The Introduction of Intramolecular Covalent Cross-linkages into Ichthyocol Tropocollagen with Monofunctional Aldehydes*

ARTHUR VEIS and MAURICE P. DRAKE†

From the Department of Biochemistry, Northwestern University Medical School, Chicago 11, Illinois

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The presence of covalent cross-linkages between the peptide chains of collagen has been postulated to account for many of the properties of collagen (1), particularly the insolubility of mature fibrous mammalian collagen in water or dilute acids at low temperature. There is as yet no direct chemical evidence as to the nature and number of the cross-linkages, although ester bonds (2-4) and aldehyde bridges (5) have been implicated. One of the strongest bits of evidence for the covalent interchain cross-linkage is that gelatins may be prepared, either from mature fibrous collagen or from mammalian tropocollagen, that can be reversibly renatured to the fibrous or segment long spacing (SLS) forms (6-9). Gelatins of the renaturable type (γ) represent a distinct molecular species and presumably consist of three peptide chains held together by cross-linkages that were imposed on the tropocollagen framework after the tropocollagen was incorporated into the fibrous collagen structure (10). The cross-linkages are thought to hold the chains in the proper register to provide nucleation sites for the reformation of the trihelical structure. It occurred to us that it might be possible to introduce intramolecular cross-linkages into a native tropocollagen molecule that would not ordinarily yield the renaturable γ-component upon denaturation, and thus synthesize a γ-component that would exhibit the proper renaturation properties. Such a synthesis would constitute a formal proof that covalent cross-linkages could account for the observed behavior of natural collagen and would provide a suitable model for investigation of the renaturation phenomena.

In this paper we report the successful synthesis of a γ-gelatin from an ichthyocol and an analysis of its stability and renaturability. Several factors influenced our choice of starting material. The collagen had to have a low content of naturally cross-linked components, it had to be already characterized or be quite similar to the collagens described in the literature, and, hopefully, it had to be available in reasonably large amount. The ichthyocol collagens as a class fulfilled the first two requirements. The buffalo fish (Ostariophysi ictiobus), a member of the sucker family and closely related to the carp, was available locally. Buffalo fish swim bladder collagen was therefore used.

The general criteria for a cross-linking reagent were that it had to be reactive in aqueous solution at neutral or acidic pH where the ichthyocol was soluble, it had to be relatively specific, and it had to be reactive at temperatures below 20° so that the collagen would not be denatured. Formaldehyde generally fulfilled these requirements. Fraenkel-Conrat and Olcott (11) found the primary reaction of formaldehyde with proteins to be the formation of the aminomethylol derivative of each free primary amino group. Cross-linkages are formed when the aminomethylol group condenses with an active hydrogen on a primary amide, guanidyl, phenol, histidyl, or indole group. The cross-linking reaction via amide or guanidyl groups occurs at room temperature and over a wide pH range (11, 12) but the cross-link is only moderately stable, being readily hydrolyzed in aqueous systems at elevated temperatures. More drastic reaction conditions are required to effect the condensation of the aminomethylol with phenol, imidazole, and indole groups (e.g. 40-70° for days or weeks), but the cross-linkages formed are more stable (12). In each case, the cross-link is a methylene bridge.

This paper, then, reports the preparation of buffalo fish swim bladder ichthyocol, the reaction of ichthyocol with formaldehyde to form cross-linked ichthyocol (CL-ichthyocol), and the characterization and physical properties of ichthyocol and CL-ichthyocol.

EXPERIMENTAL PROCEDURE

Materials and Methods

Ichthyocol Preparation—The tunica externa was stripped from fresh buffalo fish swim bladder. The acid-soluble collagen was extracted with dilute acetic acid by the method of Orekhovich et al. (13) as modified by Gallop (14). All of the extractions, dialyses, and centrifugations were carried out in a cold room at 4-10°. The tunics were extracted batchwise for 3 weeks and all extracts were combined. The acid-soluble ichthyocol was precipitated by dialysis against distilled water, was lyophilized, and was stored in the cold.

Solutions were made by stirring the dry material gently overnight in the desired solvent. The suspensions were centrifuged at 30,000 × g to remove the undissolved collagen. Only the clear supernatants were utilized in subsequent experiments.

Protein Concentration—Solution concentrations were determined by the micro-Kjeldahl nitrogen procedure of Miller and Houghton (15). The percentage error for duplicate samples assayed the same day was ±1%. Duplicate samples assayed on different days had a maximal ±3% error. Purified ichthyocol had a nitrogen content of 18.4%.
Concentrations were also determined by a hydroxyproline analysis. The method of Neuman and Logan (16) was modified as follows. Combine 2 ml of sample (0 to 25 μg of hydroxyproline), 2 ml of basic CuSO₄ reagent (0.1 M CuSO₄ and 2.5 N NaOH mixed by volume just before use), and 1 ml of 2% H₂O₂. Gently shake in a Burrell wrist-action shaker for 5 minutes, followed by vigorous shaking for 10 minutes to eliminate excess H₂O₂. Add 3 ml of 4 N H₃SO₄ and gently shake at 70° for 20 minutes. Cool in an ice bath for 2 minutes, then let stand at room temperature. Read optical density at 555 μm after 10 minutes and within 1 hour. Samples and standard were always run in duplicate. The percentage error on replicate analyses was ±2% but the error in a single evaluation could be as high as ±5%. The hydroxyproline content of the ichthyocol was 12.2%.

Cross-linking Reactions

Formaldehyde—Analytical reagent grade 37% formaldehyde was used in a ratio of 1, 2, or 3 ml of formaldehyde to 10 ml of solution to give 3.4%, 0.6%, and 0.0% formaldehyde concentrations, respectively. In every case the formaldehyde reagent was brought to the desired pH and ionic strength by the addition of acid or salt before the reagent was added, dropwise, to the protein solution. All of the reactions were carried out in the cold room at temperatures varying between 4 and 10°. The reaction solutions were then dialyzed against fresh buffer solution for 3 days, at which time the formaldehyde content of the dialysate was nil. The reaction solution was then dialyzed against fresh buffer solution containing 1.3-cyclohexane dioxide-5,5-dimethyl (dimedon) at a 0.9% concentration. Dimedon is a reagent specific for formaldehyde and removes bound but unreacted formaldehyde from the collagen (17). Finally, the reaction mixture was dialyzed against cold tap water for 2 days to remove the buffer and dimedon and to precipitate the cross-linked collagen, hereafter designated ACL-ichthyocol to indicate the product of the acid reaction. The ACL-ichthyocol fibrils that formed were collected by low speed centrifugation and were stored wet, in the cold, or were lyophilized.

Formaldehyde Cross-linking in Neutral Solution—Lyophilized ichthyocol was dissolved in 0.5 M CaCl₂ adjusted to pH 7.2 to 7.6 with dilute NaOH. The cross-linking procedure was exactly the same as that described above. However, it was difficult to dissolve CaCl₂ in the formaldehyde reagent before it was added to the collagen solution. At formaldehyde concentrations above 3.4%, the reaction mixture became turbid. If the formaldehyde was added too rapidly, local concentration effects tended to cause the ichthyocol to aggregate and precipitate out. The aggregated ichthyocol was removed by centrifugation at the end of the reaction period. Only the clear supernatant solution was subjected to the dialysis procedure. The fibrils resulting from the final dialysis against water are designated NCL-ichthyocol in the subsequent discussion.

Acetaldehyde Cross-linking—The reaction procedure and conditions were the same as with formaldehyde except that the desired amount of acetaldehyde was added directly to the collagen solutions. In most cases the acetaldehyde content was 5.0 M during the reaction. In the case of the neutral solutions, the acetaldehyde had to be added very slowly to avoid immediate precipitation of the collagen. The reaction time in neutral solution was extended to several days.

Aldehyde Assays—It was necessary to have an estimate of the formaldehyde content of the reacted collagen, exclusive of that bound in the cross-linkages. The chromotropic acid (2,7-naphthalenedisulfonic acid 4,5-dihydroxy) procedure of MacFaraday was used without modification (17). Chromotropic acid reacts with free formaldehyde present in the protein or with any formaldehyde hydrolyzed from the protein during a color development step involving heating at 100° for 30 minutes. An alternative procedure is to add dimedon to the assay mixture before heating. The free formaldehyde reacts at 5° with the dimedon, and subsequent color development at 100° gives only the reversibly bound formaldehyde. The difference between the assay in the absence and presence of dimedon gives the amount of free formaldehyde.

The formaldehyde converted to methylene bridge cross-linkages was estimated by assuming that the cross-linkages involved reaction with the ε-amino groups of lysine. The method of Blass and Raynaud (18) was used wherein the amount of lysine or hydroxylsine recoverable after treatment with fluorodinitrobenzene is a measure of the amount of unreacted, or in this case, blocked ε-amino groups. These analyses were kindly performed for us by Dr. Howard Bensusan of Western Reserve University. Ichthyocol is particularly favorable for this analysis since in the native, unmodified state, every ε-amino group reacts with the fluorodinitrobenzene and no free lysine is recovered.

Physical Properties

Sedimentation Analyses—Sedimentation coefficients were determined in a Spinco model E analytical ultracentrifuge equipped with a rotor temperature control unit. All runs were made at 59,780 r.p.m. In every case a series of runs at varying protein concentrations was made, and the reciprocals of the observed sedimentation coefficients were extrapolated to zero concentration according to a regression plot of the best straight line. Native ichthyocol and renatured CL-ichthyocol (see above) were run at 20°; denatured ichthyocol and CL-ichthyocol were run at 40°. The buffer system for all 40° runs was 0.10 M HAc plus 0.10 M KCl. The 40° runs were corrected to ε₂₀,₀ values according to the usual formula. The partial specific volume of ichthyocol as gelatin was measured in 0.05 M HAc at 40 ± 0.01° with 10-ml pycnometers. The value found was 0.709 ± 0.010 as computed from a regression plot of the density data at concentrations ranging from 0.1 to 1.0%.

In those samples in which more than one component was evident, the apparent relative concentrations of each component were determined from the areas under the refractive index gradient peaks. The values were corrected for the Johnson-Ogston effect by doing several runs at different concentrations and extrapolating the relative peak areas to zero concentration. The areas were determined from planimeter measurements of enlargements of the sedimentation pictures. The buffalo fish
ichthyocol was found to be composed of 78% one-chain a-component and 22% two-chain b-component.

Intrinsic Viscosity—Viscosities were measured in Cannon-Ubbelhode semimicro dilution viscometers with water flow times ranging from 200 to 400 seconds, depending on the temperature. The solvent was 0.10 m HAc-0.10 m KCl, pH 2.8. Measurements were made at 15° for native ichthyocol or CL-ichthyocol and at 4° for the denatured samples. Intrinsic viscosities were determined from plots of $\eta_{sp}/c$ against $c$ or $(\ln \eta_{rel}/c)$ against $c$.

Polarimetry—Optical rotation measurements were performed with a Zeiss polarimeter with a sodium arc as light source for the study of denaturation during reaction of formaldehyde with ichthyocol in acid. All other optical rotation measurements were carried out in a Rudolph model 80 photoelectric polarimeter with an oscillating polarizer prism. A mercury arc was the light source and a filter was used for isolating the 365 mp spectral line used in all measurements. Water-jacketed 1-dm tubes equipped with silica end plates were employed. The sample temperature was maintained to within $\pm 0.1^\circ$.

Thermal Denaturation

The denaturation of the various collagens was followed by measurement of the change in specific optical rotation as a function of time at various temperatures. Measurements of $[\alpha]_{200}$ were first made at 15°. The water flow in the polarimeter tube jacket was then switched from the 15° bath to a bath at the desired temperature. Approximately 2 minutes were required to bring the sample to the higher temperature. The zero time for the denaturation was taken as 1 minute after the change in water flow.

The measured rotations were plotted versus time, and smooth curves were drawn. The denaturation temperature, $T_d$, used here is defined as the temperature at which one-half the specific optical rotation change occurs within 30 minutes. The total change for native ichthyocol was from $-1360^\circ$ to $-460^\circ$ so that the material was considered to be half-denatured at $[\alpha]_{200} = -910^\circ$. The denaturation temperature was estimated from the curves drawn at the various temperatures used.

Renaturation Experiments

The collagens to be examined for renaturation ability were first dissolved in 0.1 m KCl by warming to 60° for 2 to 3 minutes. The solutions were ultracentrifuged at 40° to remove undissolved matter, and then placed at 4-10° overnight. On warming in a 40° bath, the solutions became turbid and within 30 minutes, fibrous heat precipitates developed. These were collected by centrifugation at 40°. Electron microscopy was used to verify that the precipitates were composed of collagen fibers with the native 700-A periodicity. The NCL-ichthyocol (see above) samples were renatured by this procedure.

An alternative method for the precipitation of native fiber, useful primarily with the ACL-ichthyocol and native ichthyocol, was a dialysis process. The collagen in an acetic acid solution was heat-denatured at 40° for 15 to 30 minutes and ultracentrifuged at 40°. The supernatant was dialysed against pH 3.7, 0.15 m citrate buffer in the cold. After several days, the dialysis solution was changed to a neutral 0.02 m dibasic sodium phosphate. Native type fibrils formed after overnight dialysis and were collected by centrifugation.

Segment-long-spacing (SLS) collagen was prepared by the addition of adenosine triphosphate to solutions of native or renatured collagen in 0.05 m HAc. The precipitates were allowed to stand in the cold overnight and were then harvested by centrifugation.

RESULTS AND DISCUSSION

The Cross-linking Reaction—The aim of these experiments was to produce intramolecular cross-linkages while avoiding intermolecular polymerization. An essential requirement, therefore, was to carry out the reactions at extreme dilutions so that the original ichthyocol was as monodisperse as possible. Boedtker and Doty (19) had shown that concentrations below 0.7% were required to eliminate the aggregation of native carp swim bladder ichthyocol. Accordingly, all cross-linking reactions were carried out at collagen concentrations below 0.2%. Nevertheless some intermolecular polymerization appeared to be unavoidable and the reacted collagens isolated after removal of the formaldehyde by the dimedon dialysis procedure always contained a small amount of acid-insoluble collagen. In general, high concentrations of protein and of formaldehyde and an extended reaction time resulted in increasing amounts of acid-insoluble material. The amount of insoluble collagen formed is not reflected in any of the values obtained for the CL-ichthyocols, since the insoluble fraction was always removed by centrifugation at 15,000 $\times g$ and discarded. All subsequent analyses of each preparation were made with the clear supernatant solution of this final centrifugation.

The course of the formaldehyde reaction was followed by taking aliquots of the reaction mixtures at various times, working these aliquots up to provide the formaldehyde free clear supernatant solution, and examining the sedimentation patterns of the solutions after denaturation at 40°. The sedimentation patterns of the undenatured collagens were relatively uninteresting, all giving the hypersharpeak typical of tropocollagen. Table I lists some physical properties of ACL-, NCL-, and unmodified ichthyocol in the undenatured state. The sedimentation patterns of the denatured cross-linked reaction products, however, changed in a regular and characteristic fashion with increased reaction time. Fig. 1 shows a series of sedimentation diagrams for ACL-ichthyocol formed at 0.18% in 0.1 m HAc and 3.4% formaldehyde at 4°. The zero time picture shows two components, a and b. After 20 hours, the relative a-component content was decreased, b-component increased, and a new component was evident. After 30 hours, the new component, identified as y, was present in larger amount, and after 72 hours all a- and b-components had disappeared. The sharp peak of Fig. 1d is y. Also evident is a still faster component, Z, which was never detected in the original preparation. The physicochemical properties of the denatured gelatin components are assembled in Table II.

The end point of the reaction was taken to be that situation in which the denatured cross-linked gelatin was all in the $\gamma$ form with a minimal production of acid-insoluble collagen. The optimal reaction times were found to be 15 to 18 hours at 3.4% formaldehyde in neutral 0.5 m calcium chloride and 72 hours for 0.4% formaldehyde in acidic solutions. Higher formaldehyde concentrations decreased the time for the formation of $\gamma$ but also decreased the yield as a result of the insolubilizing reaction. The more rapid cross-linking at neutral pH is in accord with the mechanism proposed by Fraenkel-Conrat and Oclett (11). The first reaction is rapid and reversible and leads to the formation
The specific optical rotation of the reaction solutions was monitored at intervals up to reaction times of 146 hours, well beyond the optimal period. There was no change in [\[alpha\rceil_2^2 from the original value for native ichthyocol. Thus, the intramolecular cross-links to form the \( \gamma \)-gelatin were imposed on the three-stranded tropocollagen molecules without disruption of their native, ordered configuration. This very clearly indicates that the groups capable of becoming cross-linked are suitably juxtaposed in the native collagen molecule. This juxtaposition places certain restrictions on neighboring group distances.

The next higher homologue of formaldehyde, acetaldehyde, also reacts with ichthyocol to produce undenatured polymers. However, intermolecular cross-linking appeared to be occurring as fast or faster than the intramolecular reaction and an optimal set of reaction conditions leading to a pure \( \gamma \) with a minimum of insolubilization could not be found. Copious cross-linked fibrous precipitates were obtained in each case, and these were not soluble in cold dilute acetic acid. The precipitates were treated in the same fashion as mature, acid-insoluble collagen fibers. They were heated in acid under denaturing conditions for 1 hour, and the resultant gelatin was collected. The gelatin was dialyzed against a high pH citrate or phosphate buffer in the cold, and the fibrous precipitate that formed was examined in the electron microscope. The fibers, partially masked by occluded amorphous gelatin, exhibited the typical collagen cross-striation indicating that both intra- and intermolecular cross-linking of the monomer units had taken place.

The Renaturation Reaction—The renaturation reaction procedures were applied to denatured unmodified ichthyocol, ACL-ichthyocol, and NCL-ichthyocol. In the latter cases the materials studied were those obtained by carrying out the cross-linking under the optimal conditions described above. These cross-linked collagen was essentially all in the \( \gamma \) or X-component form. As indicated in the bottom two rows of Table II, all three gelatins were characterized by the same value of [\( \eta \rceil_2^2 and all had very low intrinsic viscosities. The gelatins were thus thoroughly denatured in each case, with no evidence for any internal configurational order before the renaturation operation. To avoid complications with the characteristic changes resulting from collagen-fold formation in all gelatins, we restrict the usage of the term renaturation here to that process which yields water-
insoluble collagen fibers in the native form with an observable fiber periodicity of ~700 Å when viewed in the electron microscope, or the SLS form after treatment with adenosine triphosphate.

All attempts to renature the 80% α-20% β component mixture of denatured unmodified ichthyocol proved fruitless. However, the γ-rich denatured ACL- and NCL-ichthyocol preparations were readily renaturable by any of the renaturation procedures. Examination of the electron micrographs of the renatured native form fibers did not show anything of particular interest. However, the SLS (see above) electron micrographs did show a readily apparent difference between native ichthyocol-

### Table II

**Physical properties of denatured ichthyocol before and after optimal cross-linking**

<table>
<thead>
<tr>
<th>Solvent system: 0.10 M HAc; 0.10 M KCl; pH 2.8, except where noted.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Fractional composition, %</td>
</tr>
<tr>
<td>α</td>
</tr>
<tr>
<td>β</td>
</tr>
<tr>
<td>γ</td>
</tr>
<tr>
<td>X</td>
</tr>
<tr>
<td>s_90,w, Å</td>
</tr>
<tr>
<td>α</td>
</tr>
<tr>
<td>β</td>
</tr>
<tr>
<td>γ</td>
</tr>
<tr>
<td>X</td>
</tr>
<tr>
<td>[α]_20, mixture</td>
</tr>
<tr>
<td>[γ]_10, dl/g</td>
</tr>
</tbody>
</table>

* pH 4.8, 0.15 M acetate (21).
* 0.05 M HAc + 0.1% NaCl (9).
* Polymerized in pH 3.7, 0.15 M citrate.
* Polymerized in pH 2.8, 0.1 M HAc.

SLS, NCL-SLS, and ACL-SLS preparations. The native ichthyocol-SLS and NCL-SLS micrographs, Fig. 2, a and b, contained a preponderance of dimeric and trimeric end-to-end SLS aggregates, with a somewhat larger fraction of aggregates in the NCL-ichthyocol. On the other hand, the SLS preparations from renatured ACL-ichthyocol were comparatively monodisperse, with only a very few end-to-end aggregates (Fig. 2c). These differences must be taken as qualitative at this point. A detailed statistical analysis of many micrographs has not been undertaken. Another observation indicating a difference between NCL- and ACL-ichthyocol was that whereas the dialysis renaturation was virtually complete in both cases, the formation of ~700-Å spacing native fibrils by heat precipitation provided substantially lower yields of ACL- than of NCL-ichthyocol fibers.

**Physical Properties of Cross-linked Ichthyocol**—As indicated in Table I, the s_90, value reported here for the buffalo fish acid-soluble collagen is lower than that of similar carp swim bladder and cod skin collagen preparations. This probably arises from the fact that the pH 2.8 0.1 M HAc-0.1 M KCl solvent system was used rather than the more usual pH 3.5 0.15 M citrate buffers used by other investigators. Our measurements were made in the pH 2.8 system, the acid polymerization pH, to avoid further manipulation of the reacted collagen. Borcover and Veis have found that s_90 increases for the buffalo fish collagen as the pH increases at constant ionic strength or, in effect, as the net charge on the tropocollagen is decreased. Although it is difficult to compare data obtained at different pH values, the data at constant pH and ionic strength should be internally consistent and suitable for comparison. The s_90 values for unmodified ichthyocol and NCL-ichthyocol were essentially identical at approximately 2.2 Å, whereas s_90 for ACL-ichthyocol was substantially higher, 2.7 Å. There are two possibilities

D. Borcover and A. Veis, unpublished results.
that might account for this difference: the ACL-ichthyocol could have a lower net charge than either the NCL- or unmodified ichthyocol and hence have a higher sedimentation rate; or the ACL-ichthyocol could have a different average molecular size.

The optical rotation data leave no doubts that both ACL- and NCL-ichthyocol are fully organized in the collagen-fold configuration after renaturation; [α]D is higher for each of the renatured, cross-linked collagen than for native ichthyocol. Thus, each of the collagens should have the typical rod shape. Viscosity measurements show that this is indeed the case. The intrinsic viscosity of unmodified ichthyocol was 15.4, whereas before denaturation, [η]ACL was 16.0 and [η]NCL was 17.7, which indicates the cross-linked collagens were somewhat aggregated. After denaturation and subsequent renaturation, [η]NCL was unchanged but [η]ACL decreased to 5.7. These viscosity data, taken together with the electron microscopic data showing that ACL-ichthyocol gives monomeric SLS whereas NCL- and unmodified ichthyocol give end-to-end polymeric SLS aggregates, suggest that the native ichthyocol in acid solution and in neutral 0.5 M CaCl2 is partially polymerized in elongated aggregates. Reaction with formaldehyde at neutrality either stabilizes these aggregates or does not impair the ability of therenatured molecule to form new ones. Reaction with formaldehyde in acid, however, does appear to destroy the end-to-end aggregate formation in the renatured ACL-ichthyocol. If this is the case, then the intrinsic viscosity of denatured ACL-ichthyocol represents the true monomer, or γ-tropocollagen, viscosity.

The sedimentation patterns of the denatured CL-ichthyocols show the presence of a component X, which sediments faster than γ. If we assume that X represents a polymer of γ units and that the intrinsic viscosity will approximate the weight average value of the viscosities of the two components, then we can easily compute the intrinsic viscosity of each component from the mixture intrinsic viscosity and the fractional component composition. Such computations for various γ-X mixtures give [η]m = 9.6 to 13.1 and [η]X = 43.3 to 32.1 for an average [η]X/[η]m = 3.33. The high viscosity of X suggests that it is an end-to-end γ-aggregate of rodlike character and the average ratio of viscosities is close to that predicted, assuming axial ratios of 200 for tropocollagen (2800 Å X 14 Å) and 400 for an end-to-end dimer. The ratio of viscosity increments for this case is 3.5, according to the tables of Mehl, Oncley, and Simha (22).

Hodge and Schmidt (23) and Hodge et al. (24) have proposed that native tropocollagen has random nonhelical, essentially proline free peptide chain segments protruding from the helical triple-chain rod at each end. Complementary electrostatic interactions of these ends lead to end-to-end dimers and higher end-linked polymers. Our observations suggest that in the native ichthyocol solutions, end-linking occurs to give an average of approximately 20% dimer. Under the rather prolonged reaction periods required in acid cross-linking, it is probable that some of the guanidino groups form the stable (non-cross-linking) methyl derivative, reducing the effective charge on these groups in the end-chain region. This enhanced reaction with formaldehyde is reflected in the increase in formaldehyde content of ACL-ichthyocol as compared with NCL-ichthyocol in the chromotropic acid assay, Table III. As noted, the cross-linking reactions do not lead to structure breakdown. It would appear, therefore, that the chemical modification is not sufficient to disrupt the secondary structure in the end-chain region of end-linked tropocollagen molecules. Once denatured, however, the long range electrostatic organizing force is absent and the end-to-end aggregation is inhibited. The decreased charge may account for the higher [η]ACL of ACL-ichthyocol. In contrast, the presence of the X component in the denatured gelatins from NCL-ichthyocol and the complete recovery of the enhanced [η]NCL upon renaturation lead to the conclusion that covalent cross-linkages are imposed on the intact end regions under neutral reaction conditions.

As predicted by Flory (25), the imposition of cross-linkages on the ordered tropocollagen molecule leads to an increase in the denaturation temperature Tc. The data in Table I indicate that the environment does influence Tc, but a consistent difference of 1.4° was found in pH 2.8 0.1 M acetic acid. The similarity of the denaturation temperatures of the two cross-linked denatured collagens is in line with the analytical data showing that both preparations had approximately 10 cross-linkages per γ unit.

**Characterization of Denatured Gelatins** In the discussion thus far, we have assumed that the γ-component had the same molecular weight as the native ichthyocol tropocollagen monomer and that the X component was an end-to-end γ-dimer. The data obtained on the denatured unmodified, ACL-, and NCL-ichthyocol, and recorded in Table II, do not directly give evidence about the validity of these assumptions since the viscosities again refer to mixtures of the various components. However, these data are consistent with the assumptions. This can be seen by considering the hydrodynamic data in terms of the treatment of Mandelkern and Flory (26) and Mandelkern et al. (27). According to these investigators, the function S0[η]/M1 should be a constant for any particular homologous polymer series. Con-

### Table III

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Reaction conditions</th>
<th>Composition</th>
<th>Moles HCHO/10^6 g</th>
<th>Protected r-NH2 groups</th>
<th>Reaction periods required in acid cross-linking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified ichthyocol</td>
<td>pH 2.8, 0.1 M HAc, HCHO, 72 hrs</td>
<td></td>
<td>α,β</td>
<td>0.23</td>
<td>2.6</td>
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<tr>
<td>ACL</td>
<td>pH 7.4, 0.5 M CaCl2, 24 hrs</td>
<td></td>
<td>γ, X</td>
<td>1.25</td>
<td>1.00</td>
</tr>
<tr>
<td>NCL</td>
<td>pH 7.4, 0.5 M CaCl2, 24 hrs</td>
<td></td>
<td>γ, X</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>NCL</td>
<td>pH 7.4, 0.5 M CaCl2, 24 hrs</td>
<td></td>
<td>γ, X</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Insoluble steerhide collagen</td>
<td>HCHO, 48 hrs</td>
<td></td>
<td></td>
<td>0.22</td>
<td>~0</td>
</tr>
</tbody>
</table>
consider first the $\alpha,\beta$-mixture of denatured ichthyocol. Substituting the appropriate numbers from Table II for the sedimentation coefficients and taking $M_B = 2M_\alpha$, the ratio $[\eta]_B:[\eta]_\alpha$ equals 2.0. With the use of this viscosity ratio, and the mixture composition, $[\eta]_\alpha$ equals 0.49 and $[\eta]_\beta$ equals 0.98. Similarly, taking $M_\alpha = 2M_\gamma$, $[\eta]_\alpha$ equals 0.71, and $[\eta]_\gamma$ equals 0.97. For the $\alpha$-component, with the above value for $[\eta]_\alpha$, the Flory constant, $\phi_1 \rho^{-1}$, is $2.2 \times 10^4$ with $M_\alpha$ set at $10^4$, indicating that all of the parameters are in the correct range. The internal consistency of the molecular weights ($M_\alpha:M_\beta:M_\gamma:M_\delta = 1:2:3:6$) can be checked by computing $M_\gamma$ from the constant determined for $M_\delta$ and with the $\phi^2$ and $[\eta]$ data. Such a computation gives $M_\gamma$ equals 3.4 $M_\delta$. Similarly, $M_\delta$ equals 6.9 $M_\gamma$.

The intrinsic viscosity-molecular weight relationships can be examined also in terms of the Staudinger equation, $[\eta] = K M^a$. In this expression, the exponent $a$ is a parameter dependent on the properties of the polymer. A random coil molecule has $a = 1$; a spherical or network molecule has $a = 0.5$. For $\alpha$ and $\beta$, as anticipated for a single-chain random coil, $a$ has a value near 1. On the other hand the intrinsic viscosities of both the $\gamma$- and $X$ components are consistent with an $a$ value near 0.5. Thus, all of the hydrodynamic data indicate that the $X$ component has approximately twice the molecular weight of the $\gamma$-component and has the same multichain character. The high values for $M_\alpha/M_\beta$ and $M_\beta/M_\gamma$ are probably the result of a change in $\phi_1 \rho^{-1}$ with interchain cross-linking to form structures equivalent to branched polymers. In such cases, the “universal constant” $\phi_1 \rho^{-1}$ decreases from the random coil value.

Reversibility of Cross-linking Reaction—When solutions of ACL- and NCL-ichthyocol preparations were kept at elevated temperatures in the denatured state for long periods, degradative changes were evident. This degradation was followed by examining the sedimentation patterns of aliquots of an ACL-ichthyocol preparation held at pH 7.2 and 40° at various times during a 48-hour period. The sedimentation patterns, Fig. 3, show the gradual disappearance of the $\gamma$- and $X$ components and the reappearance of the $\alpha$- and $\beta$-components. The regenerated $\alpha$-component peak, however, was much less sharp than initially, clearly indicating that backbone chain cleavage and cross-linkage rupture were concurrent reactions. Thus, the aldehyde cross-linkages have a stability on the same order as the weaker peptide bonds in the collagen. As mentioned earlier, Fraenkel-Conrat and Oelott had noted that the formaldehyde cross-linking reactions between amino and amide or guanidyl groups were substantially more readily reversible than the reactions between amino groups and groups containing active hydrogen such as in phenolic or imidazole structures. It would appear, therefore, that the methylene bridge cross-linkages formed within the collagen structure by monofunctional aldehydes are between amine groups and amide or guanidyl groups.

Significance of Aldehyde Cross-linking Reaction—It is clear that intramolecular interchain cross-linkages can be introduced into the native tropocollagen monomer unit by monofunctional aldehydes without disruption of the secondary structure of the monomer unit. The imposition of the cross-links on the organized monomer unit leads to the formation of an intramolecularly polymerized molecule which behaves in a manner analogous to
The 

2010 IntrGdUCtiGn of Intramolecular Cross-links into Ichthyocol Vol. 238, No. 6


the γ-tropocollagen and γ-gelatin units isolated from connective tissues. Intermolecular cross-linkages can also be formed with the aid of monofunctional aldehydes. This set of cross-linking reactions in vitro thus establishes the point that covalent inter-chain cross-linkages could account for the appearance of the specific intra- and intermolecular polymers that have been found in gelatins derived from mature mammalian collagens. It does not, of course, prove that the natural cross-linkages in collagens are mediated by aldehydes. The fact that all connective tissues so far examined contain low concentrations of particularly reactive aldehydic metabolites (glyceraldehyde, pyruvaldehyde, acetaldehyde, glycoaalddehyde) (5), and that all of these monofunctional reagents can tan collagen (28), suggests, however, that the possibility of aldehyde cross-linking in vivo cannot be disregarded.

Under the optimal conditions for the formation of the γ unit, approximately 10 methylene bridges are introduced per tropocollagen monomer, or 3 per α-chain. One end of the cross-linkage involves the ε-amino group of lysine, and the formation of the methylene bridge renders these amino groups unavailable to fluorodinitrobenzene. The ease of formation and lability of the bonds suggests that the cross-link is either of the type

\[
R-\text{NH-CH}_2-\text{NH-C}=\text{O}
\]

or

\[
R-\text{NH-CH}_2-\text{NH-O-CH}_2-\text{NH-R}
\]

Work is in progress to determine which of the three potential residues, asparagine, glutamine, or arginine, may be participating in the cross-linking. We might guess, however, that asparagine is the most likely candidate. Studies on the alkaline treatment of mature mammalian collagen (29) indicate that all of the amide groups are not equally susceptible to hydrolysis. Cassel and McKenna (30) have estimated that there are approximately four residues of asparagine for each residue of glutamine in corium collagen. Furthermore, they indicate that all of the amide groups can be readily hydrolysed from the glutamine residues whereas the asparagine amide groups resist hydrolysis. Even alkali precursor gelatins of high molecular weight contain residual amide groups, and these molecules are thought to be branched rather than linear polymers (31). It would appear therefore that some of the amide groups might be protected by cross-linkage in the same manner as the ε-amino groups of lysine.

The peptide sequence studies of Grassmann, Hammig, and Schleyer (32), as well as electron micrographs of selectively stained tropocollagen in the SLS form (33), show that the polar groups of collagen are localized in definite regions along the length of the molecule. These regions are separated by chain segments in which there are few or no polar residues. The polar sequences contain both acidic and basic side chains in close proximity. According to models constructed to scale with the -Gly-Pro-X- repeat distances proposed by Rich and Crick (34), cross-linking may occur in a chain trial if appropriate residues are separated by no more than four or five residue repeats along the collagen fiber axis. Within a native fiber, cross-linking might occur equally as well between properly spaced residues on adjacent chains to form side-by-side aggregates corresponding to the ε-component (10).

The appearance of X, the end-to-end dimer, indicates that in the organized end chain region, each monomer contributes one of the groups necessary for the establishment of the cross-link and that these groups are appropriately positioned in the normal end-to-end aggregate. Bensusan (35) has demonstrated the...
importance of electrostatic interactions in directing the formation of end-to-end as well as of native type collagen aggregates and has shown that the guanidyl radicals are particularly influential. Our observation that the prolonged reaction times required in acid decrease the ability of the renatured monomers to form end-to-end aggregates suggests that this is the result of the chemical modification of arginine side chains and locates some of these residues in the end chain region.

Gallop, Seifler, and McIlman (2) have shown that a small number of bonds in gels are susceptible to attack by hydroxylamine and hydrazine and have therefore suggested that there is approximately 6 \times 10^{-5} equivalent of intramolecular “ester-like” linkages per g of collagen. Hörmann et al. (36) found that mature collagens contained an additional 4 \times 10^{-6} equivalent of such groups per g and theorized that the intermolecular cross-linkages in mature collagen are ester bonds formed between carboxyl groups and the hydroxyl groups of the ubiquitous hexose moieties in collagen. Blumenfeld and Gallop (37) have isolated peptides containing the hydrazide of aspartic acid from hydrazine-treated collagen. In view of the fact that the number of acid groups that can be titrated in collagen and gelatin corresponds to the number of free, nonamidated carboxyl groups indicated by amino acid analysis (38, 39), it is difficult to account for the number of acid groups that Hörmann et al. (36) suggest are involved in ester linkages. An alternative might be that the presence of K—NH—CH2— bond more susceptible to nucleophilic attack and account for the postulated esterlike character of the interchain linkages in mature collagens. This possibility is now being examined in studies with model compounds.

**SUMMARY**

1. Methylene bridge cross-linkages have been introduced into native ichthyocol tropocollagen monomers by reaction with formaldehyde.

2. The intramolecular cross-linking converts the ichthyocol monomer to the γ-tropocollagen form. Denaturation yields γ-gelatin with the molecular weight of the original tropocollagen.

3. The methylene bridge cross-linkage involves the ε-amino group of lysine and, probably, the amide function of asparagine although reaction may also occur with the guanidyl group of arginine or the amide group of glutamine. Approximately 10 such linkages are introduced per tropocollagen monomer at the optimal conditions for formation of γ.

4. The synthetic γ-gelatin has the property of renaturation, identical with the γ-gelatins extracted from mature mammalian collagens.

5. The collagen structure requires that the residues joined in the cross-link are within four to five residue repeat distances along the collagen axis. Intermolecular cross-linking can also occur. There is at least one set of groups capable of forming cross-links in the end region of the tropocollagen monomer with one member of the linkages coming from each molecule. An end-to-end dimer is formed.

6. The aldehyde-mediated cross-linkage is fairly stable to thermal hydrolysis. It hydrolyzes at a rate comparable to that of the weaker peptide bonds in collagen.

7. These data clearly show that covalent interchain cross-links can account for the appearance of the specific polymers species found in gelatin extracted from mature mammalian collagen. The participation of reactive aldehydes may be relevant to the natural cross-linking reactions in collagen during maturation.

**REFERENCES**


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Arthur Veis and Maurice P. Drake


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