Effects of Pretanning Processes on Collagen Structure and Reactivity

by

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Abstract

The cattle hide, a major byproduct of the US meat industry, is the tanner's substrate, and also the source of collagen for the food and biomaterials industries. Conversion of animal hides into leather is a multistep process that continually evolves in response to economic and environmental concerns. Processing changes are generally evaluated in terms of impact on tannery costs and quality of leather produced. Because the basis for tanning and other biomaterial applications is the stabilization of the collagen matrix, changes to the molecular characteristics of hide collagen may be expected to impact these applications. In previous studies, we began the development of protocols for production and utilization of powdered hide from specific steps in beam-house processing. In this study, the effects of pretanning processes on the structure, stability and reactivity of hide collagen with tanning materials are evaluated. The results are anticipated to assist the tanner as well as the manufacturers of collagen-based biomaterials and gelatin to better understand their substrate and changes to it that may occur when beam-house processes are altered.

Resumen

La piel vacuna, un subproducto principal de la industria de la carne de EE.UU., es el sustrato del curtidor, y también la fuente de colágeno para las industrias de alimentos y biomateriales. La conversión de las pieles de animales en cuero es un proceso de varios pasos que continuamente evoluciona en respuesta a las preocupaciones económicas y ambientales. Los cambios de proceso son generalmente evaluados en términos de impacto sobre los costos y la calidad del cuero producido. Debido a que la base para el curtido y otras aplicaciones de biomateriales es la estabilización de la matriz de colágeno, los cambios en las características moleculares del colágeno de la piel pueden esperarse que afecten a estas aplicaciones. En estudios anteriores, se inició el desarrollo de protocolos para la producción y utilización de pieles en polvo en pasos específicos en el proceso de ribera. En este estudio, los efectos de los procesos de precurtido sobre la estructura, la estabilidad y la reactividad del colágeno de la piel con materiales curtientes son evaluados. Los resultados son anticipados para ayudar al curtidor, así como los fabricantes de biomateriales a base de colágeno y gelatina para entender mejor su sustrato y modificaciones al mismo, que puede producirse cuando los procesos de ribera son alterados.

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Introduction

The US beef industry currently slaughters approximately 35 million cattle annually. About 50% of the animal's weight is sold as meat, and the remaining 50% as byproducts. Among the byproducts, the animal hide is the most valuable, serving as raw material, for the leather industry and, ultimately, because of its collagen content, the gelatin and biomaterials industries. Collagen, the most abundant protein in mammals, constituting about a quarter of the animal's total weight, is the primary component of connective tissues. The hide collagen is largely type I, one of the fibrous collagens that form insoluble scaffolds giving strength and form to the skin, tendons, bones, cornea and teeth.

The hide typically goes first to the tanner, now often located near the slaughterhouse, where the outer layer is removed for conversion to leather. The inner layers of the hide provide the raw material for the manufacture of gelatin and an array of biopolymer products. The structure of fibrous collagen, a long triple helix that further associates into fibrils, fibers and fiber bundles, makes it uniquely suitable as the basis for biomaterial engineering.[1] For the production of leather and other collagen-based biomaterials, it is essential to stabilize the collagen matrix against thermal and microbial degradation. The multistep processes used by tanners to prepare and stabilize the collagen matrix in the production of leather continually evolve in response to changes in the economic and regulatory environment. The tanner typically evaluates these changes in terms of the cost of materials coming into the tannery and income derived from the sale of leather, with little concern for changes in the molecular characteristics of the collagen that may affect both leather and other uses of collagen.

Knowledge of the effects of pretanning processes on the structure and stability of collagen will assist tanners and manufacturers of biomaterials and gelatin in understanding their substrate. Thus we are developing model systems with which to compare the molecular and fiber structure of bovine hide collagen after traditional and altered beam-house processes. The complexity of tanning related models developed in this laboratory includes the collagen microfibril model,[2,3] soluble collagen models,[4,5] and powdered hide models.[6,7]

Experimental

Materials

Fresh hide obtained from a local abattoir was sided and fleshed prior to the start of the experiments. Bacterial collagenase (361 units/mg) from *Clostridium histolyticum*, was obtained from Sigma-Aldrich, St. Louis, MO. Rat-tail, type 1 collagen was obtained from Millipore, Billerica, MA. Waynetan 175, liquid chromium sulfate, was from Elementis, LTP, Millwaukee, WI. All other chemicals were reagent grade from various suppliers.

Sample Preparation

Powdered Hide Preparation

One side of the hide was sulfide dehaired as described by Cabeza et al.[8] and the other side was oxidatively dehaired as described by Marmer and Dudley.[9] Both sides were taken to the wet blue stage as described by Cabeza et al.[8] with the following change: basic chrome sulfate (BCS) in the form of a 7.5% chromium solution, equal to 5% powdered BCS was used in the tanning step. Pieces of hide (15 x 40 cm) were taken from the backbone area, of each side for the preparation of powdered hide at six stages of the process. Powdered hide samples included the raw hide with hair removed by shaving, the dehaired, relimed, delimed-bated, pickled, and chrome tanned hide. Each piece was cut into strips, the water extracted with acetone, air dried, ground in a Wiley mill and stored under refrigeration in reclosalable plastic bags as previously described.[7]

Extracted Collagen

Collagen was extracted from powdered hide samples with 0.5 M acetic acid, centrifuged and the supernatant lyophilized as described earlier.[7] The lyophilized product was dissolved (3 mg/ml) in 0.05 M acetic acid and dialyzed overnight against two changes of 0.05 M acetic acid. Concentration of extracted collagen was estimated, from the absorbance at 218 nm with the absorption coefficient 9.43 cm^{-1}-ml-mg^{-1} (AVIV-14 spectrophotometer AVIV Biomedical Inc., Lakewood, NJ).[10] The samples were centrifuged and stored at 4°C for later analysis.

Analyses

Scanning Electron Microscopy (SEM)

Powdered hide samples were mounted on the surface of carbon adhesive tabs. Silver paint was applied to the exposed surface area around the sample to ensure conductivity and the entire specimen was sputter-coated with gold using a Scancoat Six Sputter Coater (Edwards, Wilmington, MA). Samples for SEM were imaged using a Quanta 200 FEG environmental scanning electron microscope, (FEI Company, Hillsboro, OR). SEM images were captured as described previously.[11]

Apparent Shrinkage Temperature

Hydrothermal stability of powdered hide was determined on a Multi-Cell Differential Scanning Calorimeter (DSC) (model CSC-4100) from Calorimetry Sciences Corporation, Lindon, UT, as previously described.[12] Powdered hide samples were prepared for DSC experiments by soaking in distilled water overnight and then blotting on filter paper. Pickled hide samples were soaked in 3% NaCl at pH 2. Moist, blotted samples (100 - 250 mg) were weighed into ampoules that were sealed and placed in the calorimeter. The calorimeter was programmed to record heat flow as mcal/°C while the temperature was increased from 30°C to 130°C at 1.5°C/min. The temperature at the peak of the calorimetry trace, $T_{p,}$ was considered to be an apparent shrinkage temperature.

Chromium Content

Chromium was determined as described by Cabeza et al.[8] with the following changes. Solid samples were weighed into appropriate flasks that were fitted with glass joints, 2 N HCl (50 ml) was added, and the samples hydrolyzed at 60°C for 4 hours. The hydrolyzed samples were filtered into volumetric flasks and diluted to volume with deionized water so that the chromium concentration would be between 1 and 5 ppm. Percent chromium and percent chromium oxide were calculated from original weight. Chromium was determined on a Perkin Elmer Atomic Absorption Spectrometer, Model 3300 (Norwalk, CT).

Susceptibility to Collagenase

Collagenase susceptibility of powdered hide from each treatment was analyzed in triplicate as described previously.[10] Dry powdered hide samples were suspended in 0.05 M tris(hydroxymethyl)-methyl-2-aminoethane sulfonate (TES) buffer with 0.36 mM calcium chloride, pH 7.5, and incubated with collagenase at a 1:100 ratio of collagenase to powdered hide, for 5 h at 37°C. The resulting solution was reacted with ninhydrin-citric acid and the absorbance read at 600 nm on a UV-Vis spectrophotometer. Susceptibility to collagenase was reported in terms of mmoles of amino groups released per mg of powdered hide as compared with a standard curve for leucine.[10]

Electrophoresis

Extracted collagen samples were prepared for SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate) as described by Taylor et al.[13] Separation was achieved on 4 – 15% gels with a PhastGel System (GE Life Sciences, Piscataway, NJ) using an extended protocol to assure that intact collagen could move into the gel. A broad range SDS-Standard (Bio-Rad, Hercules, CA) containing nine proteins ranging in size from 6,500 to 200,000 Daltons, and type I soluble collagen (Sigma, St. Louis, MO) were included as controls.

Thermal Stability of Extracted Collagen

The thermal stability of soluble collagen was determined from the circular dichroism spectrum as described previously.[14] Samples, 300 μl in volume, containing 0.5 - 1 mg/ml extracted collagen in 0.05 M acetic acid were placed in 1-mm-pathlength quartz cuvettes that were fitted with Teflon stoppers to prevent evaporation during the melting experiments. The ultraviolet (UV) spectrum of the sample was scanned from 300 to 190 nm against a reference solution containing all components except collagen. The cuvette was placed in the refrigerator for at least 12 hours to maximize the formation of the triple-helix structure in the sample. The thermal stability of the triple helix was determined by following the change in the circular dichroism (CD) signal at 223 nm (AVIV 420 Spectropolarimeter, AVIV Biomedical Inc., Lakewood, NJ) with increasing temperature. Melting curves were obtained by recording the CD signal at 223 nm every 0.5°C between 10°C and 55°C at a rate of ~5 min per degree. The CD signal was read in mdeg and converted to molar ellipticity as shown below:

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

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. Pretransition and denaturation temperatures (T_p and T_d) were obtained from the derivative of the melting curve.

RESULTS AND DISCUSSION

Scanning Electron Microscopy (SEM)

SEM images of powdered hide captured at 1000x magnification (Figure 1) show fiber bundles as well as individual fibers. As expected, images of the raw hide from both sides show similar fiber structure (Figure 1 upper left). Images of the sulfide dehaired powdered hide show compact fiber bundles with few separated fibers while the oxidatively dehaired powdered hide exhibits fiber bundles with relatively open structures (Figure 1 middle left) in agreement with earlier results.[10] The micrographs show little change through the relime and delime/bate steps. At the pickle, samples from the two sides look very similar, with densely packed individual fibers. After chrome tanning, more open areas are seen in the sample from oxidative dehairing.

Apparent Shrinkage Temperature

The shrinkage temperature is the most common term used to describe the hydrothermal stability of a piece of hide or leather. A primary objective of tanning is to increase the shrinkage temperature from the 65 – 70°C range characteristic of raw cattle hide to near the 100°C range characteristic of chrome tanned leather. The phenomenon measured by the shrinkage temperature is the denaturation of collagen, which can be observed by a variety of calorimetric and spectroscopic techniques. Each technique requires a different type of sample preparation, and different conditions for the measurement, resulting in values that must be qualified by reference to the method, and are apparent shrinkage temperatures. Table I details the apparent shrinkage temperatures obtained by DSC with strips of hide removed from the hide following the designated step in processing, then dried, powdered, and rehydrated. Numbers in parenthesis indicate the difference in degrees from the raw hide. Immediately after dehairing, the shrinkage temperature of the sulfide dehaired hide was indistinguishable from that of the untreated hide, while the shrinkage temperature of the oxidatively dehaired hide was more than 10°C lower. This result correlates with the SEM data that shows the more open structure of the collagen fiber after oxidative dehairing. As the tanning process proceeded, the differences became smaller.

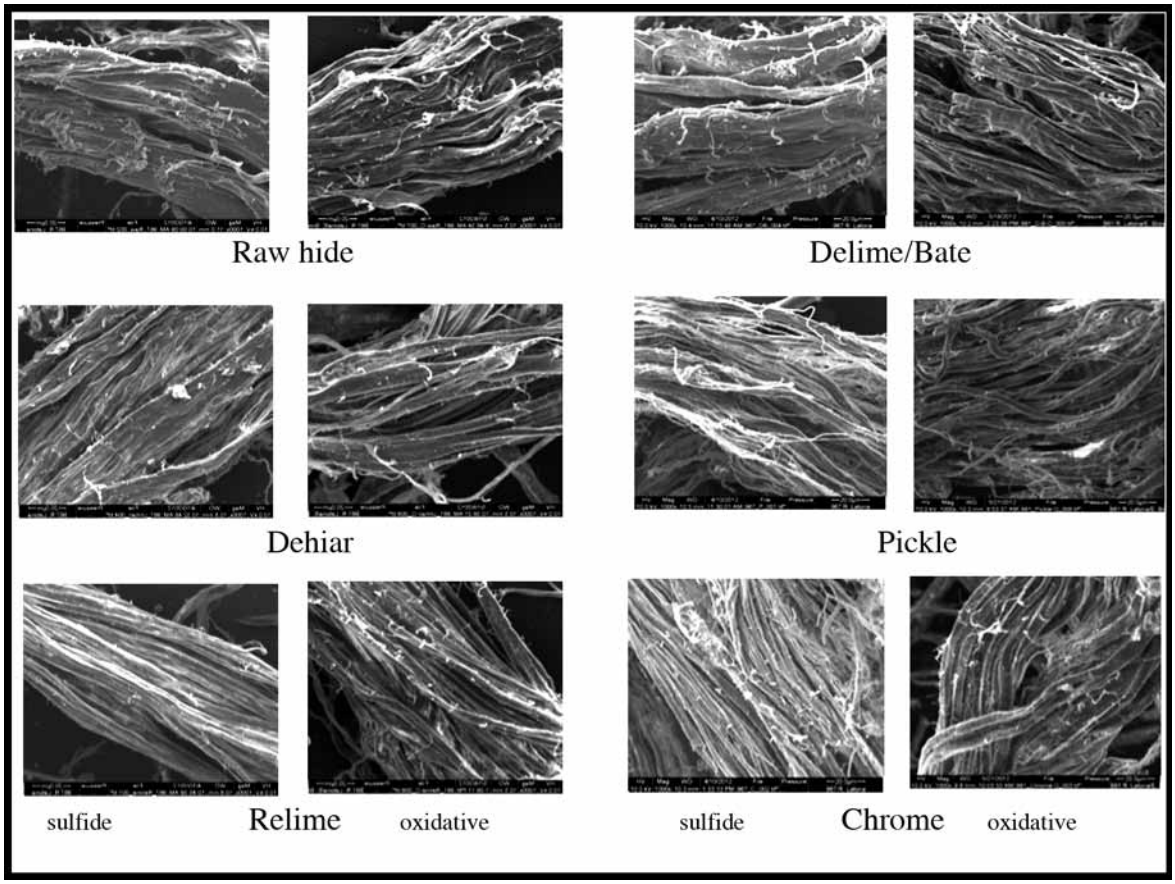

Figure 1. Scanning electron micrographs of powdered hide, 1000x magnification, bar represents 20 mm. In each pair, the left image is from the sulfide dehaired side, and the right from the oxidatively dehaired side.

TABLE I
Effects of processing on hydrothermal stability shrinkage temperature, °C.

Sample[a]	Sulfide dehaired	Oxidatively dehaired
raw hide	68.3 ± 0.1	69.4 ± 0.2
dehaired	69.1 ± 0.4 (+0.8)[b]	58.0 ± 0.7 (-11.4)
relimed	64.5 ± 0.3 (-4.0)	63.7 ± 1.8 (-5.7)
delime/bated	68.5 ± 0.7 (-0.2)	67.1 ± 0.7 (-2.4)
pickled	73.1 ± 0.6 (+4.7)	70.1 ± 0.1 (+0.6)
Cr tanned	91.4 ± 0.6 (+23.1)	95.9 ± 1.0 (+26.5)
Cr, 2nd peak	131.0 ± 0.9 (+62.7)	132.7 ± 2.2 (+63.3)

[a]Samples of powdered hide for analysis by DSC were prepared after each processing step, all values represent results obtained with at least three separate samples.
[b]Numbers in parentheses show the difference from the raw hide.

Chromium Uptake

In an earlier study,[8] chrome tanning performed with 8% commercial basic chrome sulfate (BCS) resulted in leather containing 4.52 ± 0.73% chromium calculated as Cr_2O_3. In this study, the chromium offer was lower, equivalent to 5% of the hide weight, and uptake was essentially identical for the two samples, 2.21± 0.13% by the sulfide dehaired hide, and 2.07± 0.24% by the oxidatively dehaired hide, calculated as Cr_2O_3.

Susceptibility to Collagenase

Collagenases are a class of enzymes that cleave the triple helical structure of collagen, and increase the number of available primary amino groups by exposing buried sidechain groups and creating additional N-terminal amino groups. Monomeric collagen in solution is more susceptible to cleavage by collagenase than is collagen in hide powder, and at the dehairing stage, collagen in sulfide dehaired hide powder was seen to be more susceptible than collagen in oxidatively dehaired hide powder.[10] This trend is seen to continue through the relime stage, where the sulfide dehaired material was most susceptible to collagenase (Figure 2). Interestingly, as processing progressed through the delime/bate, pickle and chrome stages, the susceptibility to collagenase of all samples declined, and was essentially identical for sulfide and oxidatively dehaired samples.

Electrophoresis

With the exception of the dehairing and chrome tanned stages, where little collagen was extracted from either treatment, collagen was more readily extracted from the sulfide dehaired hide than from the oxidatively dehaired hide. Electorphoretic patterns for the extracted collagen, prepared at each stage of processing, were similar. Typical collagen bands for the b-chain near 200 k Da, and the α1- and α2- chains slightly above the 116 k Da marker were seen, at least faintly, in all samples. Lower molecular weight bands were faint and less distinct, leading to the conclusion that the collagen extracted under these mild conditions was resistant to collagenase. A fraction of the material in most samples was in large aggregates that did not penetrate the separating gel, but remained above it in the lower density stacking gel.

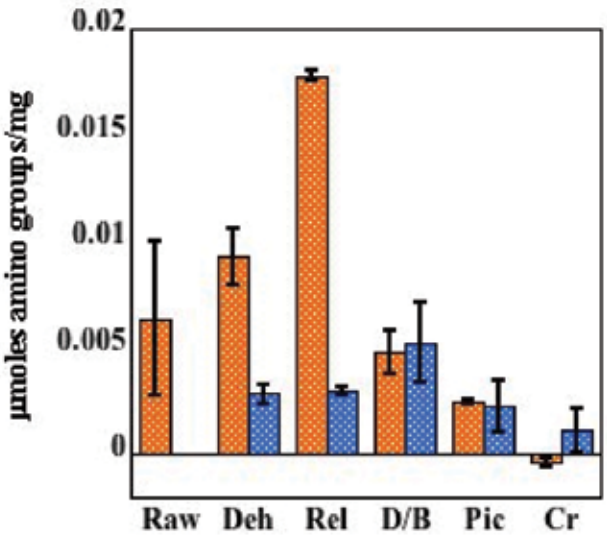

Figure 2. Available primary amino groups in powdered hide from collagenase treated sulfide (orange) and oxidatively dehaired (blue) hide. The samples from left to right are raw hide (6 tests averaged), other samples (3 each), dehaired, relimed, delime/bated, pickled, and chrome tanned.

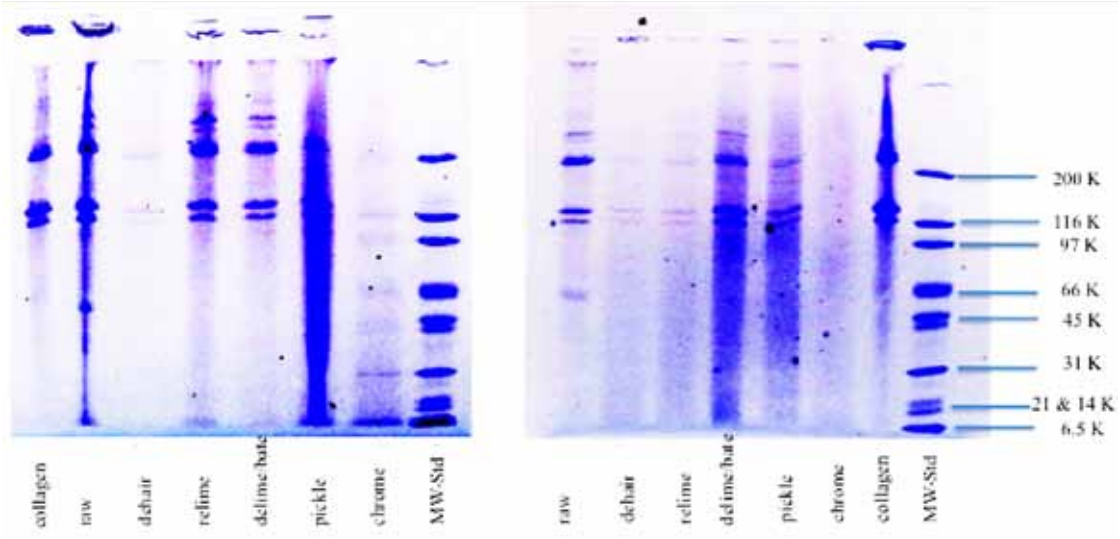

Figure 3. SDS-PAGE electrophoresis of extracted collagen from sulfide dehaired (left), and oxidatively dehaired hide (right).

Thermal Stability of Extracted Collagen

A positive band in the 220 – 225 nm range and a stronger negative band at 198 nm are characteristics of the CD spectrum of collagen in solution. In the 10 – 20°C range, where the collagen triple helix of native collagen is most stable, ($[\theta]_{223nm}$ ~ 5500 deg cm^2dmol^{-1}). At higher temperatures, the helical conformation is lost and the CD spectrum becomes featureless. Melting curves for the helix to unordered transitions of soluble collagens[14] consist of a flat region $[\theta]_{223nm}$ ~ 5500 deg cm^2dmol^{-1} representing completely helical protein, at temperatures below 20°C, and a second flat region $[\theta]_{223nm}$ ~ -1400 deg cm^2dmol^{-1} representing completely unfolded protein at temperatures above 40°C. Between these two regions, two inflections associated with the pretransition (T_p) and denaturation (T_d) were most clearly seen in the derivatives of the melting curves. CD spectra (not shown) of the collagen extracted at different stages in the tanning process from both sulfide and oxidatively dehaired hide were similar in shape to those for soluble collagen, but with less intense positive and negative bands. Melting curves (not shown) were qualitatively similar to those reported for soluble collagen.[14] The values for T_p and T_d (Table II) are 2 – 3°C higher than those for the commercial sample of soluble collage that was extracted with pepsin, and is lacking in telopeptides. Nonetheless, the 5 – 6°C separation between T_p and T_d is in agreement with the values for soluble collagen and in all cases is indicative of a classical two-stage helix to unordered transition. No significant amount of collagen could be extracted from the chrome tanned and the relimed oxidatively dehaired samples.

CONCLUSIONS

In this study, the similarities in properties between the powdered hide from sulfide and oxidative dehairing are more notable than the differences. The greatest difference between samples was at the dehairing step as would be expected. Differences decreased as the hide was processed through to the wet blue stage where tanning was complete despite a lower than typical chrome offer. Beamhouse processes that have been altered in response to environmental, economic and legislative pressures may affect the condition of the hide substance and ultimately, require changes in the tanning/finishing of leather and manufacturing processes for collagen-based biomaterials and technical gelatins. The results of this study show little lasting change to the measured properties of the hide substance and extractable collagen through the tanning process of hide dehaired by a typical sulfide treatment or the oxidative process of Marmer and Dudley.[9] The effects of other variations of beamhouse processes that are evolving throughout the industry may be different; an awareness of the possible effects on collagen fiber structure could be valuable to both tanners and biomaterial engineers.

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TABLE II
Effects of processing on thermal transition parameters for extracted collagen temperature, °C.

Sample[a]	Sulfide dehaired		Oxidatively dehaired	
	Tp	Td	Tp	Td
control	30.0	36.0		
raw hide	33.2	38.1	33.3	39.3
dehaired	32.9	38.0	33.9	38.8
relimed	33.2	38.8	N/A	N/A
delime/bated	33.3	38.4	32.8	38.9
pickled	32.3	38.1	32.1	38.0

[a]Samples of extracted collagen for analysis by CD were prepared after each processing step.

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