A Defect in the Intramolecular and Intermolecular Cross-linking of Collagen Caused by Penicillamine

II. FUNCTIONAL GROUPS INVOLVED IN THE INTERACTION PROCESS*

Kalindi Deshmukh and Marcel E. Nimni

From the Department of Medicine, School of Medicine, and the Department of Biochemistry, School of Dentistry, University of Southern California, Los Angeles, California 90033

SUMMARY

The neutral salt-soluble collagen which accumulates in the tissues of animals treated with penicillamine does not differ from normal in amino acid composition, specific optical rotation, and melting temperature. It has higher intrinsic viscosity, possibly due to a contamination with higher molecular weight aggregates originating from the depolymerization of insoluble collagen.

In contrast to the aldehyde-deficient lathyritic collagen, the penicillamine collagen has an aldehyde content greater than normal and rapidly forms stable cross-links in vitro.

Binding studies, involving film and equilibrium dialysis, revealed a significant interaction between collagen and compounds with a free γ-aminothiol structure. The binding capacity of various collagenses tested is proportional to their aldehyde content, and reduction with NaBH₄ eliminates this interaction. Reduction of these aldehydes, amidination of the ε-amino groups of lysine and hydroxylysine, or addition of sodium bisulfite (10⁻⁴ M) causes neutral salt-soluble collagen to behave like lathyritic collagen.

It is postulated that the inhibition of cross-linking caused by penicillamine in vivo and in vitro involves a reversible interaction with the aldehydes present in tropocollagen to form a thiazolidine type complex, since compounds with adjacent free sulfhydryl and amino groups are necessary for activity.

The solubilizing effect exhibited by α-aminobutyric acid or an incompletely cross-linked form of insoluble collagen as well as the generation of soluble collagen with a high aldehyde content can be attributed to the splitting of a Schiff’s base intermediate. The structure which is responsible for the initial stabilization of the collagen fiber can be reduced with NaBH₄, rendering the collagen insoluble in penicillamine.

EXPERIMENTAL PROCEDURE

Preparation of Collagen—Neutral salt-soluble collagen was obtained from the dorsal skin of normal, β-aminopropionitrile-, and d-penicillamine-treated rats. These compounds were administered mixed with the diets (4 g per kg of ground Purina rat chow supplemented with 100 mg of pyridoxine) for periods of 2 to 3 weeks (16). The skin was cleaned, cut into small pieces, and washed with cold 0.15 M NaCl overnight to extract the more readily soluble material. The collagen soluble in 0.45 M NaCl was then extracted and purified by the method described earlier (17). The final preparation was centrifuged at 105,000 × g for 2 hours. An aliquot of the supernatant was hydrolyzed and the concentration of collagen was estimated by determining hydroxyproline content by an automated version of the Stegemann procedure (18, 19).
Amino Acid Analyses—Aliquots of neutral salt-soluble collagen from normal, n-penicillamine-, and β-aminopropionitrile-treated rats were hydrolyzed with 6 N HCl and the amino acid composition was determined with a Beckman amino acid analyzer, model 120B.

Thermal Aggregation—The concentration of each type of collagen was adjusted to 3 mg per ml and the solutions were incubated at 37°C for 4 to 7 days. At the end of this incubation period, the gels were cooled at 4°C. Aggregation of collagen solution and the redissolution of the gel were followed by measuring absorbance at 530 nm at various time intervals. The opacity of the solution was used as an index of the degree of cross-linking (16).

Aldehyde Estimation—Aliquots of collagen samples were diazylized against 0.1 M glycine-HCl buffer, pH 4.0, and gelatinized by heating at 60°C for 30 min, and the aldehyde content was estimated spectrophotometrically with the use of N methylbenzothiazolone hydrazide (20).

Reduction of Collagen—Collagen was reduced by adding a 500-fold molar excess of sodium borohydride (21). The reaction was carried out at 4°C for 30 min (pH 7.5 to 8). Excess NaBH₄ was destroyed by adjusting the pH of the solution to 4.0 with HCl and dialyzing against suitable solvent. The completeness of the reaction was ascertained by aldehyde estimation.

Amination of Collagen—Collagen solutions were reacted with ethyl acetimidate by the method described by Wofsy and Singer (22). To 100 mg of ethyl acetimidate were added 0.1 ml of 5 N NaOH and 4 ml of 0.4% collagen. The mixture was adjusted to pH 8.0 and kept at 4°C for 1 hour. An additional 50 mg of reagent in 0.05 ml of 5 N NaOH were added and the pH was maintained between 8.0 and 8.5 for 1 hour. Finally, 100 mg of reagent in 0.1 ml of 5 N NaOH were added and the pH was kept between 8.5 and 9.0 for 2 to 3 hours. The reaction mixture was then dialyzed exhaustively against cold distilled water to remove excess reagent. The precipitated collagen was dissolved in 0.45 M NaCl, pH 7.0, and dialyzed against the same solvent. The ε-amino groups present in the collagen molecule were estimated before and after amination by formol titration at pH 8.5 (23). A 98% amination was achieved by this method whereas 60% amination occurred with only one addition of 100 mg of reagent before and after amidination by form01 titration at pH 8.5 (23).

Solubility of Thermillly Precipitated Collagen Reduced with NaBH₄—Neutral salt-soluble collagen (3 mg per ml) was incubated at 37°C for 24 hours. The firmly cross-linked gel was reduced with NaBH₄ and the mixture was dialyzed against water to remove excess NaBH₄ followed by dialysis against 0.02 M Na₂HPO₄. The precipitated collagen was kept shaking with 5 ml of 0.2 M d-penicillamine in 0.45 M NaCl, pH 7.0, at 4°C for 48 hours. The contents were centrifuged at 40,000 × g for 1 hour, and the precipitate and supernatant were analyzed for hydroxyproline.

Specific Rotation—This was measured with a Zeiss polarimeter with a 10-cm light path cell.

Film Dialysis—Collagen solutions (3 mg per ml) were gelatinized by heating at 60°C for 30 min. Three-milliliter aliquots were incubated for 3 hours with 15 μmoles of cysteine containing 1 μCl of DL-cysteine-3-¹⁴C (New England Nuclear, specific activity 53.6 mCi per mmole). Dithiothreitol was added to the final cysteine solution in equimolar concentration prior to incubation to keep it in a reduced form. The mixture was dialyzed at room temperature with the thin film technique suggested by Craig (25). Aliquots were removed at different time intervals and the radioactivity was measured with a liquid scintillation counter (Nuclear-Chicago). Similar experiments were performed with DL-cysteine-3-¹⁴C (Calbiochem, 17 mCi per mmole) and uniformly labeled alanine-¹³C (Nuclear-Chicago, specific activity 162 mCi per mmole) without the addition of dithiothreitol. The extent of interactions between various collagen samples and labeled amino acids was studied by measuring the rate of removal of the label during dialysis. The specific interactions of each amino acid with collagen were calculated to be the difference in interactions between these amino acids and the normal and reduced forms of collagen.

Equilibrium Dialysis—Collagen (0.5 mg per ml), 10 ml, in 0.1 M NaCl containing 0.02 M phosphate buffer, pH 7.0, was dialyzed in Teflon-lined screw cap test tubes for 96 hours at 4°C against 10 ml of 0.1 M NaCl, pH 7.0, containing 1 μmole of cysteine, 0.1 μCi of C14-cysteine, and 1 μmole of dithiothreitol. The test tubes were kept on a rotary mixer under a nitrogen atmosphere. When the equilibrium was reached, the dialysis bags were removed and wiped dry, and the radioactivity inside and outside the dialysis bags was measured. Collagen inside the bags was estimated before and after dialysis to make sure that there was no leakage.

Oxidation of Gelatin by Sodium Periodate—Commercial gelatin (Knox) was oxidized with sodium periodate in alkaline media by the method of Aronson et al. (26) and with periodate acid according to the method reported by Blumenfeld et al. (27). The increase in aldehydes was measured by the method of Paz et al. (20) and the decrease in hydroxylysine was estimated by amino acid analysis (Beckman model 120B).

RESULTS

The amino acid composition of purified neutral salt-soluble collagen from the skin of normal, n-penicillamine-, and β-aminopropionitrile-treated animals is shown in Table I. The values obtained are consistent with those found in the literature for rat skin collagen. There is no change in the amino acid composition after penicillamine or β-aminopropionitrile administration. The presence of traces of cysteine, quantitatively determined by oxidation to cysteic acid, is a consistent finding in all of our collagen fractions tested. The isolation of peptides containing cysteine from collagen has been recently reported (29, 30). Its role remains unknown but worthy of further investigations.

Solutions of salt-soluble collagen from the skin of normal rats as well as those treated with n-penicillamine or β-aminopropionitrile were compared for their capacity to form stable gels (Fig. 1). Collagens from normal and penicillamine-treated animals aggregate very rapidly and reach a maximum optical density within 10 min of incubation at 37°C. Collagen from lathyritic animals shows a retarded capacity to gel, but eventually reaches
the same maximum value for optical density. After incubation at 37° for 7 days and subsequent cooling at 2°, the gels from the control and penicillamine collagen did not show significant redissolution, while the optical density of the \( \beta \)-aminopropionitrile collagen dropped rapidly, with complete liquefaction of the gel structure. In several experiments, the penicillamine collagen showed evidence of an enhanced capacity to gel at 37° as well as a greater stability than the control when subsequently cooled at 2°.

The aldehyde contents of different neutral salt-soluble collagen preparations are given in Table II. Our findings confirm those of previous investigators (4, 5) in that the collagen from lathyritic animals is deficient in aldehydes. On the other hand, collagen from penicillamine-treated rats has significantly more aldehydes than normal. This observation reflects a major difference between the modes of action of \( \beta \)-aminopropionitrile and penicillamine.

To investigate further the role of aldehydes in the process of cross-linking neutral salt-soluble collagen from normal rats was treated with sodium borohydride whereas another sample was treated with ethyl acetoacetate hydrochloride to block all of the e-amino groups of lysine and hydroxylysine. When these two forms of modified collagen were aggregated at 37°, after the removal of excess NaBH₄ or ethyl acetimidate, their optical density reached a maximum value within 8 to 10 min, but the gels showed loose consistency (Fig. 2). Storage at 37° for 4 days followed by cooling at 4° caused rapid liquefaction of these gels with a concomitant drop in optical density, indicating that the treated collagens were unable to form stable intermolecular cross-links in vitro.

When neutral salt-soluble collagen is polymerized by incubation at 37° for 24 hours and then reduced with sodium borohydride, the fibrils retract to form a tough mass within a few minutes. Such fibrils are resistant to dissolution in 0.2 M NaCl, whereas penicillamine collagen has a higher intrinsic viscosity. These observations were made with high shear (Fig. 3) and low shear (Fig. 4) viscometers with 0.05 M acetic acid as a solvent.

Table I

<table>
<thead>
<tr>
<th>Amino acid residue/1000</th>
<th>Control</th>
<th>Penicillamine</th>
<th>BAPN*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylysine</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Lysine</td>
<td>28</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Histidine</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Arginine</td>
<td>48</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>44</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>Threonine</td>
<td>19</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Serine</td>
<td>40</td>
<td>39</td>
<td>40</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>75</td>
<td>74</td>
<td>73</td>
</tr>
<tr>
<td>Proline</td>
<td>123</td>
<td>122</td>
<td>121</td>
</tr>
<tr>
<td>Glycine</td>
<td>335</td>
<td>333</td>
<td>332</td>
</tr>
<tr>
<td>Alanine</td>
<td>101</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>Valine</td>
<td>24</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>Methionine</td>
<td>6</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>13</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Leucine</td>
<td>26</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>15</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Amide groups</td>
<td>43</td>
<td>43</td>
<td>45</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>89</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* \( \beta \)-Aminopropionitrile.

** Determined on the AutoAnalyzer with the procedure of Grant (19).

* Samples oxidized with performic acid prior to hydrolysis (28).

Table II

<table>
<thead>
<tr>
<th>Collagen sample</th>
<th>Acetaldehyde content μmoles acetaldehyde eq/100 mg collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.87</td>
</tr>
<tr>
<td>BAPN-treated</td>
<td>0.49</td>
</tr>
<tr>
<td>Penicillamine treated</td>
<td>1.60</td>
</tr>
</tbody>
</table>

* \( \beta \)-Aminopropionitrile.
Melting temperatures ($T_m$) measured by fall in viscosity (Zimm technique) were found to be $38^\circ$ for all of the types of collagen (Fig. 5). Specific optical rotation ($\alpha$) at 365 nm was similar for all of the samples tested. The ($\alpha$) values for native forms of collagen and for samples gelatinized by heating at $40^\circ$ for 30 min were $-1300^\circ$ and $-475^\circ$, respectively. These measurements indicate that no denaturation occurs during treatment with NaBH$_4$ or ethyl acetoacetate. The high intrinsic viscosity of the penicillamine collagen may reflect the presence of small amount of macromolecular aggregates which cannot be separated by high speed centrifugation.

It has been previously reported (16) that addition of $D$-penicillamine to normal salt-soluble collagen affects the rate of fiber formation during thermal aggregation. At a high concentration such as $2 \times 10^{-4}$ M it causes a complete block of the aggregation process, while at lower concentrations it delays the aggregation and causes a redissolution after cooling at $4^\circ$. Along with $D$-penicillamine, other compounds such as $\beta$-aminopropionitrile, N-acetylpenicillamine, and sodium bisulfite were tested. Fig. 6 shows that at $10^{-3}$ M concentration none of these compounds inhibits the thermal aggregation of neutral salt-soluble collagen from normal animals. Upon storage for 4 days at $37^\circ$ with subsequent cooling at $4^\circ$, the presence of penicillamine in the media causes complete liquefaction of the gel structure, whereas N-acetylpenicillamine or $\beta$-aminopropionitrile had no such effects. Addition of sodium bisulfite to the media caused the gel to dissolve completely at $4^\circ$. This compound was found to be effective at concentrations of $10^{-3}$ or $10^{-4}$ M. Such action of sodium-bisulfite can be attributed to its capacity to form addition products with aldehydes and low molecular weight ketones (31).

The binding of penicillamine to collagen was investigated in order to obtain some insight into the mechanism by which it prevents the formation of stable cross-linked fibers. Because $^{14}$C-labeled penicillamine was not available to us in a purified form, and the $^{35}$S-labeled material used in our initial experiments proved to be contaminated with significant amounts of impurities which could not be readily separated, the binding studies described here were performed with $^{14}$C-cysteine. This compound and other thiols prevent the formation in vitro of stable collagen fibrils (32).

### Table III

<table>
<thead>
<tr>
<th>Collagen sample</th>
<th>Collagen soluble in 0.2 M penicillamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control fibrils</td>
<td>%</td>
</tr>
<tr>
<td>Fibrils treated with NaBH$_4$</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>
These studies were done with film and equilibrium dialysis. Gelatin (or collagen, which behaved in the same way in these experiments) was incubated with $^{14}$C-cysteine (diluted previously with unlabeled cysteine) and the mixture was subjected to thin film dialysis against running water. The nondialyzable radioactivity was monitored at regular intervals. Affinity between cysteine and collagen is reflected by the slower rate of removal of bound label. The magnitude of these interactions, which led to the retention of labeled compound, is much greater when regular dialysis is applied to larger volumes, since the number of macromolecules interacting with the ligand prior to its passage through the dialysis membrane is proportionally increased. Various types of collagen containing different amounts of aldehyde residues were used to study the binding of cysteine with the film technique. Neutral salt-soluble collagen reduced with sodium borohydride served as a source of collagen with no detectable aldehyde residues on the molecule. Neutral salt-soluble collagen from lathyritic animals contains smaller amounts of aldehydes than normal (Table II), while collagen extracted with 0.2 M cysteamine (mercaptoethylamine) from insoluble skin of rats shows a very high aldehyde content (17). It is evident from Fig. 7 that cysteine is removed from the media at a fast rate during the initial stages of dialysis. However, the amount of cysteine that remains bound to collagen after 24 hours of film dialysis is proportional to the amount of aldehydes on the molecule. During the dialysis period, cysteamine collagen (with the highest aldehyde content, 2.5 residues/molecule) shows a maximum degree of interaction, while collagen treated with NaBH$_4$ exhibits the lowest affinity toward cysteine. On the other hand, the magnitude of interaction of cystine or alanine and collagen is independent of the presence or absence of aldehydes on the collagen molecule, indicating the lack of any specific interaction with these carbonyl groups (Fig. 8). From this experiment it can be concluded that, when the interactions attributed to the aldehyde groups on the collagen molecule are measured (binding to normal collagen — binding to reduced collagen), the only compound that interacts to any significant extent is cysteine, whereas cystine or alanine fail to show preferential binding to either form of collagen.

It seemed likely that cysteine and its analogues are binding to the collagen molecule by weak forces and that the nature of the collagen-mercaptoethylamine interaction involves an unstable binding which can be dissociated by exhaustive dialysis. Therefore, the method of equilibrium dialysis as used by Klotz to study binding of organic ions to protein molecules (33) and developed further by Hunter and Commerford (34) for interactions of amino acids with proteins was applied to this system. In addition to the collagen samples used in the film dialysis experiment peri-
FIG. 8. Specific interactions between amino acids and parent gelatin were calculated from the difference in binding of these amino acids to salt-soluble collagen before and after reduction with NaBH₄. Specific interaction (micromoles) = micromoles of amino acid interacting with 1 pmole of gelatin = micromoles of amino acid interacting with 1 μmole of reduced gelatin.

TABLE IV
Binding of cysteine-¹⁴C to collagen studied by equilibrium dialysis

<table>
<thead>
<tr>
<th>Collagen specimen</th>
<th>Observed binding ratio</th>
<th>Molar binding ratio (cysteine to tropocollagen)</th>
<th>Residues of acetylated aldehyde/tropocollagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.998</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neutral salt-soluble-reduced</td>
<td>1.017</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Lathyritic (BAPN)</td>
<td>1.043</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Gelatin (Knox)</td>
<td>1.062</td>
<td>3.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Neutral salt-soluble</td>
<td>1.076</td>
<td>4.47</td>
<td>2.6</td>
</tr>
<tr>
<td>Cysteamine-soluble</td>
<td>1.240</td>
<td>14.1</td>
<td>8.0</td>
</tr>
<tr>
<td>Alkali-oxidized gelatin</td>
<td>1.300</td>
<td>17.6</td>
<td>14.1</td>
</tr>
<tr>
<td>Acid-oxidized gelatin</td>
<td>1.355</td>
<td>22.6</td>
<td>17.2</td>
</tr>
</tbody>
</table>

* Moles of labeled cysteine inside dialysis bag (free and bound to protein)/mole outside dialysis bag.

† Assuming a molecular weight of tropocollagen to be 300,000; aldehyde estimated by the method of Pas et al. (20).

β-Aminopropionitrile.

date-oxidized gelatin served as a material rich in aldehydes. Commercial gelatin (Knox) was oxidized with sodium periodate in alkaline media or with periodic acid. The amount of aldehydes increased after oxidation and the hydroxyllysinelysine ratio changed from the normal range of 0.2 to a value of 0.04. Data in Table IV indicate that when equilibrium is reached, the binding of cysteine to collagen (or gelatin) is directly proportional to the amount of aldehydes on the molecule.

**DISCUSSION**

The exact nature and number of cross-links involved in stabilizing the collagen fibers are unknown. The study of an abnormal form of collagen induced by lathyrogens has been of significant value in beginning to understand the chemical nature of these bonds. Whereas normal soluble collagen warmed at 37° will become rapidly cross-linked and progressively insoluble with time, lathyritic collagen will never form stable cross-links (35). Lathyritic collagen differs from normal by its increased solubility in cold neutral salt solution (1) and by a decreased content of detectable aldehydes (6-9).

The neutral salt-soluble collagen which accumulates in soft tissues during treatment with D-penicillamine does not differ in amino acid composition from normal collagen or lathyritic collagen induced by β-aminopropionitrile (36). Melting temperatures and specific optical rotation also failed to show any differences. Despite of their apparent similarities, such as blocking of the formation of intramolecular and intermolecular cross-links, which causes an accumulation of α components within the collagen fibers, some of the findings reported here make it clear that the modes of action of β-aminopropionitrile and penicillamine are quite different. In contrast to lathyritic collagen, the soluble collagen obtained from D-penicillamine-treated rats, once purified by repeated precipitations, will form stable cross-linked gels when heated at 37°. Copper chelation by penicillamine does not seem to be of primary significance for the effect in vivo of penicillamine since excess copper supplemented by a different route of administration does not prevent the changes induced by penicillamine (37, 38). In addition, the aldehyde content of the collagen from the D-penicillamine-treated rat is greater than that found in normal collagen of similar solubility characteristics, again reflecting a major difference from lathyism. Similar findings involving the accumulation of α-amino adipic-β-semialdehyde have been reported for elastin isolated from the aorta of chick embryos treated with D-penicillamine (39). We have recently observed a direct correlation between the capacity of a collagen molecule to polymerize into an insoluble matrix and its aldehyde content. Soluble collagen extracted from insoluble fibers by 0.2 M cysteamine is extremely rich in aldehydes. This collagen rapidly aggregates and becomes insoluble at a faster rate than normal soluble collagen (17).

The high aldehyde content of soluble collagen present in the tissues of penicillamine-treated rats indicates that the abnormality caused by penicillamine is not related to an inhibition of an amine oxidase as may be the case with β-aminopropionitrile. It is conceivable that the prerequisite for the formation of stable covalent cross-links is the presence of an optimum level of aldehyde residues on the tropocollagen molecule. Although penicillamine does not prevent the synthesis of these aldehydes it will inactivate them by selective binding. Bornstein and Piez have shown that the recently synthesized α chains are very low in aldehyde groups and that these can be subsequently generated by oxidative deamination of specific lysine residues (4). In addition to these aldehydes a variety of α-amino aldehydes has been detected in tropocollagen (40).

Neutral salt-soluble collagen in a normal animal is a recently synthesized species of collagen, whereas the soluble collagen extracted from the penicillamine-treated tissues contains large amounts of older but non-cross-linked tropocollagen. Since at the time of synthesis collagen may contain no aldehydes, and their subsequent extracellular synthesis seems to be a time-dependent process, this could account for our high values encountered in this large pool of soluble collagen. Because of the continuous presence of penicillamine in the tissues of the treated animals their collagen does not aggregate, but upon removal of
the thiol by dialysis (in vitro) or by a relatively slower metabolic process (in vivo) its cross-linking capacity is restored to normal.

Addition of β-aminopropionitrile to a solution of normal collagen does not affect subsequent cross-linking. A derivative of penicillamine (N'-acetyl-bp-penicillamine) is also ineffective in preventing the stabilization of the collagen network, whereas penicillamine and sodium bisulfite, two compounds which have in common the capacity of reacting with aldehydes, prevent this process. In view of these findings which further suggested the participation of aldehydes in the formation of cross-links, normal collagen was modified by reducing with NaBH₄ and blocking the ε-amino groups of lysine and hydroxylysine with ethylacetimide. Reduction of the aldehyde residues gave rise to a collagen which behaves like lathyritic collagen. Blockage of the free amino groups had an effect proportional to the number of groups reacted. Whereas blocking 98% of these groups gave rise to collagen which was completely lathyritic, 60% blocking gave a collagen intermediate between normal and lathyritic collagen. These modified forms of collagen exhibited physicochemical characteristics similar to those of normal tropocollagen, as judged by solubility, intrinsic viscosity, thermal denaturation curves, and specific optical rotation.

The neutral salt-soluble collagen obtained from the β-penicillamine-treated animals showed an abnormal value for intrinsic viscosity. The value obtained with both the Ostwald and Zimm techniques (18 dl per g), which is high when compared to the normal, lathyritic and chemically modified forms of collagen (12.5 dl per g), suggests the presence of some higher molecular weight contaminants. These could arise from some fraction of the insoluble collagen present in the skin of the penicillamine-treated animals which still remains partially intermolecularly cross-linked. A small contribution of staggered tropocollagen (dimers or higher polymers) originating from such a source or derived from the spontaneous polymerization of this collagen (which has a higher capacity to polymerize than normal soluble collagen) could account for this increased intrinsic viscosity. Centrifugation at 100,000 x g for 6 hours or 150,000 x g for 2 hours was not able to alter these findings. Acetic acid- and cysteamine-soluble collagens, which we have also found to be rich in aldehydes, will polymerize more readily than neutral salt-soluble collagen (17, 41). They also have high intrinsic viscosities (25 dl per g and 22 dl per g, respectively).

All of the observations involving β-penicillamine suggested that this compound interacts with some group on the collagen molecule and prevents it from participating in the cross-linking reaction. The reversibility of the changes seen, both in vivo and in vitro, imply the existence of weak interacting forces. The experiments involving film dialysis show that the affinity of the collagen for cysteine (used as a model for thiolamines) is related to the aldehyde content of the various collagens tested. Neither this method nor molecular sieve chromatography could be used adequately to determine binding ratios quantitatively as a result of the unstable nature and the concentration-dependent variability of these interactions. Nevertheless, they give a good indication that the aldehydes on the collagen molecule were responsible for these specific interactions. The fact that the free mercaptoethylamine structure is required is illustrated by the finding that cystine, alanine, and N-acetylcyesteine do not bind specifically to a borohydride-reducible group present in collagen, as does cysteine. Information of a more quantitative nature related to the participation of aldehydes in the binding process is derived from equilibrium dialysis involving various forms of collagen. The extreme forms of modified collagens used, NaBH₄, reduced on one hand and periodate-oxidized gelatin on the other, showed no binding and maximum binding capacity, respectively. The parent gelatins derived from various types of native collagens occupied intermediate positions and these can be related to their aldehyde content.

Fig. 9 summarizes the different modes of action of penicillamine and β-aminopropionitrile (βAPN) prevent polymerization of tropocollagen.

**Fig. 9.** Mechanisms proposed for the modes by which penicillamine and β-aminopropionitrile (βAPN) prevent polymerization of tropocollagen.

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**Note:** The diagram in the image depicts the interaction between penicillamine and collagen, showing different forms of collagen and the effects of penicillamine and β-aminopropionitrile on their structures and viscosities.
amino adipic-δ-semialdehyde. The enzyme involved, which is heat-labile and inhibited by β-amino-propioniprolin, has been detected in the 100,000 g supernatant of extracts prepared from bone, aorta, and skin (47). Definite proof for this mode of action of β-amino-propioniprolin should arise from the isolation of this enzyme, determination of its capacity to increase the aldehyde content of lathyritic collagen to normal levels, and generation of the capacity to form cross-linked fibers.

The solubilizing effect in vitro and in vivo that the thiolamines exhibit on an “intermediately cross-linked” form of insoluble collagen can also be explained on the basis of the information now available. The evidence for the requirement of both aldehyde groups and ε-amino groups of lysine for adequate cross-linking seems to be quite clear and has been shown in these as well as other experiments. The stabilization of a cross-linking intermediate by reduction of recently aggregated collagen with NaBH₄ has recently been shown (48, 49). The nature of the groups involved, as well as the isolation of lysinonorleucine from elastin (50), would suggest that a Schiff’s base derived from ε-amino adipic-semialdehyde on one hand and the ε-amino group of a lysine on another may be the initial step in the formation of a stable intermolecular cross-link in collagen. Evidence for an aldol condensation of two neighboring aldehydes to generate an α,β-un satu rated aldehyde has been published (11). This new aldehyde, which should be more reactive in nature, may in turn react with an available amino group from another molecule. At the stage of a Schiff’s base intermediate, this compound could be easily split by dilute acids, thus explaining the high dispersing effect of these agents on collagen fibers. In addition, Schiff’s bases could be sensitive to nucleophilic attack by the mercapto group of penicillamine or could participate in an exchange reaction facilities, to Dr. Michael Schneir for his critical review of the article, and to Mr. David Norby for the preparation of ethyl acetimidate.

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Kalindi Deshmukh and Marcel E. Nimni

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