Collagen crosslinking in keratoconus. DONALD J. CANNON* AND C. STEPHEN FOSTER**

The examination of reducible collagen crosslinks in keratoconus cornea revealed the presence of lysinonorleucine in amounts far greater than in normal age-matched corneas. There was no indication of decreased hydroxylysine levels in keratoconus, and there were no clinical indications of a generalized connective tissue disorder. The abnormal levels of dehydrolysinonorleucine in the tissue may represent a change in hydroxylation of selected lysyl residues of normal collagen or the synthesis of abnormal collagen, perhaps an unusual type.

Keratoconus is a central noninflammatory (usually bilateral) ectasia of the cornea, of unknown etiology. Histopathologic studies have demonstrated decreased numbers of collagen fibrils which appeared morphologically normal, keratocytes with membrane anomalies, fragmentation of the epithelial basement membrane, fibrillation and disintegrations of Bowman’s membrane, and (at the electron microscopic level) degenerative changes of the basal epithelial cells. Biochemical studies have revealed decreased levels of glucose-6-phosphate dehydrogenase in keratoconus corneas, and Robert et al. have found relative decreases in hydroxylation of lysine and glycosylation of hydroxylysine, decreased total collagen, and relatively increased structural glycoprotein.

Collagen is the major macromolecular constituent of corneal stroma and is arranged in lamellae of uniform-diameter fibrils. Collagen molecules are secreted from connective tissue cells as procollagen, a biosynthetic precursor of collagen consisting of three \( \alpha \)-chains with additional amino- and carboxy-terminal extensions (pro-\( \alpha \)-chains). Fibrils are constructed from the aggregation of collagen molecules from which the procollagen peptides have been cleaved. The tissue was finely minced, washed briefly in iced 0.02M EDTA, pH 7.0, and then reduced with \(^3\)H-NaBH\(_4\) as described previously.

### Table I. Distribution of incorporated tritium in native keratoconus cornea

<table>
<thead>
<tr>
<th>Peak</th>
<th>22M</th>
<th>38F</th>
<th>40M</th>
<th>44F</th>
<th>48M</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLHNL</td>
<td>12.3</td>
<td>30.0</td>
<td>23.0</td>
<td>23.0</td>
<td>(23-36)</td>
<td></td>
</tr>
<tr>
<td>HLNL</td>
<td>13.5</td>
<td>9.5</td>
<td>12.7</td>
<td>13.0</td>
<td>(9-11)</td>
<td></td>
</tr>
<tr>
<td>LNL</td>
<td>17.6</td>
<td>17.6</td>
<td>67.1</td>
<td>8.9</td>
<td>32.0</td>
<td>(0-5)</td>
</tr>
<tr>
<td>HisHM</td>
<td>9.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>(0-4)</td>
<td></td>
</tr>
</tbody>
</table>

*Individual peaks are expressed as percent of total eluted radioactivity. The in vivo crosslinks were reduced to give the derivatives resolved as the following peaks: HLHNL, dehydrolysinonorleucine; HLNL, hydroxylysinonorleucine; LNL, lysinonorleucine; HisHM, histidinohydroxymerodesmosine.

*Range of values in normal human cornea, 15-64 years old.*

### Table II. Tritium activity in reduced lysinonorleucine

<table>
<thead>
<tr>
<th>Case, * age (yr), and sex</th>
<th>Lysine (cpm/( \mu )mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 22M</td>
<td>1,087</td>
</tr>
<tr>
<td>2. 38F</td>
<td>1,383</td>
</tr>
<tr>
<td>3. 44F</td>
<td>1,793</td>
</tr>
<tr>
<td>4. 48M</td>
<td>177</td>
</tr>
</tbody>
</table>

*Cases 1 to 3 keratoconus; case 4 normal cornea.*
Lyophilized, reduced corneal samples were then hydrolyzed to determine constituent radioactive components and amino acid content.\(^7\)

**Results and discussion.** The most striking observations of the reduced crosslink profile of keratoconus cornea are the presence and quantity of lysinonorleucine (Table I). In a previous investigation on the crosslink profile of normal human corneas as a function of age,\(^8\) lysinonorleucine accounted for at most 5% of the total eluted radioactivity. In the present experiment lysinonorleucine accounts for a major proportion of the radioactivity following acid hydrolysis, and in one striking case it was the only significant peak observed. Table II is a comparison of lysinonorleucine content in keratoconus and normal cornea on the basis of total corneal lysine content. Values for the other radioactive crosslinks in Table I are consistent with normal age-matched controls\(^6\) with the exception of dihydroxylysinonorleucine values which are lower in keratoconus.

Lysinonorleucine is a major crosslink of the protein elastin\(^9\) and has been identified in collagen.\(^10\) It exists in these proteins as dehydrolysinoonorleucine, the Schiff base condensate of lysine and the aldehyde derived from lysine, \(\alpha\)-amino adipic acid semialdehyde. It is unlikely that there is any, let alone a sufficient quantity of, elastin in keratoconus stroma to account for these results. In view of the findings of Robert et al.\(^4\) of decreased levels of lysine hydroxylation in keratoconus, the possibility exists that crosslink formation in keratoconus involving hydroxylysine would be decreased. Our results are compatible with a decrease in hydroxylysine-derived crosslinks; however, hydroxylysine to 4-hydroxyproline ratios in keratoconus were not significantly different from those of normal cornea.

A correlation between joint hypermobility and the presence of keratoconus\(^11\) has been presented as evidence for the hypothesis that keratoconus is part of a general heritable disorder of connective tissue biochemistry. However, keratoconus was not observed in a hydroxylysine-deficient collagen disease\(^12\) in which the biochemical defect was a deficiency in llsyl-protocollagen hydroxylase\(^13\) and in which the reducible crosslink pattern in a number of tissues was abnormal.\(^14\)

The specific activity of reduced keratoconus corneal collagen was somewhat higher than in normal corneas but substantially lower than in the fetus or child.\(^4\) If, as we believe, a high level of reducible bonds implies growth or rapid synthesis (as we have seen in corneal scars\(^15\)), these results imply that there is not an unusual rate of collagen synthesis in keratoconus; indeed the slightly higher specific activity detected would be expected if, as has been reported,\(^4\) catabolism is higher than normal. Nevertheless, although the rate of collagen synthesis may be close to normal, the character of the collagen must be unusual to give rise to the high level of lysinonorleucine on reduction. The collagen may be unusual because of changes in the local hydroxylation level of the type I collagen that is normal to the cornea or by the intrusion of other types of collagen not usually seen in the cornea that may have this compound as a normal crosslink. The former possibility would imply some changes in the mechanism controlling levels of hydroxylation; such controls evidently operate at a tissue-specific level because it is already established that the levels of hydroxylysine and hydroxyproline in type I collagen vary in different tissues.\(^16\) The absence of clinical signs of a generalized connective tissue disorder and the absence of decreased hydroxylysine levels suggest that the etiology of keratoconus is complex and variable.

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Key words: keratoconus, collagen, crosslinking

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Antimuscarinic effects of stereoisomers of tropicamide on rabbit iris sphincter. P. N. Patil.

The antimuscarinic activity of optical isomers of tropicamide were compared on the isolated rabbit iris sphincter. The increasing concentrations of both the (–)- and (+)-isomer shifted the dose-response curve of carbachol to the right in a parallel fashion. The competitive reversible muscarinic blocking effects of both isomers were confirmed by pA2 plots. The pA2 values from the nonpigmented irides for (–)- and (+)-tropicamide were 7.88 and 6.18, respectively. Thus the (+)-isomer has only 1/50 the blocking activity of the (–)-isomer. Although both isomers are slightly less active in the pigmented iris, the blocking effect of the active (–)-tropicamide was readily reversed by washing, whereas reversal of this isomer’s effect from the pigmented iris was relatively slow.

Tropicamide (Mydriacyl) is a rapidly acting antimuscarinic drug widely used for producing cycloplegia and mydriasis of short duration.1 The chemical structure indicates an asymmetric center in the molecule. The possibility of two optical isomers is quite obvious. Clinically, the drug is used as a racemate which contains equal parts of the levo and the dextro isomers. The pharmacologic activity of racemic mixtures usually resides in one isomer. In other words, the pharmacologic effects of drugs are stereoselective. Availability of the optical isomers of tropicamide prompted us to investigate the muscarinic blocking effects of the stereoisomers in the iris.

Materials and methods. Both albino and nonalbino (black fur) rabbits weighing 2 to 3.5 kg were sacrificed by injecting sufficient air into the marginal ear vein. Eyes were rapidly removed, and the iris was dissected in an oxygenated physiologic salt solution having the following composition in millimole: NaCl, 118; KCl, 4.7; CaCl2, 2.5; MgCl2, 0.54; Na2HPO4, 1.0; NaHCO3, 25; and glucose, 11.0. A thread was passed through the iris and tied to a hook; the opposite end of the tissue was connected by means of a silk thread to a force-displacement transducer.2 The isolated iris was suspended in a 1 ml organ bath containing the physiologic salt solution at 37°C. Throughout the experiment a baseline tension of 150 to 200 mg was maintained, and the change in tension developed by the iris sphincter in response to drugs was recorded on a Grass polygraph. During a 50 min equilibration period the tissue was washed at regular intervals; following this, two cumulative dose-response curves2 of carbachol were obtained. The first dose-response curve was done in the absence of antagonist, following which the tissue was thoroughly washed. The tissue was then exposed for 60 min to the antagonist, and a second dose-response curve to carbachol was obtained in the continued presence of the antagonist. pA2 values (which can be defined as a negative log molar concentration of an antagonist which shifts the dose-response curve of an agonist by twofold) were calculated according to the method of Arunlakshana and Schild.3 Dose-ratio (which is defined as a ratio of ED50 of carbachol in the presence and absence of antagonist) was used to calculate pA2 plots. The pA2 value indicates an apparent affinity of the antagonists to the receptor. A potent antagonist produces a high pA2 value.

In another series of experiments on the iris, re-