The Action of Hydrazine on Collagen

I. CHEMICAL MODIFICATION*

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The current view of the collagen fibril (1) pictures it as an ordered aggregate of collagen rods, which themselves consist of three intertwined peptide chains. The macromolecular monomer unit rods, tropocollagen, appear to be packed in the same way in all collagens regardless of the origin of the collagenous tissue or its age. The variations in the properties of the collagens have been ascribed in part to the presence of covalent cross-linkages between tropocollagen units (2 10) (inter-TC).† There is also good evidence for the existence of covalent cross-linkages between the individual peptide chains within the tropocollagen molecule (3, 6, 11, 12) (intra-TC). Some of the current important questions in the chemistry of the maturation of collagen are those of the chemical nature of the various cross-linkages, their number, and their distribution.

Several investigators have proposed that the cross-linkages in collagen are ester-like in character (10). The first direct evidence came from the work of Grassmann et al. (8), in which it was shown that lithium borohydride reductively cleaved a small number of covalent bonds in citrate-soluble calf skin collagen. Konno and Altman (9) reported the isolation of a glycine-carbohydrate complex, reducible with lithium borohydride, from rat muscle collagen. Major support for the presence of ester-like linkages came from the work of Gallop et al. (7), who reported that hydroxylamine and hydrazine reacted with gelatins to produce protein-bound hydroxamate and hydrazide, respectively. These investigators relied on the fact that both reagents react much more rapidly with simple esters than with simple amides or peptides in proposing that the hydrolyzable bonds were ester linkages. They found that with acid precursor gelatin, or the gelatin produced by the denaturation of calf skin or ichthyocol TC, 6 moles of hydroxamate were formed per 10⁴ g of protein. The gelatin was degraded to units with molecular weights near 20,000. Since there seemed to be neither a decrease in the number of amide groups in the gelatin, nor any increase in NH₂-terminal amino acid residues, it was concluded that native collagen and acid-pretreated gelatins contained 6 × 10⁻⁴ equivalent of weak ester-like linkages per g. Alkali-pretreated gelatine, from which approximately one-half of the amide group content was removed during pretreatment, formed only approximately 3 × 10⁻⁴ equivalent per g of bound hydroxamate. However, in this case, the molecular weight was still reduced to a value near 20,000.

The reduction of the molecular weight of the individual α-peptide chains, with the concomitant formation of bound hydroxamate, led Gallop et al. (7) to postulate that the ester-like linkages were intrachain bonds, that is, part of the gelatin backbone. From this point of view, the ester linkages could not be involved in intra-TC or inter-TC cross-linking. Gallop et al. (13) did, however, suggest that the ester linkages occurred in pairs in the α-chains and that an isomerization and rearrangement of bonds could establish ester-like cross-linkages.

The hydrazine and hydroxylamine reactions were carried out by Gallop et al. (7) at pH 9 or pH 10 in the range of 1 to 3 M reagent concentrations. Under these conditions, high concentrations of urea or thiocyanate were required for the reaction to proceed at an appreciable rate at room temperature. The reaction could be carried out in the absence of the urea at 40°.

Bello (14) attempted to apply these same reactions to fibrous steer corium collagen as well as to an acid precursor gelatin. In his experiments, the acid precursor gelatin yielded 0.5 to 1.5 × 10⁻⁴ equivalent of bound hydroxamate per g of collagen, and the insoluble corium collagen, presumably more highly cross-linked, only 2 × 10⁻⁴ equivalent of hydroxamate per g. There was no way to reconcile his data for the acid precursor gelatin with that of Gallop et al. (7). Bello found, in addition, that the insoluble collagen would not react at all with hydroxylamine or hydrazine (pH 8.6) unless the collagen was denatured before treatment with the base. Bello suggested that all the bound hydroxamate could be accounted for by the presence of carbohydrate in his collagen preparations. Hörmann (15) also examined the reaction of hydroxylamine with fibrous collagen. He confirmed that the collagen had to be denatured before it would react. Hörmann et al. (16) found that, on the average, mature steer corium collagen yielded 11 × 10⁻⁴ equivalent of bound hydroxamate per g of collagen. They also treated subpartial denaturation of the alkali-precursor gelatin stock during the pretreatment step.
Table I

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Purified collagen</th>
<th>Limed collagen</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Content of soluble components</td>
<td></td>
<td></td>
<td>Weight and hydroxyproline (Neuman and Logan (22))</td>
</tr>
<tr>
<td>Neutral salt-soluble</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid-soluble</td>
<td>0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Swelling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2.5</td>
<td>415</td>
<td>515</td>
<td>Solution uptake (Bowes (23))</td>
</tr>
<tr>
<td>pH 5.3</td>
<td>310</td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>3. Total nitrogen</td>
<td>18.08</td>
<td>17.82</td>
<td>Kjeldahl analysis</td>
</tr>
<tr>
<td>4. Amide nitrogen</td>
<td>0.66</td>
<td>0.42</td>
<td>NH₃ in partial hydrolysisate (Cassel and Kanagy (24))</td>
</tr>
<tr>
<td>5. Arginine</td>
<td>5.45–5.55</td>
<td>5.47–5.52</td>
<td>Rosenberg et al. (25)</td>
</tr>
<tr>
<td>6. Citrulline</td>
<td>Negative</td>
<td>Negative</td>
<td>Neuman test (26)</td>
</tr>
<tr>
<td>7. Hydroxyproline</td>
<td>12.9</td>
<td>12.9</td>
<td>Neuman-Logan (22)</td>
</tr>
<tr>
<td>8. Shrinkage temperature</td>
<td>62–64°C</td>
<td>58–59°C</td>
<td></td>
</tr>
</tbody>
</table>

ble TC with hydroxylamine and found $6.8 \times 10^{-4}$ equivalent of bound hydroxamate per g. They therefore concluded that there were 4 moles of ester-like linkages per $10^{-4}$ g of insoluble collagen which participated in inter-TC linkages. Following the hydroxamate formation, carried out with 0.75 M NH₂OH in 4 M LiCl at 37°C and pH 9.55 for 10 hours, the collagen was completely dissolved and degraded, presumably to an average molecular weight near 20,000. Both the collagen and TC contained a total of approximately $4 \times 10^{-4}$ mole of glucose or glucose and galactose per g. Hörmann (17) concluded that the cross-links in collagen consisted of hexoses whose two hydroxyl groups formed ester links between the different peptide chains while the hexoses in tropocollagen were bound by only a single ester group. The reducing group of the hexose was supposed to be attached to a hydroxyl side chain of one of the backbone peptide units by a glucoside-like oxygen bridge.

All of these data provide substantial evidence for the presence of hydroxylamine-sensitive, ester-like linkages in collagen but do not provide conclusive proof that the ester linkages participate in inter-TC unit bonding. The dissolution of the mature collagen fibers after hydroxamate formation could conceivably occur merely due to the extensive degradation of the individual backbone peptide chains and not because of the cleavage of interchain bonds. A clarification of this question would appear to require the selective cleavage of the inter-TC bonds while leaving intact the intra-TC bonds of weak ester-like character.

If one accepts the premise that the inter-TC bonds are ester bonds as Hörmann proposes (15–17), it is difficult to see why these groups would not be readily accessible for reaction with hydroxylamine or hydrazine.

Veis and Cohen (2, 18) and Veis et al. (3, 11) showed that collagen fibers could be selectively degraded in acid or at neutral pH to yield soluble fractions with non-gaussian molecular weight distributions and containing substantial numbers of molecules with both the peptide backbone chains and the intra-TC cross-linkages intact. These molecules could be reversibly denatured and renatured. Courts (19) showed that treatment of mature corium collagen with acid in the cold, followed by prolonged soaking in Ca(OH)₂ in the presence of salts that reduced swelling, also appeared to lead to the selective rupture of inter-TC bonds. Collagen fibers treated in this way were soluble in cold dilute acetic acid to give high viscosity solutions very similar to TC in behavior. Fibrous precipitates of this soluble collagen could be prepared by dialysis (20). These data illustrate the accessibility of the inter-TC bonds to both acid and alkaline hydrolysis but do not indicate their chemical nature.

In a search for a system in which the inter-TC bonds could be cleaved in fibrous collagen without denaturing the collagen structure, we found that hydrazine, in contrast to hydroxylamine, would react with native collagen under appropriate conditions. We therefore investigated the chemical and physical changes occurring in purified native steer corium collagen, and Ca(OH)₂-soaked, but otherwise undegraded steer corium collagen following reaction with hydrazine. This report describes the chemical aspects of the collagen-hydrazine reaction over an extended range of hydrazine concentration.

**EXPERIMENTAL PROCEDURE**

**Preparation of Collagens**—Purified steer corium collagen was prepared from fresh steer hide by the method of Veis, Anesey, and Cohen (3). The limed collagen was obtained from a similar hide subjected to a 5-day lime treatment. This material was purified by the method of Bowes and Kenten (21). Both collagens were lyophilized for storage. A number of determinations were made to characterize the collagens. These data are summarized in Table I. The short liming process did not decrease the arginine content of the collagen, and there was no indication of citrulline formation. The principal result of the liming was to reduce the amide content by 36.4%.

**Preparation of Hydrazine**—Hydrazine (Olin Mathieson Chemical Corporation, 95% + %) was redistilled and used as a standard for the preparation of hydrazine solutions of different concentrations. The concentration of the standard was determined colorimetrically by the method of Pesez and Petit (27) and was kept in the range of 96 to 98%. Dilutions of this stock solution were made in an acetone-Dry Ice bath to avoid overheating. Since aqueous hydrazine easily undergoes autoxidation, which can be accelerated by dilution, by the addition of NaOH, or by the presence of trace metals (28–30), the change in concentration of 10 and 70% aqueous hydrazine on standing in closed glass containers at room temperature was determined. There was no significant decrease in hydrazine concentrations within 6 days.

**Reaction of Collagen with Hydrazine**—Lyophilized collagen was treated with aqueous hydrazine at 25 ± 2°C for 30 hours. A ratio of 1 weight of collagen to 10 volumes of hydrazine solution was used. The moisture content of the collagen (~9%) did not cause an increase in the solution temperature on mixing. These reaction mixtures were shaken in glass-stoppered Erlenmeyer flasks. When solutions above 58.8% hydrazine were used, a wax coating was applied to the stoppers.

At the end of the reaction period the collagen was washed with many changes of cold distilled water (~7°C) for 3 to 4 days. The washings were monitored for the presence of hydrazine as follows. After each 20 hours of washing, 2 g of collagen were washed separately for 3 hours in 50 ml of distilled water. This
washing solution was tested qualitatively with salicylaldehyde (31) for the presence of hydrazine. After the first negative test, the protein was washed for another 30 hours and then lyophilized. The hydrazide content of the collagen was determined by the method described by Seifter et al. (32). Crystalline hydrazine sulfate, the reaction product of hydrazide hydrate and excess sulfuric acid, was used for the preparation of the hydrazine standards.

It was noticed that the amount of protein-bound hydrazide appeared to decrease with prolonged washing. To determine the extent of this loss, samples of collagen treated with 40% and 70% hydrazine were extracted with repeated changes of absolute ethanol for 40 hours to remove the free hydrazine. The spot test with salicylaldehyde indicated the absence of free hydrazine in the final rinse. The extracted collagen was rinsed with water to remove the ethanol, lyophilized, and then subjected to the usual aqueous wash procedure. The hydrazine content was determined at intervals during a 70-hour wash. These data are shown in Fig. 1 and indicate a gradual decrease in protein-bound hydrazide that could decrease the values reported subsequently by 5 to 10%.

Arginine Determination—Half-gram portions of collagen were hydrolyzed under reflux for 16 hours in 10 ml of 20.5% HCl. The hydrolysates were analyzed by the method of Rosenberg et al. (25). The presence of hydrazine in the amounts expected from the hydrolysis of the bound hydrazides (up to 90 equivalents of NH₂NHz per g) did not affect the arginine analysis.

**Fig. 1.** Decrease in protein-bound hydrazide during the washing procedure. Δ—Δ, collagen treated in 70% hydrazine; ○—○, collagen treated in 40% hydrazine. The upper figures on the ordinate refer to the 70% and the lower to the 40% hydrazine-treated collagens.

Citrulline Analysis—The hydrazine-treated collagens were examined for the presence of citrulline, one of the possible breakdown products of arginine. The method described by Fearon (26) was used, and no citrulline was found.

**Amino Acid Analyses**—Purified collagen and the residual and soluble fractions resulting from 40 and 70% hydrazine treatment were hydrolyzed and examined for their amino acid content by a quantitative paper chromatographic technique (33). Particular attention was given to the ornithine content. The standard amino acid mixtures contained ornithine as well as the amino acids present in collagen. The presence of ornithine increased the color yield of the lysine. Correction factors were computed for various concentrations of ornithine. These were obtained by running the standard amino acid mixture with ornithine concentrations ranging from 0 to 60.2%.

**NH-terminal Amino Acids**—Purified native collagen treated with 50% hydrazine was dinitrophenylated under the conditions described by Levy (34). The dinitrophenyl derivatives were identified qualitatively by paper chromatography.

**RESULTS**

**The Hydrazine System**

The reaction systems examined ranged from 0 to 70% hydrazine, corresponding to 0 to 21.8 M. The basicity of the solutions varies continuously over this range. Deno (35) has shown that the Hammett function, H₋, is a linear function of the hydrazine concentration. H₋ increases from a value of 10.75 for an infinitely dilute hydrazine solution to 15.93 at 18.72 M (60%). In the overlapping range up to pH 14, the H₋ and pH functions have identical values and, therefore, the pH is also a direct linear function of the hydrazine concentration. In the ensuing discussion, all the data could have been presented in terms of H₋, or pH, but are usually presented in terms of the hydrazine weight concentration since this is a more easily appreciated unit and was the experimental variable.

**Reactions during Hydrazinolysis**

**Formation of Protein-bound Hydrazide**—Hydrazine was found to react with the un-denatured fibrous collagens to yield protein-bound hydrazide over the entire hydrazine concentration range. The amount of protein-bound hydrazide in the native and limed collagen is shown in Fig. 2 as a function of the hydrazine solution concentration. The two striking features of these data are first, that the native collagen reacted with substantially more hydrazide than did the limed collagen at each hydrazine concentration, and second, that in the 40 to 50% hydrazine concentration range (H₋, 14 to 15) there was an abrupt rise in the amount of hydrazide bound to the native collagen, whereas this did not take place with the limed collagen.

The collagen fibers did not appear to be denatured by their treatment and reaction with hydrazine. Electron micrographs of the limed fibers, the native hydrazine-treated fibers, and the limed, hydrazine-treated fibers showed all of the cross-striations and periodicity of structure characteristic of native collagen as long as the hydrazine reaction solution concentration was ≤ 55%. The original limed collagen, however, showed some evidence for decreased structural integrity in having a shrinkage temperature range of 58–59° as compared with the native collagen 62–64° range (Table I). Amorphous, nonstrained, treated collagen fibers appeared at hydrazine concentration > 60%. This did not coincide with the 40 to 50% hydrazine concentration range in which the native collagen showed its enhanced formation of bound hydrazides.
**Action of Hydrazine on Collagen. I**

**Fig. 2.** The formation of bound hydrazide by collagen as a function of hydrazine concentration. ○—○, native purified collagen; ●—●, limed collagen.

**Fig. 3.** The amide group content of hydrazine-treated collagen. ○—○, native purified collagen; ●—●, limed collagen.

**Fig. 4.** The arginine content of hydrazine-treated collagen. ○—○, native purified collagen; ●—●, limed collagen.

**Table II**

<table>
<thead>
<tr>
<th>Hydrazine concentration</th>
<th>Collagen fraction</th>
<th>Arginine*</th>
<th>Ornithine†</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
<td>moles/10^4 g</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Native fibers</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>Insoluble residue</td>
<td>45</td>
<td>2.5</td>
</tr>
<tr>
<td>70</td>
<td>Insoluble residue</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>40</td>
<td>Soluble, nondialyzable</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>70</td>
<td>Soluble, nondialyzable</td>
<td>8</td>
<td>53</td>
</tr>
</tbody>
</table>

* Determined by direct analysis, method of Rosenberg et al. (25).
† Determined by paper chromatography (33). This method is subject to a larger experimental error than the arginine analysis. These data seem to be consistently high by approximately 15%.

will react to form a protein-bound hydrazide except at very low hydrazine concentrations.

**Deguanidination**—Analyses of the arginine content of the two collagens, illustrated in Fig. 4, showed that at high hydrazine solution concentrations (hydrazine > 50%, H<sub>2</sub> > 15) the arginine content was decreased. The reaction was that of conversion of the arginine to ornithine and, as shown in Table II, there was reasonably good quantitative correlation between the decrease in number of residues of arginine and the number of residues of ornithine created. There was no indication of citrulline formation, and in accord with the work of Hamilton and Anderson (36), it is safe to conclude that the direct deguanidination of arginine was the principal reaction in the presence of hydrazine. The deguanidination reaction also resulted in the formation of several amino-substituted guanidines and of sym-diaminotetrazine. This interesting aspect of the chemistry of the reaction system will be described elsewhere.

The deguanidination reaction does not involve the formation of protein-bound hydrazide, as does the cleavage of an amide bond. Therefore, although the deguanidination reaction began

to be significant in the same hydrazine concentration range as the very rapid rise in bound hydrazide in the native collagen, these two reactions were not directly related.

Hydrazinolysis of Peptide Bonds—Bradbury (37) determined the kinetics of the hydrazinolysis of simple peptides and found that the rate of cleavage of peptide bonds was finite in anhydrous hydrazine even at 2°, the freezing point of hydrazine. Rees and Singer (38) also reported that there was a continuous decrease with time of the specific viscosity of a sample of γ-globulin in anhydrous hydrazine at 25° and that this was due to peptide bond hydrazinolysis. We therefore examined the hydrazine-treated collagen for evidence of peptide bond hydrolysis and chose the 50% hydrazine-treated native collagen for this analysis. At this hydrazine concentration, the number of bound hydrazides was almost at its maximal value of $40 \times 10^{-4}$ mole bound per g, whereas only approximately 0.3 of the arginine residues had been deguanidinated and there had been no dramatic increase in amide removal. Paper chromatography of hydrolysates of the fluorodinitrophenylated, 50% hydrazine-treated collagen showed the presence of glycine, aspartic acid, and glutamic acid as new NH$_2$-terminal residues. Semiquantitatively, the NH$_2$-terminal glycine was present in lowest amount and glutamic acid in the smallest amount. These data are in accord with those of Bradbury (37, 39) who found that peptide bonds containing glycine were particularly susceptible to hydrazinolysis, and that, in addition, bonds involving serine, glutamic acid, and aspartic acid were also especially labile in insulin, lysozyme, and wool.

The number of peptide bonds cleaved by the hydrazinolysis can be determined by subtracting the number of moles of bound hydrazide formed by amide replacement from the total hydrazide content. Fig. 5 shows the results of such a calculation for both the native and limed collagen. At the maximum, approximately $22 \times 10^{-4}$ mole of peptide bonds are cleaved per g of native collagen. At the same hydrazine concentration, only $3 \times 10^{-4}$ mole of peptide bonds per g were converted to the hydrazide in the limed collagen. These data indicate that approximately $19 \times 10^{-4}$ mole of peptide bonds per g collagen were hydrolyzed during the liming process and that the same bonds were apparently involved in both liming and hydrazinolysis. Bowes (23) found evidence for an increase in both base groups and fluorodinitrobenzine-reactive groups in limed collagen. She ascribed a portion of these to newly created residues from peptide bond hydrolysis and the remainder to the presence of ornithine resulting from the deguanidination of arginine. In this study, the native and limed collagens had the same arginine content and all of the increase in reactive groups appears to be related to the hydrazinolysis of particularly labile peptide bonds. The idea that a set of such bonds exists arises because of the clear-cut maximum in hydrazide formation at 55% hydrazine (Fig. 2).

Dissolution during Hydrazinolysis

As the hydrazinolysis reaction proceeds, a number of physical changes indicate a decrease in the structural integrity of the collagen fibers. The fiber tensile strength decreases, the swelling increases, and part of the collagen dissolves. Each of these alterations will be discussed in detail in a subsequent report. For the present purpose, we can take the extent of dissolution as a measure of the degree of rupture of cross-linkages as well as of backbone peptide chain hydrazinolysis. The filled circles in Fig. 6 represent the solubility of the native collagen as a function of hydrazine treatment. The limed collagen (open circles) was somewhat more readily dissolved at low hydrazine concentrations, but in both cases the amount of collagen dissolved increased most rapidly at hydrazine concentration greater than 70%. The rapid increase in ease of dissolution, in other words, did not coincide with the abrupt increase in content of bound hydrazide at 40 to 50% hydrazine (Fig. 2) but followed the same course as the deguanidination reaction. As shown in Table II, the soluble fractions had a much lower arginine content than the insoluble fibrous residues and a correspondingly higher amount of ornithine. This observation suggests that the arginine residues...
Action of Hydrazine on Collagen. I

Vol. 238, No. 1

0 20 30 40 50 60 70 80 90 100

HYDRAZINE CONCENTRATION, %

Fig. 6. The dissolution of collagen during reaction with hydrazine. O—O, native purified collagen; •—•, limed collagen.

52 12 6 4 0 10 20 30 40 50 60 70 80 90 100

HYDRAZINE CONCENTRATION, %

Fig. 7. A summary of the modification of native purified collagen. All points except the open circles refer to the left-hand ordinate. •—•, moles of bound hydrazide; □—□, moles of amide removed; ▽—▽, moles of arginine destroyed; O—O, dissolution of the collagen.

52 12 6 4 0 10 20 30 40 50 60 70 80 90 100

Fig. 8. A summary of the modification of limed collagen. All points except open circles refer to left-hand ordinate. •—•, moles of bound hydrazide; □—□, moles of amide removed; ▽—▽, moles of arginine destroyed; O—O, dissolution of the collagen.

DISCUSSION AND CONCLUSIONS

The results reported in the foregoing are fairly clear-cut and unequivocal. The objective of this discussion is 2-fold: we wish first to correlate the state of intermolecular bonding in native steer corum collagen with its hydrazine reactivity and chemical modification; second, we wish to relate our data to the studies of others on the hydroxylamine and hydrazine reactivity of systems containing denatured fibrous collagens or gels. Because of possible chemical differences in collagens from different sources, and because of the differences in reaction conditions, this latter comparison may be considered more speculative in nature. The ensuing discussion is, therefore, somewhat artificially divided into two sections.

Reaction of Native Fibrous Collagen with Hydrazine

Evidently, three reactions take place when fibrous undenatured collagen is treated with aqueous hydrazine: deamidation, peptide bond hydrazinolysis, and deguanidination of the arginine. The first two reactions result in the formation of protein-bound hydrazide, while the deguanidination reaction does not. The extent of deamidation is directly proportional to the hydrazine solution concentration over the entire range examined. The peptide bond hydrazinolysis becomes important only when \( H_- = 14 \) and is complete, at room temperature, when \( H_- \) reaches 15. The deguanidination reaction becomes significant when \( H_- > 15 \). As the hydrazinolysis reactions proceed, the fibers begin to dissolve, and they pass rapidly into solution at \( H_- > 16 \). The course of each of these reactions is summarized in Figs. 7 and 8 for the native and limed collagens, respectively.

At hydrazine concentrations \(<40\%\) \( (H_- < 14) \) both collagens show a direct, and in limed collagen an almost one-to-one, equivalence between amide removal and the formation of protein-bound hydrazide. As noted in the previous section, the amide loss from the native purified collagen is slightly greater than the hydrazide formation at low hydrazine concentrations, indicating that some of the most reactive amide groups are undergoing hydrolysis rather than hydrazinolysis. The near equivalence of amide loss and hydrazide formation excludes the possibility that interchain imides are involved, since the breakage of such linkages would result in the formation of 2 moles of collagen-bound hydrazide for each mole of ammonia removed. Similar quantitative considerations also seem to exclude the possibility of hydrazine reaction with ester linkages. This kind of reaction would have been indicated in the native collagen if the plot of bound hydrazide in Fig. 7 had been above, rather than below, the amide removal curve.

The sharp rise in formation of protein-bound hydrazide at hydrazine \( >40\% \), and the concomitant appearance of new...
NH₂-terminal residues, indicates the presence of some particularly labile peptide bonds in native collagen. There must be a limited number of such bonds since the enhanced hydrazide formation levels off at hydrazine concentrations > 55% and since the limed collagen, in which these bonds have presumably already been cleaved, shows no such increase in bound hydrazide. The peptide bond hydrazinolysis occurs at an H⁺ of 14 to 15 and that the basicity of the liming solutions approximates 14. It is possible that the enhanced hydrazide binding includes hydrazides formed from the cleavage of ester linkages. The NH₂-terminal amino acid residue content was not determined quantitatively or directly. However, the hydrazinolysis reactions in the H⁺ 14 to 15 range do not lead to the marked solubilization of the native collagen which would be expected if ester cross-linkages were severed. Correspondingly, the limed collagen did not become markedly more soluble in this hydrazine concentration range, and the lack of solubility of the limed collagen provides evidence that the cross-linkages in the limed collagen were intact. This is in spite of the fact that approximately 20 × 10⁻⁵ mole of peptide bonds per g had been cleaved in both collagens at this point. It is difficult to imagine that weak ester cross-linkages between TC units would be less accessible to general basic hydrolysis or hydrazinolysis than would peptide bonds within the organized framework of the TC-backbone chains.

The peptide chains in collagen contain segments alternately rich in the nonpolar and imino acid residues and in the polar amino acids. The appearance of NH₂-terminal aspartic and glutamic acids on hydrazinolysis or basic hydrolysis and the lack of appearance of NH₂-terminal proline or hydroxyproline following exhaustive liming of collagen (40) provides evidence that the polar regions are most susceptible to basic hydrolysis. The imino acid-rich gelatin chain segments, on the other hand, are stable to basic hydrolysis and, in addition, give to collagen and gelatin gels their particular chain folding and organization. Gallop et al. (13) have suggested that ester linkages are located in the polar regions of the gelatin molecule. Since, in fibrous collagen, these regions are the most readily deformed and swollen and the most susceptible to general basic hydrolysis, it is difficult to see why ester linkages in these regions would not have been attacked by hydrazine. Hence, we consider the presence of inter-TC ester linkages as being rather unlikely.

The most evident correlation in Figs. 7 and 8 is that the deguanidination of arginine is paralleled by an increase in the solubility of both the limed and native collagens. Arginine has been shown to be of importance in the ordering of tropocollagen units to form the native structure (41) and in the hydrogen bond stabilization of the ordered fibrils (42). Similarly, Grabar and Morél (43) and Janus (44) have shown the importance of the guanidino group in the setting and gelation of gelatin. The reciprocal relationship between solubility and arginine content leads one to consider the possibility that, in addition to their participation in electrostatic chain interactions, the guanidino groups in collagen may be involved in the covalent cross-linkages that stabilize mature collagen. At least, the covalent cross-linkages must have approximately the chemical stability of the bond linking the guanidino group into the arginine molecule. A suitable cross-link might be that joining an e-amino group to an amide or to a guanidino group via a methylene bridge resulting from an aldehyde condensation reaction. Franckel-Courant and Olcott (45) have shown that such cross-linkages are readily formed on the reaction of aldehydes with proteins. Landucci, Pouradier, and Durante (46) claim that aldehydes occur in all collagens and gelatins. The major portion of these aldehydes are thought to be bonded to the peptide chains and in alkali-labile cross-linkages. The existence of very stable inter-TC and intra-TC cross-linkages also fits in well with the data of Courts and Stainsby (47) and Veis, Anesey, and Cohen (11, 48). These workers have each presented evidence that both alkali- and acid-precursor gelatins of high molecular weight can be obtained under fairly drastic conditions and that these high molecular weight gelatins are cross-linked multichain structures. Some peptide bonds were cleaved during the preparation of these gelatins (47), but the chain cross-linkages were still present.

In summary, the data presented here suggest that the inter-TC cross-linkages are rather strong bonds, more stable than at least one set of weak peptide bonds in the collagen backbone chains. The resistance of these interchain cross-linkages to base-catalyzed hydrolysis or to hydrazinolysis at high basicity argues against their being considered as of "weak ester-like" nature. On the other hand, a portion of the amide groups in native collagen are readily reactive with hydrazine to form protein-bound hydrazide.

Alkali-pretreated collagen contains many severed peptide chains in the polar regions of the molecule. This hydrolysis does not lead to dissolution of the collagen unless some further, more stable interchain bonds are hydrolyzed. These bonds are equivalent in resistance to hydrazine to that holding the guanidino group to arginine. Native collagen is more reactive with hydrazine than limed collagen only insofar as the weakest amide groups are removed and the weakest peptide bonds are severed. These reactions reduce native collagen to the state of limed collagen and do not render it any more soluble than the limed collagen, until at H⁺ > 16 the deguanidination reaction begins.

One possibility for a strongly basic alkali-labile bond is that of a bond joining an e-amino group of lysine to a neighboring amide group or guanidino group by condensation of an aldehyde on the e-amino group to form an aminomethylol compound that subsequently condenses to hydrolyze out water and join the two groups with a methylene or substituted methylene bridge. Intrachain cross-linkages of this type have been introduced into native ichthyocol tropocollagen showing that in the native structure the appropriate cross-linking groups are sterically in the proper configuration for reaction (49).

**Reaction of Denatured Collagen and Gelatin with Hydrazine.**

**Presence of Ester Linkages in Collagens**

Quantitatively, the data presented previously for the formation of bound hydrazides at 2 to 3 × hydrazine concentrations are in good agreement with the value reported by Bello (14) for the number of collagen-bound hydroxamic acids following the reaction of denatured flaxen collagen with hydroxylamine, but are lower than the values reported by Hörmann (15) and Gallop et al. (7). Both Gallop et al. (7, 13) and Hörmann et al. (15-17) used more vigorous reaction conditions to secure reaction of their gelatins or denatured collagens with the hydroxylamine. Hörmann, Reidel, Altschopeter, and Klunkel (16), for example, heated their reaction mixtures to 37° and pH 9.55 for 10 hours. Under these conditions, even in the absence of hydroxylamine, the isoelectric pH of acid precursor gelatin or native collagen is shifted to lower values indicating the loss of amide nitrogen. Peptide bond hydrolysis is also enhanced at pH 9.55, the first
order rate constant for gelatin peptide bond hydrolysis being some 8 times greater at pH 9.5 than at pH 7.1, at which the rate constant has its minimal value (50). It is difficult to imagine that some amide loss and peptide bond hydrolysis did not occur in the presence of the hydroxylamine at elevated temperature and high pH. In addition, our experiments indicate the ready reactivity of approximately one-half the amide groups in native collagen even at room temperature. Therefore, the results reported here, \( \sim 2 \times 10^{-4} \) equivalent of bound hydrazide per g of collagen at 3.0 \( \times \) hydrazine, must be considered as the base line for chemical modification during hydrogenolysis, excluding carbohydrate or ester reactions. Gallop et al. (7) reported that an alkali precursor gelatin that had lost 50\% of its amide groups during pretreatment also formed 50\% less bound hydrazide. The agreement of this finding with our results seems more than coincidental.

Direct chemical evidence for the occurrence of ester linkages in collagens has been given by Grassmann et al. (8) and Konno and Altman (9). Recently, Gallop et al. (13) described the isolation of amino alcohols following lithium borohydride reduction of gelatin. These amino alcohols could only have arisen from \( \alpha- \) and \( \beta- \) aspartyl ester bonds. Quantitatively, they were able to recover only 30\% of the amount expected on the basis of their hydroxylamine assay, i.e., they recovered only \( \sim 2 \times 10^{-4} \) equivalent per g of ester reaction products, which would correspond to the glucose and galactose analyses of Hörmann (17). Thus, while there seems to be little doubt that ester linkages are present in collagen, the number of such linkages is probably on the order of \( 2 \times 10^{-4} \) mole per g and the higher values reported for the hydroxylamine-sensitive bonds in denatured collagens and gels presumably include some bound hydroxamate resulting from the cleavage of weak amide and peptide bonds.

**Summary**

1. Native fibrous collagen was treated at 25\° with aqueous hydrazine solutions ranging in concentration from 5 to 70\% hydrazine (\( \sim 1.5 \) to 19 \( \times \) hydrazine). The insoluble fibrous residues were freed of excess hydrazine, and the hydrazide content was determined. Similar studies were made with limed collagen.

2. Analyses were performed to determine other possible reactions, particularly amide loss, peptide bond hydrolysis, and conversion of arginine to ornithine. The solubility of the chemically modified fibers was also determined.

3. In the low hydrazine concentration range (< 40\%), the amide loss was found to be slightly greater than hydrazide formation in the native collagen. Limed collagen formed bound hydrazide and lost amide nitrogen in exactly equivalent amounts. The bound hydrazide content of the limed collagen was one-half that of the native collagen in this range. At hydrazine concentrations in the range from 40 to 50\%, peptide bonds were cleaved in the native collagen, yielding approximately \( 19 \times 10^{-4} \) equivalent of bound hydrazide. This reaction did not occur with the limed collagen, indicating that these peptide bonds were also alkali-labile. At higher hydrazine concentrations, arginine was converted quantitatively to ornithine. Dissolution of the fibers appeared to accompany the degradination reaction.

4. The lack of collagen dissolution, under conditions in which amide and peptide bond hydrolysis occurs, suggests that the intermolecular cross-linkages in mature collagens are not ester-like in character. The ready removal of amide further suggests that amide loss should be taken into account in assessing the number of ester bonds in denatured collagens and gelsins by hydroxylamine or hydroxylamine reactivity.

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