Preparation of Intact Monomeric Collagen from Rat Tail Tendon and Skin and the Structure of the Nonhelical Ends in Solution

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Procedures for the preparation of soluble collagen from rat skin and tail tendon were reviewed and revised to permit the preparation of native monomeric collagen with intact nonhelical ends. The degree of intactness was estimated from the tyrosine content, which is present only in the nonhelical ends, and by mobility of the COOH-terminal cyanogen bromide peptide of the $\alpha_1$ chain on sodium dodecyl sulfate gels. The amount of covalently cross-linked polymeric material present was estimated by molecular sieve chromatography of denatured samples. Rapid purification in the cold was sufficient to prevent or greatly reduce proteolytic alteration. Fractionation by salt precipitation at acid pH was effective in reducing the content of polymeric material. Rat tail tendon yielded completely intact native collagen, but some high molecular weight aggregates remained. Collagen from the skin of lathyritic rats was easier to obtain free of aggregates, but contained about 1 less tyrosine residue per $\alpha_1$ chain even when isolated in the presence of enzyme inhibitors.

Proton NMR spectra of denatured acidic solutions of these preparations showed that 4 to 5 tyrosine residues per $\alpha_1$ chain were present, confirming the chemical analysis. Spectra of the native molecule showed that about the same number of tyrosine residues per chain are in rapid motion, unlike residues in the helical portion of the molecule, a result which shows that the nonhelical ends of the native molecule are unstructured in acidic solution.

It has been known for a long time that although collagen is normally insoluble because of extensive covalent cross-linking, it can be brought into solution without denaturation by cold neutral salt or low ionic strength acidic solutions from some vertebrate tissues.\(^1\) The early literature has been reviewed (1). Solution occurs because of one or a combination of several factors. (a) The collagen is newly synthesized and not yet extensively cross-linked. (b) Intermolecular aldimine cross-links that are readily opened at acid pH predominate. (c) A lathyritic agent was used which either inhibits aldehyde formation or combines with cross-link precursors and inhibits cross-linking. Rat tail tendon is a good source of collagen primarily because of factor b. Skin from young rats and other mammals is a good source primarily because of factor a and is an even better source if the animals are fed $\beta$-aminopropionitrile because of factor c. Collagen cross-linking has been most recently reviewed by Gallop and Paz (2).

However, soluble collagen preparations made by a variety of procedures suffer from two defects. First, polymeric material consisting of covalently cross-linked aggregates of native collagen molecules is always present because cross-linking cannot be completely prevented or reversed, and the swelling solvents used to extract collagen are often effective in dispersing quite large polymeric material. In fact, in most cases the “solubility” of a given collagen preparation has only an ill defined operational meaning, and it may be difficult to prepare collagen samples with reproducible properties by a given method.

The second problem is that the short nonhelical ends of the three $\alpha$ chains in the collagen molecule are subject to proteolysis during isolation. The NH$_2$-terminal region of chick skin collagen is particularly susceptible (3), but mammalian collagens also may be altered in this region. The first reported sequence at the NH$_2$-terminal end of the $\alpha_1$ chain from rat skin collagen was later found to lack the first 4 residues (4). The COOH-terminal nonhelical region is even more susceptible to proteases and it was not until denaturing solvents were used to prepare $\alpha$ chains that the existence of this region was recognized (5, 6).

In spite of these problems the chemical and structural characterization of the native collagen molecule is well advanced (7). However, in vitro studies on the interactions between collagen molecules during fibril formation and between collagen and other constituents in connective tissue may depend critically on the nature of the collagen used. The availability of intact native collagen monomer is therefore important. For this reason, we have revised standard proce-
nearly so, and on devising a method to reduce the content of tail tendon with particular emphasis on demonstrating that the COOH-terminal nonhelical ends can be kept intact or can be kept intact or, by a remittance to the order of the Journal in the amount of $1.00 per set of photocopies.

**MATERIALS AND METHODS**

**Molecular Sieve Chromatography**—Denatured collagen was analysed for its content of a chains and a chain dimers, trimers, and higher polymers by chromatography in 0.05 M CaCl₂, 0.05 M Na₂SO₄, pH 7.5, at room temperature on a column (2.3 x 120 cm) of 6% agarose. The amount of polymeric material present was estimated by partial saturates the resonances at 46°, since the measured T₁ values were obtained for all-CB₆C₁₅, the 21-residue COOH-terminal peptide, at D₂O at room temperature, lyophilized to remove exchangeable protons, and then redissolved in the same solvent. About 0.7 ml was fractions precipitating at or below 3.0% NaCl were too small to be found to be less than 1 s in denatured samples. The 3-s pulse repetition time used at high collagen concentrations (about 40 mg/ml) insures negligible saturation of spectral intensity. Therefore, relative intensities, divided by the number of magnetically equivalent protons, are proportional to the relative number of residues in denatured samples. The approximate maximal error for a single analysis of tyrosine was about +15% under these conditions. The 0.8-s pulse repetition time required to obtain spectra in a reasonable time when starting with solutions of native collagen because of the lower concentrations available (about 4 mg/ml) partially saturates the resonances at 46°, since the measured T₁ values of the aromatic and aliphatic side chain protons measured for a concentrated solution of denatured collagen at 46° are 0.7 to 0.8 s and 0.3 to 0.6 s, respectively. However, it can be assumed that the saturation effect is not temperature-dependent since the same T₁ values were obtained for α₁CB₆C₁₅, the 21-residue COOH-terminal peptide, at D₂O and denatured collagen at 46° within an experimen- tal error of about 20%. Therefore, the determination of the mobile residue content in native collagen at 22° can be made by comparing proton intensities in the spectra obtained at 22° and 46°.

**Preparation of Collagen**—Soluble collagen was prepared from the skins of lathyritic rats or from rat tail tendons by standard procedures modified to reduce the time necessary to prepare the product and improve the efficiency of individual steps. Salt fractionation by NaCl precipitation from 0.5 M acetic acid solution was utilized to reduce the amount of polymeric material. The details appear in the miniprint supplement to this paper.

**RESULTS**

**Removal of Polymeric Collagen**—Typical results obtained by salt fractionation are shown in Fig. 1. About 20% of the collagen in samples of rat tail tendon collagen prepared as described precipitated at 3.0% NaCl; about 30% precipitated at 3.0 to 3.5% and 30% more at 3.5 to 4.0%. The total recovery of about 80% is within the experimental error expected as a result of mechanical losses and weighing errors. The amount of collagen precipitating at 0.5 to 3.0% NaCl may be larger if the tissue used was from older animals, the initial extraction was more extensive, or the collagen solutions in the earlier purification steps were not properly clarified. Lathyritic rat skin collagen samples gave similar results, but the amount precipitating at 0.5 to 3.0% NaCl will depend on the degree of lathyrisn attained and may be very small if the rats were severely lathyritic.

The amount of polymeric material present was estimated by molecular sieve chromatography of a portion of denatured collagen. If very high molecular weight polymeric material was present in the sample, it did not all dissolve in the 1 M CaCl₂ solvent. The relative amounts of a chain monomers and cross-linked α chain dimers and trimers in the soluble denatured collagen can be readily estimated from the areas under the major peaks. Larger polymeric material can be approximated from the area under the curve ahead of the α chain trimers. A chromatogram of the 3.5 to 4.0% NaCl cut of a sample of skin collagen is shown in Fig. 2, and the contents of the various components in the 3.0 to 3.5% and 3.5 to 4.0% NaCl cuts of a sample of tail tendon collagen are given in Table I. Fractions precipitating at or below 3.0% NaCl were too small to study accurately in this manner, but the progression toward increased amounts of cross-linked components seen in the 3.5 to 4.0 and 3.0 to 3.5% NaCl cuts was evident.

The skin collagen sample used to obtain the chromatogram shown in Fig. 2 is unusual in having a higher content of

<table>
<thead>
<tr>
<th>NaCl fraction</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Trimer</th>
<th>&gt;Trimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0-3.5%</td>
<td>45</td>
<td>40</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>3.5-4.0%</td>
<td>60</td>
<td>33</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

*Expressed as per cent of total material recovered.

*Precipitated from 0.5 M acetic acid.

*α chain.

![Table 1: Monomer and polymer contents of denatured rat tail tendon collagen](http://www.jbc.org/content/6063/1/3234.full)

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1. Details of some of the methods and Figs. 1 and 2 are presented as a miniprint supplement immediately following this paper. For the convenience of those who prefer to obtain the supplementary material in the form of 6 pages of full size photocopies, these same data are available as JBC Document No. 76M-285. Orders for supplementary material should specify the title, authors, and reference to this paper, and the JBC Document Number, and the numbers of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.00 per set of photocopies.

2. The abbreviation used is: SDS, sodium dodecyl sulfate.
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cross-linked α chains than most samples from rats fed β-
aminoapropionitrile. If a greater degree of lathyrism is obtained,
the α chain dimers will be 10% or less of the sample and higher
polymers are nearly absent.

The molecular sieve chromatograms sometimes showed a
peak of absorbance in the excluded volume of the column (140
ml, Fig. 2) which was sometimes very large. However, in
several cases examined, desalting and lyophilization yielded
only a small residue which contained traces of hydroxyproline
after acid hydrolysis. Apparently small amounts of a high
molecular weight contaminant with a large ultraviolet extinc-
tion coefficient contribute to this peak.

Since the collagen molecule contains three α chains, cross-
linked chains larger than a trimer can only arise from inter-
molecularly cross-linked molecules after denaturation. How-
ever, the amount of material larger than a trimer will be a
minimal estimate of covalently cross-linked molecular aggre-
gates in the native sample, since intermolecularly cross-linked
collagen will give rise to α chain monomers, dimers and trimers
when denatured, if it is not fully cross-linked intramolecularly.

Intactness of Nonhelical Ends—Since tyrosine is found only
at the nonhelical ends of the collagen molecule, and it is known
that there are 2 residues in the 16-residue NH2-terminal region
and 3 residues in the 25-residue COOH-terminal region of the
α1 chain of rat tail tendon collagen obtained by 5 M guanidine
extraction (4), the tyrosine content of the native collagen and
of the α1 and α2 chains was used as an estimate of intactness.
The tyrosine distribution in the α2 chain is not fully known.
There is 1 residue in the NH2-terminal region and 2 residues in
the large COOH-terminal cyanogen bromide fragment α2-CB5
(14), but the latter value is minimal since the collagen used in
these studies was probably not intact. We assumed for the present
studies that the α2 chain is sufficiently like the α1 chain in tyrosine content and distribution that the conclusions
apply to both.

The results are shown in Table II. An old sample of rat skin
collagen is included. This sample was prepared by similar

<table>
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<th></th>
<th>Native</th>
<th>α1 Chain</th>
<th>α2 Chain</th>
</tr>
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<tr>
<td>Lathyritic rat skin collagen*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old sample</td>
<td>2.2</td>
<td>1.4</td>
<td>3.0</td>
</tr>
<tr>
<td>New sample 1, no inhibitors</td>
<td>3.8</td>
<td>3.3</td>
<td>4.5</td>
</tr>
<tr>
<td>New sample 1, with inhibitors*</td>
<td>3.9</td>
<td>3.5</td>
<td>4.4</td>
</tr>
<tr>
<td>New sample 2, no inhibitors</td>
<td>4.4</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td>New sample 2, with inhibitors*</td>
<td>4.0</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Rat tail tendon collagen*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New sample, no inhibitors</td>
<td>5.6</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td>New sample, with pepstatin*</td>
<td>5.4</td>
<td>5.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Expressed as residues per α chain. Single analyses are shown which have a maximal error of about 10%.

Salt-extracted; 3-4% NaCl cut.

20 mM EDTA, 1 mM p-mercuribenzoate, 10 μM phenylmethylsulf-
fonylfluoride in the extracting solvent.

Acid-extracted; 3-4% NaCl cut.

1 mg/4 liters in extracting solvent.

procedures to those used here except that the initial salt
extractions continued for several days, longer periods were
taken for other steps in the purification, and less attention may
have been given to other precautions. The new samples were
prepared with and without enzyme inhibitors in the initial
extracting solutions. The α1 and α2 chain samples were
prepared by CM-cellulose chromatography of the α chain
monomer fraction isolated by molecular sieve chromatography.

It can be seen that while the old skin collagen sample
averaged only about 2 tyrosines per α chain, the value
increased to about 4 in samples prepared as described here.
The presence of enzyme inhibitors did not significantly affect
this result. The rat tail tendon collagen sample shown averaged
about 5 residues per α chain, the same as the theoretically
expected value for the α1 chain. Similar results were obtained
by proton NMR (see below). The acidic protease inhibitor
pepstatin has no effect; it would not be expected to if the ends
were not altered in its absence, but the experiment provides
confirming evidence.

Since it has been reported that the COOH-terminal cyanogen
bromide peptide from the α1 chain of rat tendon or skin
collagen, α1-CB6, migrates more rapidly on SDS gels if the
COOH-terminal nonhelical region has been lost (4), we exam-
ined cyanogen bromide digests of the α1 chain from our
samples. The results show (Fig. 3) that a band appearing in the

Fig. 3. Fractionation of the cyanogen bromide peptides from the α1
chain of “old” rat skin (Tube 1) and “new” rat tail tendon (Tube 2)
collagen samples by SDS gel electrophoresis. Migration was from top
to bottom. Tube 3 contains a mixture of the two samples. The
unlabeled bands are large, incompletely cleaved fragments. CB6* indicates the proteolytically truncated form of α1-CB6, the COOH-ter-

minal cyanogen bromide peptide.
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expected position for α1.CB6 from the old collagen sample is replaced by a more slowly migrating band in preparations made by the procedure described here. The difference in position is consistent with the calculated larger size of about 10%. Tail tendon (Fig. 3) and skin collagen (not shown) prepared as described here gave identical patterns.

Proton NMR—The resonances for the ring protons of histidine, phenylalanine, and tyrosine are well resolved from other amino acids (15) in collagen, permitting their relative contents to be measured in denatured collagen. This is illustrated in Fig. 4 for the α2 chain from the 3 to 4% NaCl cut of rat tail tendon collagen. The same can be done for the methyl groups of valine, leucine, and isoleucine. Data obtained by this method and by ion exchange chromatography are compared in Table III. Denatured whole rat tail tendon collagen at 40 mg/ml showed about 5 residues of tyrosine per α chain, the same value obtained by ion exchange chromatography.

Native rat tail tendon collagen in solution gave the spectrum shown in Fig. 5b. Although the experimental error is larger because of the lower concentration and the saturation effect (see "Methods"), the downfield region showed the presence of about 2 residues of phenylalanine, 4 to 5 residues of tyrosine, but no histidine. When the sample was denatured the tyrosine resonances remained constant, the phenylalanine resonance increased and the histidine resonance appeared (Fig. 5a).

There were also large increases in intensities in the upfield region some of which can be related to those amino acids that

![Fig. 4](image_url)  
**Fig. **4. The aromatic region of the 220 MHz spectrum of the α2 chain from intact rat tail tendon collagen obtained using a π/2-τ-π/2 pulse sequence with τ = 3 s. 2,000 scans were accumulated at 48°. The α2 chain concentration was 40 mg/ml in 0.5 N [2,2,2,2-D4]acetic acid in D2O. The chemical shift scale is in parts per million from external sodium 3-trimethylsilyl [2,2,3,3-D4]propionate (TSP). The intensity scale on the ordinate is in arbitrary units.

![Fig. 5](image_url)  
**Fig. 5.** Comparison of 220 MHz spectra of intact rat tail tendon collagen obtained at two temperatures using π/2-τ-π/2 pulse sequence with τ = 3 s. (a) 46°, denatured collagen; (b) 22°, native collagen. 32,000 scans were accumulated in each case. The protein concentration was 4 mg/ml in 0.5 N [2,2,2,2-D4]acetic acid in D2O. Chemical shift is in parts per million from external sodium 3-trimethylsilyl [2,2,3,3-D4]propionate (TSP). Resonances due to solvent and spinning sidebands are shaded. The intensity scale on the ordinate is in arbitrary units. However, the inset to (a) is 4.8 times (a), and (b) is 7 times (a).

<table>
<thead>
<tr>
<th>Residue</th>
<th>NMR*</th>
<th>Ion exchange</th>
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</thead>
<tbody>
<tr>
<td>Valine + leucine</td>
<td>76%</td>
<td>80</td>
</tr>
<tr>
<td>+ isoleucine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>13%</td>
<td>13</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.5%</td>
<td>5.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.1%</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* Values normalized to 13 for phenylalanine.
* Experimental uncertainty ± 5%.
* Experimental uncertainty ± 10%.
are present in large amounts in collagen. These are: glycine H\textsubscript{a}, 3.8 to 4.0 ppm; proline H\textsubscript{a}, 3.5 to 3.7 ppm; proline H\textsubscript{a} and H\textsubscript{b}, 1.9 to 2.3 ppm; hydroxypoline H\textsubscript{a}, 3.7 to 3.9 ppm; hydroxyproline H\textsubscript{a}, 2.1 to 2.3 ppm; alanine methyl H\textsubscript{a}, 1.3 to 1.4 ppm; valine, leucine, and isoleucine methyl H\textsubscript{a}, 0.8 to 1.0 ppm. These resonances would be broadened beyond detectable limits in the spectrum of the native collagen since line widths show a monotonic increase as the rate of rotational diffusion decreases and one of the rotational diffusion coefficients of the rod-like native molecule (300 x 1.4 nm) is small.

The aromatic resonances appearing in samples of native collagen can be ascribed to the short nonhelical ends of the molecule, which then must be in rapid segmental motion and therefore unstructured. It is unlikely that the resonances could arise from a few per cent of denatured collagen since the pattern is very different from that observed for denatured collagen. Further evidence that this conclusion is correct is provided by chemical data which show that the NH\textsubscript{a}-terminal end of the a1 chain of rat tail tendon collagen contains 2 residues of tyrosine and the COOH-terminal end contains 3 residues of tyrosine, 2 of phenylalanine and no histidine (4). These are the same values within experimental error found by proton NMR, and the tyrosine value is the same as found by ion exchange analysis in this study (above).

**DISCUSSION**

We have shown that native collagen can be prepared from rat tail tendon and rat skin which is intact or nearly so and has only a small content of covalently cross-linked polymeric material. Collagen from the skin of lathyrism rats is the best source of monomeric collagen. However, the tyrosine analysis indicates that a small amount of proteolysis occurs even with the precautions taken here. Sequence studies of the homologous and very similar collagen from calf skin have shown that the COOH-terminal and the penultimate residues of the a1 chain are both tyrosine, and that the COOH-terminal tyrosine is readily lost (6). Therefore, the rat skin collagen isolated here, which has about 1 less tyrosine per a1 chain than the theoretically expected 5, may be altered in this way rather than by the complete loss of the COOH-terminal (or NH\textsubscript{a}-terminal) region from some molecules. The SDS-gel patterns of the cyanogen bromide peptides suggest the same conclusion since the truncated form of α1-CB6 was not observed in patterns obtained from rat skin collagen.

Our analyses suggest that the intact a2 chain has the same number of tyrosine residues as the a1 chain (Table II). Since 3 were found previously (14), the additional 2 are probably in a COOH-terminal nonhelical region which has yet to be characterized. They do not seem to be as susceptible to hydrolytic loss in our studies as the corresponding residues in the a1 chain. The a2 chain from skin collagen had only a fractional residue less than the a2 chain from tail tendon collagen.

The salt fractionation was reasonably effective in removing polymeric collagen from the samples. A 3.0 or 3.5% NaCl precipitation step should be included in the purification procedure when it is important to have monomeric native collagen. However, some polymeric material remains if the amount initially present is large. This is to be expected since the solubility difference between monomer and small polymers will not be large. The best way to avoid polymers is to use skin from rats in which the degree of lathyrism induced is sufficient to nearly stop cross-linking of newly made collagen. Salt fractionation and molecular sieve chromatography are useful in determining the amount of polymeric collagen present.

The purification steps used here are similar to those used by us and in other laboratories in the past. It is likely that considerable variation in the exact steps selected and their order is permissible. The important factors seem to be rapid processing and attention to detail, particularly in the early stages to remove proteases. Inhibitors are not necessary when beginning with tail tendons and the mixture tried here was not effective with rat skin.

The proton NMR studies demonstrate that this is a powerful tool for the characterization of the nonhelical ends of collagen samples that are known to be pure and free of denatured collagen. That the nonhelical ends are unstructured and mobile in acid solution is not unexpected since they do not have the triplet sequence of the helical body of the molecule and they are susceptible to proteases.

**Acknowledgments**—We are indebted to Mrs. Thelma Prather for expert technical assistance and to Mr. Guy R. Hawkins for the amino acid analyses. Some confirmatory results and more recent modifications included here were kindly provided by Doctors Barbara R. Williams and Robert Gelman.

**REFERENCES**

Intact Monomeric Collagen

Fig. 1

Tendon Collagen — The tissue was treated with a dihydrate amyllose starch derivative of sodium zein sulfate (M 1600) and extracted with 0.5 M acetic acid. The volume should be adjusted as the solution is not too viscous. A concentration of 2 mg/ml is satisfactory. The extract was filtered through a sterile filter. The extract was obtained by centrifugation at 30,000 × g for 30 min and precipitated by the addition of one-fifth volume of 30% HCl in 0.5 M acetic acid.

Salt Fractionation — Collagen from rat tendon was prepared as described above and washed with 0.5 M acetic acid to give a concentration of 1 mg/ml. All procedures were done at 4°C. Solid NaCl was added in increments of 0.5 M NaCl and the suspension was centrifuged at 30,000 × g for 30 min. The supernatant was obtained at each stage before addition of NaCl and centrifugation at 30,000 × g for 30 min. The supernatant was dialyzed exhaustively against 0.1 M acetic acid and lyophilized. The collagen in each fraction was determined by amino acid analysis.

Since salt fractionation was effective in reducing the content of polymeric collagen (see HEYER), it was useful to add an additional step to the purification procedures described above for skin and tendon collagen. At the last stage before collagen was dialyzed against 0.1 M acetic acid, the solution was dialyzed against a small portion of the volume of 50% NaCl in 0.5 M acetic acid and the precipitate was removed by centrifugation. The supernatant was then dialyzed exhaustively against 0.1 M acetic acid and lyophilized. The collagen in each fraction was determined by amino acid analysis.

The precipitate was also treated with 0.5 M acetic acid, dialyzed overnight against 0.5 M acetic acid, and centrifuged at 30,000 × g for 30 min. The supernatant was treated with 0.5 M NaCl in 0.5 M acetic acid and the precipitate was removed by centrifugation. The supernatant was then dialyzed exhaustively against 0.1 M acetic acid and lyophilized. The collagen in each fraction was determined by amino acid analysis.

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