High Molecular Weight α Chains in Acid-Soluble Collagen and Their Role in Fibrillogenesis*

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ABSTRACT: Denatured acid-soluble collagens from bovine and rat skins contain fractions which do not elute in the salt gradient in the usual carboxymethyl (CM)-cellulose chromatographic systems at 40°. These fractions are eluted with 6.0 M urea. Once isolated, these fractions show enhanced aggregation and renaturation properties typical of the cross-linked collagens. They can be dissociated into their constituent peptide chains by denaturation at 60° and the chains separated by CM-cellulose chromatography at elevated temperature. The chains so prepared have compositions different from the α1 and α2 components and, as demonstrated by disc gel electrophoresis and analytical ultracentrifugation have molecular weights 10–30% higher than α1 and α2. The composition data lead to the suggestion that the urea-eluted fractions represent α chains and polymers containing peptide extensions of noncollagen character directly adjoining the peptide chain backbones. The urea-eluted fractions renature to native fibril form and, in the presence of ATP, form very thin segment long spacing type precipitates showing a marked 300 Å head-to-tail overlap in contrast to the usual segment long spacing single spool precipitates seen in unfractionated acid-soluble collagens. It is proposed that the peptide extensions assist in the alignment and organization of monomeric collagen into the limiting microfibrils characteristic of native collagen fibers.

We have for some time maintained that isolated tissue collagens were considerably more heterogeneous than usually considered. Several lines of evidence are converging which tend to validate this view. At the peptide level, it has been shown that genetically distinct collagens exist in cartilage, skin, and dentin (Miller *et al.*, 1971; Trelstad *et al.*, 1970; Volpin and Veis, 1971a). At the molecular level, heterogeneity arises by the presence or absence of covalent attachments of noncollagenous proteins to the collagen backbone (Carmichael *et al.*, 1971). Finally, biosynthetic studies have suggested that collagen molecules, or at least their constituent α chains, are synthesized with molecular weights greater than that found in the denatured soluble collagens (Layman *et al.*, 1971; Bellamy and Bornstein, 1971; Dehm and Prockop, 1971).

With regard to the latter, Speakman (1971) has hypothesized that triple-helical collagen molecules are assembled from the larger α-chain precursors and that at some later point in the extracellular organization of these molecules into fibrillar form, the "extra" peptide region is proteolytically removed. However, the precise manner or mechanism by which the collagen monomer is incorporated into the fibrillar form has not been fully elucidated. It is this phenomenon with which we are concerned.

Recent crystallographic (Miller and Wray, 1971) and electron microscopic (Bouteille and Pease, 1971; Pease and Bouteille, 1971) studies support a microfibrillar assembly system as first proposed by Veis *et al.* (1967) and Smith (1968). If "extra" peptides are involved in the process of extracellular fibrillogenesis (Speakman, 1971), it should be possible to demonstrate this in a soluble collagen at the level of either the undenatured molecule or of the isolated, denatured subunit chains. In the present work we have taken the approach of making a detailed, quantitative analysis of the homogeneity and compositions of the subunit components from purified denatured acid-soluble bovine skin collagen. The CNBr peptides of the standard α1 and α2 chains from this collagen have already been isolated and their compositions determined (Volpin and Veis, 1971b) so that a standard for comparison of intrinsic heterogeneities is available. The well-characterized rat skin acid-soluble collagen was also used as a basis for comparison.

Materials and Methods

Preparation of Collagen Fractions. All procedures except skinning were performed at 4°.

Acid-Soluble Rat Skin Collagen. Skins from 30-day-old male Wistar rats were shaved with an electric clipper and minced with scissors. Neutral salt-soluble collagen was removed by successive extractions with 0.15 M and 0.45 M NaCl (Gross, 1957) and the acid-soluble collagen obtained by extracting the salt-insoluble residue with three portions of 0.5 M acetic acid (Piez *et al.*, 1960). The combined acid-soluble fractions were pooled and purified by precipitation with 5% NaCl, centrifugation at 13,000*g* for 30 min, and redissolution in 0.5 M acetic acid (Piez *et al.*, 1961). This process was repeated three times. Following final dissolution, the solution was desalted by exhaustive dialysis against distilled water and lyophilized.

Steer Skin Collagens. Fresh skin from a steer approximately 2-years-old was mechanically split so that the corium layer was obtained free of both hair follicles and adipose tissue. Within 30 min after the death of the animal, the corium

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layer was packed in crushed ice and subsequently frozen until needed.

The thawed corium was cut into small cubes and washed alternately with chilled water and 10% NaCl a total of three times to remove soluble protein and polysaccharide contaminants. Since the content of neutral salt-soluble collagen is on the order of <1% (Veis *et al.*, 1960), this fraction was not isolated separately.

The extraction of acid-soluble collagen, without prior removal of the neutral salt-soluble fraction, was carried out essentially according to the procedure of Piez *et al.* (1960). Purification was by the NaCl-precipitation method described above.

CM-cellulose Chromatography. The procedure employed was essentially that of Piez *et al.* (1963), as modified by Veis and Anesey (1965). Weighed samples of lyophilized collagen (~150 mg) were dissolved in ~50 ml of 3% acetic acid at 4° with stirring followed by dialysis overnight against two changes of 0.06 M acetate buffer.

Samples were denatured by incubation at 40° for 30 min, filtered through coarse sintered glass, and immediately charged to the column. (A 0.5-ml aliquot was previously removed for lyophilization to precisely determine the amount of sample introduced.) To ensure against overloading, the column was washed with 0.06 M acetate buffer for several void volumes. Collagen fractions were eluted by imposing the linear salt gradient described by Piez *et al.* (1963) at a flow rate of 180 ml/hr. Fractions were collected in 3-ml portions. After the gradient reached its limiting value, elution was continued at 40° with a solution of 0.06 M acetate buffer made 1.0 M in NaCl and 6.0 M in deionized urea (Veis and Anesey, 1965). Collection was continued until the absorbance at 230 nm fell to the base line.

Homogeneity of the fractions composing a peak was determined by acrylamide gel disc electrophoresis (see below). Using the electrophoretic patterns, the ionic strength and shape of the chromatographic peaks as criteria, fractions corresponding to the major collagen components were pooled, desalted by dialysis against water, and lyophilized. Every fraction containing protein was included in the pooling in order to check on total recovery from the resin. Since recoveries never totalled 100%, fresh CM-cellulose was used for every experiment.

Rechromatography was carried out under identical conditions except that elution of the 6.0 M urea eluate fractions was accomplished at a column temperature of 45° (Tristram *et al.*, 1965) rather than 40°.

Analytical Acrylamide Gel Disc Electrophoresis. The method of Reisfeld *et al.* (1962) as modified by Veis and Anesey (1965) was used. A stock acrylamide solution was prepared from 15 g of acrylamide and 0.6 g of *N,N'*-methylenebisacrylamide diluted to 25 ml with water. This solution was appropriately diluted with water to prepare gels ranging in concentration from 4 to 12%. A 5% gel was routinely employed for the examination of chromatographic fractions.

Samples were prepared by taking aliquots directly from column effluents and estimating concentration spectrophotometrically at 230 nm. Standard collagen solutions assayed by both hydroxyproline and Kjeldahl nitrogen determinations were used for optical density calibration. All samples analyzed contained ~25 μg of protein in up to 500 μl of 3% acetic acid and were denatured by warming at 40° for 10 min immediately before pipeting them onto the gel.

Molecular Weight Estimation. The disc electrophoretic procedure of Hedrick and Smith (1968) was used to estimate the isomer relationships and molecular weights of collagen components present in disc electrophoretograms.

Separator gels of varying pore size were prepared from the stock gel solution. Triplicates of each sample were analyzed simultaneously. The migration of the stained bands from the top of the spacer gel to the midpoint of the band was measured to the nearest 0.5 mm using a clear plastic rule. The results were expressed as absolute migration (R_a).

Hedrik and Smith (1968) found an empirical relationship between log mobility and gel concentration: proteins having different molecular sizes, but similar charges (size isomers) could be identified by nonparallel lines intersecting in the vicinity of 0% gel concentration; proteins having identical molecular sizes but different net charges (charge isomers) were characterized by parallel lines. In addition, the slopes of such lines were directly related to the molecular weights of the components (Hedrick and Smith, 1968).

To determine the isomeric relationship among collagen components, values of 100 log R_a for each component were plotted on the ordinate *vs.* gel concentration in per cent on the abscissa. At least five different gel concentrations were used for each determination. Least-squares lines were computed and the slopes and intercepts were determined.

To prepare a standard curve for molecular weight determinations, samples of α1, α2, β_{11}, β_{12}, and γ were treated as above. The molecular weights of α, β, and γ were taken as unit multiples, 10^5, 2×10^5, and 3×10^5, respectively.

Amino Acid Analyses. The total amino acid composition of the collagenous samples was determined on a JEOLCO (Japan Electron Optics Laboratory Co., Medford, Mass.) automatic analyzer employing a two-column accelerated system similar to that of Spackman (1967).

Samples of lyophilized material were hydrolyzed in 2 ml of triple-distilled HCl under nitrogen at 110° for 22 hr. For analysis of the components in the various chromatographic fractions, representative tubes were pooled using the criterion of disc electrophoresis so that relatively homogeneous subunits were analyzed where possible.

Calculations of amino acid composition did not involve corrections for the possible partial destruction or incomplete release of individual residues due to hydrolysis conditions since recent evidence (Lazlo and Olsen, 1969) showed such corrections to be near unity under conditions similar to these.

Ultracentrifuge Analyses. Sedimentation velocity measurements on denatured unfractionated collagen and on the isolated fractions were obtained using the conditions of Veis and Anesey (1965). Corrected sedimentation coefficients ($s^0_{20,w}$) were calculated by extrapolation of plots of ($1/s_{20,w}$) *vs.* concentration to zero concentration.

Electron Microscopy of Renatured Fraction. Electron microscopy was performed using a Hitachi HU-11A electron microscope. Samples of native collagen or the denatured subunit fractions in dilute solution (≤0.1%) were dialyzed exhaustively against 0.05% (v/v) acetic acid at 4°. Subsequent dialysis against a salt-free 0.4% solution of ATP in 0.05% acetic acid (pH 2.8–3.0) resulted in the precipitation of native or renatured collagen (Kühn *et al.*, 1966). The precipitates were stained either positively or negatively as described by Veis *et al.* (1970). Renaturation followed by pepsin digestion was performed as detailed previously (Veis *et al.*, 1970).

Results

Chromatographic Fractionation. Figure 1 shows the CM-cellulose chromatographic fractionation of bovine acid-

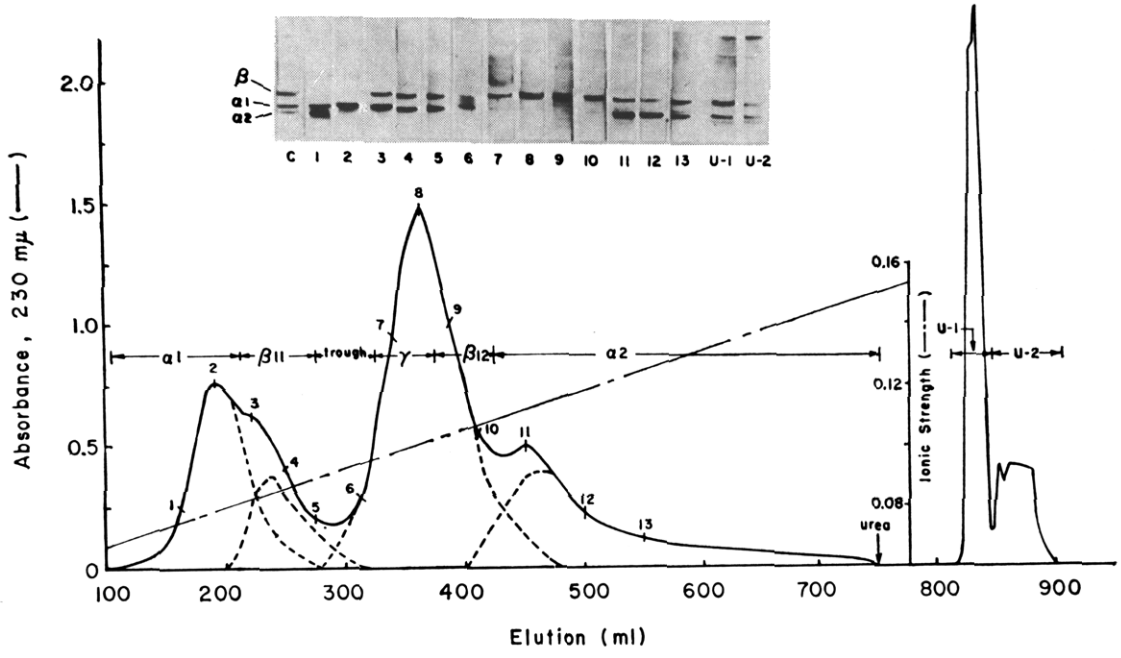

FIGURE 1: CM-cellulose chromatography of bovine acid-soluble collagen at 40°. The fractions corresponding to the numbered regions along the chromatograph were analyzed by acrylamide gel electrophoresis using the 5% gel system. The gel electrophoresis results are shown in the correspondingly numbered insets. The gel labeled C is the unfractionated acid-soluble collagen.

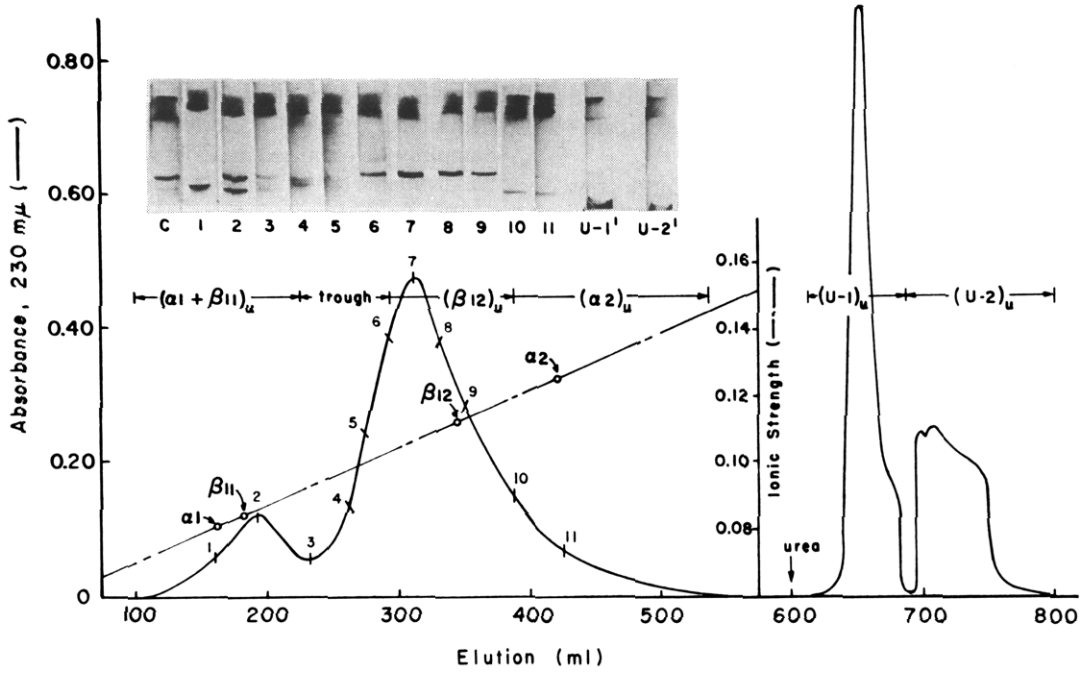

FIGURE 2: CM-cellulose rechromatography of the combined urea-eluted fractions (U-1 + U-2) from chromatography as in Figure 1. All conditions were the same as in Figure 1, except that U was denatured at 60° before chromatography and the column temperature was 45°. The gel electrophoresis was carried out with the 5% gel system.

soluble collagen. The components $\alpha 1$, β_{11}, β_{12}, and $\alpha 2$ are indicated in the salt gradient range. The two peaks, U-1 and U-2, are produced upon elution with 6.0 M urea solution with U-1 emerging at the column void volume. The numbers along the optical density plot in Figure 1 represent the locations where samples were taken for the electrophoretic analysis in the 5% polyacrylamide gel system, shown in the inset. Both optical density and direct total recovery measurements on a number of chromatograms showed that the components isolated in the linear gradient region represent only 56% of the collagen applied to the column. U-1 and U-2 comprise 13%, leaving 31% retained on the column. In contrast, control experiments with young rat skin acid-soluble collagens gave total recoveries on the order of 90%. However, even with this system, the U-1 and U-2 components comprised 10% of the collagen chromatographed.

The material in each of the regions designated by the bars in Figure 1, clearly heterogeneous in the gel electrophoretic

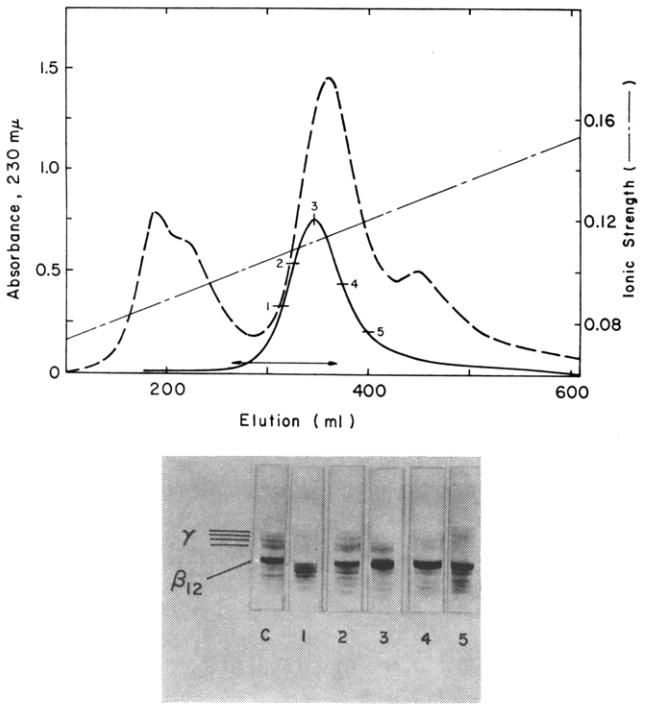

FIGURE 3: CM-cellulose rechromatography of the trough-region components obtained by chromatography as in Figure 1. Chromatography at 40°. The dashed line in A is the original chromatogram. The arrow indicates the region pooled for rechromatography and the solid line the new chromatograph. The appropriate gel electrophoretograms are shown in B.

analyses, was rechromatographed. The most homogeneous rechromatographed fractions of $\alpha 1$, β_{11}, γ, β_{12}, and $\alpha 2$ were taken for amino acid analysis and were used as the standards for molecular weight calibration in the gel electrophoretic technique of Hedrick and Smith (1968). Similar fractions were also isolated from rat skin acid-soluble collagen for independent molecular weight calibration standards.

In terms of the examination of the heterogeneity of the collagen, two areas appeared to be of particular interest—the region designated as the "trough" in Figure 1 on the leading edge of the β_{12} peak and the urea-eluted fractions.

Compared with the subunits eluted in the salt gradient range, U-1 and U-2 showed enhanced aggregation properties. Upon dialysis of U-1 or U-2 against H_2O at 4° to remove the urea, precipitates usually developed. These could be dispersed in 3% acetic acid but the suspensions were unstable. Subsequent dialysis of the acetic acid suspensions against the starting 0.06 M acetate, pH 4.8, chromatography buffer at 4° again resulted in precipitation. The precipitate would not dissolve completely in the chromatography buffer, even at 40°. However, by heating the pH 4.8 suspension of collagen to 60° for 15 min (Tanzer, 1967), the denatured collagen did dissolve. The 60° solubilized components did not behave in the fashion of usual collagen subunit chains in that reaggregation was evident at 40° and yields from CM-cellulose chromatographic analyses carried out at 40° were nearly zero in the salt gradient range. Elution of these chromatographic columns with 6.0 M urea again yielded the U-1 and U-2 fractions.

Tristram *et al.* (1965) had reported that an "aggregate" fraction from soluble bovine collagen could be disaggregated by heating at pH 4.8 for 15 min at 60° and that this fraction could be kept disaggregated at temperatures of 43° and above.

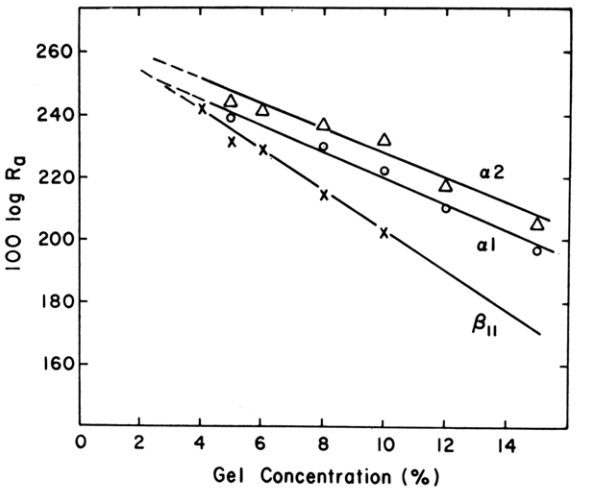

FIGURE 4: A plot of log absolute migration distance *vs.* gel concentration for isolated $\alpha 1$, $\alpha 2$, and β_{11} components from bovine acid-soluble collagen; $\alpha 1$ and $\alpha 2$ represent charge isomers, $\alpha 1$ and β_{11} size isomers.

The same behavior was evident for U-1 and U-2 and the rechromatography of the total urea eluate fraction (U = U-1 + U-2) was carried out at 45° with the usual salt gradient and urea elution conditions. The chromatographic results are shown in Figure 2, along with the corresponding disc electrophoretograms. Slightly over half the total U was eluted in the salt gradient, a small amount was still present as components requiring 6 M urea for elution and almost 30% could not be recovered by these procedures.

The salt gradient eluted fractions, as seen in Figure 2, appear similar to the α and β components obtained in the original salt gradient elution chromatogram. This similarity led to the conclusion that only weak urea or heat-labile linkages holding the aggregates together were being broken. An alternative approach would be to consider that the α-chain peptide backbones themselves were being hydrolyzed by the 60° treatment and that the fragments, held together by cross-linkages, were coincidentally eluting chromatographically, and migrating in gel electrophoresis, at positions similar to the standard α and β components. This is a very

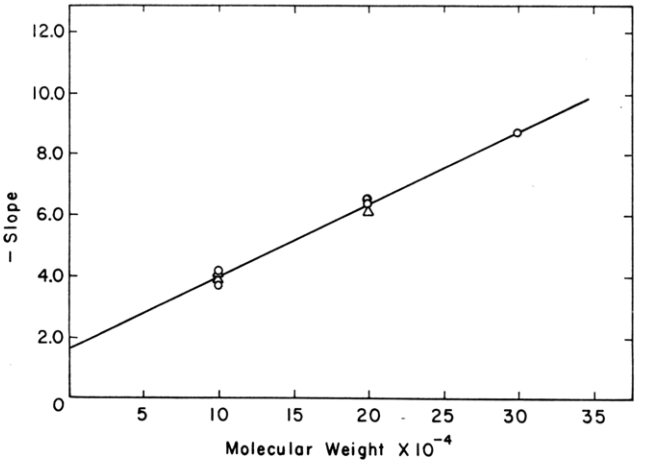

FIGURE 5: Calibration plot of molecular weight *vs.* slope from log absolute migration *vs.* gel concentration plots as in Figure 4. (○) Bovine acid-soluble $\alpha 1$, $\alpha 2$, β_{11}, β_{12}, and γ_{112}; (△) rat acid-soluble collagen $\alpha 1$, $\alpha 2$, β_{12}.

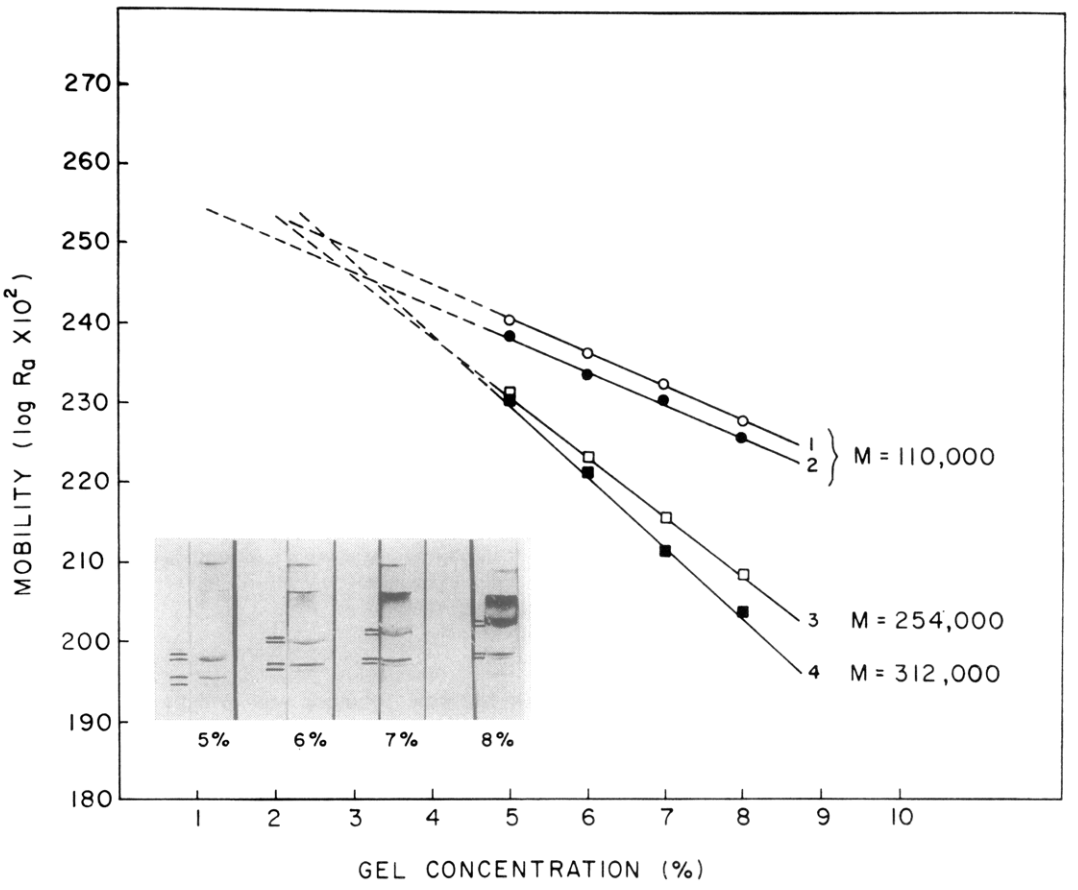

FIGURE 6: Mobility *vs.* gel concentration plots for the U-1 peak components of bovine acid-soluble collagen. The molecular weights estimated from the calibration line (Figure 5) are shown directly on the figure, as are the gel electrophoresis data from which computations were made. The plots are numbered corresponding to the migration distance, band 1 is the most rapidly moving band, band 4, the least.

unlikely situation. Tanzer (1967), for example, has reported that β and γ components stabilized by *intramolecular* reduction with borohydride do not show the appearance of any new components or α-chain degradation products following the 60°, 15 min, pH 4.8 treatment. Tristram *et al.* (1965) also found no evidence for α-chain peptide backbone degradation under these conditions. Three types of control studies further demonstrate that the disaggregation upon brief heating at 60° is reversible and is accomplished without peptide chain rupture. First, disc electrophoretic analyses of soluble rat skin collagen, in which the majority of the collagen is present as α- and β-chain subunits, comparing the usual 40° denatured collagen with collagen heated to 60°, shows no evidence for α-chain degradation. Second, exclusion chromatography of rat skin isolated β and γ components, carried out on Agarose 1.5 columns by the method of Piez (1968), and in direct comparison of 40°- and 60°-treated collagen samples, showed no evidence of peptide backbone degradation. Finally, disc gel electrophoretic studies of the total U fraction from bovine skin collagen carried out with 6 M urea in the gels showed that the effect of urea was to reversibly dissociate U into α- and β-like subunits.

Rechromatography of the trough and γ regions (gels 6 and 7 of Figure 1) from the initial salt gradient elution did not resolve the complexity in heterogeneity (Figure 3 A,B) but demonstrated that a number of minor components migrating in the regions between the α and β positions, as well as a multiplicity of γ components, were present.

Gel Electrophoretic Analysis of Molecular Weight. Analytical disc gel electrophoresis of U-1 and U-2 (Figure 2) indicated that the urea-eluted components after denaturation at 60° contained α and β components migrating quite closely to the standard α and β components in the 5% acrylamide gel system. However, sedimentation velocity studies on U showed (Table I) that the sedimentation coefficients of the urea-eluted components were uniformly higher than the comparable standard salt gradient eluted components. Due to the small amounts of isolated U-1 and U-2 and the necessity to retain as much as possible for chemical analyses, we resorted to the method of Hedrick and Smith (1968) for molecular weight determinations. A plot of log absolute migration *vs.* gel concentration (Figure 4) shows the expected results for charge isomers $(\alpha 1)_s$ and $(\alpha 2)_s$[1] and size

TABLE I: Sedimentation Coefficients of Bovine Collagen Subunits.

	$s^0_{20,w}$ (S)	
	α	β
"Standard" acid-soluble collagen subunit	2.81 ± 0.04	3.86 ± 0.07
U-1–U-2 mixture	3.26 ± 0.13	4.06 ± 0.06

[1] For convenience in designating component source, we shall subsequently use subscript s for the standard salt gradient eluted com-

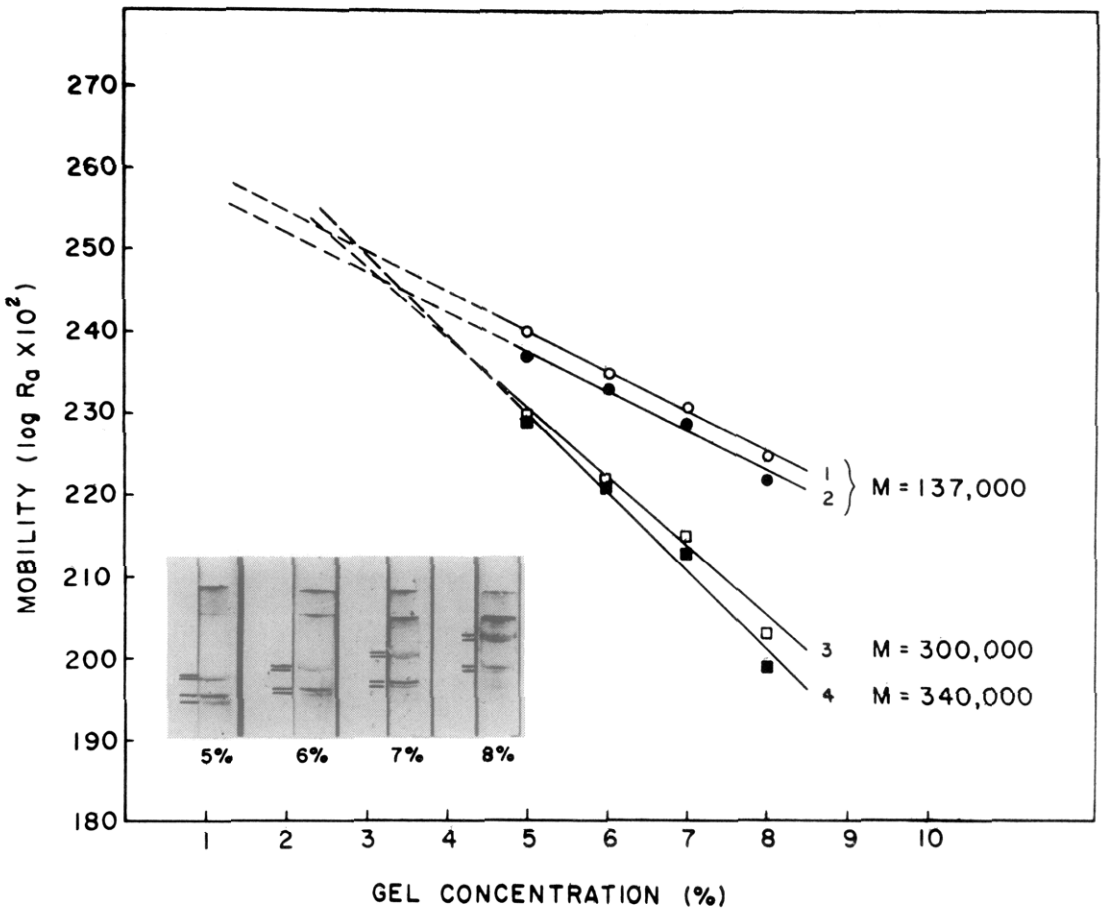

FIGURE 7: Mobility *vs.* gel concentration plots for the U-2 peak components of bovine acid-soluble collagen. The data are presented as in Figure 6.

isomers $(\alpha 1)_s$ and $(\beta_{11})_s$. The slopes of the lines are then plotted (Figure 5) against molecular weight to yield a straight line giving a calibration plot for molecular weight and experimentally determined slope. Figure 5 includes data for the isolated, rechromatographed components from both rat skin and bovine skin. The slopes, as deduced from least squares, are reproducible from run to run to ± 0.1 in the units utilized. The sodium dodecyl sulfate gel technique was not used for this analysis because it has recently been shown (Tung and Knight, 1971) that electrophoretic mobilities in sodium dodecyl sulfate can reflect charge differences. Indeed, in contrast to the present method which yields identical weights for $(\alpha 1)_s$ and $(\alpha 2)_s$, the sodium dodecyl sulfate technique shows different weights for these components (Sykes and Bailey, 1971).

The mobility data for U-1 and U-2 components are shown in Figures 6 and 7. Due to the very marked reduction in mobility for the higher weight components (labeled 3 and 4 in each figure) at the higher gel concentrations and the fact that the apparent weights exceed 300,000 for component 4, these data are less reliable than those for the components with weights less than 300,000. However, there is no uncertainty in the values for components 1 and 2, in each case. In agreement with the sedimentation coefficient data, both the U-1 and U-2 $(\alpha)_u$ components have molecular weights higher than the corresponding $(\alpha)_s$ components. In the 5% gel system, bands 1 and 2 (Figure 6), respectively, correspond to $(\alpha 2)_u$ and $(\alpha 1)_u$, which are isolated upon rechromatography of U (Figure 2). Similar analyses of the urea-eluted components from rat skin acid-soluble collagen show the $(\alpha)_u$ to have molecular weights of 114,000 and the $(\beta)_u$ molecular weights of 218,000 and 239,000. The appearance of the urea-eluted components, with molecular weights 10–20% elevated above that of the salt-eluted components is a general phenomena, not restricted to the bovine collagen system.

Amino Acid Compositions of Rechromatographed Fractions. The amino acid compositions of $(\alpha 1)_s$, $(\alpha 1)_u$, $(\alpha 2)_s$, and $(\alpha 2)_u$ are listed in Table II. The fractions were taken for analysis from the appropriately labeled regions indicated in Figures 1 and 2. The compositions of $(\alpha 1)_s$ and $(\alpha 2)_s$ are in excellent correspondence with the total of CNBr peptides from these fractions (Volpin and Veis, 1971b) when computed on the same molecular weight basis and converted to residues per 1000 total residues. On the other hand, $(\alpha 1)_u$ and $(\alpha 2)_u$ have relatively lower contents of hydroxyproline. In $(\alpha 1)_u$ proline is also reduced. Equally important, in comparing $(\alpha 2)_s$ and $(\alpha 2)_u$, the sum of proline and hydroxyproline is less in $(\alpha 2)_u$, indicating that the reduction in hydroxyproline is not the result of the underhydroxylation of an $\alpha 2$ chain. Both $(\alpha 1)_u$ and $(\alpha 2)_u$ are also markedly lower in methionine. This may be due to formation of unidentified methionine oxidation products (Keutmann and Potts, 1969). However, Volpin and Veis (1971a) have observed a collagen fraction with the same low methionine content in total CNBr digests of insoluble bovine skin collagen. This fraction resists CNBr cleavage in

ponents, *e.g.*, $(\beta_{11})_s$, and the subscript u for the urea-eluted fractions, *e.g.*, $(\alpha 1)_u$.

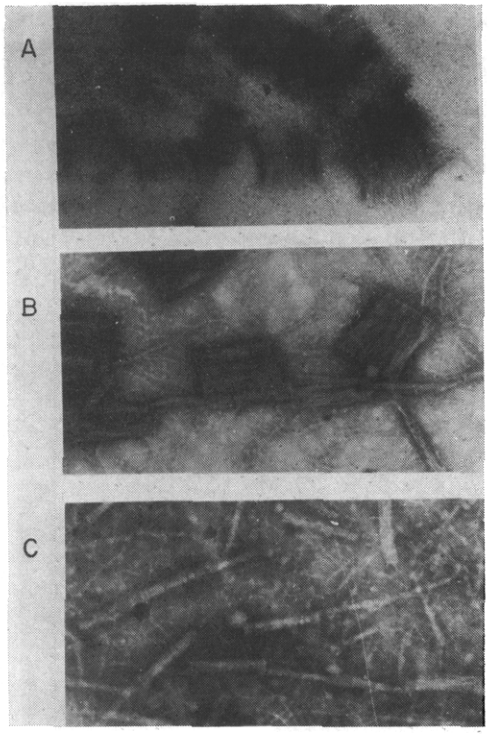

FIGURE 8: Electron micrographs of various ATP, segment long spacing type precipitates of the collagen fractions. (A) Unfractionated bovine acid soluble collagen, positive stain with phosphotungstic acid (pH 2.3). (B) Unfractionated bovine acid-soluble collagen, negative stain with phosphotungstate (pH 7.0). (C) Renatured combined (U-1 + U-2) fraction, negatively stained with phosphotungstate (pH 7.0). All conditions for preparation of C identical with conditions for B.

70% formic acid at 25° and is insoluble in the acid CNBr digestion mixture. Thus, the CNBr peptide analysis of the urea eluate fraction is not an entirely appropriate way to attack the structure of the U components and may be the reason that such components have heretofore been missed, neglected, or thought to be nonexistent. Both $(\alpha 1)_u$ and $(\alpha 2)_u$ contain more tyrosine and histidine than their counterpart $(\alpha)_s$ chains and show other small compositional differences.

Electron Microscopic Investigations of Renatured Urea Eluate. To examine the question of organization of the subunits within the urea eluate and their heightened aggregative ability, segment long spacing aggregates were prepared by renaturing the unfractionated urea eluate components and then dialyzing salt-free solutions against ATP. This particular mode of aggregation is a very sensitive means of detecting intact monomeric and polymeric collagens (Hodge and Schmitt, 1960). As a control, unfractionated bovine skin acid-soluble collagen was carried through the same set of renaturation and precipitation operations. The unfractionated acid-soluble collagen formed copious segment long spacing precipitates, whereas the urea eluate components formed thin, threadlike structures with only a small amount of precipitate.

Electron micrographs, Figure 8, show the distinctly different behavior of the two preparations. The acid-soluble collagen forms typical segment long spacing spools which approach 1000 Å in width, indicating the presence of many collagen monomers in side-by-side register. Negative staining, Figure 8B, reveals that the predominant segment long spacing spools lie in a matrix of very fine fibrils along which some monomer units are aligned in such a way that there are A–B overlaps of about 300 Å. These fibrils, which are a minor element in the unfractionated acid-soluble collagen, are the major constituents of the renatured urea eluate fraction. There are very few monomers (Figure 8C). Because of the thinness of the filaments (widths <300 Å), positive staining did not reveal these structures well. Kühn *et al.* (1966), Zimmerman *et al.* (1970), and Veis *et al.* (1970) have noted similar head-to-tail or A–B overlap aggregations in polymeric collagens. The absence of prominent individual segment long spacing spools is further confirmation of the asymmetrically linked, polymeric nature of the urea eluate components.

Figure 8C shows that the segment long spacing polymeric fibrils are surrounded by apparently unorganized material, probably resulting from incompletely renatured chains. When renatured collagen chains are treated with pepsin (Kühn and Zimmerman, 1966), any nonrenatured molecules or regions of molecules are digested and the resulting ATP precipitate is much cleaner. Positive staining of such preparations (Figure 9) shows the retention of very prominent end-overlap regions and suggests that renaturation begins in these regions. Since renaturation begins most readily in the region of covalent interchain cross-linkages (Drake and Veis, 1964; Harrington and Rao, 1970), the end-overlap regions must be one locus of intermolecular cross-linkages, as well as the site of microfibrillogenesis.

TABLE II: Bovine Corium Collagen Subunit Compositions. Comparison to Rechromatogramed Urea Eluate.[a]

	Orig Acid-Soluble	$(\alpha 1)_s$	$(\alpha 1)_u$	$(\alpha 2)_s$	$(\alpha 2)_u$
Hydroxyproline	88	92	79	88	71
Aspartic acid	48	42	42	49	50
Threonine	17.3	16.0	17.4	18.0	18.4
Serine	33	34	39	35	40
Proline	133	138	128	117	119
Glutamic acid	76	76	77	74	75
Glycine	329	319	337	328	311
Alanine	106	126	130	98	124
Valine	22.4	17.0	13.9	31	21.5
Methionine	5.0	6.0	2.3	3.7	2.4
Isoleucine	12.5	8.8	8.7	16.3	14.7
Leucine	26	21	22	32	36
Tyrosine	3.8	2.7	5.8	2.3	4.0
Phenylalanine	13.3	12.7	12.2	13.7	18.0
Hydroxylysine	6.4	5.3	5.8	8.8	5.9
Lysine	26	30	31	23	31
Histidine	5.2	3.0	5.2	6.2	8.1
Arginine	48	50	46	55	50

[a] Residues per 1000 total amino acid residues.

Discussion

Three principal points may be made from the data presented above. First, soluble collagens contain small, but significant (~10%) amounts of collagenous components not generally recovered in CM-cellulose chromatography by the usual procedures. This fraction exhibits heightened aggregative properties, readily renaturing and forming native-type collagen fibrils. The electron microscopic evidence suggests

that this fraction contains intermolecular covalent bonds and that these bonds are within a 300 Å head-to-tail overlap region. Second, the soluble intermolecularly cross-linked fraction has a significantly different composition from that of the predominantly intramolecularly cross-linked fractions eluted from CM-cellulose chromatography in the linear salt gradient range. The soluble intermolecularly cross-linked fraction in mature bovine collagen is reduced in both hydroxyproline and methionine and enriched in tyrosine and histidine. This is indicative of the inclusion of a protein or polypeptide moiety in close association with the intermolecularly linked soluble collagen. Finally, rechromatography of the dissociated urea eluate fraction showed the presence of components corresponding to the standard α chains of the acid-soluble collagen. However, the isolated $(\alpha)_u$ chains are of higher molecular weight than the usual $(\alpha)_s$ and, as shown in Table II, reflect the differences in composition noted above, that is, lower methionine and hydroxyproline and elevated tyrosine and histidine. Thus, the extra-collagenous material is associated directly with the individual collagen α chains. Since these components chromatograph and electrophorese as essentially homogenous molecules in the denatured state, it can be concluded that the "extracollagenous" portions are covalently linked to the α chains. The presence of α chains with covalent polypeptide extensions in denatured collagen preparations presupposes the existence *in vivo* of a population of intact extracellular collagen molecules with molecular weights greater than 300,000 as well as the larger set with molecular weight equal to 300,000.

Current investigations on collagen biosynthesis (Dehm and Prokop, 1971; Bellamy and Bornstein, 1971) are leading to the conclusion that the α chain precursors of the collagen molecules are synthesized with molecular weights greater than $(\alpha)_s$. In these studies the high molecular weight α chains have been detected in minute amounts in soluble collagen from newborn rat calvaria as radioactively labeled peaks eluting in the CM-cellulose system ahead of $(\alpha 1)_s$ [and hence designated pre-α1] (Bellamy and Bornstein, 1971) or from cell cultures as a highly insoluble fraction requiring drastic treatment for solubilization (Dehm and Prokop, 1971). Speakman's hypothesis (1971) proposes that such peptide extensions register the α chains for triple-helix formation, stabilizing the formed molecules during transport and are finally cleaved proteolytically upon fibril formation. Bellamy and Bornstein (1971) support these ideas and pose the additional thought that the attachments serve to inhibit intracellular fibrogenesis.

The relationship between the components observed by Bellamy and Bornstein (1971) and those presented here is unclear. The $(\alpha)_u$ components and their polymers are present in larger amounts than the pre-α components, are detectable upon chromatography by the usual absorbance measurements at positions not corresponding to the pre-α1 peak, and are isolated from mature tissues not undergoing rapid growth or turnover. Thus, if the $(\alpha)_u$ and their polymers contain extracollagenous peptide extensions as continuations of the α-chain peptide backbones, one must conclude that all of the peptide attachments to $(\alpha)_s$ are not removed by proteolysis upon fibril formation. The alternative to this is to consider that the peptide attachments in $(\alpha)_u$ are attachments added to the α chains at some later point in the sequence of events in fibrillogenesis. However, studies with [^{14}C]glycine pulse-labeled rat calvaria collagen and *in vivo* [^{3}H]proline pulse-labeled rat skin collagen (A. Veis, J. Anesey, and J. E. Arwin, in preparation) indicate that the U fractions are highly labeled and suggest that the $(\alpha)_u$ attachments are direct extensions of the peptide backbone.

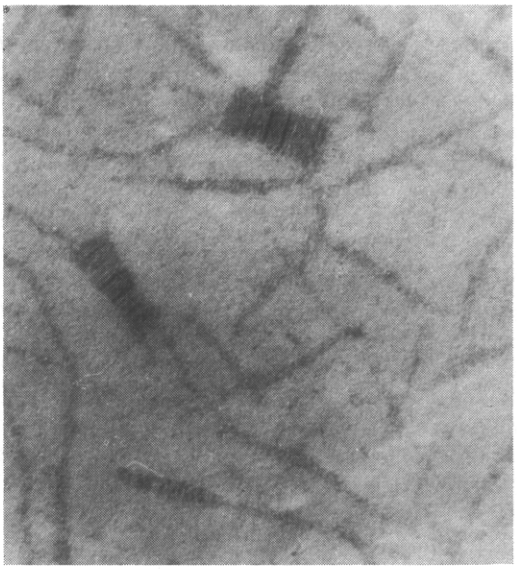

FIGURE 9: Combined (U-1 + U-2), denatured in 0.25 M citrate buffer, then renatured at 25°, pH 3.7. Digested with pepsin (10:1, collagen–pepsin) 18 hr, 25°. Fibrils precipitated by dialysis against H_2O, stained with phosphotungstate (pH 2.5.). The end-overlap regions are evident as the most stable, pepsin resistant aggregation areas.

The concept of a "registration" peptide is equally as valid in the mechanism of fibrillogenesis as it is in considering the initial collagen triple-helix formation. Veis (1967) and Veis *et al.* (1967) have proposed that collagen fibers are built from smaller microfibrils of 30–50 Å diameter. The 700-Å axial shifting of adjacent molecules is established within these limiting microfibrils as is the most fundamental set of intermolecular cross-linkages (Veis *et al.*, 1970). The microfibril model has been modified by Smith (1968) and essentially substantiated in the recent work of Veis *et al.* (1970), Miller and Wray (1971), Bouteille and Pease (1971), and Pease and Bouteille (1971). According to this model, fibrillogenesis is at least a two-step process: (1) the formation of unit assemblies in precise 700-Å staggering and localized in the limiting microfibrils; and (2) the aggregation of the microfibrils into fibrils with an overall matching of 700-Å periodicity but no specific microfibril-to-microfibril shift (Veis *et al.*, 1970). The first step would appear to require some very specific localization of interactions to form the microfibrils with proper registration and to limit the side-by-side accretion of molecules to form large diameter nonmicrofibrillar fibers typical of reprecipitated soluble monomer collagen (see review by Cox and Grant, 1969).

The electron micrographs of renatured ATP precipitated U in Figure 8C and pepsin-treated U in Figure 9 emphasize the prevalence of head-to-tail junctions between adjacent molecules in contrast to the lack of such overlaps in reprecipitated native acid-soluble collagen which has only a small content of U components. From these observations plus similar electron micrographs from polymeric dispersed microfibrils (see Figure 6, Veis *et al.*, 1970), it is likely that the fundamental limiting microfibrillar arrangement is set by that proportion of molecules bearing the $(\alpha)_u$ attachments and that these are bound at, or direct intermolecular binding at, the head-to-tail overlap region. In this model the unit assembly element would be the end-overlapped dimer.

The U fractions represent a portion of the fiber system which is trapped on the CM-cellulose columns as high molecular weight, asymmetric aggregates. The bonds joining the molecules into aggregates are evidently reversible labile bonds, disrupted by urea and/or elevated temperatures. From the chromatographic properties of borohydride-reduced native collagen (Tanzer, 1968) and the lability of certain collagen cross-links (Bailey, 1968; Bailey and Lister, 1968), it can be inferred that the labile bonds of the fraction of collagen yielding U are of the Schiff's base type. The end-overlap intermolecular binding implies that the α1-CB6 peptide would be involved in the stabilized end-overlap region. The presence of the extension peptide in this region could account for the marked loss of the α1-CB6 peptide in CNBr digests of cross-linked collagen and the heterogeneity noted in the composition of this peptide (Volpin and Veis, 1971a; Stark *et al.*, 1971; Rauterberg and Kühn, 1968).

References

Bailey, A. J. (1968), *Biochim. Biophys. Acta 160*, 447.

Bailey, A. J., and Lister, D. (1968), *Nature (London) 220*, 280.

Bellamy, G., and Bornstein, P. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1138.

Bouteille, M., and Pease, D. C. (1971), *J. Ultrastruct. Res. 35*, 314.

Carmichael, D. J., Veis, A., and Wang, E. T. (1971), *Calcif. Tissue Res. 7*, 331.

Cox, R. W., and Grant, R. A. (1969), *Clin. Orthop. 67*, 172.

Dehm, P., and Prockop, D. J. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1196.

Drake, M. P., and Veis, A. (1964), *Biochemistry 3*, 135.

Gross, J. (1957), *in* Connective Tissue, Turnbridge, R. E., Ed., Oxford, Blackwell, p 61.

Harrington, W. F., and Rao, N. V. (1970), *Biochemistry 9*, 3714.

Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.

Hodge, A. J., and Schmitt, F. O. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 186.

Keutmann, H. T., and Potts, J. T., Jr. (1969), *Anal. Biochem. 29*, 175.

Kühn, K., Fietzek, P., and Kuhn, J. (1966), *Biochem. Z. 344*, 418.

Kühn, K., and Zimmerman, B. (1966), *Biochem. Z. 344*, 123.

Laszlo, F., and Olsen, B. R. (1969), *Eur. J. Biochem. 11*, 140.

Layman, D. L., McGoodwin, E. B., and Martin, G. R. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 454.

Miller, A., and Wray, J. S. (1971), *Nature (London) 230*, 437.

Miller, E. J., Epstein, E. H., Piez, K. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 1024.

Pease, D. C., and Bouteille, M. (1971), *J. Ultrastruct. Res. 35*, 339.

Piez, K. A. (1968), *Anal. Biochem. 26*, 305.

Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2*, 58.

Piez, K. A., Lewis, M. S., Martin, G. R., and Gross, J. (1961), *Biochim. Biophys. Acta 53*, 596.

Piez, K. A., Weiss, E., and Lewis, M. S. (1960), *J. Biol. Chem. 235*, 1987.

Rauterberg, J., and Kühn, K. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 230.

Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.

Smith, J. W. (1968), *Nature (London) 219*, 157.

Spackman, D. H. (1967), *Methods Enzymol. 11*, 3.

Speakman, P. T. (1971), *Nature (London) 229*, 241.

Stark, M., Rauterberg, J., and Kühn, K. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 101.

Sykes, B. C., and Bailey, A. J. (1971), *Biochem. Biophys. Res. Commun. 43*, 340.

Tanzer, M. L. (1967), *Biochim. Biophys. Acta 133*, 584.

Tanzer, M. L. (1968), *J. Biol. Chem. 243*, 4045.

Trelstad, R. L., Kang, A. H., Igarashi, S., and Gross, J. (1970), *Biochemistry 9*, 4993.

Tristram, G. R., Worrall, J., and Steer, D. C. (1965), *Biochem. J. 95*, 350.

Tung, J.-S., and Knight, C. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 1117.

Veis, A. (1967), *in* Treatise on Collagen, Vol. 1, Ramachandran, G. N., Ed., London, Academic Press, p 367.

Veis, A., and Anesey, J. (1965), *J. Biol. Chem. 240*, 3899.

Veis, A., Anesey, J., and Cohen, J. (1960), *J. Amer. Leather Chem. Ass. 55*, 548.

Veis, A., Anesey, J., and Mussel, S. (1967), *Nature (London) 215*, 931.

Veis, A., Bhatnagar, R. S., Shuttleworth, C. A., and Mussell, S. (1970), *Biochim. Biophys. Acta 200*, 97.

Volpin, D., and Veis, A. (1971a), *Biochem. Biophys. Res. Commun. 44*, 804.

Volpin, D., and Veis, A. (1971b), *Biochemistry 10*, 1751.

Zimmerman, B. K., Pikkarainen, J., Fietzek, P. P., and Kühn, K. (1970), *Eur. J. Biochem. 16*, 217.