

Some Properties of Collagen Modified by the Mannich Reaction

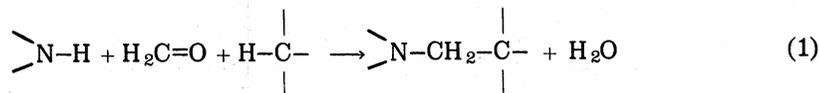
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Synopsis

The Mannich reaction of several aliphatic active hydrogen compounds with formaldehyde and collagen, the principal protein of animal hides and skins, was investigated. These reactions were all carried out under extremely mild conditions: aqueous solutions buffered at about pH 4 and at or slightly above room temperature. The sites of the reaction in the protein were found to be the lysine, hydroxylysine, and histidine residues. The extent of the reaction and the effects of various factors on the reaction also were determined. Although polyfunctional active hydrogen compounds were used, there was little indication that cross-linking had taken place. With acetylacetone and methyl acetoacetate the evidence indicated that dihydropyridines were formed. The extent of such a reaction was calculated from ultraviolet absorption data and from the amino acid analysis of the chemically modified proteins. The results of the two methods were in extremely good agreement with each other. With malonic acid a product was formed that offers potential for practical application. A number of new compounds were found in the hydrolyzate of the product. Two of these were identified as N_{ϵ} -(β -carboxyethyl)-L-lysine and $N_{\epsilon}, N_{\epsilon}$ -bis(β -carboxyethyl)-L-lysine.

INTRODUCTION

Perhaps one of the most useful reactions of primary and secondary amino groups is the Mannich reaction (1). This reaction, which takes place under a variety of conditions, involves the condensation of the amino group with an aldehyde and an active hydrogen compound, as shown:



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This is a simplified equation, and more complex reactions can and do occur. For example, if the original amino group is primary, the initial product is a secondary amine, which will react again in the same manner. Further, if the original active hydrogen compound contains more than one active hydrogen, all of these can react. In synthetic work these complex reactions can present serious problems. However, as will be apparent later, they were expected to be beneficial for our purposes.

In the application of this reaction to the chemical modification of proteins the predominant reaction is with the ϵ -amino groups of lysine and hydroxylysine residues. The aldehyde, which is used extensively in this reaction, is formaldehyde, an aldehyde also well known for its tanning action (2). The latter reaction has also been considered a Mannich type of reaction (3). A variety of active hydrogen compounds will take part in the Mannich reaction. These include aromatic compounds and aliphatic compounds. The former type of compound has already been implicated as taking part in a Mannich reaction with formaldehyde and proteins (4).

It was the intent of the present investigation to study the reactions of a variety of aliphatic active hydrogen compounds with collagen, the major protein of animal hides and skins, with the hope of either finding new tanning agents or attaching new functional groups that would have a beneficial effect on subsequent treatments utilized in leather manufacturing.

EXPERIMENTAL

Preparation of Collagen

The collagen was prepared from the central portion of a limed cowhide, from which the grain and flesh portions had been removed by splitting. This central portion was extracted with lime, acetic acid, and salt solutions, to remove extraneous material. It was finally washed free from electrolytes, dehydrated with acetone, and ground in a Wiley mill.

Mannich Reaction on Collagen

The collagen (3 g.) was suspended in a solution consisting of 29.5 ml. of water, 1.1 g. of sodium chloride, 0.75 g. of sodium formate, and 0.6 ml. of a 10% sulfuric acid solution. This suspension was shaken for 30 min., during which time the pH was 3.95. Formalin (equivalent to 10.7 mmoles of formaldehyde) and the active hydrogen compound (6.1 mmole) were added, and the suspension was shaken for 24 hr. In some cases methanol was added, so that only one liquid phase was present.

The product was isolated by filtration, washed first with water and then with acetone, and then allowed to dry on the funnel.

Mannich Reaction on Pickled Cabretta Skins

A piece of a pickled cabretta skin (25 g.) was placed in a solution consisting of 50 ml. of water, 1.25 g. of sodium chloride, and 1.25 g. of sodium formate in a 1 qt. bottle. This was placed on a tumbling machine and tumbled for 30 min. At the end of this time the desired amounts of formalin and active hydrogen compounds were added, and the tumbling was continued for 18 hr. The skin was then removed, washed thoroughly with running water, and allowed to dry at room temperature. For determinations of shrinkage temperature the sample was taken before drying. For ultraviolet analyses portions of the dried sample were submitted to hydrolysis in 2N hydrochloric acid solution for 4 hr.

Cyanoethylation of Collagen

The collagen (3 g.) was suspended in 30 ml. of a 2N sodium carbonate solution, and the resulting suspension was shaken for 0.5 hr. The pH of the resulting suspension was 11.1. Acrylonitrile (1.25 ml., 1 g.) was added, and the shaking was continued for 4 hr. more. The pH of the resulting suspension was 11.0. The shaking was continued while glacial acetic acid was added in 0.5 ml. portions. The pH was checked after each addition. When the pH of the solution had stabilized at 5.6, the shaking was stopped, the reaction mixture was filtered, and the residue was washed well with water, methanol and, finally, acetone. It was then allowed to dry on the funnel.

Analytical Determinations

The amino acid analyses were run on a Piez-Morris ion-exchange column with a continuous, gradient, elution buffer (5). The ultraviolet absorption spectra were measured on a Bausch and Lomb Spectronic 505 Model automatic recording spectrophotometer. The shrinkage temperatures of the ground samples were determined by the method of differential thermal analysis (6). The shrinkage temperatures of intact skins were determined on freely suspended samples as described by Fein et al. (7). Formaldehyde was determined in the protein by the method of Highberger and Retzsch (8) and, in the spent reaction mixtures, by a modified iodometric method (9).

RESULTS AND DISCUSSION

The active hydrogen compounds used in this investigation were acetyl-

TABLE I

Amino Acid Content of Chemically Modified Collagen

Amino acid ^a	Collagen control	Formaldehyde alone	Modified with formaldehyde and:			
			Acetylacetone	Methyl acetoacetate	Malonic acid	Dimethyl malonate
HOPro	71.0	63.8	70.5	78.8	111.0	66.9
Asp	48.2	48.0	44.6	44.1	48.1	44.9
Thr	16.6	16.6	15.7	15.3	17.0	15.5
Ser	33.5	32.4	32.4	32.4	36.0	30.5
Glu	74.8	72.3	69.5	69.5	75.9	65.0
Pro	130.2	130.0	122.9	118.7	136.3	127.1
Gly	362.6	353.5	326.1	329.2	332.5	332.0
Ala	114.5	115.4	107.1	104.2	112.8	108.7
Val	22.5	24.2	20.8	19.5	21.9	23.0

Meth	1.3	6.0	5.5	4.8	7.0	5.4
Ileu	12.9	13.0	11.9	11.8	14.0	13.4
Leu	25.6	26.0	23.7	23.6	25.2	24.5
Tyr	3.6	0.0	0.0	0.0	0.0	0.0
Phe	14.0	13.4	13.4	12.1	12.4	14.0
HOLys	7.5	7.1	3.5	4.6	7.5	6.7
Lys	25.7	25.3	6.3	8.7	0.9	14.3
Hist	5.9	5.6	3.5	2.9	0.0	1.7
Arg	50.7	50.6	48.7	46.7	49.1	48.3
Total N, ^b %	18.10	18.22	17.02	16.27	17.31	17.24
Ts, ^c °C.	67.9	86.8	72.8	68.8	77.5	82.6

^a Millimoles per 100 g. of sample.

^b Kjeldahl.

^c Shrinkage temperature; see under "Experimental."

acetone, methyl acetoacetate, malonic acid, and dimethyl malonate. This is by no means an exhaustive list of active hydrogen compounds nor even of functional groups that could be introduced by this method, but these compounds were selected for reasons that will become obvious when each is discussed.

Several reasons may be cited for the selection of the conditions for the general chemical modification reaction. One of the more important of these reasons is the fact that collagen, besides intact animal hides and skins, is completely stable in a salt solution of the concentration used, buffered at the chosen pH. Another reason is based on the finding that these were the optimum conditions for the treatment with malonic acid and formaldehyde, which appear to give the most promising product. A third reason is that formaldehyde alone reacts with collagen to a considerable extent under these conditions (2).

For most of the analytical work a purified collagen preparation was used (see under "Experimental"). This material had already been subjected to strongly basic conditions (lime and sulfide) in order to remove the hair; therefore, it cannot be considered native collagen. However, it does conform fairly closely to collagen in amino acid composition. The analytical data for this material are given in Table I along with the corresponding data for some of the chemically modified products.

The other material used as a source of collagen was the pickled skin of a South American hairsheep, cabretta. This was used completely in the early work and at all times when it was desired to determine the effect of a chemical modification reaction on the final leather.

In all our attempts at chemical modification by using the Mannich reaction a comparable reaction was always run with formaldehyde alone with the protein, so that the properties imparted by the formaldehyde could be compared with the properties imparted by the chemical modification. Therefore, analytical data for formaldehyde-treated collagen are included in Table I.

Acetylacetone

This compound of all those tested had the greatest possibility of producing crosslinks because of the large number of active hydrogen atoms. It reacted rapidly with the protein and formaldehyde, as indicated by the color produced in the protein. This took on a deep yellow color initially and finally turned brown. That the protein had been modified was indicated by a number of other observations. First, the shrinkage temperature of the product is 14 C.° lower than that of the formaldehyde-treated sample. Second, the total nitrogen content of the product is more than 1% lower than those of collagen or the formaldehyde-treated sample. Third, the lysine and hydroxylysine contents were substantially reduced, the former by 70 to 75% and the latter by about 50%. The histidine content

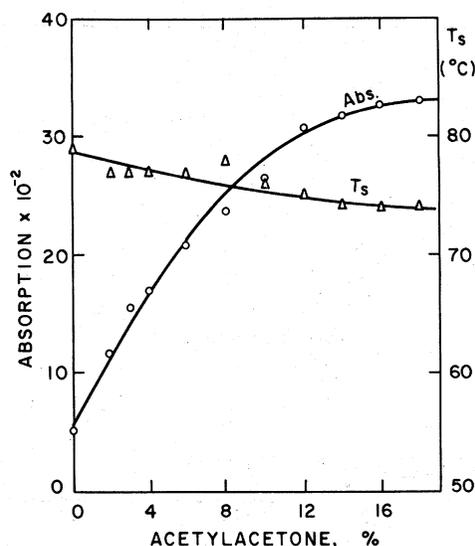


Fig. 2. Effect of acetylacetone concentration (4.8% formaldehyde) on (o) the ultraviolet absorption and (Δ) shrinkage temperature of modified collagen.

Although the reaction conditions used for the chemical modification are not considered optimum for this conversion, they are not too far removed from those recommended by Haley and Maitland (11). Moreover, the fact that the environment at any specific location within the protein may be considerably different from the conditions existing in the external solution must be taken into consideration.

Compounds of the type shown (3,5-dicarbonyl-1,4-dihydropyridines) have fairly strong ultraviolet absorption (12). Therefore, a portion of the chemically modified sample was submitted to a mild acidic hydrolysis, and the ultraviolet absorption spectrum of the hydrolyzate was measured. The spectrum is reproduced in Figure 1. The essential features of this spectrum agree with those of the spectra of the above-mentioned types of compound (12).

With the information from the ultraviolet absorption spectrum and the amino acid analysis some estimated comparisons of the extent to which this reaction may have taken place can be made. A few calculations based on the extinction coefficient expected for this type of compound indicate that there are about 16 mmoles of these ultraviolet-absorbing residues per 100 g. of protein. By using the data from the amino acid analyses it can be calculated that a combined 23 mmoles of lysine and hydroxylysine per 100 g. of protein have reacted. This is a surprisingly close agreement, considering the experimental difficulties and the assumptions made in the calculations.

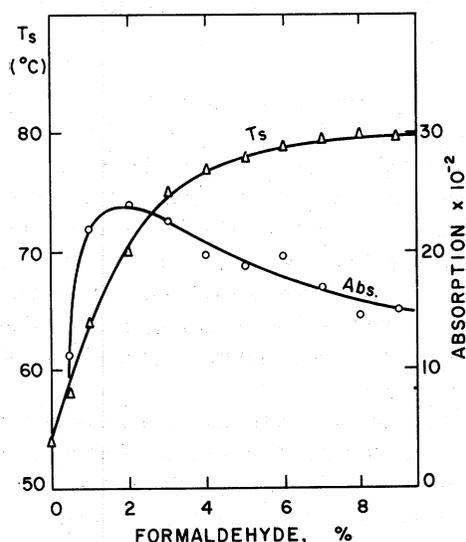


Fig. 3. Effect of formaldehyde concentration (4% acetylacetone) on (○) ultraviolet absorption and (△) shrinkage temperature of modified collagen.

To substantiate our proposal that the ultraviolet-absorbing material that is formed is not involved in crosslinking, we studied the effect of varying the amount of the two reagents, formaldehyde and acetylacetone, on the ultraviolet absorption of the hydrolyzates of the chemically modified products and also on the shrinkage temperature of the same product. The results are shown in Figures 2 and 3. In these experiments pickled cabretta skins were used. Therefore, although the chemistry involved is the same, the actual values of the ultraviolet absorption are lower because of the presence of extraneous materials in these skins. However, these values are of the same order of magnitude, and the results are still valid. As can be seen from Figure 2, increasing the acetylacetone concentration for a constant formaldehyde concentration caused a decrease in the shrinkage temperature of the chemically modified products and a corresponding increase in the ultraviolet absorption of the hydrolyzates of the products. Increasing the formaldehyde concentration while the acetylacetone concentration was held constant caused a continual increase in the shrinkage temperature to a maximum of about 80°C., while the ultraviolet absorption of the hydrolyzates underwent a rapid increase, reached a maximum at about 2% formaldehyde, and then gradually decreased. This is shown in Figure 3. The conclusions that may be drawn from these data are: that, as stated above, the ultraviolet absorbing product that is formed is not involved in crosslinking, that its formation actually interferes with crosslinking by the

formaldehyde, and that both formaldehyde and acetylacetone are involved in its formation.

Methyl Acetoacetate

This compound reacted in a manner very similar to acetylacetone. This is not surprising in view of their similar chemical properties. In this case 66% of the lysine reacted plus 39% of the hydroxylysine. There was a loss of histidine, too. The calculations were made from the data in Table I. These values always have to be viewed with some caution because of the possibility of elution of a new compound from the column at the same position as a known compound. There was almost a 2% loss in total nitrogen.

Methyl acetoacetate can also take part in a Hantzsch dihydropyridine synthesis (10) in a manner analogous to that of acetylacetone. The reaction involved is similar to that in eq. (2). The essential details of the ultraviolet spectrum (see Fig. 1) also agree with the literature (12); the indications are that 13 mmoles of the dihydropyridine residue were formed per 100 g. of protein, while a combined 20 mmoles of lysine and hydroxylysine reacted: again, a surprisingly close agreement.

One important fact that should be mentioned is that the amino acid chromatograms of the products resulting from chemical modifications caused by formaldehyde and acetylacetone and by formaldehyde and methyl acetoacetate revealed that a reaction had taken place, as indicated by the losses of certain amino acids, but failed to indicate the presence of any new compound. This is in contrast to the ultraviolet absorption of the hydrolyzates of these products. Two explanations of this are possible. One is that the materials responsible for the ultraviolet absorption do not have primary amino groups capable of reacting with ninhydrin; the other is that these materials responsible for the ultraviolet absorption were not eluted from the ion-exchange columns in the amino acid analyzer.

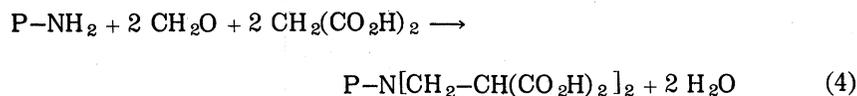
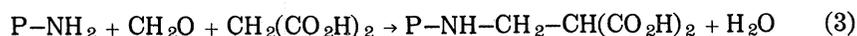
Malonic Acid

The results obtained with this compound were the most significant from a practical point of view. Under the conditions used malonic acid and formaldehyde reacted readily with collagen, as indicated by the amino acid composition (Table I). More than 95% of the lysine reacted. The data seem to indicate that none of the hydroxylysine reacted; however, the peak representing this compound on the chromatogram does not resemble the peak which is usually obtained. The peak usually observed is that of the partially resolved isomers present in collagen, but the peak observed here is that indicative of a single compound. It appears, therefore, that the hydroxylysine has also reacted to a considerable ex-

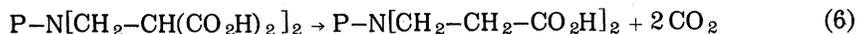
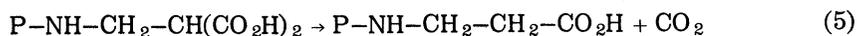
tent and that the peak observed here represents a new compound formed during the reaction or the subsequent hydrolysis. Histidine, in this case, reacted completely. The reason for the increased value for the hydroxyproline is not known, but this increase is probably caused by a new compound eluting at the same time. There were no significant differences in the amounts of the other amino acids. The total nitrogen was reduced by almost 1%, and the shrinkage temperature was reduced, compared with that of the material treated with formaldehyde alone.

The practical significance of this modification arises when this modified material is caused to react with solutions of Cr^{3+} ions, commonly used in chrome tanning, under conditions normally used for this process. The product thus obtained had a shrinkage temperature of 119.4°C ., an increase of 41.9°C . on chrome tanning. The corresponding product that had been pretreated with formaldehyde only had a shrinkage temperature of only 109.6°C ., an increase of 22.8°C . on chrome tanning. This effect of the chemical modification with malonic acid and formaldehyde may be of value to the tanning industry. Analysis of these chrome-tanned products for Cr^{3+} oxide indicated that the chemically modified product contained on the average 10% more than the product that had reacted with formaldehyde only. These data indicate that a chemical change had taken place in the protein, which increased both its binding capacity for the complex chromium ions present in the chrome tanning solution and its ability to utilize these ions for crosslinking. The technical aspects of this have already been reported (13).

The primary products that would be expected to be formed (assuming unipoint fixation) in the reaction are shown in the following:



Compounds of these types usually undergo decarboxylation quite easily, as shown in the following equations:



This is analogous to the known reactions of malonic acid and substituted malonic acid with simple amines and formaldehyde (14). The possibility of crosslinking with formaldehyde and malonic acid by the reactions indicated in eqs. (7) and (8) cannot be excluded completely,

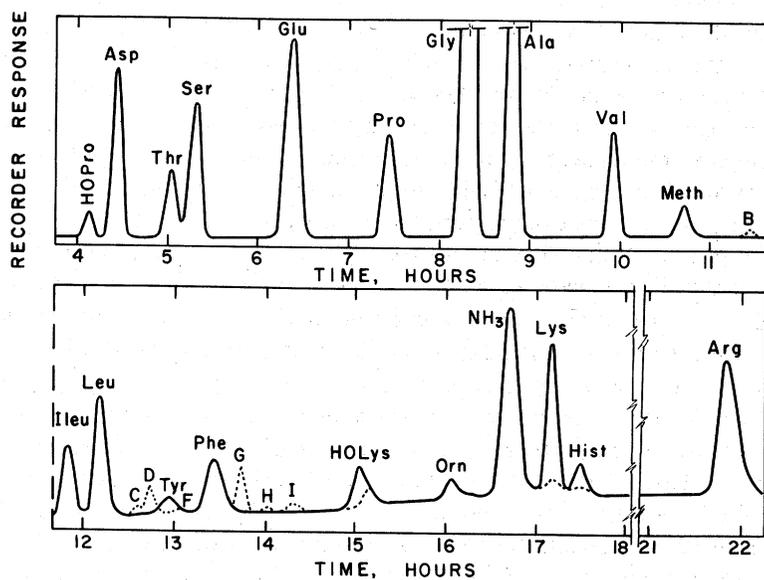
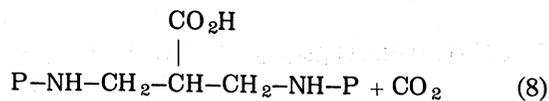
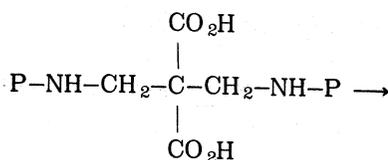
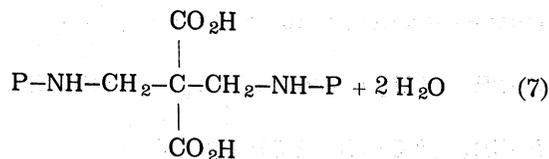
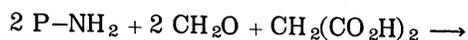


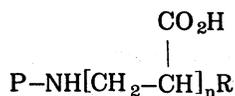
Fig. 4. Chromatogram of amino acids of collagen and collagen modified with formaldehyde and malonic acid. The seven new compounds are labeled with letters B, C, D, F, G, H, and I.



since malonic acid is known to react in this manner (14). However, the indications are that no significant crosslinking occurred. It should be noted that the products shown in eqs. (7) and (8) are secondary amines and capable of undergoing further reactions of the same kind.

Both lysine and hydroxylysine residues are capable of taking part in these reactions via their ϵ -amino groups; although the amino acid analysis indicated that histidine had reacted, the nature of this reaction is not known.

If a few assumptions are made, some interesting calculations can be performed. First, taking the amounts of the three amino acids that have reacted and assuming that each free primary amino group (lysine and hydroxylysine) could react with two molecules of formaldehyde in the Mannich reaction, and each imidazole group (histidine) could react with one molecule of formaldehyde, the maximum amount of formaldehyde that should have been consumed can be calculated. This amounts to about 68 mmoles per 100 g. of collagen. Analyses of the spent reaction mixtures for formaldehyde revealed, however, that 125 mmoles had reacted per 100 g. of collagen. This is corrected for the formaldehyde that is bound to the protein by itself and is almost twice the value calculated on the basis of the three amino acid residues involved in the reaction. The only explanation readily apparent is that polymerization has taken place to form structures such as



where R may be $-\text{H}$, $-\text{CH}_2-\text{OH}$, or $-\text{CH}-\text{NH}-\text{P}$, and n has an average value of about 2. The secondary amino groups could, of course, react further.

Taking all of these reactions into consideration, one would expect a complex mixture of products to be formed. This is indeed the case, as shown by the amino acid analysis, Figure 4, which reveals the presence of at least seven new compounds in the hydrolyzate of the collagen sample chemically modified with formaldehyde and malonic acid. In this figure the solid line represents a chromatogram of a hydrolyzate of collagen, and the dotted line represents the modifications made by treatment of the collagen with formaldehyde and malonic acid. The decreases of the lysine and histidine are apparent, as is the modification of the hydroxylysine peak. Two of these new compounds have been identified: they are $\text{N}_\epsilon, \text{N}_\epsilon$ -bis- $[\beta$ -carboxyethyl]-L-lysine (compound B) and N_ϵ - $[\beta$ -carboxyethyl]-L-lysine (compound H). They were reported by Friedman and Cavins (15) in connection with their work on the cyanoethylation of amino acids and proteins and correspond structurally to the products of the reactions shown in eqs. (5) and (6). In this connection we found that cyanoethylation of collagen by their procedure gave rise to these same two compounds besides three additional ones on hydrolysis of the cyanoethylated collagen. One of the latter three compounds elutes between valine and methionine, a second one elutes in the vicinity of com-

pound D, and a third one elutes after compound H. Except for compounds B and H, these new compounds are as yet unidentified.

Dimethyl Malonate

This was an interesting compound in that, although it did not react to as great an extent, the hydrolyzate of the collagen modified with it and formaldehyde had some of the same new derivatives as those found in the hydrolyzate of the product from interaction of collagen with malonic acid and formaldehyde. These derivatives included compounds B, D, G, and H. In addition there was a new compound (A) eluting between methionine and compound B and another (E) eluting where tyrosine usually does.

We should like to thank M. Friedman and J. F. Cavins, of the Northern Utilization Research and Development Division, ARS, Peoria, Illinois, for a generous gift of $N_{\epsilon},N_{\epsilon}$ -bis- $[\beta$ -cyanoethyl]-L-lysine, the precursor of the two β -carboxyethyl derivatives of lysine that were observed.

Mention of brand or firm names does not constitute an endorsement, made by the Department of Agriculture, over others of a similar nature not mentioned.

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