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EFFECTS OF NEUTRAL SALTS ON COLLAGEN STRUCTURE AND CHROMIUM-COLLAGEN INTERACTIONS*

by

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ABSTRACT

Tanning, the multi-step process whereby complex salts of Cr(III) crosslink collagen fibrils, is more an art form than a science and the mechanism of the chromium-collagen interaction is poorly understood. The long range goal of this research is to develop an experimental model with which to study the interactions of Cr(III) with collagen that are characteristic of tanning. In the present study, the thermal stability of acid soluble collagen from bovine skin is studied by circular dichroism spectropolarimetry. The melting curve for the triple helix of calf skin collagen in 0.05 M acetic acid was analyzed in terms of a pre-transition followed by complete denaturation, with a characteristic Tp = 34.0°C and Td = 40.7°C. For pepsin solubilized, adult bovine dermal collagen, slightly lower denaturation temperatures were observed — Tp = 31.0°C, Td = 37.7°C probably due to the removal of the nonhelical telopeptides in the solubilization of adult skin collagen. The addition of salts, NaCl or KCl, initially had a destabilizing effect on the triple helix, lowering both Tp and Td nearly in parallel. At low concentrations of NaCl, preferential binding occurred with apparent dissociation constants Kd(Tp) = 0.034 M and Kd(Td) = 0.017 M for calf skin collagen at 1 mg/mL in 0.05 M acetic acid, suggesting the possibility of site preferential interactions contributing to the observed melting behavior. As the salt concentration was increased between 0.1 M and 0.5 M, little further destabilization was observed, but Tp and Td became indistinguishable. At 0.4 M NaCl (0.5 M KCl) the samples began to gel. Here, water-structuring effects may play a role. In contrast, under conditions of the model tanning system, where Cr(III) was added at pH 2 and the pH was then adjusted to 4.5 with bicarbonate, the 'tanned' collagen gelled at a salt concentration of 0.1 M.

INTRODUCTION

Chrome tanning has a long history and has been empirically optimized to 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. The long range goal of this research is to develop an experimental model for observing at the molecular level the interactions of Cr(III) with collagen that are characteristic of tanning. Previously, using a very simplified model tanning system based on soluble collagen in dilute acetic acid, we showed that partial denaturation of collagen was necessary prior to crosslinking with Cr(III).[1]

Individual chains of the fibril-forming collagens, in particular types I and III, the major protein components of bovine hide, have short nonhelical regions, telopeptides, at either end of a tripeptide repeat (Gly-X-Y) that is about 1000 amino acid residues in length. In this tripeptide repeat, 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

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the center of the triple helix with the X and Y residues directed outward from the helix.[2] Individual chains have a left handed twist with three residues per turn. Three chains wrap around each other to form a right handed supercoiled triple helix. In the formation of this 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 with the X and Y side chains on the surface. Several characteristics of the primary structure may contribute to the stability of collagen at various levels. Proline and hydroxyproline side chains in the X and Y positions of the tripeptide limit the flexibility of the polypeptide chain. The nonrandom distribution of ionizable and hydrophobic side chains along the repeating unit results in the occurrence of charged and hydrophobic patches on the surface that contribute to stabilization of higher order structures through electrostatic and hydrophobic interactions.[2] Naturally occurring telopeptide-based bi- or multifunctional crosslinks contribute to fibrillar stability.[3]

In chrome tanning, the collagen fibrils of a hide become crosslinked by coordinate bonds between bi- or polynuclear Cr(III) ions and carboxylate side chains of the protein.[4,5] Basic chromium sulfates, which form polynuclear complexes, are the basis for commonly used tanning agents. In an earlier study, we proposed a model tanning system that used $KCr(SO_4)_2$ and soluble collagen in dilute acetic acid. In this low ionic strength environment, we demonstrated that partial destabilization of the collagen triple helix was necessary prior to crosslinking with Cr(III).[1]

In the present study, the proposed model tanning system is expanded to include neutral salts such as are used in typical pretanning processes. The curing of hides with salt (NaCl) has been the traditional method for preserving hides prior to tanning. Because of the problems associated with the disposal of large quantities of effluent containing sodium chloride, a procedure for curing hides with potassium chloride was recently developed.[6] Potassium chloride is a plant macro-nutrient; thus the effluent from KCl curing of hides could be applied directly to agricultural land. In the refinement of the proposed model tanning system, the effects of NaCl and KCl on the stability of the collagen triple helix are evaluated.

EXPERIMENTAL

Materials

To establish a base line for the spectropolarimetric description of the triple helix and its thermal stability, the calf skin collagen (CSC) used in our earlier study[1] was compared with Vitrogen 100 (Collagen Corporation, Palo Alto, CA). Vitrogen is a purified, pepsin-solubilized adult bovine dermal collagen (BDC) and was supplied as a sterile solution in 0.012 M HCl at a concentration of 3 mg/mL. Other chemicals were reagent grade. Stock solutions were prepared in 0.05 M acetic acid.

Spectroscopy

Samples, 300 µL in volume, containing less than 3 mg/mL collagen were placed in 1 mm pathlength cuvettes. The cuvettes were made of far-ultraviolet (far-uv) transparent quartz, and fitted with Teflon stoppers to prevent evaporation during the melting experiments. The uv spectrum of the sample was scanned (AVIV 14 Spectrophotometer, AVIV Associates, Lakewood, NJ) at ambient temperature from 300 nm to 190 nm against that of a reference solution containing all components except the collagen. The concentration of collagen in solution was estimated from the absorbance at 218 nm using the molar absorption coefficient (ε = 883,129 cm^{-1}-L-$mole^{-1}$) determined by Na.[7] The stoppered cuvette containing the sample was then refrigerated at 4°C for at least 12 hours.

The sample compartment of the AVIV 60DS Circular Dichroism Spectropolarimeter (AVIV Associates, Lakewood, NJ) contains an aluminum block through which circulating water may be pumped. The block is designed so that the cuvette containing the sample is positioned properly in the light beam and at the same time is largely surrounded by the temperature controlled metal block. A circulating water bath is connected to the sample block and the temperature of the bath is programmed from the spectropolarimeter in response to signals from a thermistor placed in the block.

CD studies of collagen were done in the following way. After equilibration of the sample block at 10°C, the cuvette containing the sample was quickly transferred from the refrigerator to the instrument. An initial scan of the sample between 300 nm and 185 nm was made to determine the appropriate instrument settings to provide useful data for that sample. The instrument was then programmed with a scan-melt-scan algorithm so that the sample was scanned at 10°C from 250 nm to 215 nm in 1 nm steps using a 2 sec time constant. Melting curves were obtained by recording the CD signal at 223 nm every 0.2 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:[8]

$[\theta]_\lambda = \theta_\lambda / ncd$ deg cm^2 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 first derivative of the melting curve.

Simulated Tanning Procedure

A four step process modified from the data of Taylor and co-workers[9] was used to simulate a model tanning system. The collagen was first dissolved in 0.05 M acetic acid at pH 4 containing the appropriate concentration of neutral salt. Second, the solution was acidified to pH 2 by the dropwise addition of 1 M H_2SO_4. Third, chromium, in the form of a 10% $KCr(SO_4)_2$ 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 aliquots of 0.4 M $NaHCO_3$. 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.

Results

The triple helix to random coil transition in soluble collagen is a temperature and time dependent process that can be monitored by several physical techniques, including calorimetry[9] and spectropolarimetry (CD).[1,2] 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 triple helical conformation in collagen.

Characterization of Soluble Collagen

The CD spectrum of triple helical collagen is characterized by a positive band around 223 nm and a much stronger negative band at 198 nm. In a previous paper,[1] we attempted to monitor both bands and use the absolute value of the difference in magnitude between them as a measure of the amount of triple helical structure. The noise level on the CD signal increases dramatically as the far-uv limit of the spectropolarimeter optics (185 nm) is approached. Also, at low wavelength substances such as salts that are not optically active, but do absorb light, contribute to higher noise levels. The decision was thus made to focus on the 223 nm band where noise levels are inherently lower, fewer substances absorb significantly, and higher concentrations of collagen may be studied. In order to establish a baseline for the description of the triple helical content of collagen in terms of the 223 nm CD signal spectra were obtained for each of the collagens at several concentrations in the 0.5 - 2 mg/mL range at 10°C and at 50°C. Figure 1a shows the melting curves for the BDC preparation at concentrations of 0.8, 1.0, and 1.5 mg/mL collagen in 0.05 M acetic acid. Although there is a small variation in the maximum ellipticity, the flatness of the curves below 20°C and above 40°C suggests that these regions of the curve represent two conformational extremes, completely folded and completely unfolded protein. From 16 separate determinations using BDC and CSC, at 10°C, the average for 100% helix was $[\theta]_{223nm} = 5600$ (± 400) deg cm^2 dmol^{-1} and at 50°C for 0% helix it was $[\theta]_{223nm} = -1400$ (± 150) deg cm^2 dmol^{-1}. Although a small, concentration dependence to the maximum ellipticity can be seen in Figure 1a, the magnitude of the dependence was within the limits of experimental variation.

On the other hand, the shape of the curve between 25°C and 40°C is more dependent on the collagen concentration. The helix to coil transition of bovine skin collagen in dilute acidic solution is characterized as a two step process, a predenaturation transition followed by a more complete denaturation. At low collagen concentrations the two step nature of the melting is clearly seen in the melting profile (Figure 1a). As the concentration of collagen is increased above 1 mg/mL, less detail can be seen in the melting curve. The temperatures associated with the pretransition (Tp) and denaturation (Td) were obtained from the first derivative of the melting curve (Figure 1b). These temperatures were nearly constant as the concentration of collagen was increased in the range between 0.5 and 2.0 mg/mL. For BDC, Tp was 31°C (± 0.6°C) and Td was 37.7°C (± 1.1°C) over the 0.8 - 1.6 mg/mL concentration range. For CSC, Tp was 34°C (± 0.1°C) and Td was 40.7°C (± 0.4°C) over a 1 to 2 mg/mL concentration range, in excellent agreement with Tp = 35°C (± 0.5°C) and Td = 40.3°C (± 1.0°C) determined calorimetrically over a 0.25 - 1.75 mg/mL concentration range for calf skin collagen in a similar solution.[10] It seems likely that the larger differences observed between the calf skin collagens and BDC arise from differences in the preparations of the protein. Although both Tp and Td for BDC were 2 - 3°C lower than for the calf skin collagens, the material behaved otherwise the same, and because a larger supply of a single batch of BDC was available, it was used for much of this work.

Effects of Salts

At a collagen concentration of 1 mg/mL in 0.05 M acetic acid, the addition of salt, NaCl or KCl, at a level of

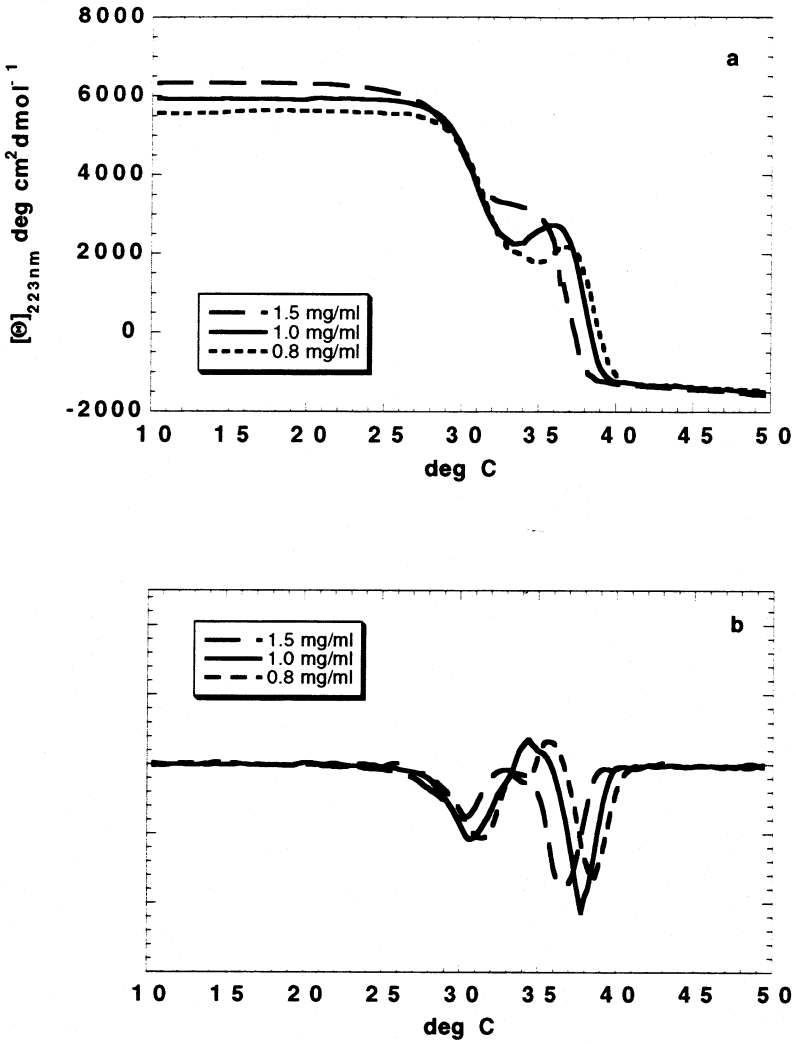

FIGURE 1. — Effect of protein concentration on melting curve of BDC in 0.05M acetic acid. a. Molar ellipticity at 223 nm recorded every 0.2°C over the 10°C to 50°C range with a time constant of 10 sec and a 3 deg/hr heating rate. b. First derivative plots of the melting curves.

0.017 M caused a drop of about 3 deg in both Tp and Td, but essentially no change in the shape of the melting curve (Figure 2a). Figure 2b is a plot of Tp and Td for CSC at 1 mg/mL as a function of salt concentration from 0 to 0.2 M NaCl; this plot has the appearance of an inverted kinetics curve. Using the kinetics analogy, apparent dissociation constants Kd = 0.017 M for complete denaturation and Kd = 0.034 M for the pretransition were determined.

Changes in the shape of the melting curve for 1 mg/mL BDC in 0.05 M acetic acid became apparent when the salt concentration was between 0.1 M and 0.5 M KCl (Figure 3a,b). At salt concentrations less than 0.4 M identical results (not shown) were obtained with NaCl. As the salt concentration was increased above 0.3 M NaCl or 0.4 M KCl, A_{218nm} decreased and the viscosity of the sample increased dramatically. In 0.4 M NaCl, only Td could be discerned, and in 0.5 M NaCl, the sample formed a gel in the cuvette. Although some melting occurred above 30°C, the behavior was in no way comparable to samples that remained fluid. Analysis of the first derivative curves for the melting of BDC (1 mg/mL in 0.05 M acetic acid) in increasing concentrations of KCl (Figure 3b), showed a decrease of about 7°C in Td as the concentration of KCl was increased from 0 to 0.5 M. At KCl concentrations up to 0.4 M, the pretransition temperature Tp decreased less than 2°C, but above 0.4 M the pretransition disappeared entirely. The change in the shape of the melting curve, particularly the derivative curve, could easily be seen as the KCl concentration was increased. In contrast to the effect of increasing collagen concentration, no shift in relative area from Tp to Td was observed until the pretransition vanished above 0.4 M KCl or 0.3 M NaCl.

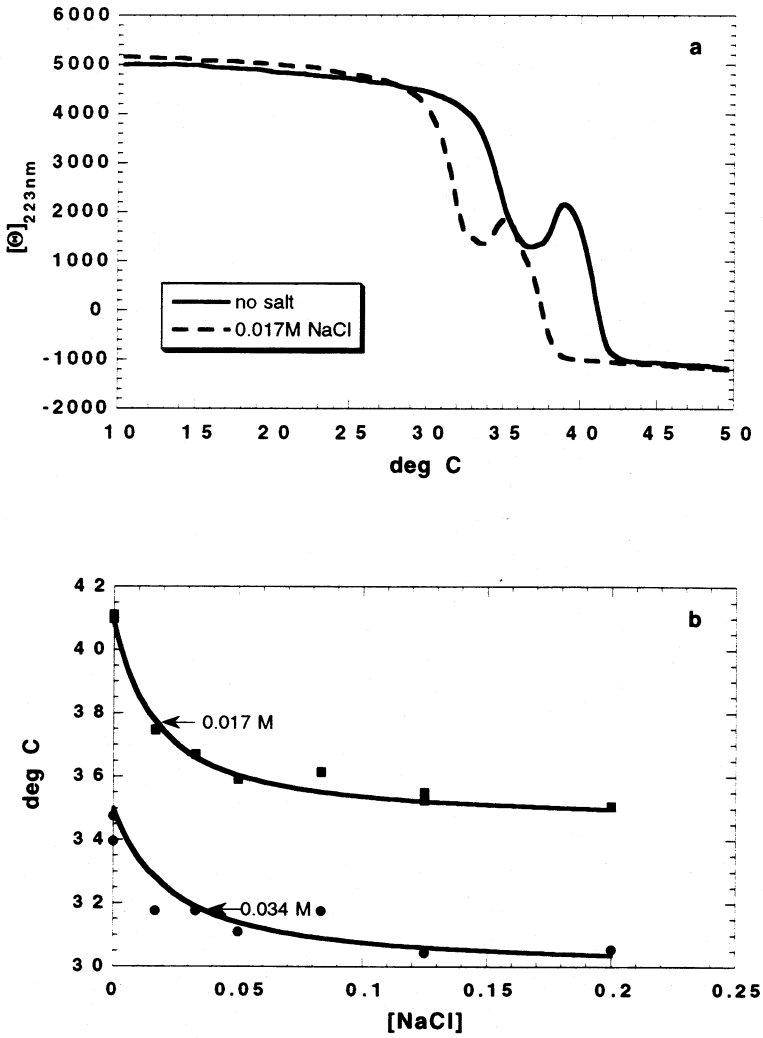

FIGURE 2. — Effect of low concentrations of salt on thermal stability of collagen. a. Solid line represents the melting curve for collagen at 1 mg/mL in 0.05 M acetic acid. Dashed line show the effect of 0.017 M NaCl on the melting behavior of collagen. b. Tp (circles) and Td (squares) for CSC vs [NaCl], 0 - 0.2 M NaCl. Kds were determined from a rectangular hyperbola fit of the data, after inversion of the temperature scale.

Simulated Tanning

Figure 4a shows the melting curves for the first three steps of the simulated tanning process using BDC at 1 mg/mL in 0.07 M NaCl. For the baseline, BDC in the 0.07 M NaCl, 0.05 M acetic acid solution, Tp and Td were 29.9 and 35.2°C respectively. When the sample was acidified to pH 2, Tp was essentially unchanged at 29.3°C and Td decreased to 32.3°C. The addition of $KCr(SO_4)_2$ at pH 2 caused the disappearance of Tp and a further slight decrease in Td to 31.7°C. After the addition of NaHCO3 to raise the pH to 4.5 the 'tanned' collagen was too insoluble to be put into the cuvette for spectroscopic studies. In experiments at these concentrations, no differences were noted between the effects of NaCl and of KCl. In contrast, without additional salt, the 'tanned' collagen was soluble, however the initial helical content was reduced by about 25%, and the melting curve lacked any discernible transition points (Figure 4b).

Discussion

The collagens are a biologically diverse family of proteins based on the deceptively simple sequence of amino acids (Gly-X-Y)100-400. This sequence where X or Y is Pro or Hyp in about 25% of the tripeptide repeats imposes a rigid elongated coil type structure on the individual chains. This Gly-X-Y amino acid pattern gives the entire family of collagens a very high level of homology. However, there is still considerable variation in function and structure allowed by the varying lengths of the tripeptide repeats and by the arrangement of amino acids (not Pro or Hyp) in the

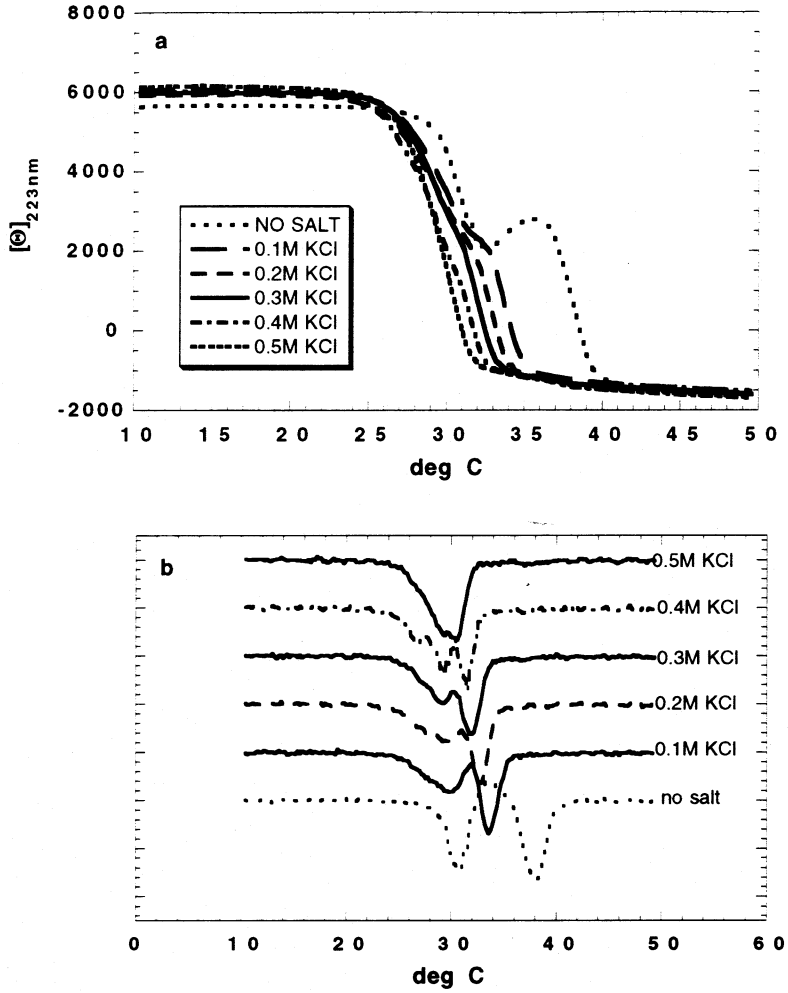

FIGURE 3. — Effect of salt concentration on the melting behavior of BDC. a. Molar ellipticity at 223 nm recorded every 0.2°C over the 10°C to 50°C range with a time constant of 10 sec and a 3 deg/hr heating rate; salt concentrations were 0 to 0.5 M, the solvent was 0.05 M acetic acid. b. First derivative plots of the melting curves.

X and Y positions. At the present time at least 20 genetically different collagens have been identified and may be classed as either fibrillar or nonfibrillar. In all collagens, single strands containing the Gly-X-Y sequence tend to associate in a triple helical conformation. In the fibrillar collagens, of which types I and III are the primary skin or hide collagens, these triple helices further associate to form the microfibrils and fibrils that give strength to connective tissues.

The thermal behavior of fibrillar collagens is complex and not thoroughly understood despite numerous studies over the past thirty years.[11-13] Multiple thermal transitions at temperatures between 50°C and 110°C have been reported for intact fibrillar collagens. The mechanisms responsible for those transitions may be related to the thermal stability or shrinkage temperature, which is a primary characteristic of the conversion of hide to leather. In the context of tanning, the shrinkage temperature of a raw hide is between 50°C and 60°C, while that of a suitably tanned leather is in the vicinity of 100°C or higher.

Underlying the denaturation of the collagen fibril are the thermal characteristics of individual collagen molecules. A range of thermal behaviors have been reported for acid-soluble collagens using calorimetric and spectroscopic methods.[10, 14-17] The work of Komsa-Penkova et al.[10] clearly shows that the thermal denaturation characteristics of soluble collagen are dependent on protein concentration, heating rate, and pH as well as the effects of added salts. Thus it is not surprising that a range of thermal behaviors have been documented for collagens from different sources in the presence of salts, alcohols and other substances.[14-17] Collagens from older animals (adult bovine dermal collagen) are likely to have more natural crosslinks, which might be expected to increase the denaturation temperatures.

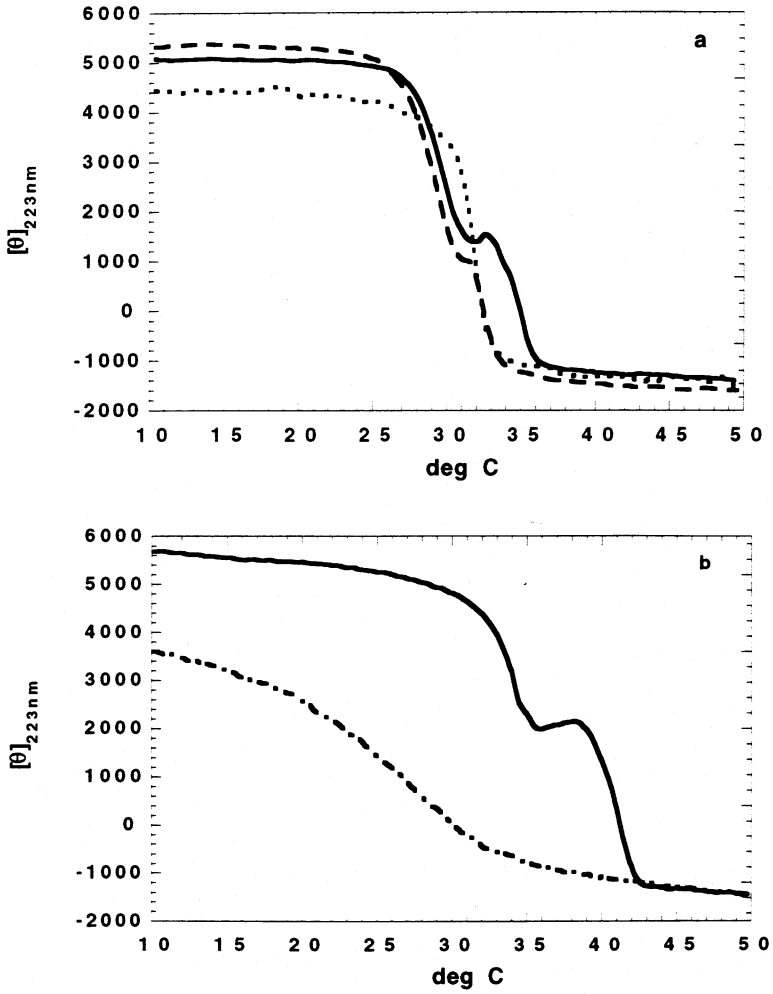

FIGURE 4. — Melting curves for soluble collagen under conditions of simulated tanning. a. Collagen in 0.07 M NaCl, 0.05 M acetic acid (solid line); after adjusting the pH to 2.0 with of 0.1 M H_2SO_4 (dashed line); after addition of $KCr(SO_4)_2$ at pH 2 (dotted line). b. Collagen in 0.05 M acetic acid, no added salt (solid line); after adjusting to pH 2 with H_2SO_4, adding $KCr(SO_4)_2$, and adjusting to pH 4.5 with $NaHCO_3$ (dash-dot line).

However, the treatment necessary to extract a soluble collagen from the adult hide may remove more of the telopeptides, those non-helical regions at either end of the triple helix, thus producing a triple helix with slightly reduced thermal stability as observed here.

Although it has often been convenient in calculations to treat collagen melting as a single phase transition, implying that a molecule is either in the triple helical structure or is isolated as a single strand in an unordered or random coil conformation, in reality, one might expect the path from triple helix to single unordered strand to involve multiple steps. The measured Tm of 37°C for CSC in dilute acetic acid at pH 4 reported previously[1] has now been separated, by way of the first derivative of the melting curve, into a pretransition Tp at 34.0°C and a denaturation Td at 40.7°C. These temperatures are in excellent agreement with the calorimetric data[9] recently published for a similarly prepared calf skin collagen. In their calorimetric study, Komsa-Penkova et al.[10] also investigated the effects of neutral salts on collagen stability. The close agreement of their data with the CD data reported here strongly suggests that both techniques are measuring the same phenomenon.

The salting out effects seen first with NaCl above 0.3 M and above 0.4 M with KCl were clearly visible in the CD study, where they were accompanied by a decrease in spectrophotometrically observed concentration, combined with a gelling of the sample. The effects of these higher salt concentrations were the same on both CSC and BDC. A similar gelling or salting out by NaCl and KCl was observed calorimetrically by Komsa-Penkova et al.,[10] but only at slightly at higher salt concentrations. The destabilization of the triple helix by the addition of neutral salts suggests that at least part of the structural stability of collagen is due to electrostatic interactions. At low salt (<0.2 M)

preferential binding may occur with a destabilizing effect. It is well known that ionizable and hydrophobic side chains tend to be grouped in patches along a collagen triple helix.[18,19] At high salt concentrations there is the possibility of stabilizing effects from electrostatic and hydrophobic interactions,[16,17] as well as from hydrogen bonds and the electron withdrawing character of Hyp.[20]

The addition of even low concentrations of NaCl or KCl to the simulated tanning system, containing collagen at the 1 mg/mL level, reduced the solubility of the system significantly so that the addition of $KCr(SO_4)_2$ causing the pretransition to become smoother and basification leading to nearly complete insolubility. These results are undoubtedly more realistic than those obtained in the absence of salt where the 'tanned' collagen was soluble, but with the initial helical content reduced about 25%, and with a melting curve that lacked any discernible transition points.[1]

Conclusions

The melting profile of acid-soluble collagen in dilute acetic acid may best be described in terms of a pretransition followed by a more complete denaturation. The characteristic temperatures (Tp and Td) for these transitions may be easily obtained from the first derivative of the CD melting curve. The addition of neutral salts at low concentrations to acid-soluble collagen, in solution, at pH 4 reduced the thermal stability of the collagen triple helix as seen by a 3°C decrease in both Tp and Td. Here salt binding may play a role as noted by the Kd (0.017-0.034 M). As the salt concentrations were increased, Tp and Td blended into a single peak and above 0.3 M NaCl or 0.4 M KCl they became indistinguishable and the solution began to gel. Here cosolute-solvent effects may play a role.[17] Except for the greater solubility of the KCl-collagen system, no differences were noted between NaCl and KCl. The melting behavior observed in the CD spectra for CSC appears to be the same as that reported in the most recent differential scanning calorimetry (DSC) study.[10] This suggests that adding this technique to our experimental studies will expand the usefulness of our model tanning system because decreased solubility limits the effectiveness of spectrophotometry.

References

1. Brown, E. M., Dudley, R. L. and Elsetinow, A. R.; A conformational study of collagen as affected by tanning procedures. *JALCA* **92**, 225-233, 1997.
2. Piez, K. A.; Molecular and aggregate structures of the collagens. in *Extracelluar Matrix Biochemistry*, (K. A. Piez and A. H. Reddi ed.) Elsevier, New York, 1984.
3. Bailey, A. J. and Paul, R. G.; Collagen: A not so simple protein. *J. Soc. Leather Tech. Chem.* **82**, 104-110, 1998.
4. Harlan, J. W. and Feairheller, S. H.; Chemistry of the crosslinking of collagen during tanning. in *Protein Crosslinking: Biochemical and Molecular Aspects*, (M. Friedman ed.) Advances in Experimental Medicine and Biology, Vol. 86A, p. 425-440, 1977.
5. Chagne, V., Silvestre, F. and Gaset, A.; Review of chrome tanning. Part 1, *Leather*, p. 17 - 26, 1996.
6. Bailey, D. G. and Gosselin, J. A.; The preservation of animal hides and skins with potassium chloride. *JALCA* **91**, 317-333, 1996.
7. Na, G. C.; UV Spectroscopic characterization of type I collagen. *Collagen Rel. Res.* **8**, 315-330, 1988.
8. Algorithm provided by Aviv Associates with the Model 60DS spectropolarimeter.
9. Taylor, M. M., Diefendorf, E. J., Artymshym, B., Hannigan, M. V., Phillips, J. G., Feairheller, S. H. and Bailey, D. G.; The chemical and physical analysis of contemporary thru-blue tanning technology. pp. 86, 1984.
10. Komsa-Penkova, R., Koynova, R., Kostov, G. and Tenchov, B. G.; Thermal stability of calf skin collagen type I in salt solutions. *Biochim. Biophys. Acta* **1297**, 171-181, 1996.
11. Wallace D. G., Condell, R. A., Donovan, J. W., Paivinen, A, Rhee, W. M. and Wade, S. B.; Multiple denaturational transitions in fibrillar collagen. *Biopolymers* **25**, 1875-1893, 1986.
12. McClain, P. E. and Wiley, E. R.; Differential scanning calorimeter studies of the thermal transitions of collagen: Implications on structure and stability. *J. Biol. Chem.* **247**, 692-697, 1972.
13. Finch, A. and Ledward, D. A.; Shrinkage of collagen fibres: A differential scanning calorimetric study. *Biochim. Biophys. Acta* **278**, 433-439, 1972.
14. Privalov, P. L. and Tiktopulo, E. I.; Thermal conformational transformation of tropocollagen. I. Calorimetric study. *Biopolymers* **9**, 127-139, 1970.
15. Russell, A. E.; Effect of alcohols and neutral salt on the thermal stability of soluble and precipitated acid-soluble collagen. *Biochem. J.* **131**, 335-342, 1973.

16. Gekko, K. and Shozo, K.; Increased thermal stability of collagen in the presence of sugars and polyols. *J. Biochem.* **94**, 1983.

17. Danielsen, C. C.; Precision method to determine denaturation temperature of collagen using ultraviolet difference spectroscopy. *Collagen Rel. Res.* **2**, 143-150, 1982.

18. Hulmes, D. J. S, Miller, A., Parry, D. A. D., Piez, K. A. and Woodhead-Galloway, J.; Analysis of the primary structure of collagen for the origins of molecular packing. *J. Mol. Biol.* **79**, 137-148, 1973.

19. King, G., Brown, E. M. and Chen, J. M.; Computer model of a bovine type I collagen microfibril. *Protein Engineering* **9**, 43-49, 1996.

20. Holmgren, S. K., Taylor, K. M., Bretscher, L. E. and Raines, R. T.; Code for collagen's stability deciphered. *Nature* **392**, 666-667, 1998.

DISCUSSION

Steve Wren, Schill & Seilacher – I noticed that when you were preparing the collagen you used acetic acid first and then you produced a pickling system with sulfuric acid. This procedure may have had some affect on the way that the chrome took up because you would have altered the complex. Do you think that this is significant?

Yes it is very significant. If you simply add the chromium in the acetic acid you don't get any effect whatever. If you add the chromium in the sulfuric acid and don't add the bicarbonate or something to bring the pH up then you get no effect over what you got with the sulfuric acid alone.