that with bacterial protein fractions in the cell-free system, the amount of cation bound was close to that obtained with whole, intact cells (1, 2). However, the ability to discriminate between different monovalent cations was greatly reduced in the cell-free system. The selectivity aspect was not looked at closely in the present study, but from our preliminary experiments it appeared that some selectivity for potassium over sodium ions was retained by corneal proteins in a cell-free environment.

The values for potassium binding by corneal macromolecules are higher than those given by Damadian for bacterial protein fractions (1). This might be a reflection of different amounts and/or easier accessibility of fixed charges on corneal versus bacterial (*E. coli*) intracellular proteins.

The difference in the passive binding of potassium ions by intracellular macromolecules from separate corneal layers offers a simple explanation for such a well-known phenomenon as the accumulation of relatively large concentration gradients of cations in corneal cells and tissues. Although membrane-situated pumps have been proposed as mechanisms for cellular ion accumulation in the cornea (9, 11), a concept of cell polyelectrolytes serving as ion-exchanger resins may contribute to a possible explanation of the movement of ions against "apparent concentration gradients" and to an understanding of differences known to exist between the transport activities of corneal epithelial and endothelial layers (9, 11).

Summary. The experimental evidence described in this report shows that the electrophoretic mobility differences between epithelial and endothelial proteins appear to be a general phenomenon for mammalian corneal tissues. The characteristic behavior in an electrical field of epithelial and endothelial proteins was demonstrated to be a result of net charge rather than molecular-size differences by using agar and polyacrylamide as support media for electrophoresis. In both techniques the majority of endothelial proteins was grouped closer to the anode.

Data are given to show that the observed net-charge differences are reflected in the ion-exchange properties of epithelial and endothelial protoplasmic proteins. In the cell-free environment, the endothelial macromolecules bound about ten times more K^+ than the epithelial macromolecules. These binding differences could account, at least partially, for the net accumulation of ions against an "apparent concentration gradient."

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1. Damadian, R., Science **165**, 79 (1969).
2. Damadian, R., Biophys. J. **11**, 739 (1971).
3. Damadian, R., Biophys. J. **11**, 761 (1971).
4. Whiteside, T. L., Hamada, S., and Manski, W. J., submitted for publication.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. **193**, 265 (1951).
6. Scheidegger, J., J. Int. Arch. Allergy **7**, 103 (1955).
7. Chrambach, A., Reisfeld, R. A., Wycoff, M., and Zaccari, J., Anal. Biochem. **20**, 150 (1967).
8. Duke Elder, S., and Wybar, K. C., "The Anatomy of the Visual Systems." C. V. Mosby Co., St. Louis (1961).
9. Maurice, D. M., *in* "The Eye" (Hugh Davson, ed.), Vol. 1, p. 489. Academic Press, New York (1969).
10. Cope, W. F., and Damadian, R., Nature (London) **228**, 76 (1970).
11. Maurice, D. M., J. Physiol. **221**, 43 (1972).

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