

CROSSLINKING OF COLLAGEN WITH ACRYLAMIDE DERIVATIVES II: N,N'-METHYLENEBISACRYLAMIDE AND HIGHER HOMOLOGS

by

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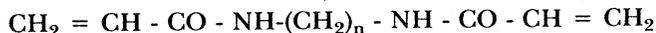
Abstract

Two symmetrical derivatives of acrylamide, namely: N,N'-methylenebisacrylamide and N,N'-dimethylenebisacrylamide, were found to crosslink hide collagen and, with proper control of the reaction conditions, served as tanning agents. The reactions take place under alkaline conditions and swelling is controlled by addition of sodium sulfate. Shrinkage temperatures over 80°C were obtained and the products exhibited good resistance to chemical and enzymic attack. The products were made into white leather on a small scale. Two higher homologs, N,N'-trimethylenebisacrylamide and N,N'-hexamethylenebisacrylamide, could not be made to react under the same conditions nor under modified conditions designed to improve their solubility.

Introduction

In the first paper of this series⁽¹⁾ we described the crosslinking of hide collagen with N-hydroxymethylacrylamide and N,N'-(1,2-dihydroxyethylene)-bisacrylamide. From the results we obtained it was apparent that these two compounds react with hide collagen as tanning agents by crosslinking the hide collagen. Two different kinds of reactive groups are present, however, in both of these compounds and they can react with the collagen by different means. We also reported on the reaction of acrylamide itself with hide collagen, demonstrating almost complete reaction of this compound with lysyl residues. Acrylamide, itself, does not provide crosslinking; we showed, however, that the acrylamide residues in the two compounds mentioned above would react in the same manner with hide collagen. In the present paper, we describe the crosslinking of hide collagen with the two symmetrical derivatives of acrylamide, N,N'-methylenebisacrylamide (MBAA) and N,N'-dimethylenebisacrylamide (DMBAA), both of which are capable of reacting with hide collagen by only one mechanism, that is, by the same mechanism as does acrylamide itself⁽²⁾. These two compounds, however, can react at both ends of the molecule and when this occurs crosslinking takes place. We also describe our attempts to cause N,N'-trimethylenebisacrylamide (TMBAA) and N,N'-hexamethylenebisacrylamide (HMBAA) to react in the same manner.

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MBAA $n=1$

DMBAA $n=2$

TMBAA $n=3$

HMBAA $n=6$

These compounds are used as comonomers in the polymerization of acrylamide to provide crosslinking of the resulting polymers and they are readily available. Since these compounds are derivatives of acrylamide and one, MBAA, is also a derivative of formaldehyde, appropriate caution must be exercised in their use. Anyone desiring to use them is referred to the respective Manufacturers Safety Data Sheets for information covering their use.

In this paper we describe our attempts at crosslinking hide collagen with these four compounds under conditions similar to those described in the first paper of this series⁽¹⁾ as well as with modified conditions used to overcome the low water solubilities of the higher homologs.

Experimental

MATERIALS

MBAA was obtained from Kodak Co.* as a fine white powder and DMBAA, TMBAA, and HMBAA were obtained from Polysciences, Inc.* Other chemicals used in these studies were all standard research-grade laboratory chemicals. Limed cowhides and limed sheepskins were either obtained from local tanneries or were produced in our experimental tannery using standard leather-manufacturing procedures. Further processing of our experimentally-developed products was also accomplished in our experimental tannery using similar procedures.

Methods and Procedures

For laboratory-scale experiments, the limed hides or skins were cut into pieces measuring approximately 45 mm by 50 mm. Each piece weighed 50 g. All percentages are based on the limed weights of the hide-and-skin pieces. All experiments were carried out in 1L glass bottles on a tumbling machine at 48 rpm and at room temperature (20-25°C) for appropriate amounts of time as described below. Sodium sulfate was used in various amounts to control swelling under the alkaline conditions used in most experiments. Triton X-100* (0.3%) was used to aid in the dispersion of the acrylamide derivatives.

In a typical experiment, a 50g sample of limed hide or skin was placed in a 1L bottle with 300 ml of the 0.3% Triton X-100 solution along with an appropriate amount of sodium sulfate and the mixture was tumbled for 30 minutes. The desired amount of the acrylamide derivative was then added and the bottle was tumbled for various amounts of time. Samples were taken at various intervals for measurement of the pH of the solution and of the shrinkage temperature of the sample.

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

In order to improve the solubilities of DMBA, TMBA, and HMBAA in the reaction mixtures, a 1:1 mixture of water and isopropyl alcohol was used, in place of water alone, both with and without added sodium sulfate.

Amino-acid analyses were carried out on a Beckman Model 119CL Analyzer. Shrinkage-temperature measurements were carried out on strips of treated hides or skins using typical industry equipment. Digestions of the products with acid and alkali were carried out by immersing the samples in 10 times their weight of the appropriate solution at 65°C for 1 hr. The enzyme digestions were carried out at pH 7 and 37-38°C for 4 hr. The residues were washed, air-dried, and weighed.

These methods and procedures are the same as those reported in the first paper⁽¹⁾.

Results and Discussion

We discussed in the first paper⁽¹⁾ that the introduction of crosslinks into hide collagen increases the hydrothermal stability of the collagen (as measured by the shrinkage temperature), the resistance of the collagen to swelling, and the resistance of the collagen to enzymic attack. These same measurements are used in this paper.

We investigated the use of several salts for their effectiveness in reducing the swelling of the limed hide pieces at the 30% level with 3% of MBAA and the results are reported in Table I. Sodium sulfate, sodium chloride and potassium sulfate appeared to be equally effective in achieving the final shrinkage temperature of 79°C but sodium sulfate was chosen for further work because of its better ability to control alkaline swelling. The results obtained with ammonium chloride illustrate two points both of which have most likely influenced the results obtained. First, the ammonium chloride effectively lowered the pH of the reaction mixture within the first thirty minutes to about pH 7, a condition under which the desired reaction will not take place. Also the ammonia present in equilibrium with the ammonium ions can compete with the amino groups in the protein for the acrylamide residues of the reactant and reacts in exactly the same manner thus consuming the reactant. As it turned out, no detectable crosslinking of the hide collagen took place when this salt was used. Although the pH fell to about the same level in all of the other reactions shown in Table I, it did so - in all these other cases - over the course of the entire reaction, i.e. 24 hours.

TABLE I

Salts Used to Control Swelling (with 3% MBAA)

<u>Salt (30%)</u>	<u>Shrinkage Temperature °C</u>
No Added Salt	77
Sodium Sulfate	79
Sodium Chloride	79
Potassium Sulfate	78
Ammonium Chloride	59

The effect of time on the reaction with 3% MBAA and two levels of sodium sulfate, 30% and 60%, is shown in Figure 1. The reactions are not as rapid as those found with N-hydroxymethylacrylamide⁽¹⁾ and the final shrinkage temperatures are not as high. The results, however, are still respectable. At least 15 to 18 hr. are required to obtain the maximum shrinkage temperature.

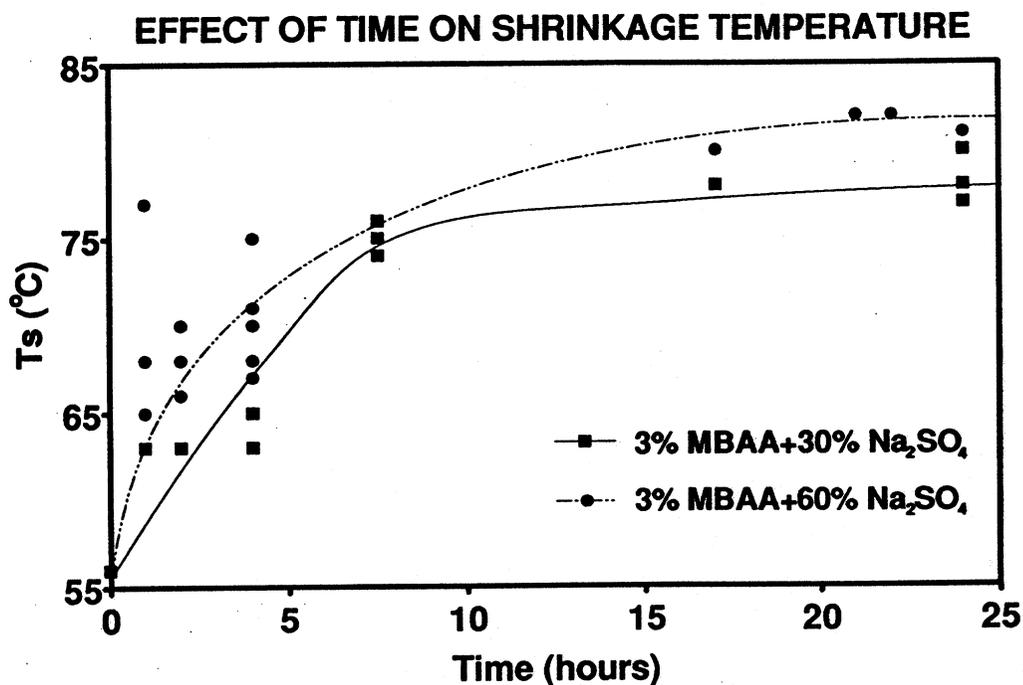


FIG. 1. — Effect of time on the crosslinking of cattlehide collagen by MBAA at two levels of added sodium sulfate as measured by shrinkage temperature. Multiple points at the same time represent the results of separate experiments.

—■— With 30% added sodium sulfate.
 —●— With 60% added sodium sulfate.

The effect of sodium sulfate on the reaction is shown in Figure 2. When used with just 3% MBAA, all four levels of sodium sulfate provided higher final shrinkage temperatures after 24 hr; a maximum of 85°C was obtained with both 60% and 90% sodium sulfate. Even the lowest amount of sodium sulfate, 18%, increased the rate of reaction.

As we discussed in the first paper⁽¹⁾, the sodium sulfate is obviously controlling the alkaline swelling of the hide collagen and opening up the collagen fibers, providing an improved condition for the chemicals to penetrate and react.

Figure 3 shows the results of using different levels of MBAA, this time in the presence of two levels, 30% and 60%, of sodium sulfate. The amounts of 3% MBAA and 60% sodium sulfate provided the best results. It is worth noting that amounts of MBAA in excess of 5% provided lower shrinkage temperatures. When high levels of MBAA are used the reaction of each available amino group with a separate MBAA molecule is favored over crosslinking. The available amino groups of the hide collagen, therefore, are consumed with free MBAA and are not available for crosslinking. We made use of this result in an earlier study⁽³⁾ when we wanted to introduce polymerizable vinyl groups onto collagen for further graft-polymerization reactions.

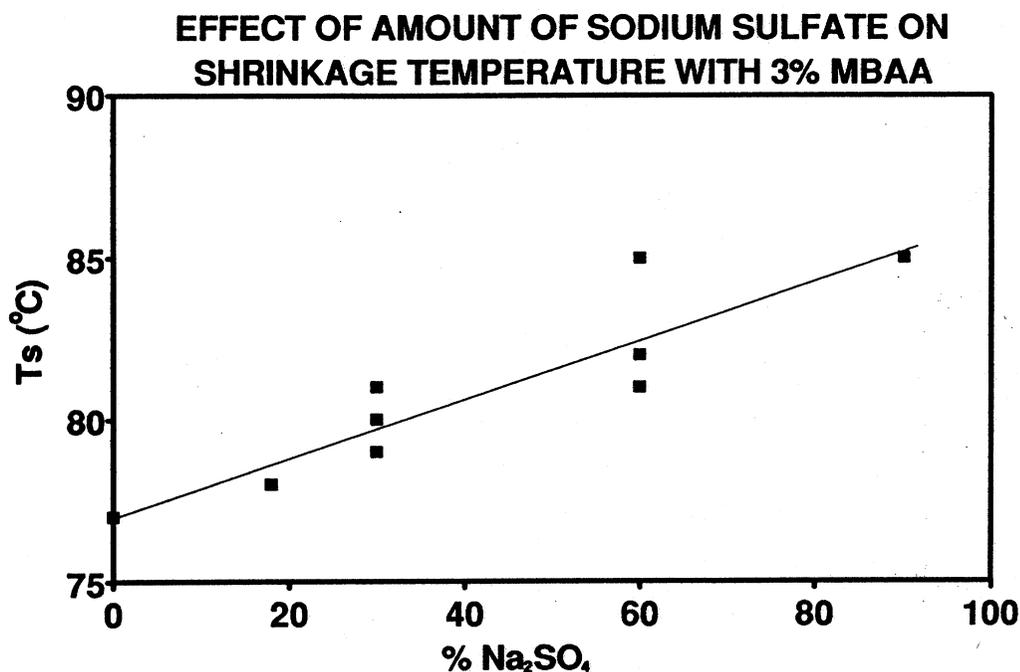


FIG. 2. — Effect of amount of added sodium sulfate on the crosslinking of cattlehide collagen by 3% MBAA after 24 hr. Multiple points for the same amount of sodium sulfate represent the results of separate experiments. A final shrinkage temperature of 85°C was obtained only when pH of reaction mixture not permitted to fall below 9 by addition of alkali (see text).

No independent study of the effect of pH on the reaction was carried out with these compounds; as mentioned above, however, the pH of the reaction mixtures fell as the reaction proceeded when no effort was made to control it. The final pH was normally close to 8 and the shrinkage temperature was about 80°C to 82°C in these cases. In several instances, we added sodium carbonate or magnesium oxide to the reaction mixtures to lessen the fall of pH and achieved a final pH of between 9 and 10 in these cases. This resulted in a slightly higher final shrinkage temperature of 85°C. These results are illustrated in Figures 2 and 3 by the experimental points at this temperature as noted.

Our attempts to use the three higher homologs of MBAA were not very successful. DMBAA, 3%, was used with 60% sodium sulfate in two separate attempts and, after 24 hr., shrinkage temperatures of around 70°C were achieved. Similar attempts with TMBAA and HMBAA were completely unsuccessful. Considering the possibility that the lack of solubility of these compounds in water was the major problem, the reactions were repeated with a 1:1 mixture of water and isopropyl alcohol, first with added sodium sulfate, then without it, on the assumption that the alcohol would control the swelling. Neither was successful. These results were disappointing because we felt that the different methylene chain lengths of the compounds would give us a measure of the distances between reacting amino groups in the collagen. We do not feel that the completely negative results obtained should be taken as demonstration that the optimum chain length is one methylene group, though that may be the case. We had hoped, also, that the longer chain lengths in the crosslinks, if they had been formed, would not suffer from the same susceptibility to acid as do the MBAA crosslinks—to be discussed later.

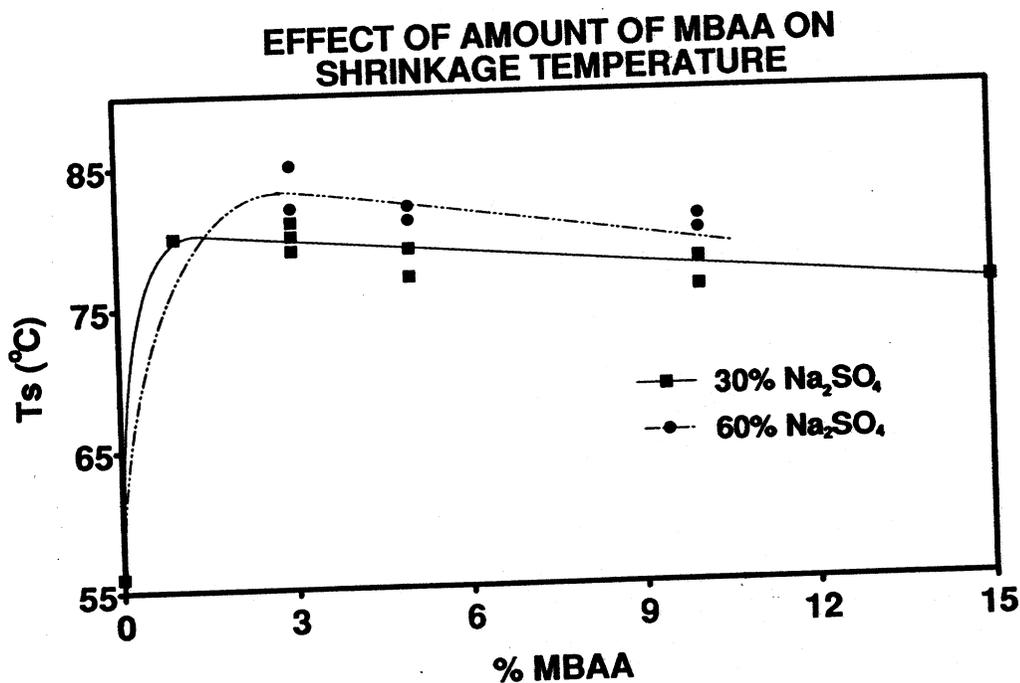


FIG. 3. — Effectiveness of different amounts of MBAA after 24 hr on the crosslinking of cattlehide collagen at two different levels of added sodium sulfate as measured by shrinkage temperature. Multiple points at the same amount of MBAA represent the results of separate experiments. A final shrinkage temperature of 85°C was obtained only when the addition of alkali prevented the pH of the reaction mixture from falling below 9 (see text).

- With 30% added sodium sulfate.
- With 60% added sodium sulfate.

The stability of the tannages with MBAA and DMBAA to exhaustive washing in running tap water was excellent. The product tanned with MBAA experienced no loss in shrinkage temperature even after 72 hr. Surprisingly, the product tanned with DMBAA realized an increase in shrinkage temperature of 2°C. This is best explained by considering that further crosslinking is taking place under these conditions from the reaction of the second reactive group of the DMBAA molecules that had only reacted at one end during the initial reaction. In comparison, the results obtained with the two compounds described in our first paper⁽¹⁾, where the shrinkage temperatures fell, certainly demonstrate that two different types of crosslinking are involved.

The resistance of these products to acid, alkali and enzymic attack was measured and compared to that of untreated controls. The results are shown in Table II. When compared with the control sample, the MBAA and DMBAA products are more stable to both alkaline and acidic conditions. The susceptibility of the $-\text{CO}-\text{NH}-(\text{CH}_2)_n-\text{NH}-\text{CO}-$ groups to any acid hydrolysis, however, indicates the need to consider this in further leather-manufacturing processing steps with these products. Further investigation of this is underway. Both MBAA and DMBAA provided resistance to enzymic attack but the results obtained with DMBAA were considerably better than those with MBAA. The reason for this is not known.

The analyses for selected amino acids of the product made with MBAA are reported in Table III and compared with the results for the product made with acrylamide (AA). While an 80% reduction in the amount of lysine was realized with acrylamide, a 90% reduction was realized with MBAA. A 75% reduction was realized in the amount of hydroxylysine found as compared with a 58% reduction with acrylamide. The lysine derivatives to be expected from these reactions⁽¹⁾ were identified chromatographically in the hydrolysates. The bis-lysinyll derivative was by far the more abundant.

TABLE II

**Resistance to Acid, Alkali and Enzymes:
Percent Loss in Weight When Treated With**

	0.1N Hydrochloric Acid	1.0N Hydrochloric Acid	0.1N Sodium Hydroxide	Collagenase
Control	17	76	81	41
MBAA	7	51	5	19
DMBAA	7	65	24	1

TABLE III

Amino Acid Analyses of Modified Proteins

Amino Acid	Mole Percent (Normalized on Phenylalanine)		
	Control	AA	MBAA
LYS	2.03	0.39	0.18
HLY	0.64	0.26	0.16

Finally, it is interesting to compare the effectiveness of the two acrylamide derivatives in crosslinking hide collagen with that of the two compounds described in the first paper of this series⁽¹⁾ and with that for acrylamide also included. N-Hydroxymethylacrylamide and N,N'-(1,2-dihydroxyethylene)-bisacrylamide obviously provided the highest shrinkage temperatures and reacted very rapidly. It is almost certain that for either of these two compounds to be used as tanning agents, this rapid initial reaction would have to be moderated in some manner. MBAA reacted more slowly and did not achieve as high a shrinkage temperature while DMBAA reacted even more slowly, probably because of its more limited solubility. This may prove to be beneficial for use as a tanning agent. The sensitivity of these tannages to acids is, also, of major concern because of the necessary exposure of the products to acidic conditions in further leather-manufacturing steps. These results indicate the need for additional studies of these and similar compounds and the direction those studies should take.

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Discussion

DR. DAVID BAILEY (U.S.D.A.) discussion leader: Thank you very much, Steve. Do we have any questions?

MR. ARTHUR AMBROSINO (Independent Leather): Steve, Frank alluded to the use of acrylamides and acrylonitriles as fabric sizing materials coming under increased scrutiny. Do you see it as worthwhile looking at chemicals which might later become concerns because of their toxicity? Secondly, do I understand that you are tanning without a prior pickling operation?

DR. FEAIRHELLER: The answer to the second question is 'yes'. These tannages were conducted directly on limed stock which was washed after liming and then put into the sodium sulfate solution. Now, to get back to your first question, acrylamide is something you would not want to use in your tannery, as well as some of those lower-molecular-weight compounds we talked about last year, the N-methylol acrylamides. The methylenebisacrylamide is used, as are these other bisacrylamides, in the polymer industries for cross-linking polyacrylamide. As you get to higher molecular weights, the toxicity of these compounds goes down. That was another reason for looking at these higher-molecular-weight compounds. Because of our inability to produce products with acceptable physical properties, however, we plan to discontinue this research. The fact is that there are also other compounds now that we have become aware of with less hazardous properties that we will investigate in the future. We have demonstrated indeed that they do behave, as we thought they would, as crosslinking agents and by derivatizing the protein we can improve its ability to accept chrome and thus obtain a higher shrinkage temperature, but there are too many negative factors involved here.

DR. ECKHARDT HEIDEMANN (Technische Hochschule Darmstadt): There is a maximum in one curve at about 5% of monomethylene. What is the relationship to the number of lysines involved in your first table?

DR. FEAIRHELLER: I didn't do the calculation but we obtained 90% reaction of the free lysine residues using 3% methylenebisacrylamide. The optimum has to be just in about that range.

DR. HEIDEMANN: From this finding one can conclude that more or less all of the other lysines are inhibited. That is very important to see.

DR. FEAIRHELLER: That is true for better than 10% under these alkaline conditions.

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