plement in the anterior chamber. The mechanism of "blocking" antibody would then be to block the afferent arc of the immunologic reflex by covering surface antigens.

The results of this study clearly demonstrate a direct cytotoxic effect of heterologous complement-activated antibodies on rabbit corneal endothelial cells in vitro when serum in high titer or undiluted complement are used. The mechanism of cell damage is most probably associated with an increased cell wall membrane permeability because the intracellular enzymes are accessible to the nitroblue tetrazolium. To date, no one has been able to demonstrate by transmission electron microscopy any "holes" in the cell walls of rejected endothelium. Specific fixation of these cytotoxic antibodies to rabbit corneal endothelium has been demonstrated by fluorescein conjugated antibody staining and by decreased serum antibody titers following adsorption with rabbit corneas. It is probably a matter of time before cytotoxic antibodies can be identified by electron microscopy and their role in the corneal graft rejection process elucidated.

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The glycosaminoglycans of isolated rabbit corneal stroma, clamped between two lucite plates at near normal hydration, were digested with testicular hyaluronidase in saline solution. After equilibration with 0.9 per cent saline solution alone the sodium and chloride content of the stroma was determined. Chloride was in equilibrium with both normal and hyaluronidase-treated stroma, allowing use of the Donnan calculation for excess or bound sodium to be made. Normal stromas contained 200 mEq. bound sodium per kilogram of dry weight calculated from the Donnan calculation; hyaluronidase-treated stromas contained 110 mEq. bound sodium per kilogram of dry weight. The data show that about half of the bound sodium in the corneal stroma is on nonsaccharide binding sites. Quantitative verification of the loss of glycosaminoglycans was performed.

It has been demonstrated that the polyanionic glycosaminoglycan structure of the stroma is responsible for a significant amount of the total tissue cation binding capacity. A detailed mathematical analysis of the ion binding data has indicated, however, that about one-half of the sodium which is bound by the stroma is on nonsaccharide sites, a point of importance in relation to stromal physiology.

Glycosaminoglycans can be degraded by various methods and recent examples of such procedures, relevant to the present study, are those which have been used on the arterial wall to examine the relationship of ion binding to glycosaminoglycan content in this tissue. In the present study a digestive procedure was used in an at-
at each of the four corners of the plates, 1 and flasks were shaken for at least 12 hours and were dried to constant weight at 105° C. and percent NaCl, stromas were removed from the two lucite plates held apart with 400 μm spacers and at specific increasing concentrations of the magnesium chloride concentration is increased, there is competition between the magnesium and the dye for the negative anionic glycosaminoglycan sites and at specific increasing concentrations of the magnesium specific glycosaminoglycans can be serially identified. Photographs of the tissues were taken under identical illumination and magnification and the silver removed from pieces of the exposed film. The silver was then determined quantitatively in a Perkins-Elmer Atomic Absorption Spectrophotometer. This procedure allows both the identification and quantification of glycosaminoglycans in tissue.

**Results.** The results of the ionic analysis are shown in Table I. Stromas of normal hydration, when immersed in 0.9 per cent NaCl, show a bound sodium concentration of about 200 mEq. per kilogram of dry wt,1 with chloride usually at a slightly lower concentration than found in the ambient solution (Table I). Hyaluronidase treatment resulted in stromas having a chloride concentration close to equilibrium. Applying the Donnan equation to the hyaluronidase data shows that the bound sodium concentration is about half the normal bound sodium concentration (Table I). The net fixed negative charge, a measure of the difference between the concentration of negative and positive sites in the stroma per mass of dry tissue irrespective of whether they bind ions or not, is also reduced compared to the normal values, indicating that there is a net loss of stromal charge (Table I). Treatment with hyaluronidase results in a decrease in the amount of bound sodium on a wet and dry weight basis and suggests that the change in the glycosaminoglycans leaves only the nonsaccharide sites binding sodium. The histochemical analysis revealed that there was significant loss of both hyaluronic acid and sulfated glycosaminoglycans to values about 10 to 15 per cent of those found in normal tissues.

### Discussion.
It was suggested from findings on arterial wall that hyaluronidase causes depolymerization of the sulfated glycosaminoglycans, and the quantitative histochemical evidence sustained this suggestion. Such a change causes a reduction in the molecular weight of the side chains to less than 20,000 and results in a loss of the cation binding properties of such treated tissues. Testicular hyaluronidase was used successfully to degrade glycosaminoglycans in both arterial wall and in corneal stroma. The latter results indicated that, after digestion, the degree of swelling by corneal stroma was greatly reduced from that occurring in normal tissue.

### Materials and methods.
Corneas were removed from 3 to 4 kilogram albino rabbits, denuded of the bonding membranes by scraping with a spatula and 6 mm. diameter buttons trephined from the whole stromas. The chemical treatment utilized was testicular hyaluronidase digestion. Eight stromas were immersed in 50 ml. of 0.9 per cent NaCl containing 500 mg. testicular hyaluronidase (Type IV from Sigma Chemical Company, St. Louis, Mo. 820 NF units per milligram) for 24 hours to depolymerize glycosaminoglycans. The stromas were clamped at all times between two lucite plates held apart with 400 μm spacers and at each of the four corners of the plates, and were equilibrated with 250 ml. of 0.9 per cent NaCl for three hours after the treatment period. Eight untreated, normal stromas were also used in a parallel experiment. After equilibration in 0.9 per cent NaCl, stromas were removed from the clamp, blotted lightly on filter paper, and weighed immediately in tared Erlenmeyer flasks. Tissues were dried to constant weight at 105°C and electrolytes were extracted in 0.1 N HNO₃. The flasks were shaken for at least 12 hours and analyses made for sodium and chloride using a Beckman DU spectrophotometer with a flame attachment and a Buchler-Cotlove chloridometer, respectively.

Some corneas were treated as above, then fixed, embedded in paraffin, and sectioned at 5 μ. The treated tissue was examined using photographic densitometry after staining of the sections with Alcian Blue in different concentrations of magnesium chloride, which is a recognized method of identifying glycosaminoglycans. As the magnesium chloride concentration is increased, there is competition between the magnesium and the dye for the negative anionic glycosaminoglycan sites and at specific increasing concentrations of the magnesium specific glycosaminoglycans can be serially identified. Photographs of the tissues were taken under identical illumination and magnification and the silver removed from pieces of the exposed film. The silver was then determined quantitatively in a Perkins-Elmer Atomic Absorption Spectrophotometer. This procedure allows both the identification and quantification of glycosaminoglycans in tissue.

### Table I. Hydration and ionic analysis of normal and hyaluronidase-treated rabbit corneal stroma, with calculated bound sodium and Δ, the net fixed negative charge in the tissue.

<table>
<thead>
<tr>
<th></th>
<th>H (gm. water/gm. dry weight)</th>
<th>Na⁺ (mEq./Kg. dry weight)</th>
<th>Cl⁻ (mEq./Kg. dry weight)</th>
<th>Bound Na⁺ (mEq./Kg. dry weight)</th>
<th>Δ⁻ (mEq./Kg. dry weight)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.1 ± 0.2</td>
<td>219.2 ± 3.2</td>
<td>149.6 ± 1.9</td>
<td>194.5</td>
<td>216</td>
<td>8</td>
</tr>
<tr>
<td>Hyaluronidase-treated</td>
<td>3.5 ± 0.1</td>
<td>182.0 ± 5.9</td>
<td>155.8 ± 3.1</td>
<td>110.6</td>
<td>114</td>
<td>8</td>
</tr>
</tbody>
</table>
time to the enzyme was 24 hours at a concentration of 8,200 units per milliliter which would provide both an adequate contact time and enzyme concentration for glycosaminoglycan degradation. The quantitative examination of stromal tissue sections for glycosaminoglycans revealed that hyaluronic acid was lost entirely from the stroma and other sulfated compounds were also lost from the tissue. The relationship between hyaluronidase degradation and ion binding has been adequately demonstrated in the arterial wall, and a similar situation appears to exist in the stroma; that is that the capability for sodium binding by glycosaminoglycans is markedly reduced.

The free acid sites in the stroma are one of the major contributing factors to the swelling pressure of the stroma, and direct measurements reveal that this Donnan (or electrostatic) pressure is about one-half of the total swelling pressure of rabbit stroma in physiological solution and at near-normal hydration. After digestion with hyaluronidase the swelling capacity of the stroma is markedly inhibited, thus indicating that a major factor normally causing swelling has been negated. Enzymatic digestion with testicular hyaluronidase, therefore, appears to remove glycosaminoglycans from the corneal stroma very effectively and offers a means of studying the behavior of corneal stroma which is free of acidic sites generated by the glycosaminoglycans.

If the depolymerization of glycosaminoglycans is complete, and the quantitative histochemical data indicates a loss of about 85 to 90 per cent of these compounds, the reduction of the bound sodium value to one-half that of a normal stroma indicates that about one-half of the total bound sodium is located on anionic sites elsewhere in the stroma. The reduction in net fixed charge and the reduction in total bound sodium, both found here, provides direct experimental verification of the previous data based on the results from a large number of stromas under various ambient bathing conditions and at different hydrations, which revealed that approximately one-half of the total stromal bound sodium is on non-saccharide sites.

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Key words: ion binding, rabbit, corneal stroma, glycosaminoglycans, hyaluronidase degradation, sodium binding sites.

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Pathways of the eye’s response to topical nitrogen mustard. LEE M. JAMPOL, ALAN AXELROD, AND HOWARD TESSLER.

We studied the effect of prior corneal herpes simplex infection with its resultant corneal hypesthesia on the irritative response of the rabbit eye to topical nitrogen mustard. Both the miosis and the breakdown of the blood-aqueous barrier that follow the application of topical nitrogen mustard were diminished in eyes infected three weeks previously with herpes simplex virus. Nonspecific corneal scarring did not affect the response. This suggests again that an axon reflex requiring intact sensory innervation mediates the response.