

**CROSSLINKING OF COLLAGEN WITH
ACRYLAMIDE DERIVATIVES I:
N-Hydroxymethylacrylamide and
N, N'-(1,2-Dihydroxyethylene)-Bisacrylamide**

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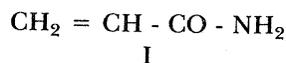
Abstract

Two derivatives of acrylamide, namely: N-hydroxymethylacrylamide and N,N'-(1,2-dihydroxyethylene)-bisacrylamide, were found to react rapidly and, at least in part, irreversibly with hide and skin collagen. As measured by shrinkage temperature, the two compounds effectively crosslinked the collagen and, with proper control, could serve as tanning agents. The reaction takes place under alkaline conditions but swelling of the protein was controlled by the addition of sodium sulfate to the reaction mixtures. Shrinkage temperatures of 85°C and higher were obtained. The products exhibited the required resistance to chemical and enzymic attack and, on a small scale, were made into white or pale yellow leather, respectively. The results were compared to those obtained with formaldehyde and glyoxal.

Introduction

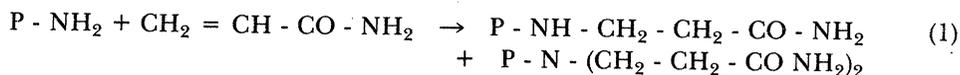
As discussed in detail by Harlan and Fairheller⁽¹⁾, the tanning of hides is accomplished by the crosslinking of the major hide protein, collagen. Tanning with trivalent chromium compounds is by far the most widely used and most effective tanning agent for most leathers. Concerns over suspected environmental problems associated with the use and disposal of trivalent chromium compounds and possible restrictions on the availability of chrome ore imported from other countries, however, have caused research projects to be initiated in a number of laboratories to find alternatives^(2,3). This research has included studies of other metal tannages as well as attempts to develop new organic tannages. The only truly effective organic tannages are the aldehyde tannages using formaldehyde and glutaraldehyde. Each aldehyde, however, has certain drawbacks associated with its use. Formaldehyde, in addition to having restrictions on its use because of concerns over its effects on the health of those using it, does not produce stable crosslinks since its reaction with proteins is reversible. Glutaraldehyde does provide stable crosslinks but is suitable only for certain types of leather. Neither aldehyde, moreover, is satisfactory as a primary tannage for all types of leather.

The development of new monomers by the polymer manufacturing industry and their use as crosslinking agents for hide collagen using reaction conditions known in classical organic chemistry has provided us with the possibility of developing a new group of tanning agents. The monomers are all derivatives of acrylamide (I)



and the reaction conditions are those adapted from the classical Michael Reaction⁽⁴⁾. These conditions are the same as those described in a recent paper on the chemical modification of hide collagen to improve chrome tanning⁽⁵⁾.

Acrylamide reacts readily and almost completely with the side-chain amino groups of lysyl residues in hide collagen as shown in equation (1)⁽⁴⁾. This

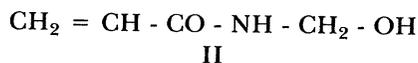


reaction with acrylamide itself obviously does not provide crosslinking. Derivatization of the $-\text{NH}_2$ group of acrylamide with other reactive groups can provide compounds that will crosslink hide collagen and this paper is the first of a series in which we will describe our research on such multifunctional compounds.

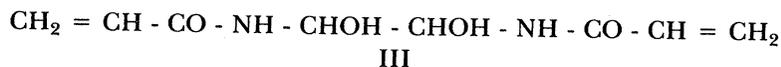
Up until now, the availability in commercial quantities of organic compounds with at least two reactive groups that will crosslink hide collagen has been limited. In addition to availability, such compounds must also:

1. Dissolve or disperse easily in water without reacting with it and readily penetrate the hides or skins under conditions normally used or achievable in the leather-manufacturing process.
2. React readily and uniformly with hide collagen at room temperature forming relatively stable crosslinks and providing a suitably high shrink temperature.
3. Be safe for use in a tannery.

In this paper we shall describe the crosslinking of hide collagen with N-hydroxymethylacrylamide (II) (HMAA)



and N,N'-(1,2-dihydroxyethylene)-bisacrylamide (III).



Compound II is a product of the reaction of acrylamide or acrylonitrile with formaldehyde^(6,7). (Because it is formed with formaldehyde and is a potential source of formaldehyde under certain conditions, care must be taken in its use. Users are referred to the Manufacturer's Safety Data Sheet for this compound for the proper conditions for handling it.) This compound has the reactive $-\text{NH} - \text{CH}_2 - \text{OH}$ group which, in addition to the $\text{CH}_2 = \text{CH} - \text{CO} -$ group, reacts rapidly with proteins. The one reaction was shown by amino-acid analysis to be a Michael Reaction; the other is almost certainly a condensation

reaction involving the hydroxymethyl groups. Compound III is a product of the reaction of acrylamide with glyoxal and possesses the same type of reactivity but has in total four sites for reaction instead of two. The conditions through which we have caused these compounds to react with hide collagen and our results are described below.

Experimental

MATERIALS

N-hydroxymethylacrylamide was obtained as a 48% by weight solution in water and N,N'-(1,2-dihydroxyethylene)-bisacrylamide was obtained as the dry solid, both from Aldrich Chemical Company.* Other chemicals used were standard reagent grade laboratory chemicals. Limed cattlehides and limed sheepskins were obtained from local tanneries or were produced in our experimental tannery using standard procedures.

METHODS AND PROCEDURES

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

In a typical experiment, a 50g sample of limed hide or skin was placed in a 2L 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 appropriate amount of the acrylamide derivative was then added and the bottle was tumbled for various lengths of time. Samples were taken at specified intervals for measurement of solution pH and the shrinkage temperature of the sample. To evaluate the effect of pH, citric acid in various amounts was added to the reaction mixtures, which were then tumbled for 30 minutes and the pH was measured before the acrylamide derivative was added.

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 and on ground hide collagen using a Perkin-Elmer Model DSC-1 differential scanning calorimeter. Digestions of the products with acid or alkali were carried out by immersing the samples in 10 times their weight of the appropriate solution at 65°C for 1 hour. Enzyme digestions were carried out at pH 7 and at 37-38°C for 4 hours. The residues were washed, air-dried, and weighed.

Results and Discussion

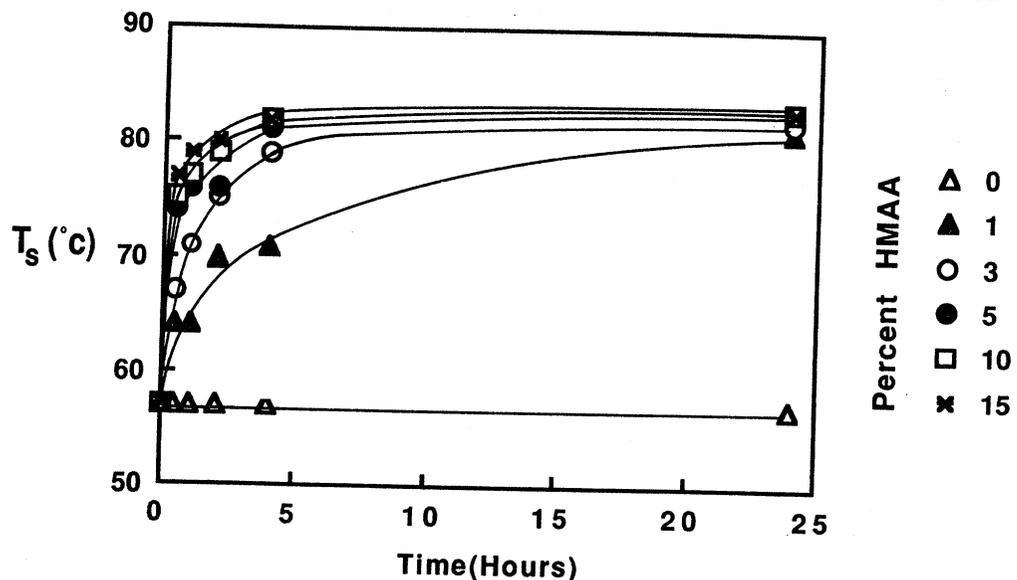
Crosslinking of the polypeptide chains of collagen has been accepted as the mechanism of tanning and the stability of the tannage is directly related to the chemical stability of the crosslinks. The introduction of these crosslinks increases the hydrothermal stability of the collagen (as measured by the shrinkage temperature), the resistance of the collagen

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

to swelling and the resistance of the protein to enzymic attack⁽⁸⁾. The value of the shrinkage temperature as a measure of the effectiveness of a tanning agent has been well recognized by both the practical tanner and the leather research worker⁽⁹⁾ and is used regularly for this purpose.

As a first step, the effect of applying various amounts of compound II to limed cattlehide samples for different periods of time with no added salts was studied and the results are shown in Figure 1. As judged by the significant increase in shrinkage temperature, which is directly related to both the amount of compound II offered and the time allowed for reaction, compound II is a crosslinking, or tanning, agent. A maximum shrinkage temperature of 83-84°C was obtained in 24 hours with 5% of compound II although shrinkage temperatures of 80°C or higher were obtained in much shorter periods of time and with less compound II. With 10% of compound II, essentially no further increase in shrinkage temperature was obtained after 4 hours. The pH of these reaction mixtures fell with increasing concentrations of compound II as shown in Figure 2 indicating that some delimiting was also taking place.

FIGURE 1
EFFECT OF HMAA ON SHRINKAGE TEMPERATURE



The effect of three different levels of sodium sulfate on the reaction of limed cattlehide samples with 3% of compound II is shown in Figure 3. Even the lowest amount used, 5%, provided a more rapid reaction, and all three levels provided slightly higher shrinkage temperatures overall. Under these alkaline conditions, sodium sulfate is obviously reducing the swelling of the hide collagen and opening up the collagen fibers providing an improved condition for the crosslinking chemical to penetrate and react. A level of 10% sodium sulfate was chosen for further experiments and results from a repeat of some of the experiments reported in Figure 1 are presented in Figure 4. As can be seen, shrinkage temperatures above 85°C were obtained after just 1 hour with just 5% of compound II. With higher amounts of compound II and a 20 hour reaction time, the shrinkage temper-

FIGURE 2
EFFECT OF HMAA ON pH

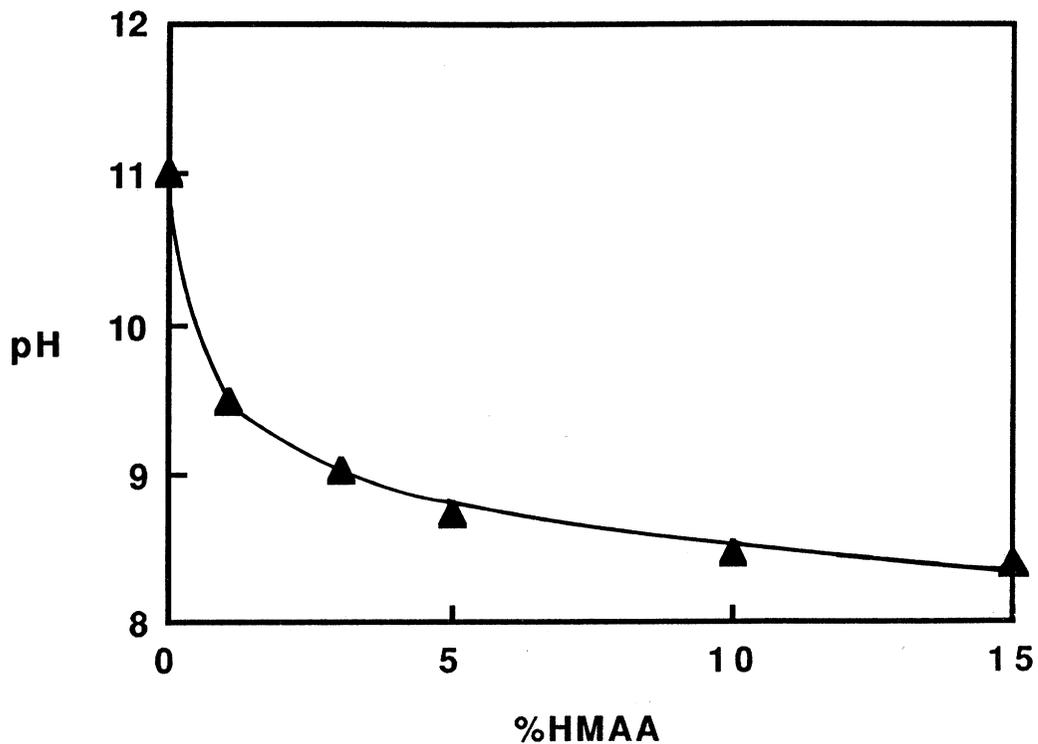


FIGURE 3
EFFECT OF SODIUM SULFATE ON SHRINKAGE TEMPERATURE

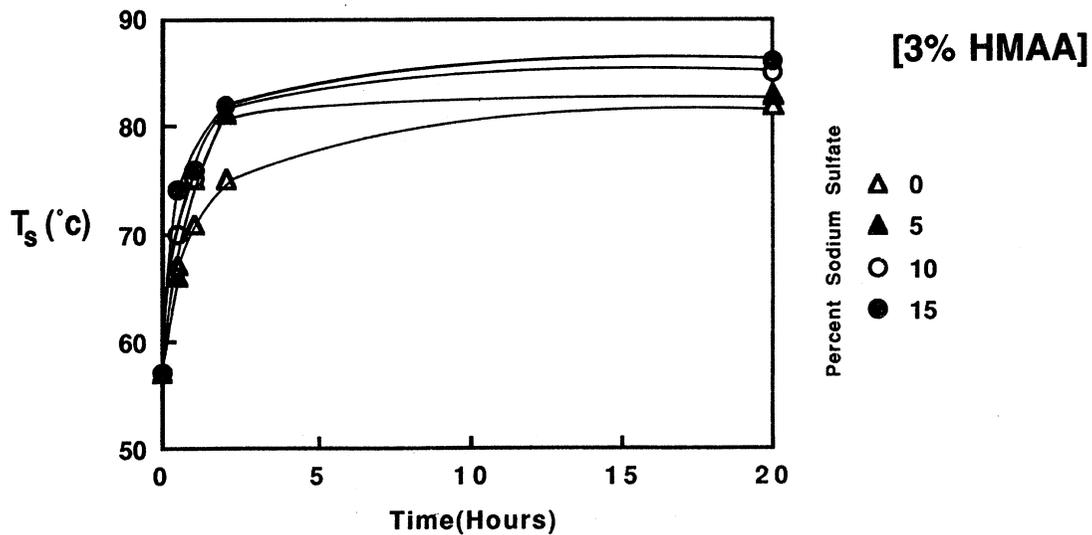


FIGURE 4

EFFECT OF HMAA ON SHRINKAGE TEMPERATURE
IN PRESENCE OF 10% SODIUM SULFATE

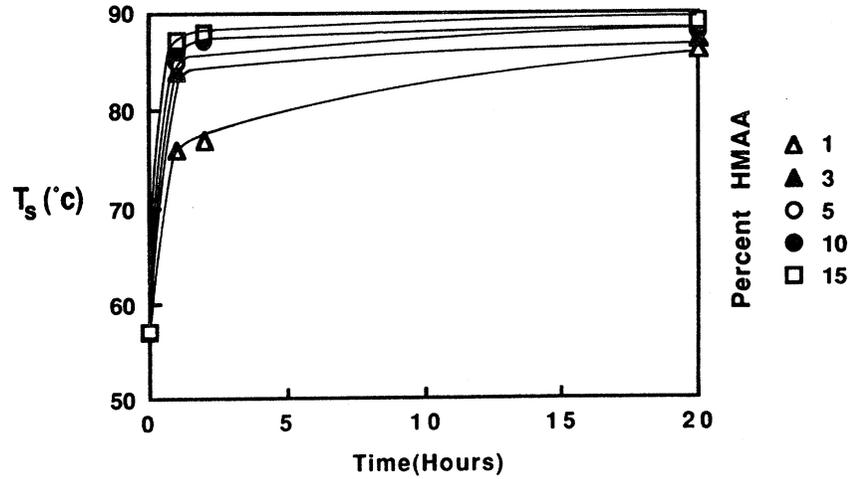
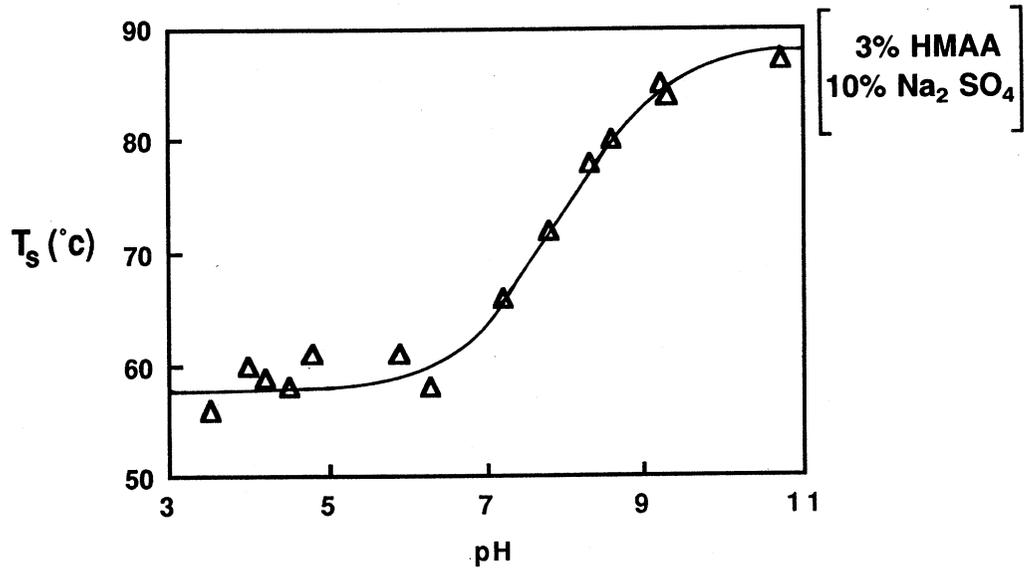


FIGURE 5

EFFECT OF pH ON SHRINKAGE TEMPERATURE



atures approached 90°C. Similar results were obtained with limed sheepskins where a maximum shrinkage temperature of 84°C was reached.

The effect of pH on the reaction using 3% of compound II and 10% sodium sulfate and running the reactions for 24 hours is shown in Figure 5. As was anticipated from the nature of the Michael Reaction taking place, an alkaline pH of at least 9 is required and the maximum effect is not realized until the pH is raised above 10.

Compound III gave similar results except that the products were light yellow instead of pure white. Treatment of limed cattlehides with 3% of compound III in the presence of 10% sodium sulfate resulted in a shrinkage temperature of 82°C after 2.5 hours and 83°C in 4 hours. The shrinkage temperature increased only to 84-85°C after 24 hours so there appears to be little advantage to continuing the reaction beyond about 4 hours.

We felt that because of the relationship of compounds II and III to formaldehyde and glyoxal, respectively, it was necessary to compare the products obtained with those obtained with the two aldehydes. Indeed, the product prepared with an amount of formaldehyde equivalent to 10% of compound II using identical conditions to those reported in Figure 4 had a shrinkage temperature of over 90°C, about 5°C higher than the product prepared with compound II. Similarly, the product obtained with glyoxal had a shrinkage temperature about 3°C to 4°C higher than that obtained with compound III. However, the results presented and discussed below indicate clearly that the products obtained with compounds II and III, while having shrinkage temperatures lower than those obtained with formaldehyde and glyoxal, respectively, were chemically different and superior in other ways.

Several methods were used to test and compare the stability of these tannages. First, simple washing of samples of cattlehide and sheepskin treated with 3% of compound II with running tap water for 72 hours resulted in only 1°C decreases in shrinkage temperatures. Other samples of treated cattlehide were tested for susceptibility to digestion with acid (two different concentrations), base, and the enzyme collagenase⁽¹⁰⁾. The results are reported in Table I. It is clearly evident that the resistance to digestion under all four sets of conditions is significantly improved over the results obtained with the control and, in those conditions tested, over the results obtained with the corresponding aldehyde.

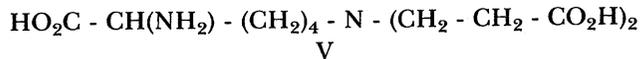
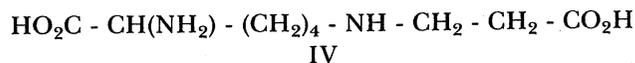
TABLE I

Resistance to Acid, Alkali and Enzymes

Tanning Agents	Percent Loss in Weight When Treated with			
	0.1 N Hydrochloric Acid	1.0 N Hydrochloric Acid	0.1 N Sodium Hydroxide	Collagenase
Control (i.e. untanned)	16.88	75.53	81.00	41.36
II	5.43	24.17	20.5	5.61
Formaldehyde	14.10	—	11.61	—
III	3.24	38.13	9.29	2.78
Glyoxal	11.23	—	13.10	—

Amino-acid analyses of these samples confirmed that the primary site of reaction is with the lysyl residues of the collagen. The results are summarized in Table II. Reaction of hide collagen with compound I under identical conditions as used for compounds II and III resulted in an 80% reduction in the amount of lysine found by amino acid analysis of the hydrolysate of the modified protein sample. Reaction with either compound II or III

resulted in 25% reductions in the lysine as measured by the same method. Similar reductions were realized for hydroxylysine. The products expected from the acid hydrolysis of protein lysyl residues reacted with compound I, as shown in Equation I, are compounds IV and V⁽⁴⁾;



indeed, the chromatogram for the hydrolysate of this sample had peaks corresponding to reported elution times for these two compounds. The peak corresponding to compound V was the larger of the two indicating that the reaction proceeded toward complete modification of the lysyl residues. Compounds IV and V could not be quantified due to the lack of pure compounds that could serve as standards. Their presence in the hydrolysates is simply indicated by a + in Table II. The hydrolysates of the samples modified with compounds II and III had peaks corresponding mainly to compound IV indicating that the lysyl residues reacted mostly only once and not twice as they did with compound I. When the hide protein was allowed to react with formaldehyde under the same conditions and then hydrolyzed for amino-acid analysis, the amounts of these three amino acids were essentially the same as those in the control.

TABLE II

Amino Acid Analyses of Modified Proteins

Amino Acid	Mole Percent of Amino Acid after Tannage with the agents shown					
	CONTROL	I	II	FORM	III	GLYOXAL
LYS	3.05	0.60	2.28	3.08	2.48	2.30
HLY	0.96	0.40	0.71	0.83	0.76	0.56
ARG	5.40	5.75	5.44	5.57	3.37	1.55
IV		+	+		+	
V		+			Trace	
UNK*					+	+

*Unknown

It is interesting that reaction of the hide collagen with compound III resulted in a 42% reduction in arginine. Whether or not reaction of this amino acid would result in crosslinking is not known. When the hide protein was allowed to react with glyoxal under the same conditions, comparable reductions in the amounts of lysine and hydroxylysine were found. In addition, the amount of arginine was reduced by over 70%. There was an additional peak in the chromatogram of the hydrolysate of protein modified with both compound III and glyoxal referred to as UNK in Table II.

From the results reported above, it is apparent that compounds II and III are reacting as tanning agents by crosslinking the hide protein. The alternative conclusion that these compounds are undergoing hydrolysis to the respective aldehydes which are acting as the tanning agents appears to be refuted by several of the results reported. The effect of pH

on the reaction is not consistent with a formaldehyde tannage, and the stability of the tannages, as measured by the several tests above, is greater. Also, the results of the amino-acid analyses clearly indicate that the lysyl residues of the hide protein are reacting irreversibly with compounds II and III. We have not ruled out the possibility that we might be realizing a combination of effects including some limited hydrolysis to the aldehydes which are also reacting. This latter may explain the loss of arginine found upon treatment with compound III.

The reduction of these reactions to practical tannages and the properties of the resulting leathers will be reported in a later paper as will a consideration of modifications required in other unit process steps. Their use to make leather has only been carried out on a small laboratory scale to date.

Acknowledgement

Author Li Ying would like to express his deep appreciation to his best friends, Dr. and Mrs. William Ma, for their support in daily life during the period of his stay at the Eastern Regional Research Center. He would also like to express his appreciation to Dr. Robert Tu for his kind assistance in planning this visit to the United States of America and to the Eastern Regional Research Center for making it possible for him to conduct these research studies at the Center in collaboration with his coauthors.

The authors appreciate the assistance given them by Edward J. Diefendorf in conducting small-scale leather production studies and by Harold J. Dower, who carried out the amino-acid analyses.

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