APPROACH TO A TANNING MECHANISM: STUDY OF THE INTERACTION OF ALUMINUM SULFATE WITH COLLAGEN*

by

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ABSTRACT (IN ENGLISH AND SPANISH)

The production of leather from animal hides or skins is accomplished through the use of tanning agents that stabilize the collagen matrix. The molecular characteristics of tanning agents are quite varied, and led to the expectation that leathers produced with different tanning agents would have fundamentally different structures. Current proposals suggest that the structural similarities between leathers produced with different tanning agents are greater than the differences. The goal of this work is to contribute to the elucidation of a general mechanism for tanning. Although complex salts of Cr(III) are currently the most effective tanning agents, salts of other metals, including aluminum, have been used either alone or in combination with vegetable tannins or other organic chemicals. In the present study, the interaction of aluminum sulfate with collagen is investigated. In a model system, using soluble collagen, ^{13}C NMR spectroscopy showed that Al(III) formed a complex with carboxyl groups on collagen. ^{27}Al NMR revealed the formation of a second sphere complex between collagen and aluminum. The effects of Al(III) binding on the thermal stability of collagen were studied by circular dichroism spectroscopy of soluble complexes and differential scanning calorimetry of insoluble complexes. Comparison of Al(III)-collagen interactions with Cr(III)-collagen interactions is expected to provide insight into a more generalized mechanism for tanning.

La producción del cuero a partir de pieles de animales se logra mediante el uso de agentes curtientes que estabilizan la matriz del Colágeno. Las características moleculares de agentes curtientes son muy variadas, y esto ha conducido a la apreciación que cueros producidos con diferentes agentes curtientes poseen estructuras químicas fundamentalmente distintas. Propuestas actualizadas sugieren que las estructuras de cueros producidos por diferentes curtientes son mas parecidas que diferentes. El objetivo de este trabajo es contribuir a elucidar un mecanismo generalizado para la Curtición. Aun que las sales de Cr(III) acomplejado, son en la actualidad los agentes curtientes mas efectivos, sales de otros metales, aluminio incluido, se han empleado ya sea individualmente o en combinación con taninos de origen vegetal o otros productos orgánicos. En el presente estudio, la interacción entre sulfato de aluminio con colágeno se investiga,. Utilizando como modelo un sistema con colágeno disuelto, la Espectroscopia Magnética Nuclear del carbono 13 demostró que Al(III) formó un complejo con los grupos carboxílicos colagénicos. Espectrografía Nuclear Magnética del isótopo 27 del aluminio reveló la formación en la segunda esfera de coordinación, complejación entre el aluminio y colágeno. Los efectos de los enlaces del Al(III) sobre la estabilidad térmica del colágeno fueron estudiados por Espectroscopiá Circular Dichróica (luz polaizada) de los complejos solubles y los insolubles por Calorimetría por Barrido Diferencial. Comparaciones entre las interacciones del Al(III)-colágeno con las de Cr(III)-colágeno son de esperar producir una visión clave hacia un mecanismo generalizado.

INTRODUCTION

The tanning of hides or skins to make leather was perhaps the earliest manufacturing process. Early leathers were

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most likely made by 'tanning' the hide with the brain of the animal, or by soaking it in extract of tree bark. During the twentieth century mineral (Cr, Al, Fe, Ti, Zr), organic (aldehyde, acrylate, polyphenol) and combination tannages came into use. Each process was developed more or less independently, and explained generally in terms of chemical bonding where the tanning agent would form a bridge between amino acid residues on collagen fibrils.

On a fundamental level, tanning is a multistep process whereby collagen is modified to enhance its functionality. The perishable animal hide is converted into leather, which resists microbial attack and may last indefinitely. Tanning processes have developed empirically and remain more art form than science. During the past hundred years, chromium (Cr(III)) came to be the most widely used and studied tanning agent. Although Cr(III) is a very efficient tanning agent for the production of high quality leather, the chromium-collagen interaction is still poorly understood. Until recently, most attempts at describing the chemistry of chrome tanning focused on the crosslinking of collagen fibrils. The primary chrome-tanning agent is a mixture of Cr(III) complexes, where two or more chromium atoms connected by oxygen and/or hydroxyl bridges have varying numbers of associated sulfate ions.[1] One role of chromium in the tanning of leather is to crosslink the collagen fibrils by forming complexes with the carboxyl groups of glutamic and aspartic acid side chains on collagen.[2,3] Other mineral tannages have generally been assumed to follow the chromium model.

Vegetable tannins were assumed to react with collagen by means of both electrostatic and coordinate forces[2] and to crosslink or cluster between basic sidechains. Glutaraldehyde was considered to interact with collagen through lysine to lysine crosslinks via either the monomer or a condensed polymer as the tanning agent.[4] Acrylamide was shown to bind to lysine residues and derivatives such as N,N'-methylenebisacrylamide and higher homologues could crosslink lysines.[5]

Recent proposals (Covington and Song,[6] and Ramasami,[7]) that tanning is a matter of protein modification, not just collagen crosslinking, are supported by our recent finding[8] that hydrothermal stability of leather is a function of total bound chromium, not just a fraction that forms 'productive crosslinks'. The long-range goal of this research is to contribute to an experimental model for examining at the molecular level the interactions of tanning materials with collagen. Previously, we have examined the interaction of Cr(III)[9] with collagen in dilute acetic acid (HAc) and the effects of neutral salts[10,11] on the stability of the collage triple helix. In those studies we used spectroscopic methods to monitor the effects of 'tanning' processes on soluble collagen. Changes in thermal stability, expressed as the temperature coefficient of the helix to coil transition, could be estimated from the circular dichroism (CD) spectra. ^{13}C NMR spectroscopy was used to show that Cr(III) did indeed interact with the carboxyl groups of aspartic and glutamic acids.[9] Ideally one would like to directly observe the change in chemical shift, of a peak in the NMR spectrum, upon the addition of a metal ion to a collagen solution. With ^{13}C NMR spectroscopy, it was possible to observe the effects of Cr(III) on the conformation of a water-soluble collagen, but paramagnetic line broadening made it impossible to obtain information about the Cr-collagen complex.[9]

The aluminum-collagen interaction is attractive because although not a solo tanning agent, aluminum is thought to interact with collagen in a fashion similar to that of chromium.[12] Aluminum salts, in solution, are colorless and thus interfere less in optical spectroscopy. As an NMR probe, the advantages are that Al is not a paramagnetic species and that one isotope, ^{27}Al, is directly observable by NMR spectroscopy. The ^{27}Al spectral data can provide direct information about the Al-collagen complex. Spin lattice relaxation times (T_1's) of ^{27}Al species in solution are directly related to the motion of the Al-collagen complex and indirectly to the size of the complex.

EXPERIMENTAL

Materials

Pepsin-solubilized adult bovine skin collagen (BSC) was purchased from Cohesion (Palo Alto, CA) as Vitrogen 100, a sterile solution in 0.012 M HCl at a concentration of 3 mg/mL. Bovine tendon (BTC) and rat-tail tendon (RTC) collagen type I from Upstate Biotechnology (Waltham, MA) were supplied as sterile solutions in 0.02 M HAc at concentrations of 3 - 4 mg/mL. All collagen samples were dialyzed in 10,000 MW cutoff dialysis cassettes (Pierce, Rockford, IL) against 0.05 M HAc 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 (Beckman Coulter, Fullerton, CA) preparative ultracentrifuge at 25°C for one hour to sediment any large aggregates. The supernatant from this centrifugation contained the soluble collagen used in further experiments. Other chemicals were reagent grade.

Spectroscopy and thermal stability

Samples, 300 µL in volume, containing 1 - 2 mg/mL collagen were placed in 1 mm pathlength cuvettes. The cuvettes were made of far-ultraviolet transparent quartz, and

fitted with Teflon stoppers to prevent evaporation during melting experiments. The ultraviolet (UV) spectra of the samples were scanned (AVIV 14 Spectrophotometer, AVIV Biomedical, Lakewood, NJ) at ambient temperature from 300 nm to 190 nm against dialyzate. The concentration of collagen in solution was estimated from the absorbance at 218 nm using the molar absorptivity (ε = 883,129 cm^{-1}-L-$mole^{-1}$) determined by Na.[13] The stoppered cuvette containing the sample was then refrigerated at 4°C for at least 12 hours to maximize the formation of triple helical structure.

The thermal stability of the triple helix was determined by following the change in the CD signal at 223 nm (AVIV Model 215 Circular Dichroism Spectrometer, AVIV Biomedical, Lakewood, NJ) with increasing temperature. The instrument was programmed with a scan-melt-scan algorithm so that the sample was scanned at 10°C from 250 nm to 200 nm in 1 nm steps using a 2 sec time constant. Melting curves were obtained by recording the CD signal at 223 nm every 0.5 deg between 10°C and 50°C with a time constant of 10 sec and a heating rate of 3 deg per hour. A final scan of the sample at 50°C was made. The CD signal was read in mdeg and converted to molar ellipticity:

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

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. Pretransition and denaturation temperatures (Tp and Td) were obtained from the derivative of the melting curve.

Simulated tanning procedure

A four-step process modified from the data of Taylor and co-workers[14] was used to simulate a model-tanning system. The starting reaction mixture consisted of 1.0 - 1.5 mg collagen in 1 mL of 0.05 M HAc as the primary solvent and the 'tanning' steps described previously.[9] Briefly, for the second step, the collagen (BSC, RTC, or BTC) solution was acidified to pH 2 using microliter drops of 1 M H_2SO_4. In the third step, aluminum, in the form of a 20% $Al_2(SO_4)_3$ solution in water, was added to the acidified collagen at a 1:100 ratio. In the fourth step, the Al collagen mixture was slowly neutralized to pH 4 by the hourly addition of 5 µL aliquots of 0.4 M $NaHCO_3$. Neutralization was considered to be complete when pH 4 was stable for at least one hour. Spectroscopic analysis was carried out at each stage of the simulated tanning process. Solutions for spectroscopy were degassed under vacuum.

NMR spectroscopy

Samples for ^{13}C NMR were prepared with 16.67 mg collagen in 0.60 mL 0.012 N HCl. For studies of the Al-collagen complex, 1.67 mg of $Al_2(SO_4)_3$ hydrate was

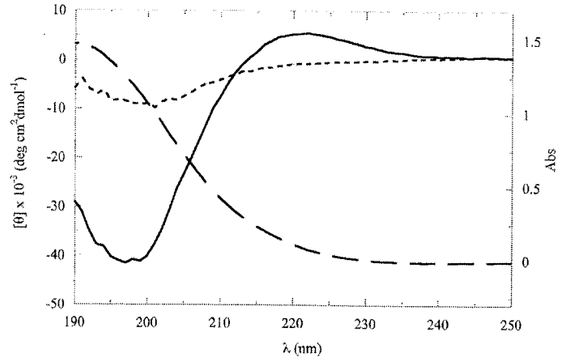

Figure 1. - UV absorbance (- - -), CD (—) spectra at 10°C and (— —) at 50°C of soluble calfskin collagen in 0.05 M HAc.

added to form a 1:10 Al:collagen wt:wt mixture; a second equal addition of $Al_2(SO_4)_3$ hydrate produced a 1:5 mixture. The pH of NMR samples was estimated to be in the 2.5 - 3.5 range, an exact value could not be determined in the H_2O - D_2O solvent mixture. All ^{13}C NMR spectra were obtained on either a Varian (Varian Corp., Palo Alto, CA) Gemini-200 or Varian Unity + 400 NMR spectrometer operating at 50 or 100 MHz, respectively. In a typical NMR experiment 20,000 to 60,000 (8K data points) transients were collected using a 90° ^{13}C pulse (23.5 µsec with the Gemini-200 and 10.5 µsec with the Unity + 400 spectrometer) and a 1s recycle time, proton decoupling was achieved using WALTZ-16 proton decoupling. Tetramethyl silane (TMS) was used as the instrument standard, and the

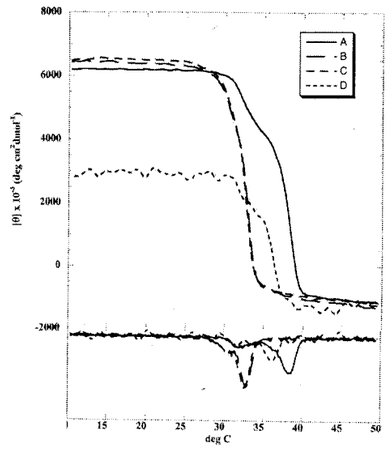

Figure 2. - Melting curves (A - D) for soluble collagen under conditions of simulated tanning. Sample conditions for the melting curves were as follows: A - collagen in 0.05 M HAc at pH4.3; B - sample A after acidification to pH 2 with H_2SO_4; C - sample B after addition of $Al_2(SO_4)_3$; and D - sample C after neutralization to pH 4 with $NaHCO_3$. Upper curves - Molar ellipticity at 223 nm recorded every 0.5°C over the 10°C to 50°C range with a time constant of 10 sec and a 3 deg/hr heating rate. Lower curves - First derivative plots of the melting curves.

TABLE I
Effects of Aluminum on Thermal Transitions in Collagen

Collagen	Tp	Td	Tp area	Td area
Collagen	34.2 ± 0.7	40.2 ± 0.7	1500 ± 60	4300 ± 100
pH 2		33.0 ± 0.7		5700 ± 90
pH 2 + Al		33.0 ± 0.7		5700 ± 150
Neutralized	33.7 ± 0.3	37.9 ± 0.5	700 ± 300	800 ± 700

C_4 (C-OH) peak of hydroxyproline as the internal chemical shift standard (71.3 ppm from TMS). Chemical shifts are reported as ppm from TMS. Samples for ^{27}Al NMR were the 1:5 Al:collagen complex from ^{13}C NMR studies and an $Al_2(SO_4)_3$ solution without collagen. The ^{27}Al NMR spectra were obtained on the Unity + 400 NMR spectrometer operating at 104.7 MHz. Quartz NMR tubes were used to decrease the aluminum background peak in the ^{27}Al spectrum. In a typical experiment, 16 to 128 (8K data points) transients were collected using a 90° ^{27}Al pulse (8.15 µsec) and a 1s recycle time. The ^{27}Al T_1 data were obtained using an inversion-recovery pulse sequence (180° - *tau* -90° - *acq*). Each ^{27}Al T_1 experiment was repeated 10 times at each temperature and the standard deviations for the ^{27}Al T_1's were less than 2.5%. All NMR data were analyzed off-line using the NUTS 2D program (Acorn NMR Inc., Livermore, CA). Line widths of the ^{27}Al peaks were determined from the fitting of the spectral data using the line fit sub-routine. The ^{27}Al T_1's were calculated from the inversion-recovery experiment using the Varian software package.

RESULTS

Spectroscopy and thermal stability

Figure 1 shows the absorbance spectrum at ambient temperature and the circular dichroism (CD) spectra at 10°C and at 50°C of a typical soluble collagen sample at pH 4.3 in 0.05 M HAc. The absence of tryptophan residues and the low levels of tyrosine give the near-UV region of the absorbance spectrum a flat appearance. In the concentration range used for this study, 0.5 to 3.0 mg/mL, with short pathlength (1 mm) far-UV quartz cuvettes the relationship between absorbance and concentration was linear. A positive band around 223 nm and a stronger negative band at 198 nm characterize CD spectra of triple helical collagen. These features are best observed with solutions that have been cooled to 10°C or lower for several hours. At higher temperatures, the helical conformation is lost and the CD spectrum becomes relatively featureless above 45°C.

The apparent melting curve obtained by recording the CD signal at 223 nm as a function of temperature between 10°C and 50°C gives an indication of the stability of the helical conformation in collagen. In an earlier study[9] we attempted to monitor both the 223 nm and 198 nm bands and to use the absolute value of the difference in magnitude between them as a measure of the percent helical structure. However, the CD signal becomes less reliable in the far-UV where baseline absorbance contributes to higher noise levels. In the present study we focus on the 223 nm band where noise levels are inherently lower, fewer substances absorb significantly, and higher concentrations of collagen can be studied.

The relative flatness of the melting profile (Figure 2, Curve A) at temperatures below 25°C and above 45°C suggest that collagen in dilute solution is essentially native below 25°C and denatured at temperatures above 45°C. The helix-to-coil transition of bovine skin collagen in slightly acidic solutions (pH 3 - 5) is characterized as a two-step process, a pretransition (Tp) followed by complete denaturation (Td). The two-step nature of the melting is clearly seen in the melting profile (Figure 2, Curve A). As the acidity of the collagen solution is increased to pH 1 - 2, Td approaches Tp, and a single transition is seen. The temperatures associated with Tp and Td were obtained from the derivatives of the melting curves (Figure 2, Table I). For the various collagen samples used in this study, Tp = 34.2°C (± 0.7°C) and Td = 40.2°C (± 0.7°C) under conditions that favor a native conformation. These values are in excellent agreement with values determined calorimetrically.[15]

Simulated tanning

Figures 2 shows melting curves and their derivatives, respectively, for BSC as affected by the simulated tanning process using $Al_2(SO_4)_3$. Curve A representing native collagen at pH 4.3 clearly shows the two step melting transition. Acidification of the collagen solution to pH 2 (Curve B) had little effect on the ability to form a triple helix at low temperature. The thermal stability of this helix was, however, significantly destabilized. The pretransition represented by Tp disappeared completely, and denaturation temperature Td was reduced to 33.0°C (± 0.7°C). The addition of $Al_2(SO_4)_3$ at pH 2 (Curve C) had no effect on the conformation, or the conformational stability of the collagen helix, in agreement with our previous chrome

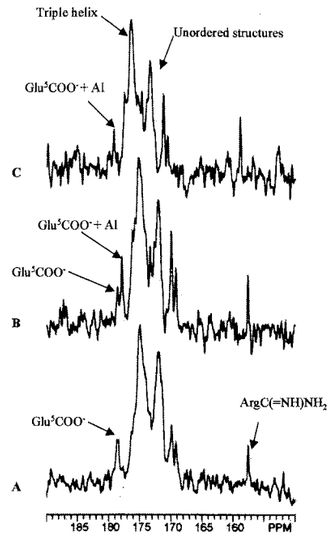

Figure 3. - ^{13}C NMR spectra of the region 190 to 150 ppm of (A) collagen (2.9 x 10-4 M), (B) collagen plus 4.89 x 10^{-6} moles $Al_2(SO_4)_3 \cdot 18H_2O$ and (C) collagen plus 1.02 x 10^{-5} moles $Al_2(SO_4)_3 \cdot 18H_2O$. All spectra were obtained on a Varian Gemini-200 at 40°C. The chemical shift scale is given as ppm from TMS.

tanning results.[9] When the BSC - $Al_2(SO_4)_3$ mixture was neutralized to pH 4.5 by the slow stepwise addition of $NaHCO_3$, a two phase melting curve (Curve D) was obtained, with slightly lower transition temperatures Tp = 33.7°C (± 0.3°C) and Td = 37.9°C (± 0.5°C) than were observed for the collagen before addition of H_2SO_4 and $Al_2(SO_4)_3$. Without the addition of a tanning species, the process was completely reversible. When BSC was acidified and then neutralized as described above without the addition of $Al_2(SO_4)_3$, the final CD spectrum (not shown) was identical with the initial spectrum (Tp = 35°C, Td = 40°C).

The relative areas of the peaks in the derivative curves (Figure 2) were calculated and are presented in Table I. These are dimensionless numbers, but because the melting profiles (Figure 2) are presented in degrees ellipticity per mole, based on the triple helical collagen molecule, the areas under the peaks in the derivative curve are proportional to the heat input required for denaturation of the triple helix. The sum of the areas associated with Tp and Td for collagen at pH 4.3 was essentially the same as the areas associated with the single peaks under more acidic conditions. In contrast, the sum of the areas associated with Tp and Td for the Al collagen complex at pH 4.3 was considerably less. The initial helical content of the Al complexed collagen, curve D, is reduced about 50% from that of the samples represented by Curves A - C. This suggests that the Al-collagen interactions partially disrupt the helix. However, the remaining helix behaves much like helices that are not complexed by a tanning agent. The Al-complexed collagen is a clear, rather viscous material, not quite a gel.

^{13}C NMR

The carbonyl region of the ^{13}C NMR spectrum of collagen is shown in Figure 3. Based on NMR assignments for carboxyl groups in proteins by Tollinger et al.,[16] the peak at 179.2 ppm in the collagen spectrum (Figure 3-A) was assigned to Glu^5COO^-. With the addition of $Al_2(SO_4)_3$ to the collagen at a 1:10 wt:wt ratio (Figure 3-B), the Glu^5COO^- peak was chemical-shifted upfield to 178.5 ppm. In addition, this peak was split into two component peaks, the more upfield component assigned to a Glu-$Al_2(SO_4)_3$ complex and the lower field component to glutamic acid residues that were influenced by the presence of $Al_2(SO_4)_3$, but not complexed with it. The line widths for these two carboxyl peaks were identical and approximately the same as the line width observed for carboxyl peaks in the ^{13}C NMR spectrum of collagen. The carboxyl peaks, in the spectrum with added aluminum, do not appear to be exchange-broadened as was reported for the Glu peak of a nonapeptide fragment of collagen when $Al(NO_3)_3$ was added.[17] After a second equal addition of $Al_2(SO_4)_3$ to produce a 1:5 Al-collagen mixture (Figure 3-C), the upfield shift was complete, and the single peak at 178.5 ppm was assigned to the Glu-$Al_2(SO_4)_3$ complex. These aluminum-influenced upfield shifts of carboxyl carbon peaks are consistent with those reported for both Al-oxalic acid[18] and Al-Leu^5-enkephalin complexes.[19]

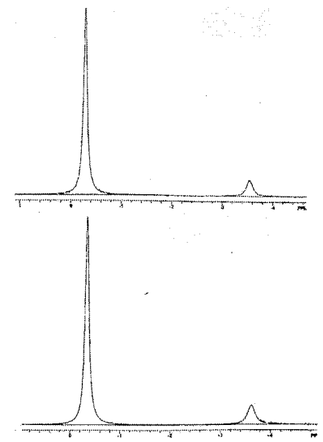

Figure 4. - ^{27}Al NMR spectra of (a) $Al_2(SO_4)_3$ in D_2O and (b) collagen plus 1.02 × 10^{-5} moles $Al_2(SO_4)_3 \cdot 18H_2O$. Both spectra were obtained on a Varian Unity + 400 NMR spectrometer at 30°C.

TABLE II
Effects of Collagen on ^{27}Al NMR Spectrum of $Al_2(SO_4)_3$

	$Al_2(SO_4)_3$			Collagen plus $Al_2(SO_4)_3$		
	Line widths (Hz)[a]		Peak ratio[b]	Line widths (Hz)[a]		Peak ratio[b]
Temp °C	Peak 1c	Peak 2d	P1/P2	Peak 1[c]	Peak 2[d]	P1/P2
10	7.89	13.57	17.8	7.86	10.38	20.9
20	11.89	21.14	7.8	7.65	12.9	10.5
30	9.35	18.70	5.25	8.83	16.39	6.5
40	11.18	33.32	2.9	11.82	27.48	3.8
50	21.13	57.00	1.8	21.6	46.47	2.6
60	52.22	105.49	1.2	ND[e]	ND[e]	

[a]All line widths were determined from the simulation of the ^{27}Al NMR spectrum.
[b]All peak areas were determined from the simulation of the ^{27}Al NMR spectrum.
[c]The chemical shift of this peak was 0.0 ppm and the peak was assigned to $[Al(H_2O)_6]^{3+}$.
[d]The chemical shift of this peak was -3.3 ppm and the peak was assigned to $[Al(H_2O)_5(SO_4)]^+$.
[e] Not determined.

Direct observation of the effects of $Al_2(SO_4)_3$ on the Asp^4COO^- groups in collagen was not possible because the Asp^4COO^- peak is buried under the broad peak at 174.7 ppm that is assigned to the collagen triple helix. The splitting (Figure 3 - B and C) between the peaks at 174.7 ppm and 172.0 ppm that is assigned to non-helical portions of the collagen backbone,[20] in the presence of $Al_2(SO_4)_3$ may be indicative of an interaction between aluminum and Asp^4COO^-. The only other peak that can be readily assigned in the ^{13}C NMR spectra is the peak at 157.7 ppm assigned to $C(=NH)NH_2$ of arginine.[17,21] An estimate of the percent helix was made by determining the area under the two peaks at 174.7 and 172 ppm. From the fit of the spectral data it was estimated that 60% of the sample had a triple helical conformation; this estimate did not change in the temperature range (29°C - 45°C) of the ^{13}C NMR experiments. NMR experiments require a much higher concentration of collagen than CD experiments (10 mg/mL vs. 1 mg/mL), they were done at low pH to assure the solubility of the concentrated collagen. At the higher concentrations of collagen, external, helix-to-helix bonds stabilize the helix to a greater degree than occurs in a very dilute solution.[22] Under the conditions typical in NMR experiments Consonni *et al.*[23] observed the triple helix in the range from 0 to 47°C.

^{27}Al NMR

^{27}Al NMR spectroscopy was used to observe the effects of Al-collagen interactions on the aluminum species. In an aqueous solution, $Al_2(SO_4)_3$ dissociates to $[Al(H_2O)_6]^{3+}$ and $[Al(H_2O)_5(SO_4)]^+$, with a sulfate ion replacing one of the inner sphere waters to form the inner sphere sulfate anion complex. It was thought that perhaps a carboxylate group on collagen might compete with the sulfate ion in the formation of an inner sphere complex. The ^{27}Al NMR spectra (Figure 4) of Al-collagen and $Al_2(SO_4)_3$ without collagen were nearly identical. The NMR spectra contained only two peaks and no other ^{27}Al species were observed even when the sweep width was increased and the base line of the spectrum was carefully examined. The downfield peak (0.0 ppm) is assigned to $[Al(H_2O)_6]^{3+}$ and the upfield (-3.3ppm) peak to $[Al(H_2O)_5(SO_4)]^+$.

$$SO_4^{2-} + [Al(H_2O)_6]^{3+} \rightleftharpoons [Al(H_2O)_5(SO_4)]^+ + H_2O$$

These species are present in equilibrium, with the direction of the equilibrium being dependent on pH, temperature, and

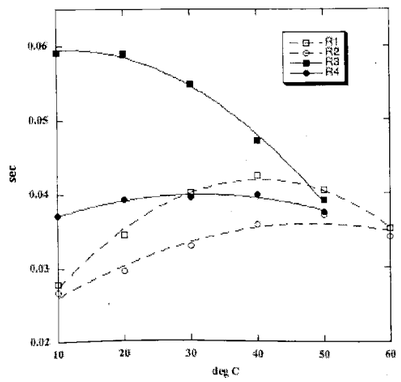

Figure 5. - Plots of relaxation times T1 vs. temperature for $Al_2(SO_4)_3$ with and without collagen. R1. $[Al(H_2O)_6]^{3+}$[aqueous $Al_2(SO_4)_3$], R2. $[Al(H_2O)_5(SO_4)]^+$ [aqueous $Al_2(SO_4)_3$], R3. $[Al(H_2O)_6]^{3+}$ in the presence of collagen, and R4 $[Al(H_2O)_5(SO_4)]^+$ in the presence of collagen.

Figure 6. - The gap region, between arrows, of the ERRC collagen microfibril model anchored by short segments of the adjoining overlap regions. The peptide backbone is represented by single strand ribbons, acidic sidechains (glutamic and aspartic acids) are represented by dark balls and basic sidechains (lysine and arginine) by light colored balls.

the concentrations of $Al_2(SO_4)_3$ and $(SO_4)^{2-}$. In addition, the temperature dependence of the rate of exchange is represented by the change in ^{27}Al line widths (Table II). The line widths of the peaks are almost identical for both spectra shown in Figure 4, and they were not easily measured. The spectra were simulated and the simulation yielded the line widths that are given in Table II. An increase in temperature shifts the equilibrium to the right and increases the exchange rate.[24] The line widths for the peaks in the ^{27}Al NMR spectrum of Al collagen were narrower than those observed in the corresponding spectrum of $Al_2(SO_4)_3$ without collagen, indicating that the exchange rate was slower in the presence of collagen. The line widths of both Al^{3+} species in the presence or absence of collagen increased with increasing temperature, indicating more rapid exchange at higher temperatures. The presence of collagen also caused the equilibrium to shift to the left, indicating a greater concentration of $[Al(H_2O)_6]^{3+}$, suggesting that some of the sulfate groups may associate directly with the collagen.

The spin lattice relaxation times (T_1's) of the ^{27}Al species present in solution can provide information about the motion of the complex in solution and indirectly about the size of the complex. The relationship between T_1 and the rotational correlation time τ_2 is given by:

$$R_1 = R_2 = \frac{3}{40}\frac{2I+3}{I^2(2I-1)}\left(\frac{e^2Qq}{\hbar}\right)^2\tau_2$$

Where $R_1 = 1/T_1$, $R_2 = 1/T_2$. I is the nuclear spin (5/2 for ^{27}Al) and $\left(\frac{e^2Qq}{\hbar}\right)^2$ is the quadrupole coupling constant.[25]

Because of the relationship between T_1 and T_2, it is possible, in principle, to obtain information about the correlation time from T_1. In these experiments, T_1's were measured directly and plotted against temperature (see Figure 5). The individual plots are convex, not linear, which suggests that two relaxation mechanisms are occurring. One mechanism is likely to be the tumbling of the complex aluminum ion in solution and the other, the exchange of inner sphere water with a SO_4^{2-}. The presence of collagen perturbs the relaxation times. The relaxation time of $[Al(H_2O)_5(SO_4)]^+$ in the presence of collagen is longer than the relaxation time of $[Al(H_2O)_5(SO_4)]^+$ in aqueous solution without collagen. In the temperature range of 10°C to 30°C, the curves are nearly parallel, but at temperatures greater than 30°C the relaxation times of $[Al(H_2O)_5(SO_4)]^+$ in a collagen solution tend to approach those of $[Al(H_2O)_5(SO_4)]^+$ in aqueous solution. It is likely that $[Al(H_2O)_5(SO_4)]^+$ forms a weak complex with collagen at lower temperatures and that this complex is not stable at temperatures greater than 30°C. On the other hand, $[Al(H_2O)_6]^{3+}$ forms a much stronger complex that is more heat stable than the $[Al(H_2O)_5(SO_4)]^+$ collagen complex. From the T_1 data, it would appear that the $[Al(H_2O)_6]^{3+}$ collagen complex is not stable at 50°C.

Under neutral conditions, the collagen helix is stabilized by salt bridges.[26] At low pH, the glutamic and aspartic acid sidechains are protonated and the salt bridges are disrupted. As the pH is raised, these sidechains become ionized and salts of Al may act as counter ions. Figure 6 shows the distribution of acidic and basic amino acid side chains in the vicinity of the gap region of microfibrillar collagen. These sidechains may participate in salt bridges or be sites for the binding of counter ions.

DISCUSSION

The thermal behavior of fibrillar collagen is complex and not thoroughly understood despite numerous studies over at least the past thirty years. The triple helical structure of collagen is well established from X-ray diffraction patterns of solid samples. Nevertheless, the conformation is unstable at room temperature in dilute solutions.[27] When a dilute solution of native collagen at pH 4-5 is refrigerated at < 10°C for several hours, the primarily triple helical conformation is stabilized.[9] When a solution of triple helical collagen is heated, the collagen denatures to a less ordered conformation. The denaturation or helix-to-coil transition of collagen can be followed by monitoring the CD signal at a single wavelength (223 nm) to generate a melting profile. Ranges of thermal behaviors have been reported for acid-soluble collagens using calorimetric and spectroscopic methods. The work of Komsa-Penkova *et al.*[15] clearly showed that the thermal denaturation characteristics of soluble collagen were dependent on protein concentration, heating rate, and pH as well as the effects of added salts. By raising the temperature at a rate no greater than 4°C per hour, one can approximate the rate of unfolding and minimize the effect of time dependence on melting temperature.

Although it has sometimes been convenient to treat the melting of collagen as a single-phase transition, implying that a molecule is either in the triple helical structure or is

separated as a single strand in an unordered conformation, in reality, one might expect the path from triple helix to single unordered strand to involve multiple steps. The derivatives of the melting curves clearly suggest a pretransition (Tp) followed by a more complete denaturation (Td) for collagen at about 1 mg/mL in a dilute HAc solution. The areas under the two peaks suggest that about one-fourth of the energy required for denaturation is consumed in the pretransition step. Under more acidic conditions, the single step denaturation occurs at a temperature closer to that of the native collagen pretransition, and requires about the same energy as the two-step denaturation of native collagen. As was seen in our earlier study[9] using a Cr(III) sulfate complex, the addition of a mineral ion complex at low pH, followed by neutralization resulted in a lower degree of helicity before the start of heating and less fine structure observable in the melting profile. This profile does hint at the two-step transition seen for native collagen, but in the case of the Al collagen, Tp and Td were at slightly lower temperatures.

The dimensional stability of collagen is closely related to its organized molecular structure, and results from the interplay of electrostatic, hydrophobic, and van der Waals interactions in addition to hydrogen and covalent bonds. Although it is not clear at this time which factors contribute to the two-step melting of native collagen, the agreement in the peak area calculations between collagen at pH 4.3 and at low pH suggests that the acidic conditions serve to facilitate the denaturation of collagen in solution at a concentration of 1 - 2 mg/mL. The presence of electrostatic interactions may well contribute to the pretransition at pH 4 - 5. At higher acidity (pH 1 - 2) in low concentrations of collagen, the exposure of side chains to solvent would disrupt salt bridges as did the addition of neutral salts to serve as counter ions.[10] The differences in shape between the melting profiles of collagen at pH 4 -5 and the Al-collagen complex under the same conditions suggest that perhaps when the pH is raised, the aluminum ions serve as counter ions for carboxyl groups on collagen and interfere with the complete recovery of helical structure. The ^{13}C and ^{27}Al NMR data were collected at a collagen concentration of 16 mg/mL. At this concentration, one may expect a higher degree of self association of the collagen molecules as evidenced by greater helical stability. The estimated 60% helix appeared to be stable in the (29°C to 45°C) temperature range used for ^{13}C NMR, even when $Al_2(SO_4)_3$ was added at low pH. This result is consistent with the report from calorimetric studies that soluble collagen at 20 mg/mL had a single melting transition in the 45°C to 50°C range.[27] For intact fibrillar collagens, multiple thermal transitions at temperatures between 50°C and 110°C have been reported.[28,29] Underlying the denaturation of the collagen fibril are the thermal characteristics of individual collagen molecules.

The hydrothermal stability of a manufactured biomaterial such as leather will reflect the stability of collagen structures at the molecular level as well as at several levels of supramolecular structure. The hydrothermal stability in solution or in a fibrous matrix can be influenced by a variety of chemical or physical modifications to collagen. Hydrated aluminum sulfate has been shown to interact with collagen in solution. The ^{13}C NMR data suggest that the interaction may be preferentially at the glutamate side chains. Melting curves of Al-tanned collagen in dilute solution suggest that the Al-collagen interaction has a slight destabilizing effect on the collagen helix at 30°C. ^{27}Al NMR data also confirm dissociation of the complex at temperatures above 30°C. Another recent report[30] demonstrated that the treatment of soluble collagen with aluminum sulfate, complexed with citrate as a masking agent, decreased the viscosity of the collagen solution, particularly above 30°C.

SUMMARY

The CD spectrum of dilute (1 mg/mL) soluble collagen at pH 2 was not altered by the addition of an aluminum sulfate/acetate complex. When this solution was neutralized to pH 4, the apparent helical content of the collagen was reduced by about 50%, and the thermal stability of the remaining helix was decreased from 35°C to 33°C. At higher collagen concentration (~28 mg/mL), the effect of collagen on the ^{27}Al NMR spectrum confirms the formation of an Al collagen complex which becomes unstable above 30°C. The data reported here provide a partial explanation for aluminum's poor performance as a solo tannage, and its value in combination tannages.

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