

We expected that the acylation of amino groups furnished by lysine residues present in collagen would proceed readily and that the use of N-hydroxysulfosuccinimide esters of dicarboxylic acids (1) would lead to formation of crosslinks and also be accompanied by an increase in shrinkage temperature. By use of a series of dicarboxylic acids with varying numbers of methylene units between the carboxylic acid functions, we hoped to study the effect of chain length of the crosslinking agent on the shrinkage temperature of the hide. Furthermore, we wished to utilize our recently developed three-dimensional molecular model of Type I collagen^(6,7) to correlate spacing between potential binding sites with the chain length of these dicarboxylic acids.

Experimental

MATERIALS

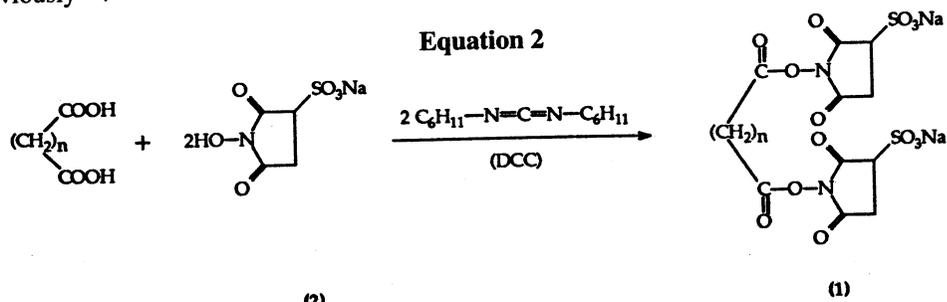
N-Hydroxymaleimide, sodium metabisulfite, 1,3-dicyclohexylcarbodiimide, N,N-dimethylformamide, and all dicarboxylic acids were purchased from Aldrich Chemical Company.*** Pickled sheepskin was obtained from a local tannery.

SYNTHESIS OF ACYLATING AGENTS:

The procedure followed was essentially as described by Staros⁽⁴⁾ in the following sequence.

(a) N-hydroxysulfosuccinimide sodium salt (2). A solution of 1.68 g (0.00884 mole) of sodium metabisulfite in 15 ml water was added dropwise with stirring under nitrogen to 2.0 g (0.01768 mole) of N-hydroxymaleimide dissolved in 25 ml absolute ethanol. The mixture was stirred at room temperature for 2 hours and the alcohol and water were removed by evaporation under reduced pressure. The bright yellow residue was dissolved in 50 ml water, filtered, and lyophilized overnight. The dry yellow powder was triturated with 100 ml ethyl ether, filtered, and recrystallized from 90% ethanol. Yields of 95% were obtained.

(b) Dicarboxylic acid N-hydroxysulfosuccinimide esters (1). A mixture of 1.82 g (0.0084 mole) of N-hydroxysulfosuccinimide sodium salt (2), 1.90 g (0.0092 mole) of 1,3-dicyclohexylcarbodiimide (a 10% excess) and 0.0042 mole of the appropriate dicarboxylic acid in 25 ml dry N, N-dimethylformamide was stirred at room temperature overnight. The reaction is outlined below in equation (2). The mixture was cooled to 3°C for 2 hours, filtered to remove the precipitated dicyclohexylurea. Then 200 ml ethyl acetate was added to the filtrate to precipitate the succinimide ester. Filtration led to isolation of the succinimide esters. They were extremely hygroscopic and were used without further purification. Characterization of these reagents by HPLC and fast atom bombardment mass spectroscopy has been described previously^(4,8).



*** Reference of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

CROSSLINKING OF SHEEPSKIN

The following standard procedure was used in all crosslinking experiments. Pickled sheepskin (20 g) was mixed with 200 ml water, 20 g sodium sulfate and the dicarboxylic acid N-hydroxysulfosuccinimide ester (1) obtained from 1.82 g (0.0084 mole) of N-hydroxysulfosuccinimide. The esters were completely water soluble. The mixture was adjusted to pH 7.4 by addition of solid sodium bicarbonate and tumbled at 48 rpm at room temperature for 2 hours, sampled to determine shrinkage temperature and tumbled an additional 18 hours before sampling again. The pH of the final mixture was usually about 8.0. The crosslinked hide was chrome tanned, retanned and finished into crust leather.

Sybyl (Tripos, St. Louis, MO) software on a SGI-4D/35 (Silicon Graphics, Mountain View, CA) workstation was used to construct and manipulate computer models of the succinimide esters (1) with $n = 6, 8$ and 10 . The models were energy minimized using the Tripos force field at 0°K and then subjected to 1,000 fs of dynamics to simulate motions at 300°K . The distance between the carboxyl groups was measured before and after the dynamics simulation to suggest appropriate distances between crosslinking sites.

Results and Discussion

A series of N-hydroxysulfosuccinimide esters of dicarboxylic acids was prepared with varying numbers of methylene groups between the carboxylic acid functions. The numbers of intervening methylene groups varied from 1 (malonic acid) to 14 (1,14-hexadecanedioic acid). Table I lists the esters prepared, as well as the shrinkage temperatures obtained after 2 hours, 20 hours, and after chrome tanning. For comparison similar information is provided for an untreated sample of sheepskin processed under identical conditions.

The data in Table I also illustrate the effect of crosslinking on shrinkage temperatures of sheepskin samples with changes in methylene units of the dicarboxylic acid acylating agent. Highest temperatures ($68-72^\circ\text{C}$) (before chrome tanning) were obtained with dicarboxylic acids containing 7-12 carbons atoms (5-10 methylene groups) between carboxyl groups. Also noteworthy was the fact that the increases in shrinkage temperature occurred rapidly, within 2 hours, with relatively little additional increase after 20 hours. This is in accord with previous observations that these reagents react rapidly.

In a separate experiment, an additional quantity of acylating agent (C-8 derivative) was added after 20 hours and the reaction was allowed to proceed another 2 hours. No increase in shrinkage temperature was observed, indicating that hydrolysis of the reagent as a competing reaction with the acylation of the collagen was not a limiting factor.

The amount of acylating agent used was also investigated. Using the same weight of C-8 derivative in each case, varying amounts of sheepskin were used as the substrate (10 g, 20 g, 40 g, 60 g). Results are shown in Table II.

It appears that shrinkage temperatures remained high even when the amount of sheepskin was increased and that the amount of the acylating agent used in our standard procedure (see Experimental section) was sufficient to achieve maximum benefits.

The finished leathers had a good appearance, with no visible defects regarding discoloration, brittleness, flexibility or temper when compared with a control. However, it should be emphasized that the obvious expense required to synthesize the crosslinkers renders their practical use as tanning agents questionable.

Dimensions of the crosslinks derived from C-8, 10 and 12 are summarized in Table III. Each of these dicarboxylic acids effective as a crosslinker of collagen. The energy minimized models for these crosslinks increased in length with increasing numbers of methylene groups expected.

However, after dynamic simulation of motions at 300°K, each of these moieties approached a length of 9 Å. The length of the C-8 molecule was extended slightly during the dynamics simulation while the C-10 and C-12 molecules were shortened. In the 36-residue long segment of collagen Type I microfibril⁽⁹⁾, the distances between ε-amino groups of the 14 lysine residues range from 4.2 to 51.7 Å. Ten of the inter-residue distances are between 7 and 11Å, making suitable targets for these kinds of crosslinks. In the skin sample the much larger collagen fibrils would provide greater numbers of potential bindings sites.

At the present time, the data from Tables I and III suggest that crosslinks derived from C-7 to C-12 dicarboxylic acids can be accommodated by collagen microfibrils and lead to highest shrinkage temperatures (68-70°C) observed in sheepskin before additional chrome tanning.

TABLE I

Shrinkage temperatures (T_s) of hide following reaction with dicarboxylic acid esters of N-hydroxysulfosuccinimide (1)

Dicarboxylic acid	T_s (2 hours)	T_s (20 hours)	T_s (Cr tanned)
C-3*	50°C	49°C	98°C
C-4	50	50	103
C-5	59	59	108
C-6	63	64	105
C-7	69	70	105
C-8	70	70	104
C-9	70	70	103
C-10	70	70	105
C-11	72	70	102
C-12	68	70	103
C-13	65	65	103
C-14	61	61	103
C-16	54	57	104
Control	52	52	104

*n = 1

TABLE II

Constancy of T_s with increasing amounts of sheepskin

Acylating agent	Wt. (g) Sheepskin	T_s (2 hours)	T_s (20 hours)	T_s (Cr tanned)
C-8	10	71°C	70°C	106°C
C-8	20	70	70	104
C-8	40	68	68	105
C-8	60	67	67	103

TABLE III
Dimensions of crosslink

Dicarboxylic acid	Length of crosslink* Å	Length after dynamics Å
C-8	8.7	8.92
C-10	10.3	9.53
C-12	11.1	9.46

* Initial lengths are the carboxyl to carboxyl distance in the energy-minimized model of the crosslink

References

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Discussion

Dr. William Prentiss, Retired: Frank, you said at the beginning that part of the objective of this work was to try to correlate the structural models of collagen with the dimensions of crosslinking agents by preparing and evaluating these crosslinking compounds of different chain length. Are you able, at this point, to correlate the dimensions of the C₈-compound, that gave you near optimal results on your shrinkage temperatures, with any of the reactive groups in the collagen structures?

Answer: At the present time, no. This is something we are aiming for, it's the ultimate goal; but, I don't have any further information at this time.

Professor Eckhart Heidemann, Technische Hochschule, Darmstadt, Germany: The compounds you used for crosslinking are not very stable in aqueous solution. Have you followed the activity of these active esters over time in the reactions? What is the loss of activity with time?

Answer: We have not made these measurements; however, the stability of the compounds in aqueous solutions has been studied. While it is true that they do react with water, they are many

times more reactive with amines including the free amino groups in proteins - there is a vast difference. They do react with water; but, in competition with the amino groups, there's no comparison.

Professor Heidemann: But, you haven't proved that in these reactions?

Answer: No.

Professor Heidemann: How long were the crosslinking reactions?

Answer: A maximum of 24 hours; however, the maximum shrink temperatures were obtained within the first 2 hours. I believe that the reaction was over within 15 minutes.

Professor Heidemann: Could it be that there is no further reaction after the first 2 hours because most of the active reagent is exhausted, hydrolyzed?

Answer: Yes, that could be; however, we postulate that the maximum crosslinking that can occur with these compounds has occurred in that time.

CROSSLINKING OF COLLAGEN WITH DICARBOXYLIC ACIDS

by

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Abstract

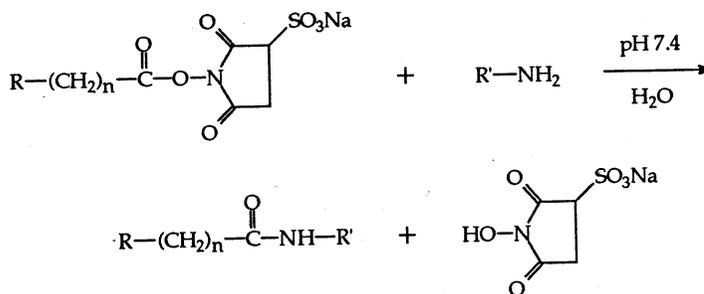
We have studied the crosslinking of collagen in sheepskin by treatment with N-hydroxysulfosuccinimide esters of dicarboxylic acids. The acylating agents are prepared from dicarboxylic acids including suberic (C-8), azelaic (C-9) and sebacic (C-10) acids. These agents are extremely soluble in water and react readily with sheepskin, resulting in increases in shrinkage temperatures before chrome tanning. A description of the synthesis of the acylating agents and a discussion of the physical properties of the resulting leathers are presented.

Introduction

The tanning of hides is carried out by crosslinking of the protein collagen and has been the subject of considerable research at our laboratory. Most recently, the crosslinking of collagen with acrylamide derivatives^(1,2,3) was shown to lead to increases in shrinkage temperatures.

In the currently reported work, we used N-hydroxysulfosuccinimide esters of dicarboxylic acids (1) as acylating agents of amino groups present in collagen. Succinimide esters have been used extensively as acylating agents for proteins⁽⁴⁾. In addition to their reported ready solubility in water, they have the advantage of rapid reaction with proteins, as shown in equation (1). The esters have been shown to have slow rates of hydrolysis as compared with their rates of reaction with nucleophiles, especially nitrogen nucleophiles in proteins⁽⁵⁾. In order to insure complete water solubility and rapid reaction, these reagents typically have a sulfonate group attached to the succinimide portion of the molecule. Thus: Reaction with proteins (R^1-NH_2)

Equation 1



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