

A CONFORMATIONAL STUDY OF COLLAGEN AS AFFECTED BY TANNING PROCEDURES*

by

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ABSTRACT

Chrome tanning of animal skins to produce high quality leather is a well established industrial process. The multistep process whereby complex salts of trivalent chromium (Cr(III)) crosslink collagen fibrils is more an art form than a science. One of the first steps in this process is the interaction of chromium with collagen and this reaction is poorly understood. Our goal is to develop a scientific basis for improving the effectiveness and efficiency of tanning technology. In the present study, we use spectroscopic methods circular dichroism (CD) and ¹³C nuclear magnetic resonance (¹³C NMR) and transmission electron microscopy (TEM) to characterize the effects of various steps in the chrome tanning process on the conformation of soluble collagen. Partial denaturation of collagen prior to chromium binding appears to be necessary. The ratio of the ¹³C NMR peaks due to free carboxyl groups of aspartic and glutamic acids was altered when chromium was bound to the collagen. A network of soluble crosslinked collagen could be seen in transmission electron micrographs of chromium-collagen complexes.

INTRODUCTION

Effective crosslinking of collagen in animal hides produces leather, having properties of strength and resistance to organisms that would otherwise attack and destroy the hide.

For more than one hundred years, the most commonly used crosslinking agents have been complexes of trivalent chromium (Cr(III)). In recent years, tanners have empirically developed "high exhaust" tannages that decrease the amount of chromium released into sewage or solid waste treatment processes. Tanning, however, remains more an art form than a science and the mechanism of the chromium-collagen interaction is poorly understood. Our goal is to develop a scientific basis for improving the effectiveness and efficiency of tanning technology. In the present study, we use spectroscopic methods circular dichroism (CD) and ¹³C nuclear magnetic resonance (¹³C NMR) and transmission electron microscopy (TEM) to characterize the changes in soluble collagen at various stages of the chrome tanning process.

Individual chains of the fibril forming collagens, in particular types I and III, the major protein components of bovine hide, have stretches of about 1000 amino acid residues with a repeating triplet amino acid sequence of Gly-X-Y, where X and Y are often proline and hydroxyproline. This sequence pattern leads to the formation of a unique triple-helical structure in which the glycine residues are oriented towards the center of the triple helix with the X and Y residues directed outward from the helix.¹ The three chains each have a left handed twist with three residues per turn, together they form a right handed supercoiled triple helix. In the formation of the triple helix, the individual chains are staggered by a single residue. Thus, the glycines, of the three chains, form a shallow helix up the center of the triple helix. In the outer X and Y positions of collagen type I, glutamic acid, histidine, leucine, and phenylalanine

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show a (>86%) preference for the X position, threonine, lysine, arginine, methionine, and glutamine show a preference (60% - 80%) for the Y position while alanine, aspartic acid, asparagine and serine are nearly evenly distributed between the two positions.

In chrome tanning, the collagen fibrils of a hide become crosslinked by coordinate bonds between bi- or polynuclear chromium ions and carboxylate side chains of the protein.^{2,3} Chromium sulfate and chromium chloride have been used in tanning studies. Although polynuclear complexes are formed in both systems, the sulfate ion gives better penetration of the hide, making it the more effective tanning agent. Oxygen bridged bi- and trinuclear chromium sulfate complexes appear to be the most effective chrome tanning agents. In the present study, we have used $\text{KCr}(\text{SO}_4)_2$ and soluble collagen as a model system to examine the effects of tanning steps on the conformation of collagen.

EXPERIMENTAL

Fetal calf skin collagen isolated for an earlier study⁴ had been lyophilized and stored frozen for several years. Soluble collagen used for these studies was recovered essentially as described by Williams and coworkers.⁵ The lyophilized material was suspended in 5 mM acetic acid (pH 4) at concentration of 3 mg/ml. The suspension was dialyzed in 12,000 MW cutoff Spectrapor** tubing against 5 mM acetic acid overnight at 4°C, with one change of dialyzate, to remove small peptide degradation products and to assure an equilibrium solvent composition. The material was then centrifuged at 165,000g in a Beckman L8-70 preparative ultracentrifuge at 25°C for one hour to sediment any large aggregates. The supernatant from this centrifugation contained the soluble collagen.

Simulated Tanning Procedure

A four step process modified from the data of Taylor and co-workers⁶ was used to simulate the tanning process. The collagen was first dissolved in 5 mM acetic acid at pH 4. Second, the solution was acidified to pH 2 using microliter drops of 1 M H_2SO_4 . Third, chromium, in the form of a 10% $\text{KCr}(\text{SO}_4)_2$ solution, was added to the acidified collagen at a 1:100 ratio. Finally, the pH of the Cr-collagen mixture was slowly raised to pH 4 by the hourly addition of 5 μL 0.01M NaHCO_3 . Although the binding of chromium to soluble collagen would not require a time scale of this

magnitude, the procedure more closely approximates the time scale needed to assure penetration of a hide by the chemicals. Spectroscopic analysis was carried out at each stage of the simulated tanning process.

Optical Spectroscopy

The concentration of collagen in solution was determined from the absorbance at 218 nm using the molar absorption coefficient ($\epsilon = 883,129 \text{ cm}^{-1}\text{-L-mole}^{-1}$) determined by Na.⁷ Samples were placed in 1 mm pathlength cells and reference solutions containing all components except the collagen were prepared. CD spectra between 250 nm and 185 nm were obtained on an AVIV 60DS Circular Dichroism Spectrophotometer. In the CD spectrometer, plane polarized light is decomposed into right (R) and left (L) circularly polarized wave components. Absorption of the R and L circularly polarized waves to different extents by an optically active sample generates a CD spectrum. The CD signal is read in mdeg and converted to molar ellipticity.⁸

$$[\theta]_{\lambda} = \theta_{\lambda}/ncd \text{ deg cm}^2 \text{ dmol}^{-1}$$

where n is the number of amino acid residues in the protein chain, c is the molar concentration, and d is the pathlength in millimeters. The collagen CD spectrum¹ is characterized by a positive band at 223 nm, $[\theta] = 7500 \text{ deg cm}^2 \text{ dmol}^{-1}$ and a negative band at 198 nm, $[\theta] = -53,000 \text{ deg cm}^2 \text{ dmol}^{-1}$. To obtain a CD spectrum of our soluble collagen, an aliquot of solution (0.5 mg/ml) in a 1mm pathlength, far uv-quartz, Teflon stoppered cuvette was scanned from 250 nm to 190 nm in 1 nanometer steps using a 2 sec time constant. Melting curves were obtained by recording the CD signal at 223 nm every 0.1 deg between 25°C and 45°C using a time constant of 10 sec and a heating rate of 3 degrees Celsius per hour.

Nuclear Magnetic Resonance Spectroscopy

¹³C NMR spectroscopy was used to follow the effects of tanning steps on the peptide backbone of collagen and the carboxylate side chains. Collagen (5 mg/ml) was initially dissolved in D_2O containing 5 mM acetic acid. The simulated tanning procedure described above was followed with adjustments for the higher concentration of collagen. ¹³C NMR spectra were obtained at 37°C on a Varian Gemini-200 instrument operating at 50 MHz. In a typical experiment, 8k data points were collected with a pulse width of $90^\circ = 25 \text{ ms}$. and a recycle time of 2.5 s using

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WALTZ-16 proton decoupling. The C₄ (C-O-H) of hydroxyproline was used as an internal chemical shift standard (71.3 ppm).

Transmission Electron Microscopy

An aliquot of 10 μL from each protein sample was applied to a thin film of amorphous carbon supported by a 400 mesh Cu screen for 30 to 60 seconds. The sample was washed off the surface of the carbon film with a controlled stream of droplets of 2% uranyl acetate solution, and the residual stain was adsorbed from the surface of the screen with Whatman #1 filter paper. After air-drying, the negatively stained regions of the sample were examined in a Model CM12 scanning-transmission electron microscope (Philips Electronics, Mahwah, NJ) operating in the bright field imaging mode. Photographic images were recorded at an instrumental magnification of 22000x.

RESULTS

Characterization of Soluble Collagen

Experiments were performed at least 4 times; the results shown are average values. Collagen samples from several preparations were used in this study. All were subjected to the clean up procedure described above. The absolute value of the ratio of the CD signals at 223 nm and 198 nm was used as a check on the degree to which the protein was in a native conformation at the start of the tanning process.

$$R = |(\theta_{198} / \theta_{223})|$$

For all samples used, the average value of R was 7.8 ± 0.3 . Sample concentrations of about 0.4 mg/ml were used to give an absorbance at 218 nm of 0.4 A in a 1mm pathlength cell. This concentration allowed us to record the CD spectrum to at least 190 nm. Figure 1 shows the UV and CD spectra of soluble collagen in 5 mM acetic acid. The collagen triple helix is characterized by a small positive band at about 223 nm, and a larger negative band at 198 nm.

CD Spectra

Figure 2 shows the average CD spectrum recorded at 25°C of collagen at each stage of the simulated tanning process. At pH 2, the CD detectable triple helical structure at 223 nm is about 10% less than at pH 4. The conformational change was completely reversible in the absence of chromium (data not shown). The addition of chromium at pH 2 appeared to cause a further decrease of about 8% in the triple helical content. Under binding conditions, pH 4 in the presence of chromium, the CD detectable triple helical structure was

reduced to less than 50% of the starting value. The addition of chromium to collagen in 5 mM acetic acid at pH 4 had little effect on the CD spectrum of the collagen (data not shown).

Helix to Coil Transition

The triple helix to random coil transition in soluble collagen is a temperature and time dependent process that may be monitored by several physical techniques, including CD spectroscopy.¹ The apparent melting curve obtained by recording the CD signal at 223 nm as a function of temperature between 25°C and 45°C gives an indication of the stability of the helical conformation in collagen. Figure 3 shows melting curves for collagen under the conditions of simulated tanning. The ellipticity at 25°C gives a measure of the extent to which the protein is in a native conformation, while T_m, the midpoint of the helix to coil transition, is an indication of the stability of the conformation. T_m values are listed in Table I.

¹³C Nuclear Magnetic Resonance Spectroscopy

Figure 4a shows the full ¹³C NMR spectrum of collagen at the start of the simulated tanning. Figure 4b focuses on the effects of tanning steps on the 150 to 190 ppm region of the spectrum which encompasses peaks due to both carbonyl groups⁹ and backbone carbons in either triple helical or random coil conformation.¹⁰

Electron Microscopy

Transmission electron micrographs were obtained for each of the four tanning steps. Figure 5 shows micrographs of the starting native collagen solution and the final chromium-collagen complex. Under acid conditions, micrographs (not shown) were similar to the those of the native collagen in solution. Crosslinking appeared on return to a more neutral pH.

DISCUSSION

The procedure of Williams and coworkers⁵ produced reasonably high quality collagen from an isolate that had been stored frozen for several years.⁴ The CD spectrum of the collagen in 5 mM acetic acid is in excellent agreement with that of Piez and Sherman¹¹ for collagen isolated from rat tail under the same conditions. Native collagen in solution < pH 5 denatures when it is heated. The denaturation, or helix to coil transition, can be followed by monitoring the CD signal at a single wavelength (223 nm). This melting can be treated as a phase transition with a characteristic midpoint (T_m). The denaturation is both time and pH

TABLE I
Effects of Tanning on the Helix to Coil Transition in Soluble Collagen

	T_m	std. dev.
pH 4	37.0	0.4
pH 2	32.0	0.5
pH 2 with Cr	32.6	1.2
pH 4 with Cr	35.0	2.3

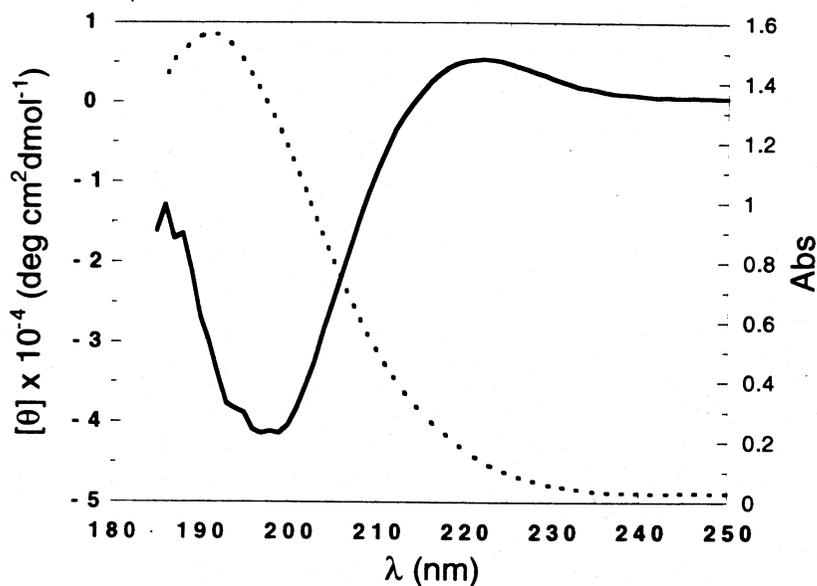


FIGURE 1. — UV absorbance (-----) and CD (——) spectra of soluble calf skin collagen in 5 mM acetic acid.

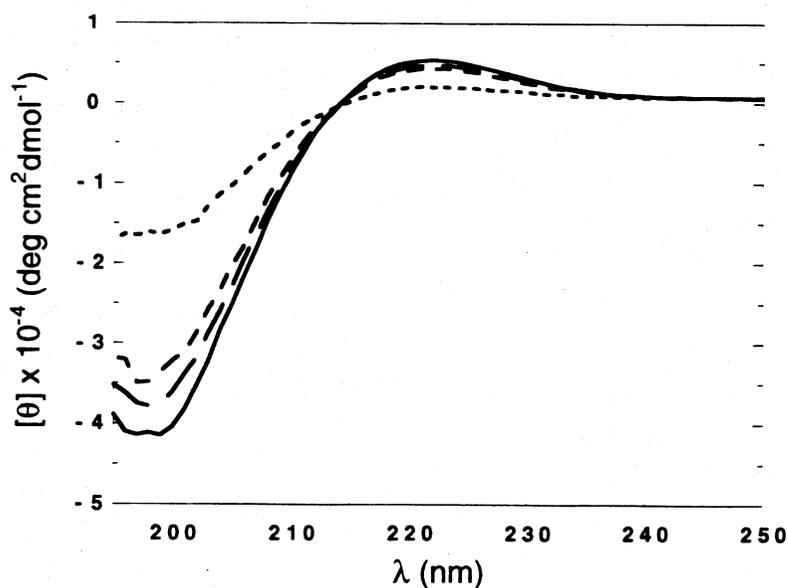


FIGURE 2. — CD spectra between 250 nm and 190 nm of soluble collagen after each of the four tanning steps. The plot are as follows: solid line (——) the spectrum of collagen in 5 mM acetic acid; long dash (— — —) after acidifying the collagen solution to pH 2 with 0.1 M H_2SO_4 ; shorter dash (-----) after addition of Cr(III) at pH 2; and shortest dash (-----) under crosslinking conditions with Cr(III).

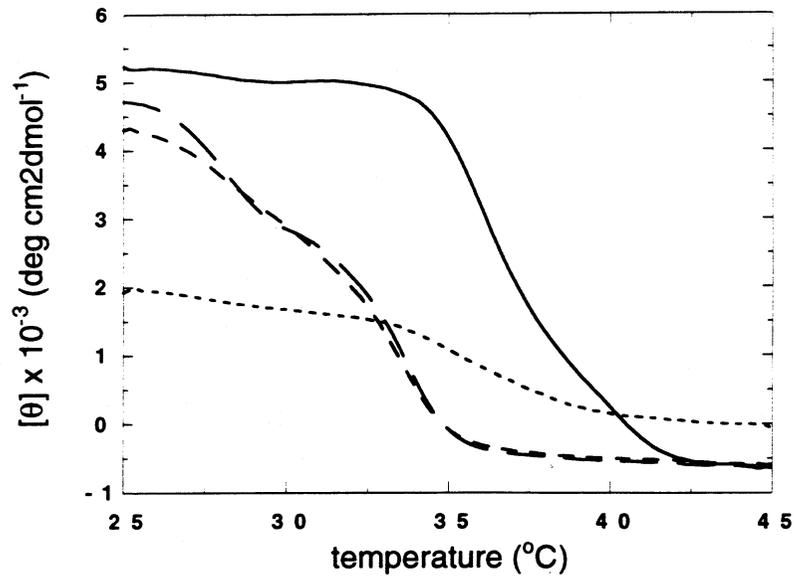


FIGURE 3. — Melting curves for soluble collagen after each tanning step. Individual plots are designated as in Figure 2.

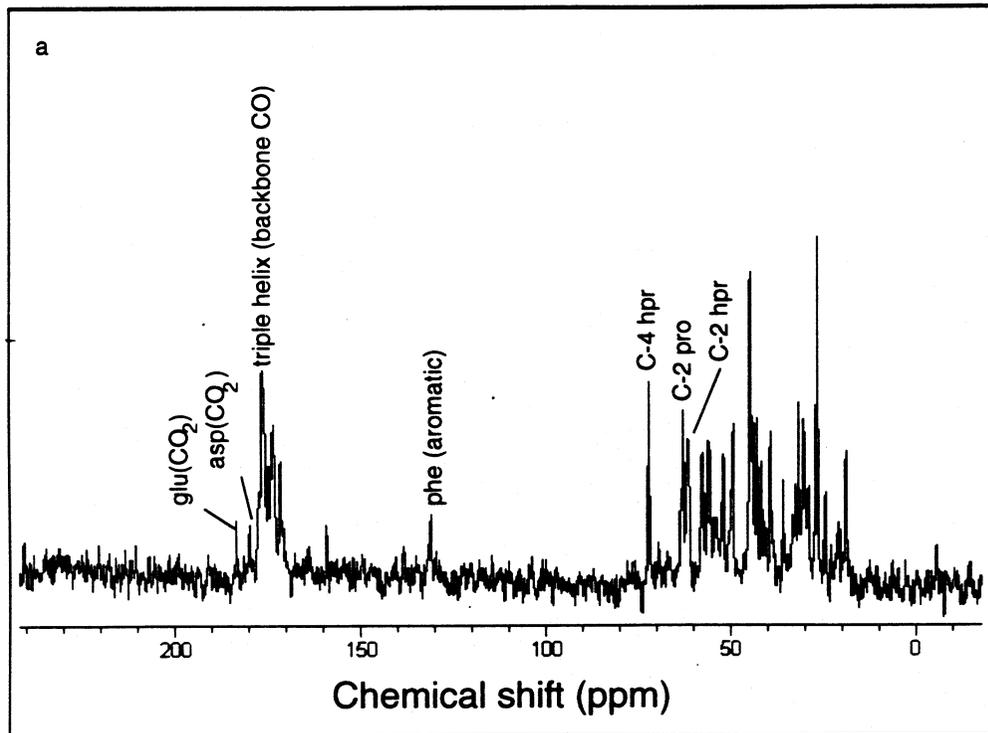


FIGURE 4a. — ^{13}C NMR spectrum of soluble collagen in D_2O /acetic acid.

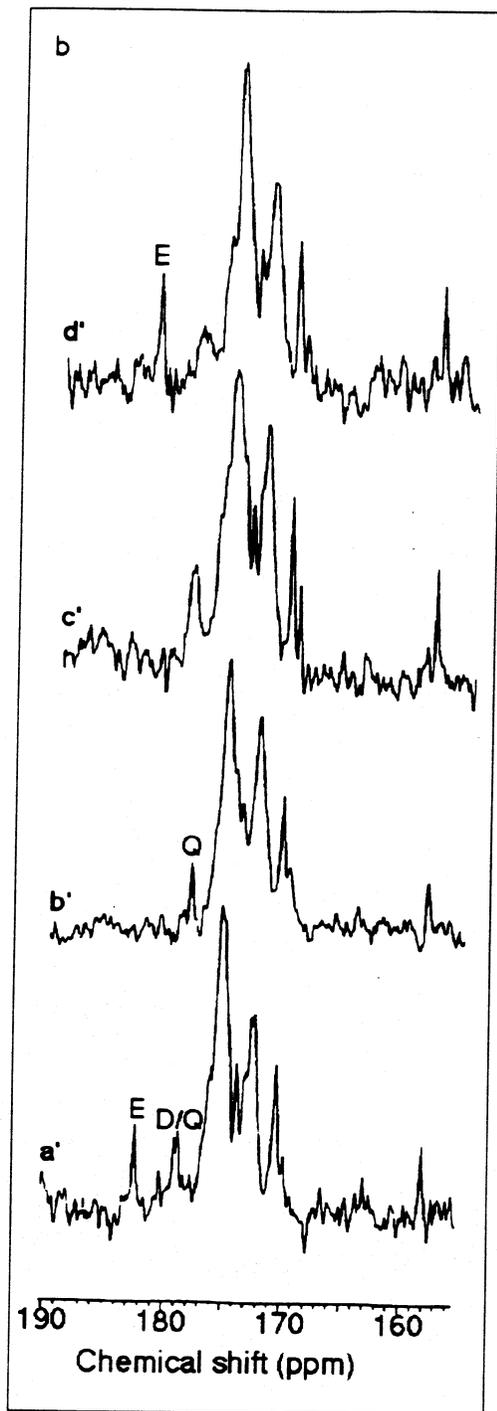


FIGURE 4b. — Expanded view of the carbonyl region of the ^{13}C NMR spectrum at each stage of the model tanning process. Curve a' is expanded from 4a, b' was obtained after acidifying the collagen solution to pH 2, c' after the addition of Cr(III) at pH 2, and d' under crosslinking conditions with Cr(III). D, E and Q are the single letter designations for aspartate (asp), glutamate (glu) and glutamine (gln).

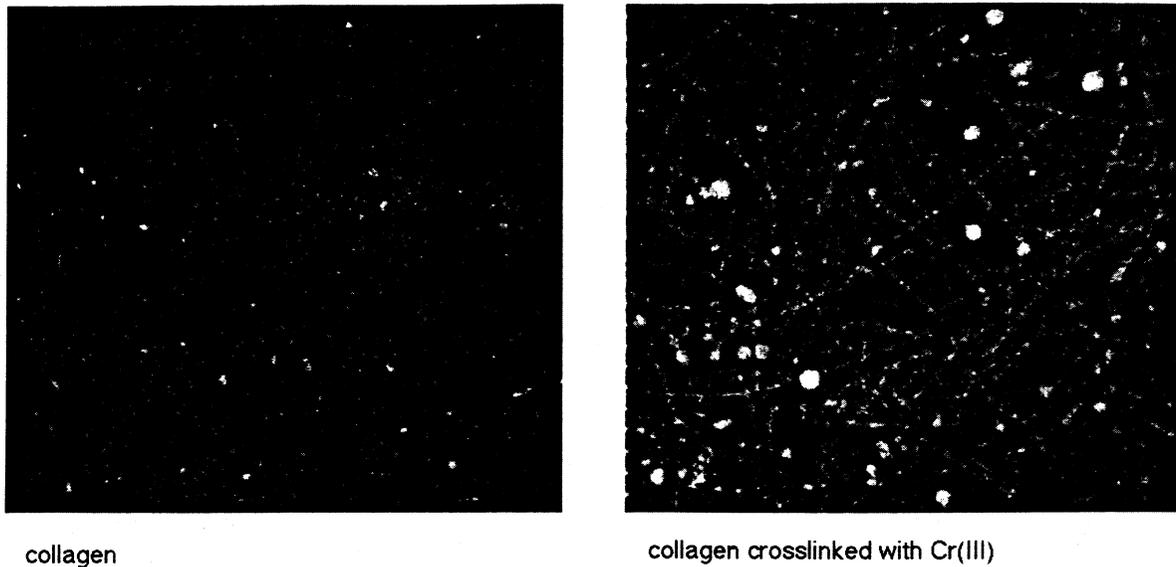


FIGURE 5. — Transmission electron micrographs, on the left, of soluble collagen in 5 mM acetic acid, on the right, of the collagen plus Cr(III) under the conditions of crosslinking. The particle like spots on the right may be crystals of Cr(III) salts.

dependent. By raising the temperature at a rate no greater than 4°C per hour, the apparent time dependence could be minimized. In the pH 4 - 5 range T_m is just slightly higher than the normal body temperature of the animal. Under more acidic conditions, T_m is several degrees lower.¹

Although it is convenient for the calculation of T_m to treat melting as a single phase transition, implying that a molecule is in either a helical or a random coil conformation, in reality, some parts of a collagen molecule will denature more readily than others. It is well known that ionizable and hydrophobic side chains tend to be grouped in patches along a collagen triple helix.^{12,13} The measured T_m of 37°C for collagen at pH 4 and 32°C at pH 2 is consistent with earlier studies.¹ There is a hint of multistep melting in the pH 4 curve that is more pronounced at pH 2. Although the addition of chromium at pH 2 resulted in a slight decrease in the initial helical content, it had no significant effect on either the T_m value or the shape of the melting curve. The binding or fixation that accompanied the slow basification of the chromium-containing collagen solution resulted in a smaller amount of helical structure at the start of the melt, but with a melting curve qualitatively more like that of the native protein. It appears that chromium crosslinks may form primarily in regions where the helical structure has been destabilized by the acid conditions. Thus the crosslinks may stabilize a less than native conformation of collagen.

The carbonyl region of the ¹³C NMR spectrum of collagen, roughly between 170 and 185 ppm, contains several peaks. Two of these peaks can be assigned to the carboxylate carbons of glutamate (182.3 ppm) and aspartate (178.8 ppm) side chains.⁹ Additional peaks due to the amide carbons of glutamine (178.5 ppm) and asparagine (175.3 ppm) as well as backbone carbonyls in triple helical (173.5 ppm) or random coil (171.0 ppm) conformations contribute to the spectrum in this region. The chemical shifts of the carboxylate carbons of free glutamic and aspartic acids are pH dependent,¹⁰ shifting to 172.4 ppm and 174.4 ppm respectively when fully protonated. In Figure 4b (curve a'), the ¹³C NMR spectrum of our starting material, the 182.3 ppm peak is attributed to glutamate, while the doublet at 178.8 - 178.5 ppm is attributed to aspartate and glutamine. Upon acidification, Figure 4b (curve b'), the peaks assigned to the glutamate and aspartate side chains are shifted down field, falling under the broader backbone peaks in the 171 ppm to 174 ppm region and contributing to the small shift of the sharpened glutamine peak to 178.2 ppm. With the addition of chromium, Figure 4b (curve c'), the 178 ppm peak is broadened, suggesting that a species contributing to this peak is interacting with chromium. Finally, after basification to crosslinking conditions, Figure 4 (curve d'), the glutamate peak reappeared and the aspartate peak was significantly broadened suggesting a possible preference for this side chain.

Although there was no apparent precipitate in the reaction vial containing the chromium-collagen complex at pH 4, the question of the solubility of this complex was considered. Electron microscopy was employed to examine the solubility of crosslinked collagen. If the crosslinked collagen was insoluble one would expect to see either no protein material in a 10 μ L aliquot, or possibly one or a few large aggregates floating in solvent. The rather uniform network (Figure 5b) was evenly dispersed throughout the 10 μ L aliquot, suggesting that at a concentration of 0.5 mg/ml collagen, even collagen crosslinked with chromium is significantly soluble.

CONCLUSIONS

A very simplified model was developed to enable us to look at molecular aspects of the mechanisms of chrome tanning. With this model, measurable effects on the conformation and conformational stability of soluble collagen under the conditions of chrome tanning could be seen. Partial denaturation of the collagen triple helix prior to chromium binding appears to be necessary. The partially denatured conformation appeared to be stabilized by crosslinking and the chromium-collagen complex remained soluble at the concentration used in this study. The carboxylic acid groups of aspartate and glutamate side chains were affected to different extents when chromium was bound to the collagen. The question of a chromium preference for aspartate side chains or simply for carboxyl groups at the Y position should be explored both experimentally and with the ERRC collagen molecular model.¹⁴ This study provides some basic information on specific steps in chrome tanning. It forms a basis for the inclusion of conditions more like those of tanning in future experimental and theoretical studies.

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DISCUSSION

William Prentice, retired – Eleanor, often times we run into a problem known as acid swelling when we go through the pickling step. That is the reason for putting salt in that system before chrome tanning. I would expect that at the low pH of 2 the system would be in a highly charged cationic state. Am I correct? In which case, your molecule structure might be different and you might get something similar to a

denaturation simply from that effect. In the presence of a little bit of salt you may see quite different results. Would it be worthwhile to repeat the experiment with salt added or do you plan to do that?

We actually have done some experiments with sodium chloride as added ions and we haven't seen a whole lot of difference. We haven't really completed that series yet.