the study was terminated 10 weeks following keratoplasty. Sera from the recipient rabbits were examined intermittently for hemagglutinating activity directed at donor antigens, but no satisfactory test for hemagglutination in rabbits could be obtained. Grafts were considered rejected when they became too hazy for iris detail to be seen. One ocular infection occurred, and this rabbit was eliminated from the study. Vessels eventually reached the graft in all eyes except one.

Results. Eight of the 38 rabbits were eliminated from the study according to the above criteria. The results in the remaining 30 rabbits are presented in Table I. No statistically significant difference in rejection rate or mean survival time was found between the two groups. When the paired samples were considered (13 of the original 19 pairs of rabbits remained intact), again no significant difference was found.

Discussion. Our results do not in any way negate those of the previous studies showing the effects of splenectomy in inbred rats, but demonstrate that quite different results can be obtained when different experimental animals are used. In addition, our animals were outbred rather than inbred, and the histocompatibility differences are not uniform. The studies in rats are applicable to penetrating corneal grafts only if a significant amount of the graft antigen first presents itself to the immune system of the recipient via the anterior chamber and intravenous route, bypassing the regional lymphatics. Antigen injected into the corneal stroma apparently does not travel by this pathway, although that from penetrating grafts may. In any event, we have shown that prior splenectomy has no effect upon the rejection rate of penetrating corneal transplants in rabbits. The significance of the contrast between our findings in rabbits and those of Kaplan and Streilein in rats requires further investigation.


Key words: Corneal allograft, splenectomy, rabbit penetrating keratoplasty, corneal graft rejection, keratoplasty enhancement.

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Corneal glycogen synthesis. I. Evidence for a gluconeogenic pathway in beef cornea. J. STEVENS ANDREWS.

Beef eye anterior chambers were perfused with media containing radiolabeled glycogen precursors. Incorporation of 14C from L-alanine-U-14C into corneal epithelium glycogen suggested the presence of a gluconeogenic pathway in the eye. Failure to isolate radioactive glucose from L-alanine-U-14C-containing perfusate after passage through the anterior chamber strongly suggests a corneal site for this pathway.

Glycogen is an important metabolic energy reserve in those tissues which synthesize and store it. The observations that glycogen is present in corneal epithelium1 and metabolically active1,2 prompted an investigation of the pathways of synthesis in beef cornea.

Since most, if not all, glucose utilized by the corneal epithelium comes from the aqueous humor of the anterior chamber,2 a method of perfusing beef eye anterior chambers with chemically defined media was devised. Incorporation of 14C-labeled glycogen precursors in the perfusion medium permitted a preliminary assessment of the pathways of glycogen synthesis.

Methods. Chemicals used in this investigation were the purest grade commercially available and were used without further purification. Radiolabeled substrates were purchased from New England Nuclear, Inc., Boston, Mass. Beef eyes were obtained at a local abattoir.

Perfusion media were prepared according to Dikstein,4 using the basal salt solution with glutathione (BSSG). The medium was placed in a single reservoir and a mixture of water-saturated 5 per cent CO2:20 per cent O2:75 per cent N2, bubbled into the reservoir. After division of the single perfusion stream into eight channels, the medium was pumped by an eight-channel pump through beef eye anterior chambers at a rate of either 170 or 60 ul per minute. In certain experiments, two reservoirs were used consecutively and a two-way Teflon stopcock was used to switch from the first to the second reservoir.

REFERENCES


The perfusion medium passed from the pump into a clear plastic box with maintaining a water-saturated atmosphere. Each perfusion channel entered a beef eye anterior chamber through a 20 g. needle and exited from the eye by the same method. The eight exit channels were collected into a single stream and pumped into a collection vessel located approximately 15 cm., above the eyes. The eyes were placed in the indentations of plastic egg carriers manufactured for camping use. The entire apparatus was placed in an incubating oven maintained at 37° C.

Upon removal of the needles from an individual eye, the corneal surface was immersed in a beaker of water at 90° C. for approximately 20 seconds. After this treatment, the epithelial layer could be easily and cleanly removed from Bowman's membrane and placed in a tube containing 1 ml. of 2N NaOH. Immersion for 30 minutes at 60° C. was sufficient to completely digest the epithelial samples. After cooling, 2 ml. of ethanol was added, the solutions were mixed, and were stored overnight at 5° C. Appropriate blank and serum albumin samples were also prepared, digested, and precipitated in conjunction with the epithelial samples.

The next morning the samples were centrifuged and the supernatant fluids transferred individually to clean test tubes for protein assay. Aliquots of 20 µl were assayed for protein by the procedure of Lowry and colleagues.13

The precipitated glycogen was washed four times with 3 ml. of 95 per cent ethanol and given a final wash with chloroform:methanol (2:1). All washings were discarded. The samples were dried at 60° C. for 20 minutes and then hydrolyzed with 0.5 ml. of 2N HCl at 105° for 2 hours. A 0.3 ml. aliquot was assayed for radioactivity by liquid scintillation and a 10 µl aliquot for glucose by an enzymatic method.14 Glycogen concentration was calculated as micromoles of glycogen glucose per milligram of epithelial protein.

In one experiment, the perfused fluid containing l-alanine-U-14C was collected in successive 3 ml. samples after an initial perfusion period of 30 minutes. Four drops of 4N HCl were added to each sample followed by 600 mg. of BioRad AG3-xA (OH) and 300 mg. of BioRad AG50W-xS8(H).15 Each sample was mixed on a vortex mixer for 30 seconds, shaken for 30 minutes, and remixed as before. After centrifugation, a 0.3 ml aliquot of the supernatant was assayed for radioactivity.

Results. In order to evaluate the distribution of the perfusion fluid in the anterior chamber, a perfusion experiment was conducted with 0.2 per cent 2,7-dichlorofluorescein in the medium and the eyes were photographed at 1 minute intervals after the perfusion had been begun at 60 µl per minute. Color photographs were taken at 0, 1, 2, and 3 minutes. After 2 minutes, the photographs demonstrate an even distribution of the dye over the posterior surface of all corneas without regard to needle placement in the anterior chamber.

Fig. 1 illustrates glucose-1-14C incorporation into epithelial glycogen from the perfusion medium. For the glucose-specific activity employed in these two experiments, approximately an hour must elapse before significant incorporation of glucose into epithelial glycogen takes place.

The results obtained in a series of experiments employing a constant level of l-alanine-U-14C and decreasing levels of unlabeled glucose are presented in Table 1. In these experiments the eyes were initially perfused with a medium containing unlabeled glucose. Thirty minutes later the second medium containing both unlabeled glucose and l-alanine-U-14C was started. Epithelia were removed from the eyes at the times indicated after starting the first perfusion medium. Over the experimental period, the steady-state rates of l-alanine carbon incorporation into epithelial glycogen appear to be approximately the same with the possible exception of a lower rate in the presence of glucose perfused at 120 mg. per 100 ml. It is of interest that some glycogen synthesis is taking place at all levels of glucose perfused. In order to estimate the quantitative contributions of alanine to the glucose pool, an experiment
replicating the one described above, but employing glucose-U-14C and unlabeled l-alanine in the second perfusion medium, was run. For convenience the results of both experiments are presented in Table I.

Next, the possibility that glucose was synthesized from l-alanine by another eye structure and subsequently incorporated into epithelial glycogen was examined by collecting perfusate and removing labeled polar compounds by resin treatment. The results are presented in Table II and demonstrate that the gluconeogenic glucose could only have been synthesized in the cornea.

Assuming that there is no compartmentation of the glucose-6-phosphate pool, then the ratio of the moles contributed by the perfused glucose and by the gluconeogenic l-alanine estimates the relative contribution of each pathway to the synthesis of glycogen. Thus, as the perfused glucose concentration decreases from 120 to 35 mg. per 100 ml., the alanine incorporation remains approximately the same but the relative contribution of alanine to the synthesis of glycogen decreases from 12 to 10 per cent over the period of time examined.

Since epithelial glycogen levels in rabbit corneas have been reported to be sensitive to mild trauma, a summary of the glycogen concentrations observed in each of the eyes in these experiments is presented in Table III. With the exception of the alanine experiment at the lowest glucose level, no consistent drop in glycogen concentration was observed over the experimental period. An analysis of variance of these values shows no significant effect on glycogen concentration due to perfused glucose concentration or to time of perfusion. Thus, it can be concluded that

### Table I. Incorporation of l-alanine-U-14C and glucose-U-14C from anterior chamber perfusate into epithelial glycogen

<table>
<thead>
<tr>
<th>Elapsed time (min.)</th>
<th>l-Alanine-U-14C* mmoles incorp. mg. protein</th>
<th>Glucose-U-14C* mmoles incorp. mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Perfused glucose in mg. per 100 ml.</td>
<td>Perfused glucose in mg. per 100 ml.</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>105</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>135</td>
<td>0.035</td>
<td>0.035</td>
</tr>
<tr>
<td>150</td>
<td>0.042</td>
<td>0.064</td>
</tr>
<tr>
<td>165</td>
<td>0.061</td>
<td>0.087</td>
</tr>
<tr>
<td>180</td>
<td>0.074</td>
<td>0.107</td>
</tr>
<tr>
<td>195</td>
<td>0.084</td>
<td>0.099</td>
</tr>
<tr>
<td>210</td>
<td>0.103</td>
<td>0.102</td>
</tr>
</tbody>
</table>

*The l-alanine concentration was 0.84 mmolar and had an specific activity of 522 d.p.m. per nmole. The glucose concentration was 35 mg. per 100 ml."

### Table II. Conversion of l-alanine-U-14C to glucose-U-14C during passage through the beef eye anterior chamber

<table>
<thead>
<tr>
<th>Medium treatment</th>
<th>l-Alanine-U-14C* mmoles incorp. mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonperfused Glucose-U-14C*</td>
<td>5,584 4,392 79%</td>
</tr>
<tr>
<td>Nonperfused Lactate-3-14C</td>
<td>5,092 0 0</td>
</tr>
<tr>
<td>Nonperfused l-Alanine-U-14C</td>
<td>68,756 869 1.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Perfused*</th>
<th>l-Alanine-U-14C* mmoles incorp. mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>170 29 17</td>
</tr>
<tr>
<td>2</td>
<td>137 0 0</td>
</tr>
<tr>
<td>3</td>
<td>10,433 70 0.6</td>
</tr>
<tr>
<td>4</td>
<td>30,833 158 0.4</td>
</tr>
<tr>
<td>5</td>
<td>36,754 340 0.8</td>
</tr>
<tr>
<td>6</td>
<td>43,403 291 0.5</td>
</tr>
<tr>
<td>7</td>
<td>46,707 187 0.3</td>
</tr>
<tr>
<td>8</td>
<td>44,579 180 0.3</td>
</tr>
<tr>
<td>9</td>
<td>42,464 179 0.4</td>
</tr>
<tr>
<td>10</td>
<td>39,850 218 0.5</td>
</tr>
</tbody>
</table>

*The l-alanine concentration was 0.84 mmolar and had a specific activity of 522 d.p.m. per nmole. The glucose concentration was 35 mg. per 100 ml. The usual two-stage perfusion was performed at 60 ml per minute.

### Table III. Epithelial glycogen concentrations

<table>
<thead>
<tr>
<th>Perfusate glucose in mg. per 100 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusate glucose in mg. per 100 ml.</td>
</tr>
<tr>
<td>A* G A G A G A G</td>
</tr>
<tr>
<td>35 50 85 120</td>
</tr>
<tr>
<td>105 0.37 0.32 0.30 0.37 0.34 0.40 0.43 0.33</td>
</tr>
<tr>
<td>120 0.31 0.41 0.30 0.37 0.40 0.33 0.29 0.40</td>
</tr>
<tr>
<td>135 0.33 0.36 0.35 0.38 0.31 0.33 0.39 0.38</td>
</tr>
<tr>
<td>150 0.29 0.36 0.32 0.38 0.31 0.32 0.26 0.40</td>
</tr>
<tr>
<td>165 0.25 0.31 0.42 0.40 0.31 0.39 0.41 0.37</td>
</tr>
<tr>
<td>180 0.27 0.31 0.29 0.40 0.33 0.38 0.39 0.32</td>
</tr>
<tr>
<td>195 0.27 0.38 0.36 0.36 0.35 0.31 0.37 0.37</td>
</tr>
<tr>
<td>210 0.25 0.31 0.35 0.38 0.33 0.38 0.28 0.43</td>
</tr>
</tbody>
</table>

*The headings A and G refer to the radioactive substrate perfused in that particular experiment (A = alanine and G = glucose).
mild trauma due to manipulation of the cornea had no consistent effect on epithelial glycogen concentration during the experiment.

Discussion. Eyes perfused in the manner described above appear "normal." The perfusion medium remains clear, as does the cornea, and the pupil contracts during the experiment. Occasional blockage of the outflow path results in increased intraocular pressure and loss of corneal transparency.

Beef corneal epithelium glycogen concentrations as the eyes arrive in the laboratory are quite variable but average 0.416 μmoles of glycogen glucose per milligram of protein. Perfusion with normal levels of glucose stops the continued decrease that occurs and results in an equilibrium value of approximately 0.22 μmoles of glycogen glucose per milligram of protein within 60 minutes. The actual value attained was characteristic of the particular eye, that is, the level of glucose perfused and the initial concentration of glycogen. In those experiments using glucose-U-14C no relationship could be established between the equilibrium glycogen concentration and the amount of labeling found. Thus, it is apparent that within the range of concentrations presented in Table III, the amount of precursor incorporated is related to the amount of precursor present rather than the concentration of the end product.

Since changes in glycogen concentration were anticipated, an initial perfusion period with the experimental glucose concentration was used in order to stabilize epithelial glycogen concentrations. An analysis of variance of all the glycogen concentration data (Table III) supports attainment of these objectives. An F test for the error due to perfusion time is nonsignificant, as is the F test for glucose levels. Although there is some variability present in the data for l-alanine-U-14C incorporation into glycogen, an analysis of variance of the data in Table I demonstrates a significant dependence on perfusion time and confirms that the amount of l-alanine incorporated is proportional to the concentration of the end product.

Treatment of the glucose incorporation data in exactly the same manner revealed that the level of perfused glucose was directly proportional to the rate of glucose incorporation into glycogen but that the length of the perfusion period was not proportional to the amount of glucose incorporated. Examination of Table I reveals the presence of two consecutive high values of glucose incorporation during the initial perfusion period with 85 mg. per 100 ml. of glucose. It seems likely that these two values are spurious and probably result from some experimental deficiency such as incomplete removal of free glucose-U-14C from the precipitated glycogen.

Essentially similar rates of l-alanine incorporation suggest that the enzymatic steps between glucose-6-phosphate and glycogen are not saturated by the concentrations of glucose-6-phosphate attained and, therefore, the rate of incorporation from the precursor pool is proportional to the precursor concentration. Division of the estimated rates of glucose-U-14C incorporation (μmoles per hour) by the perfused glucose concentration (μmoles per liter) results in similar incorporation figures for each of the glucose experiments. Since l-alanine was perfused at the same concentration in all experiments, similar rates of l-alanine-U-14C incorporation at all glucose concentrations should be observed.

The perfused concentration of alanine (0.84 mmolar) is higher than that reported for rabbit (0.6 mmolar)19 and human (0.3 mmolar)20 aqueous and was chosen somewhat arbitrarily to correspond to assumed conditions of starvation. Recent investigations have shown that the aqueous-plasma ratio of l-alanine in man is 1.020 and in rabbits exceeds 1.0.20 Thus, although the experiments with perfused l-alanine in the presence of high levels of glucose are physiologically artificial, the results show that the gluconeogenic pathway in the cornea is available to l-alanine at all concentrations of aqueous glucose.

From the Department of Ophthalmology, Vanderbilt University School of Medicine, Nashville, Tenn. This project was supported in part by NIH Research Grant EY00048, awarded by the National Eye Institute, PHS/DHEW. Submitted for publication Sept. 11, 1975. Reprint requests: J. Stevens Andrews, Ph.D., Department of Ophthalmology, School of Medicine, Vanderbilt University, Nashville, Tenn. 37232.

Key words: cornea, beef, l-alanine, glycogen, perfusion, anterior chamber, gluconeogenesis.

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Hydrostatic pressure effects on deswelling of de-epithelialized and de-endothelial-
ized corneas. KAREN A. BOWMAN AND KEITH GREEN.

The effect of varying hydrostatic pressure on the thinning rate of preswollen de-epithelialized 
or de-endothelialized corneas has been determined in the specular microscope. The appropriate mem-
brane was removed, the cornea given access to Ringer to swell, and then fluid exchange at that 
surface blocked with oil. De-epithelialized corneas thin more slowly as hydrostatic pressure on the 
posterior surface is increased, until fluid movement ceases at 60 to 70 mm. Hg. Fluid movement can 
occur, therefore, against a considerable hydrostatic pressure. De-endothelialized corneas thin at a 
high rate as hydrostatic pressure is increased; this effect is probably a mechanical one with in-
creasing pressure forcing fluid out across the epithelium.

Since its introduction, the specular microscope has been used extensively in the study of corneal physiology.1-3 With this system, the cornea can be excised atraumatically from the eye and mounted in appropriate chambers for determina-
tion of thickness to an accuracy of ± 2 μm. With little variation most investigators have elected 
to use hydrostatic pressure of about 15 mm. Hg4-5 on the endothelial surface, although lower pres-
sures have been used, e.g., 8 mm. Hg. One study has reported no effect of hydrostatic pressure variation between 6 and 37 mm. Hg on the deswelling rate of the de-endothelialized cornea.5

The present work was a study of the effects of varying the applied hydrostatic pressure be-
tween 5 and 50 mm. Hg on the deswelling rate of both de-epithelialized and de-endothelialized corneas.

Materials and methods. Adult albino rabbits, 2 to 3 kilograms, were killed with an overdose of 
sodium pentobarbital and the eyes enucleated with the lids as described previously.1 Corneal ex-
cision and mounting in the appropriate chambers also followed the standard procedure.1-3

De-epithelialized corneas. For convenience the epithelium was removed from the cornea with a 
Gill corneal knife prior to enucleation and mounting. The stroma, therefore, was allowed access to fluid over a time period of about 10 minutes prior to placement in the chamber. Krebs-bicarbonate-Ringer’s solution,1 with added adeno-
sine (5.0 mM.) and reduced glutathione (0.3 mM.),4 was placed on the exposed stromal sur-
face and the stroma allowed to swell for 15 minutes after mounting; the fluid was removed and the stroma covered with silicone oil (No. 20 CSKS Dow Corning, Midland, Mich.) to pre-
vent further fluid exchange across the exposed stromal surface. Thickness was measured by al-
ternately focusing on the anterior stromal surface and the endothelium. The endothelial surface was 
perfused with modified Krebs-bicarbonate-Ringer’s solution at 37° C. at a rate of 33 μl per minute.

A control series consisted of thickness measure-
ments at 15 mm. Hg for 5 hours. The experi-
mental series consisted of an initial 1 hour of 
15 mm. Hg, for comparison with the controls, fol-
lowed by one of three other subsequent pressures 
5, 30, or 50 mm. Hg applied for the second hour, a different pressure from this series ap-
plied for the third hour, and the remaining uns-
used pressure from this series applied for the 
fourth hour, which allowed at least four corneas 
to be subjected to each possible sequence of these 
three pressures. A final period of 15 mm. Hg 
followed between hours 4 and 5. Each pressure 
was applied for 1 hour, and the deswelling rate 
determined over the final 30 minutes of each hour, since initial experiments showed that the 
thickness adjustment became regular within 30 minutes.

De-endothelialized corneas. The endothelium was removed by gentle scraping with the cornea 
atached to the mounting rod. The posterior sur-
face of the cornea was given free access to Ringer’s solution and allowed to swell for 20 minutes. The normal mounting procedure was then fol-
lowed except that silicone oil was perfused across 
the exposed posterior stromal surface and the 
epithelial surface was perfused with modified 
Ringer’s solution. The perfusion system delivered 
Ringer’s at 1 ml. per minute and 32° C. to the 
epithelial surface and the fluid level was main-
tained with a vacuum overflow. The normal vol-
ume needed to fill the anterior space bordering 
the epithelium was 1 ml. without immersion of 
the objective, thus the turnover was 1 volume 
per minute, sufficient to prevent stagnation of 
the epithelial fluid bath which, by evaporation, 
could become more hypertonic. The objective was 
immersed into the perfusing solution every 30 
minutes for thickness determinations.

A similar experimental protocol to that out-
lined above for the de-epithelialized corneas was 
followed; that is, at least two corneas were used.

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