A study of enzyme activity in corneal repair

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The use of nitroblue tetrazolium to stain corneal dehydrogenase enzymes indicates several things:

An enzyme profile of a given group of cells may be a mirror of the normality of the cells and may be a guide to the viability of tissue.

When a central button of rabbit cornea is killed by freezing, the healing tissue has a higher concentration of oxidative enzymes than the normal cornea. Healing is a process which requires oxidative energy.

In the normal cornea the oxidative enzymes appear more concentrated in the superficial third of the corneal stroma. This suggests that the enzyme concentration and the metabolism may depend on the availability of oxygen from the air.

When one eye of a rabbit is sutured shut, after 48 to 72 hours the concentrations of DPN and TPN diaphorase, lactic, alpha glycerophosphate, malic dehydrogenases, and probably succinic dehydrogenase, decrease.

Tetrazolium stains permit the histochemical demonstration of dehydrogenase enzymes in tissue sections. These stains are soluble compounds which act as hydrogen acceptors developing insoluble formazan precipitates in the reduced state. Blue tetrazolium was first used by DeBerardinis to study frozen corneal tissue sections and subsequently has been used by Kuwabara and Cogan, who studied the dehydrogenase activity of intact corneas. A more recent study by Baum employed nitroblue tetrazolium (NBT), a more sensitive indicator of dehydrogenase activity. With this agent, Baum was able to demonstrate a variety of dehydrogenases in frozen corneal sections. The methods employed in this study are similar to those used by him.

Two major uses of this technique have been studied in this laboratory. First, a series of enzyme stains (an enzyme profile) has been used to indicate the health or normality of cells. Cells that are dead or severely damaged do not have normal enzyme patterns. This method has provided a valuable tool for the study of the preservaton of corneal tissues. These studies will be presented in detail elsewhere. Second, a survey of dehydrogenase activity has provided interesting information on the energy requirements of wound healing. These studies will be outlined.

Methods

In order to produce areas of damage to the cornea of sufficient size to permit study, a method previously employed by Maumenee was used. The
central cornea of both eyes of 1 to 2 kilogram New Zealand white rabbits was frozen, with a brass rod tapered to have a 5 mm. diameter end. The rod was cooled in a dry ice—alcohol bath, dried, and applied for 5 seconds to the protruded eye of a rabbit anesthetized with Nembutal. When the effect of lid closure on corneal repair was studied, one eye was closed by suturing the lids shut with 4-0 silk. In these lid closure experiments, animals were sacrificed at 24 hour periods for 7 days after the initial trauma (Figs. 1 to 4).

Eyes for enzyme studies were enucleated and, in general, were immediately frozen in liquid nitrogen. They were compared with eyes which were not frozen in liquid nitrogen but were placed in a freezer kept at -70° C. No difference was seen in the enzyme staining patterns between the liquid nitrogen frozen and the -70° C. frozen eyes. Some eyes in this study were frozen at -70° C. All eyes were stored at this latter temperature, and in most cases the eyes were sectioned within a few days after enucleation. Controlled studies indicate that storage of the eyes for 2 to 3 months did not affect the staining results.

The basic staining media was stored in 4 ml. aliquots at -20° C. At this temperature, preservation was adequate for at least one month. However, storage at -4° C. was not adequate. Substrates were added in liquid or powder form to make a final concentration as shown in Table I. Coenzymes were added in powder form to provide the concentrations indicated.

In order to study enzyme profiles of corneal stroma from serial sections, the corneas were removed while the eyes were still frozen and were placed on cryostat mounts. The corneal sections were removed from the cryostat knife by touching drawn capillary tubes to the tissue or its surrounding ice. Care was taken to limit the amount of ice present when the cornea was transferred to the slide. The sections were then placed under a heat lamp for about 45 seconds to achieve a temperature of 36 to 40° C. Immediately after drying, 1 ml. of the staining solution was placed on the slide, and the incubation time was regulated to achieve the desired intensity of deposit. After tetrazolium staining, the slide was washed in several changes of distilled water, fixed in buffered formalin for 3 minutes, and mounted in glycerol.

Endothelial studies were accomplished by staining whole corneas which were frozen and then submerged in a vial containing 4 ml. of the basic medium plus the substrate and coenzymes. During and after the staining process, the endothelium was easily studied under the dissecting microscope while still in situ. Permanent preparations were made by immersing the corneas in 10 per cent buffered formalin, dehydrating in alcohols and an alcohol—ether mixture, and immersing the cornea in collodion for 20 minutes to 1 hour. In the dehydration process, it must be borne in mind that the formazan precipitate is soluble in alcohols and ether, and some of the stain will be lost. A minimum time in the alcohols should be used (about 5 minutes in 70, 80, 95, and 100 per cent alcohol and a similar time in the 50 per cent alcohol to 50 per cent ether mixture). After immersion in collodion, the cornea was inverted and a stream of air was applied over the endothelial surface to remove excess collodion and dry the superficial film. The cornea was then placed in water for at least 5 minutes and the collodion was peeled off, taking with it the endothelium. Collodion sheets were cleared in terpineol, dehydrated in xylool, and mounted. In some studies the endothelium was peeled off without the use of collodion and was then mounted in glycerol (Fig. 5).

In all cases in which the enzyme concentrations in open and closed eyes were compared, the eyes were enucleated at the same time and kept frozen.

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Molarity</th>
<th>NBT (mg./ml.)</th>
<th>Buffer</th>
<th>Mg</th>
<th>CN</th>
<th>PVP (%)</th>
<th>pH*</th>
<th>H₂O (ml.)</th>
<th>Coenzyme</th>
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<tr>
<td>None (control)</td>
<td>1</td>
<td>.25</td>
<td>.005</td>
<td>.05</td>
<td>6</td>
<td>7.16</td>
<td>DPN</td>
<td></td>
<td></td>
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<tr>
<td>Sodium lactate</td>
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<td>.05</td>
<td>6</td>
<td>7.15</td>
<td>4</td>
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<td>α-glycerophosphate</td>
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<td>.05</td>
<td>6</td>
<td>7.11</td>
<td>4</td>
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<td></td>
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<tr>
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<td>.25</td>
<td>.005</td>
<td>.05</td>
<td>6</td>
<td>7.20</td>
<td>4</td>
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<td></td>
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<tr>
<td>Sodium succinate†</td>
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<td>.005</td>
<td>.05</td>
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<td>DPNH</td>
<td>15 mg./ml.</td>
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<td>.005</td>
<td>.05</td>
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<td>7.10</td>
<td>4</td>
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<tr>
<td>TPNH</td>
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<td>.005</td>
<td>.05</td>
<td>6</td>
<td>7.10</td>
<td>4</td>
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<tr>
<td>Glucose-6-phosphate</td>
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<td>.25</td>
<td>.005</td>
<td>.05</td>
<td>6</td>
<td>7.10</td>
<td>4</td>
<td>TPN</td>
<td></td>
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<tr>
<td>Alcohol (absolute ethanol)</td>
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<td>.005</td>
<td>.05</td>
<td>6</td>
<td>7.10</td>
<td>4</td>
<td>DPN</td>
<td></td>
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<tr>
<td>α-glyceraldehyde</td>
<td>.5M</td>
<td>.25</td>
<td>.005</td>
<td>.05</td>
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<td>7.10</td>
<td>4</td>
<td>DPN</td>
<td></td>
</tr>
</tbody>
</table>

*The pH for these stains was critical.
†Phenazine methosulfate used was 1 mg. milliliter to enhance staining.
Fig. 1. Brass rod used for freezing the cornea, 5 mm. diameter end.

Fig. 2. Rabbit cornea immediately after freezing.

Results

The results to be summarized represent observations on more than 200 eyes. The photomicrographs shown are representative. Although there are some variability, most of the changes noted were obvious and easily repeatable.

After acute cell death, such as occurs with sudden freezing, about 2 hours were required for the loss of enzyme staining from corneal tissue kept at room temperature. This enzyme loss occurred whether the tissue remained in situ or whether it was incubated in a balanced salt solution. Presumably the loss of staining was caused by the leakage of the dehydrogenase enzymes from the damaged cells. Hematoxylin staining of the cell nuclei persisted for 12 hours although some alterations in nuclear structure could be noticed earlier. Normal cells, on the other hand, maintained enzyme patterns whether in situ or in balanced salt solution for at least 8 hours.

Endothelium

The normal endothelium was easily stained for lactic and malic dehydrogenases as well as for DPNH and TPNH diaphorase. After freezing of the cornea, no endothelial cells were seen in the wounded area until 1 to 2 days after injury when healing had begun. By 2 to 3 days after the initial wounding of the cornea, the patterns of enzyme staining in the re-
generating endothelium were apparent and striking. Cells in the healing area showed much darker staining and much higher concentrations of all of the enzymes studied than did the normal endothelial cells. The border of the healing cornea was usually sharp, and the cells in the area undergoing mitosis and regeneration were heavily colored when stains were performed for the oxidative dehydrogenase enzymes. By approximately 5 days after wounding, however, these higher concentrations of enzymes in the healing area disappeared and the new endothelium assumed the char-

Fig. 3. Rabbit cornea one-half hour after freezing. The cornea has thawed and edema is slight.

Fig. 4. Rabbit cornea 1 day after freezing. Progressive edema is developing which becomes most severe at about 3 days after freezing.

Fig. 5. Normal corneal endothelium stained for lactic dehydrogenase. (×250.)
Fig. 6. Corneal endothelium 3 days after freezing. The normal cornea on the left shows a relatively small amount of lactic dehydrogenase staining. The healing cornea on the right is very heavily stained, indicating that high concentrations of the enzyme are present. (×250.)

Stroma

Studies of the corneal stroma were similar, in general, to those of the endothelium. Cellular regrowth seemed to take place first in the region above the endothelium and then just below the epithelium, with the areas in the midstroma healing most slowly. In all preparations the diaphorases stained very heavily and quickly, a heavy stain generally being seen within 3 minutes. Lactic dehydrogenase (LDH) also stained quickly and heavily, the staining time usually being 5 to 8 minutes. When staining was regulated so that there were no maximal deposits, there was a clear-cut difference between the normal cornea and the keratocytes in the regenerating area, enzyme concentrations in the newly healed area being higher in the stroma as they were in the area of endothelial repair. These differences were seen with diaphorase stains and stains for lactic, malic, and alpha glycerophosphate dehydrogenase.

One striking observation was that, especially in the normal cornea, there seemed to be a concentration of both the diaphorases and the dehydrogenases in the superficial layers. The marked concentration of stain for oxidative enzymes in the superficial corneal layers suggested that oxygen from the air might be involved in the stromal metabolism, especially that of the superficial cornea. This finding, combined with the apparent fact that repair of the cornea required higher concentrations of enzymes and presumably a higher production of energy, inspired experiments to contrast corneal repair in the open eye to which oxygen from the air was readily available with that in the eye with the lids closed.

Stromal repair in open and closed eyes

Clinically there was a definite difference between eyes which remained open and
eyes with the lids sutured shut. After the initial freezing, the corneal edema increased during the first 3 days and was similar in the open and closed eyes (Figs. 1 to 4), although the closed eyes might have been slightly more edematous. After the third day, however, the open eyes began to clear rapidly, the corneal opacity usually disappearing between the third and fourth day. By the fourth day, the corneas of the open eye were slightly thickened but much thinner than those of the closed eyes, and

Fig. 7. Corneal endothelium 3 days after freezing, stained for malic dehydrogenase. The normal endothelium on the left contains a relatively small amount of enzyme, but the healing endothelium on the right is densely stained. (×400.)

Fig. 8. Tritiated thymidine uptake in stromal cells as seen in autoradiography. A, Four days after freezing, eyes open. (×40.) B, Four days after freezing, eyes closed. (×40.) In the open eye there are many dark cells which have taken up the thymidine. In the closed eye only one cell in the healing area has taken up tritiated thymidine, and this is not near the surface of the cornea.
corneal edema was obviously more severe in the closed eyes. Examination of rabbits 5 days after injury revealed no opacity in the opened eye while that in the closed eye often persisted as a faint nebula. In the open eye the thickness of the cornea was sometimes as much as a third more than normal, but in the closed eye the cornea was usually swollen 1½ to 2 times normal. By the sixth day, the edema in both eyes was usually much less. By the seventh day, both eyes had healed, the clinical improvement in the closed eyes generally being 1 to 2 days slower than that in the open eyes.

Serial sections of the wounded cornea stained by hematoxylin and eosin showed results somewhat similar to those found on clinical examination. The healing of the opened eye was usually 1 to 2 days in advance of that of the closed eyes, in terms of the size of the central defect and cellularity, but this was exceedingly difficult to quantitate.

A further indication of this delay in healing was provided by studies with tritiated thymidine. Almost all stromal cells taking up tritiated thymidine were near the advancing border of keratocytes as they migrated into the acellular center of the wound. The cells nearest the acellular area appeared to be dividing most rapidly and in the open eyes many cells taking up tritiated thymidine were found near the edge of the defect extending from the subepithelial area down to the endothelium. The tritiated thymidine uptake by keratocytes of closed eyes was markedly decreased. The number of cells taking up the thymidine was reduced considerably at 3, 4, and 5 days. It was very rare to find dividing cells in the subepithelial area, most cells being in the midstroma or in the area near the endothelium.

Since the repair of corneal defects seemed to require larger amounts of energy, it was not unexpected that any change in the normal metabolism which would affect energy production might influence the healing of these wounds. Present evidence indicates that the bulk of metabolism of the

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Fig. 9. Sections of corneal stroma in open and closed eyes stained for malic dehydrogenase 3 days after injury. Both sections were placed on the same slide, stained simultaneously, and photographed under identical conditions. The open eye on the left has higher concentrations of enzyme in the healing area than in the normal area. There is stratification such that the enzyme concentration appears highest in the superficial layers of the cornea, and the enzyme stain of the open eye is much heavier than that of the closed eye. (×40.)

Fig. 10. Corneal stroma stained for malic dehydrogenase 5 days after injury. The opened eye (left) has higher concentrations of enzyme than the closed eye. In the closed eye, the enzyme concentration which seemed higher in the superficial layers of the normal eye now appears nearly uniformly distributed throughout the corneal stroma. (×40.)
corneal stroma is oxidative. Previous evidence suggests that this oxygen comes, to some extent, from diffusion via the anterior chamber, but the primary source of oxygen appears to be from the air. It has already been demonstrated that closing the eyes decreases the availability of oxygen to the cornea. Corneal repair, therefore, was studied by staining the dehydrogenases in paired eyes of the same rabbit, one eye open and one closed. When enzymes were studied, serial sections from both eyes were treated identically and paired sections were stained on the same slide in order to be certain that results were comparable.

The heaviest and most rapid staining in all cases was seen when diaphorase was studied and either DPNH or TPNH was used as the substrate. Although it is possible that transhydrogenase enzymes might function to make the demonstration of diaphorase less specific, the addition of either DPNH or TPNH resulted in prompt, strikingly dark staining. In order to demonstrate differences, it was necessary to stain the eyes in a very short period of time. When this was done differences were apparent. At 2 to 3 days, a decrease could be seen in the diaphorase content of both normal and injured closed eyes. The higher concentration of diaphorase in the superficial stroma of open eyes was no longer apparent after the eyes had been closed for 3 to 4 days, the distribution then being nearly homogeneous throughout the normal cornea. In the closed eyes, not only did diaphorase content decrease but the number of diaphorase positive cells in the healing area was reduced as was the cellularity when measured by other parameters.

The study of lactic dehydrogenase yielded results that were generally similar to those with diaphorase. Although the lactic dehydrogenase took considerably longer to stain than did the diaphorase, the apparent amount of lactic dehydrogenase also decreased after 3 to 4 days of closure of the eyes. The stratification and higher concentration of the enzyme initially present in the subepithelial areas were no longer obvious. The total enzyme content, therefore, appeared to decrease with closure of the eyes. The results of the staining of alpha glycerophosphate dehydrogenase...
were virtually identical to those with lactic dehydrogenase.

The staining of malic dehydrogenase was very satisfactory, although color development required longer than was required for a similar intensity with either lactic dehydrogenase or diaphorase staining. With malic dehydrogenase, differences between the open and closed eyes were perhaps the most striking. As with other enzymes, the concentration of malic dehydrogenase in the more superficial layers of the stroma decreased as the eye remained closed. The migration of enzyme-containing cells into the wounded area was slower when the eyes were closed. There was a marked difference in total enzyme content of the cornea between the open and closed eyes, the closed corneas being markedly depleted of this oxidative enzyme as well as the others. Photographs of malic dehydrogenase taken from sections stained on the same slide under identical photographic conditions show that there is a marked difference in staining for this enzyme between corneas which were covered by the lids and those which were freely exposed to the oxygen of the air. The healing of the cornea seemed to correlate well with the intensity of the enzyme stains.

It was possible to stain stromal succinic dehydrogenase, especially when phenazine methosulfate was used. In our hands, however, this stain was not completely satisfactory and it was not sufficiently reliable to permit changes in concentration to be judged with assurance. It was our impression that the changes of concentration in succinic dehydrogenase were similar to those of the other oxidative enzymes, but this cannot be stated with certainty. The study of succinic dehydrogenase is desirable, since this enzyme does not depend on DPN or diaphorase for its hydrogen transfer; however, the prompt and dense staining with diaphorase seen in all sections strongly suggests that diaphorase was never the limiting factor in the staining of other enzymes. Similarly, our sections suggest that the alterations in diaphorase and malic dehydrogenase after closing the eyes may be more marked than the decrease in alpha glycerophosphate dehydrogenase and in lactic dehydrogenase (these enzymes, though aerobic, are part of the glycolytic pathway), but this technique appeared too imprecise to permit a definite conclusion.

Stains for glucose-6-phosphate dehydrogenase were made but did not demonstrate sufficient enzyme to draw conclusions.

**Discussion**

In previous reports, the mechanism of action and special considerations of tetrazolium staining have been explored in great detail. Although the corneal enzymes observed in our preparations have been observed previously in normal cornea, it seems apparent that the use of these in the histochemical portrayal of enzyme profiles provides one measure of the normality of cells, and can serve as an indication of cellular damage. This should be a valuable guide in the study of tissue preservation.

Many excellent studies of corneal metabolism have been done by conventional biochemical techniques. Present evidence indicates that the metabolism of the cornea is primarily oxidative. The hexose monophosphate shunt seems to play little role in the metabolism of the corneal stroma. Under normal circumstances, when the eye is open, glycolysis seems to be relatively unimportant. In fact, studies by Kuhlman suggest that, depending on the relative concentrations of glucose and lactate in which the cornea is incubated, the cornea may under some circumstances preferentially oxidize lactate. Langham has already provided evidence that the normal cornea depends on oxygen in the air and that closing the eye can alter the corneal metabolism. In excellent studies, he has shown that suturing the lids of one eye of a rabbit closed for 3 to 4 hours caused no increase in corneal lactic acid concentration; however, 24 hours of lid closure increased the corneal lactic acid to 114 per cent of the value of the other eye.
other studies in which rabbits breathed oxygen via an endotracheal tube but remained in an anaerobic nitrogen atmosphere, the lactic acid concentration of the corneas in the anaerobic atmosphere rose to 133 per cent of normal. These results indicated that with the eye open the cornea was able to use atmospheric oxygen and that this was an important source of oxygen for the oxidative metabolism. In the closed eye it seemed possible that some oxygen might diffuse to the cornea from the vessels of the closed lids; however, the oxygen available was certainly less than with the eye open, and lactate accumulated. In addition to oxygen from air or, to a much lesser extent, from the closed lids, it is likely that some oxygen is available to the cornea from diffusion through the anterior chamber. Although oxygen may be available to some extent from the lid vessels and from the anterior chamber, there is no question that in Langham's experiments the oxygen supply to the cornea was depleted when the eyes were closed and that lactic acid concentration increased.

In the absence of oxygen, the cornea rapidly shifts its metabolism from an aerobic one to an anaerobic one after the depletion of available oxygen. Our studies have shown that even the enzymes required for oxidative metabolism begin to decrease in amount in an obvious way after the eyes have been closed for 2 to 3 days. The relative concentration of these aerobic enzymes in the more superficial layers of the normal corneal stroma seems to be caused by the availability of oxygen in this area since, even in the normal cornea, when the eyes are sutured shut this differential concentration disappears. It seems likely that when the cornea is deprived of a significant proportion of its available oxygen, not only does the metabolism shift toward a glycolytic one, as might be expected from the Pasteur effect, but the total energy which the cornea can mobilize is probably decreased.

It has been well documented in bone, brain, and elsewhere that the healing of wounds is accompanied by an increase in metabolism and an increase in aerobic enzymes. This appears to be true in the cornea also. In the endothelium there is a marked increase in the malic and lactic dehydrogenase as well as in diaphorase. This is restricted to the healing area and is very striking indeed. In the stroma, the differential is less obvious but no less definite. The differential rate of wound healing of the endothelium between open and closed eyes was not studied, but it seems likely that the moderate slowing of healing in the corneal stroma of closed eyes is caused, at least in part, by the relative deprivation of oxygen occurring when lids are sutured shut. Although many variables take place when the lids are sutured shut for a period of days, the persistence of edema and clouding of the cornea, the relative slowing of cellular ingrowth as estimated by standard histologic techniques, and the decrease of division of cells as measured by tritiated thymidine uptake suggest that even though the epithelium has completely regrown within about 12 hours and protects the cornea, the stroma of the cornea does not heal at a normal rate unless oxygen is freely available. It must be emphasized that although the healing of the cornea may have been moderately retarded, it was by no means stopped. Energy available from glycolysis and from the oxygen supply by diffusion through the anterior chamber and perhaps from the lids would be expected to permit corneal metabolism to continue and reparative processes to occur. It was only the rate of repair which appeared to be moderately slowed.

Summary

The use of nitroblue tetrazolium to stain corneal dehydrogenase enzymes indicates several things:

1. An enzyme profile of a given group of cells may be a mirror of the normality of the cells and may be a guide to the viability of tissue.
2. When a central button of rabbit
cornea is killed by freezing, the healing tissue has a higher concentration of oxidative enzymes than the normal cornea. Healing is a process which requires oxidative energy.

3. In the normal cornea the oxidative enzymes appear more concentrated in the superficial third of the corneal stroma. This suggests that the enzyme concentration and the metabolism may depend on the availability of oxygen from the air.

4. When one eye of a rabbit is sutured shut, after 48 to 72 hours the concentrations of DPN and TPN diaphorase, lactic, alpha glycerophosphate, malic dehydrogenases, and probably succinic dehydrogenase, decrease. The relative abundance of these enzymes in the superficial layers of the cornea is no longer seen. With this decrease in oxygen supply to the cornea and with the decrease in enzyme concentrations, there is a moderate retardation of wound healing as indicated by persistence of corneal edema and clouding, slight delay in cellular ingrowth into the wounded area, and a decrease in the number of cells dividing as measured by uptake of tritiated thymidine. Even with the eye closed, corneal healing continues but seems to lag 1 to 2 days behind the healing of the cornea of the open eye. Wound healing requires energy, and, since some of the necessary corneal oxygen appears to come from the air, closure of the eyes appears to decrease the concentration of enzymes required for oxidative metabolism and to cause a modest delay in corneal repair.

REFERENCES

Discussion
Virginia Weimar, Atlantic City, N. J. I have found most interesting Drs. Kaufman and Capella's comments on a possible new means of evaluating the viability of cells. This has been a problem of major importance to cell physiologists and to those studying the influence of carcinogenic agents on cells.

I wish to commend the authors on the very careful work which they have done in the preparation of tissues for histochemical study. However, analysis of the healing cornea by histochemical means presents special difficulties with reference to the study of the stroma.

The epithelium and the endothelium are easily evaluated. As the authors have indicated, the healing endothelium becomes very rich in diaphorase and dehydrogenase activity which is probably considerably underestimated, as they say, because of the necessity in their procedure of dehydrating through solvents that remove some of the diformazan formed. Probably the only reasonable
estimate of the extent of loss of activity by such
dehydration and clearing treatments can be made
by evaluating pairs of sections, one of which is
dehydrated and cleared through the alcohols and
ether or xylol, and the other of which is mounted
in an aqueous mounting medium. We have found
that in such pairs of sections a great deal of
diformazan is actually removed by the alcohol-
ether or xylol treatments. This loss is not notice-
able, however, in very rich preparations.

The chief point, however, concerns the inter-
pretation of enzyme activity of any type in the
healing stroma. Work which has been done by
others on the histochemistry of healing connective
tissues shows that the enzyme activity found is
primarily in the invading white blood cells.

For example, Gedigk and Fischer1 histochem-
ically evaluated 12 different enzymes in 6 different
cell types in the formation of new connective
tissues in response to injury. The major enzyme
activity found was in the various types of white
blood cells. They did find some DPN diaphorase
activity in proliferating mesenchymal cells, but
found that the greatest activity was present in the
monocytes with weaker activity in lymphocytes
and polymorphonuclear leukocytes.

White blood cells are rich in many enzymes,2 to
cite a few: lactic dehydrogenase, glutamic acid
dehydrogenase, glyceraldehyde-6-phosphate de-
hydrogenase, acid and alkaline phosphatase, β-
glucuronidase, and many others too numerous to
mention.

In our work on the enzymology of the healing
corneal stroma, we have found that it is imprer-
tive to counterstain so that the cell type having
the enzyme activity may be determined.3 During
the first 4 or 5 days of corneal stromal repair, we
have found that the enzyme activity of the in-
vading white blood cells of all types may com-
pletely mask that of the keratocytes.

For this reason it is not possible to interpret the
data presented by the authors on the healing
corneal stroma as indicative of enzyme activity
in the keratocytes until the results of suitable
counterstaining of the cells involved are available.
It may very well be that the enzyme activity is
indeed in the keratocytes, but, in view of the
known intense enzyme activities of the invading
white blood cells, we must await further studies
on this point.

I should like to make a point concerning the use
of tritiated thymidine as an indicator of cell
division. It has been shown by Pelc4 that tritum
labeled thymidine may be taken up extensively by
cells which do not undergo cell division. For ex-
ample, in one case Pelc cites a labeling index of
45 per cent and a mitotic index of 1.2 per cent.
He suggests that the total amount of DNA of a
cell can be removed without cell division.

It makes no difference in this particular paper
how the uptake of the tritiated thymidine is inter-
preted. I want to take this opportunity to register
a protest against the widespread, uncritical use of
tritiated thymidine as indicative of cell division.
It is intriguing to contemplate the possibility that
aside from DNA synthesis prior to cell division,
other exciting changes in DNA metabolism may
be taking place in response to injury. Certainly
there are changes in the morphologic appearance
of cell nuclei and their staining properties in
response to injury.

Especially interesting are the findings with
reference to both the enzyme activity and the
clinical appearance of the injured cornea when the
lid is open or closed during the healing process.
I have been asked many times what the possible
effect of such a procedure might be, and I am very
glad someone has finally carried out the experi-
ment.

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2. Braunsteiner, Herbert, and Zucker-Franklin,
Dorothea: The physiology and pathology of
leukocytes, New York, 1962, Crune & Stratton,
Inc., chap. 7.
3. Weimar, Virginia, and Haraguchi, Kenneth:
Presented at the Western Regional Conference
on developmental biology, Friday Harbor, May

Dr. Herbert E. Kaufman (closing). Dr. Weimar's
discussion is perceptive as we would expect. One
point in our paper, however, has led to some
confusion. We described the method of making
permanent dehydrated preparations not because
these were used in our evaluation but rather to
point out the difficulties that we discovered when
this method was attempted. Only aqueous prep-
arations mounted in glycerol or studies of endo-
thelium in situ on the cornea were used for evalua-
tion. Permanent preparations could be made which
would exhibit the same changes observed under
the more natural circumstances; but Dr. Weimar
is quite right that the processing of this material
leads to difficulties, and permanent preparations
alone cannot be used for evaluation, and were not
so used in this study.

Dr. Weimar makes the point that it is crucial to
separate the invading cells of hematogenous origin
from the keratocytes. Our original hope was that
enzymatic differences in cells of different origin
might provide an additional marker for such cells.
This did not prove to be the case. However, in our
studies we did employ numerous counterstains and
serial section staining techniques to differentiate
cells of hematogenous origin from the true keratocytes. Although our morphologic abilities are limited, we felt reasonably confident that we could separate these two types of cells. This impression was confirmed by the fact that the normal keratocytes exhibited the same general response to anoxia (closing of the lids) that was seen in the healing keratocytes. It is true that in a few preparations there was considerable invasion by cells of hematogenous origin, especially in some of the closed eyes, but these were not considered in drawing our conclusions, and I think that there can be little question that the obvious and extensive changes observed both in the healing area and in the normal area, with and without anoxia, are real.

Although it is conceivable that the uptake of tritiated thymidine may occur under unusual circumstances in which DNA synthesis does not result in cell division, certainly most authors have agreed that this is an excellent index of cell division and may well be much more reliable than the mitotic index. In any case, we can state with great assurance that DNA synthesis was considerably inhibited by the anoxia of lid closure as compared with controls.