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# UNIVERSITY OF CINCINNATI

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I hereby recommend that the thesis prepared under my supervision by Ignacio S. Salcedo entitled A Study of the Collagen-Formaldehyde Reaction

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

John H. Higbidge



A Study of the Collagen-Formaldehyde Reaction

A dissertation submitted to the

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of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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## Table of Contents

	Page
Introduction	1
Removal of the Uncombined Formaldehyde.	5
The Role of Polymerization on the Collagen-Formaldehyde Reaction.	8
The Nature and Stability of the Collagen-Formaldehyde Reaction.	13
The Application of the Law of Mass Action to the Collagen-Formaldehyde Reaction.	21
The Effect of Aging.	26
The Relation of Shrinkage Temperature and Degree of Shrinkage to Formaldehyde Tannage.	28
The Collagen-Formaldehyde Reaction in Organic Solvents	36
Summary and Conclusions	41
Bibliography	42

## Introduction

Formaldehyde as a fixing agent has been used for many years and was patented as a tanning agent by Payne and Pullman in 1893. Since then a number of investigators have studied the action of various aldehydes upon both collagen and gelatin and have developed practical uses of aldehyde tanning. Formaldehyde has been found useful as a tanning agent for many white leathers, for glove leather and as a pretanning agent in other tannages.

In connection with an important investigation of aldehyde tannage, Thomas, Kelly, and Foster (13) made a thorough review of the literature. A summary of the studies made on the mechanism of formaldehyde tannage made by Highberger and Retzsch (7) is as follows:

"Studies of the mechanism of formaldehyde tannage carried out in the past have been seriously hampered by the lack of a method for determining the amount of formaldehyde present in the tanned leather. On this account various more or less indirect methods have been resorted to in investigating the conditions necessary for tannage. Gerngross and Gorges, for instance, used as an index of the amount of tannage the resistance of the leather to hydrolysis by hot water, as measured by the amount of soluble nitrogen obtained under standardized conditions. Theis and Schaffer, on the other hand, have applied to the same problem the measurement of the increase in the shrinkage temperature, and this method

has also been used by Casaburi and Cantarella. Since investigations such as these are based upon the evaluation of properties of importance in the finished leather, they have yielded valuable results on the conditions necessary for tannage. No quantitative information concerning the amounts of formaldehyde combined with the collagen can be obtained from them, however, and qualitatively only that which can safely be inferred on the assumption, not always entirely justified, that the change in the property measured is directly proportional to the amount of tanning agent fixed.

Thomas, Kelly, and Foster attempted to study the formaldehyde tannage of hide powder quantitatively, using a weight increase method. They found, however, that their tanned samples invariably showed a loss rather than a gain in weight. In experiments with gelatin these workers reported results obtained by two methods: a difference method in which the amount of formaldehyde fixed was calculated from the difference in concentration between the initial and the final solutions, and a "direct" method, in which the fixation of formaldehyde was obtained as the difference between 100 and the percentage of protein in the dry, tanned material. The application of these methods to hide powder, however, yielded results which were so erratic as to make their publication unjustified, although a sample tanned in 20 per cent formaldehyde at pH 9.0 for 24 hours was reported to show 13.5 per cent formaldehyde by the

"direct" method. The results of these investigators showed, however, that the tanning action of formaldehyde was greatest at pH values between 6 and 10, and that it increased with increasing concentration of formaldehyde.

"Apparently the only other available results of a quantitative nature are those reported by Leunier and Schweikert, who tanned hide cubes, both normal and deaminised, in 3 per cent formaldehyde at pH values between 5 and 10, estimating the formaldehyde fixation by the difference in concentrations of the initial and final solutions. Their figures, which are rather meager, seem to show that formaldehyde fixation is low in acid solutions, increasing to a maximum at about pH 7, and falling off again at pH values above 9. The same general behavior, with a decreased amount of fixation was shown by the deaminised hide cubes."

Following the development by Hightberger and Metzsch (3) of a convenient and accurate method for determining formaldehyde in formaldehyde tanned leather, an investigation into the quantitative relationships was begun. In previous papers (7, 8) the results of a quantitative study of the combination of collagen with formaldehyde were reported. The results may be summarized as follows:

In formaldehyde concentrations of 1 per cent or less, and at pH values lower than 7 to 8, the reaction is confined to the free amino groups provided by the lysine of the collagen, each free amino group reacting with one molecule

of formaldehyde. Only the undissociated groups react, and in order to ensure the reaction of all the lysine groups a pH value of 7 to 8 is necessary. At more alkaline reactions the additional fixation of formaldehyde is due to the reaction of the formaldehyde with the guanidino groups of arginine.

With increasing concentration of formaldehyde greater amounts of formaldehyde are fixed by the collagen. The pH-fixation curves for these higher concentrations are similar to those already found for the concentrations of 1 per cent or less, except that the breaks occur at higher fixation values. There is a tendency for the flat portion of the curve to shift slightly to more acid reactions with increasing formaldehyde concentration indicating a mass action effect. The increase in fixation with increasing concentration is relatively small, suggesting that the increased combination is due to the reaction of the free amino and guanidino groups with monomeric rather than polymeric formaldehyde molecules.

The object of the present work is to obtain further quantitative data regarding the collagen-formaldehyde reaction, to study the factors affecting the reaction and to correlate, if possible, shrinkage temperature and degree of shrinkage to the formaldehyde tannage. The experimental methods used and the discussion of the results will be presented separately under the following subtitles:

1. Removal of the Uncombined Formaldehyde
2. The Role of Polymerization on the Collagen-Formaldehyde Reaction.
3. The Nature and Stability of the Collagen-Formaldehyde Reaction.
4. The Application of the Law of Mass Action to the Collagen-Formaldehyde Reaction.
5. The Effect of Aging.
6. The Relation of Shrinkage Temperature and Degree of Shrinkage to Formaldehyde Tannage.
7. The Collagen-Formaldehyde Reaction in Organic Solvents.

1. Removal of the Uncombined Formaldehyde

The experimental methods used, unless otherwise modified, were identical with those described in detail in a previous paper (7). Collagen powder (4) was tanned for twenty-four hours, with constant agitation, in definite concentrations of formaldehyde in 0.1 M phosphate buffer solutions. Potassium hydroxide was used instead of sodium hydroxide in making the buffer solutions, the glass electrode being less sensitive to potassium ions. The equivalent of 2.000 grams of dry, ash-free collagen was used with 100 ml. portions of solution. To facilitate the study of the rate at which the uncombined formaldehyde is removed from the tanned collagen samples, it was necessary to modify the method of washing previously employed. The formaldehyde content was then determined on

the undried sample by the Highberger and Retzsch method (6).

The method of washing adopted was an intermittent one. After tanning, the collagen sample was removed to a Wilson-Kern extractor, washed well with distilled water using suction, and covered with 100 ml. of the wash liquor. It is then allowed to stand for definite periods of time, each washing together with a 50 ml. rinse of distilled water being analyzed for formaldehyde by the Highberger and Retzsch method.

One-half hour, one hour, and two hour intervals were tried, the collagen mixture being stirred with a glass rod before being allowed to stand. The washing was continued until only negligible amounts of formaldehyde were being washed out and which approach a constant value. Washing experiments were carried out using distilled water, methyl alcohol, ethyl alcohol, dilute sodium hydroxide solution, buffered solutions, and different concentrations of sodium bisulfite and sodium sulfite.

Washing with distilled water and acid buffered solutions is slow and gives too high results. The uncombined formaldehyde washes out readily the higher the pH value of the wash liquor. Methyl alcohol and ethyl alcohol did not prove to be much more efficient than distilled water and the buffered alkaline solutions of ethyl alcohol are no more efficient than the buffered aqueous solutions of the same pH value. Dilute sodium hydroxide solution and alkaline buffered

solutions are good washing agents but their high pH value causes an increase in the amount of combined formaldehyde for samples tanned at much lower pH values.

Different concentrations of sodium bisulfite solutions did not prove of much help either. Sodium sulfite solutions seem to be the best washing agents so far studied. Sodium sulfite has a dual role in the washing. It dissolves any polymerized formaldehyde and reacts with the uncombined monomeric formaldehyde to form  $\text{HOCH}_2\text{SO}_2\text{O}^-$ . The alkalinity of the sodium sulfite solution (pH of 0.02 M sodium sulfite = 8.90) creates an electrostatic repulsion between the  $\text{HOCH}_2\text{SO}_2\text{O}^-$  ions and the non-diffusible  $\text{H}_2\text{N}-\text{P}-\text{COO}^-$  ions. Hence there is created a driving force to expel uncombined formaldehyde. This is obviously the reason why sodium bisulfite did not prove to be effective. In acid medium (pH of 0.1 M sodium bisulfite = 4.80) there will be an electrostatic attraction since the protein will be mostly in the form  $\text{HOOC}-\text{P}-\text{NH}_3^+$ .

Sodium sulfite has also a decided advantage over the other alkaline washing agents mentioned above in that the error due to further formaldehyde combination of samples tanned at more acid pH values is minimized due to its relatively low pH value and to its reaction with the uncombined formaldehyde. To further minimize this error samples tanned at a pH value lower than that of sodium sulfite are first washed with distilled water to remove part of the uncombined formaldehyde.

After trying out different concentrations of sodium sulfite the following procedure of washing was adopted:

The tanned collagen sample is transferred to a Wilson-Nern extractor, washed free of adhering formaldehyde solution with distilled water using suction and covered with 100 ml. of 0.02 M sodium sulfite. The mixture is stirred with a glass rod, allowed to stand for two hours, drained out with the aid of suction and rinsed with 50 ml. of distilled water. This operation is repeated three times, then followed by a fifteen-hour overnight wash, three two-hour washes, a two-hour wash with distilled water and an overnight wash with distilled water. The washing with distilled water is to remove most of the sodium sulfite from the collagen fibers. A collagen sample tanned at a pH value greater than 11 after being washed by this method, will lose approximately 0.05 to 0.06 millimols of combined formaldehyde for the first additional day of washing, the amount slightly decreasing the second day. For a sample tanned at a pH value greater than 7 this value is approximately 0.03 to 0.04 millimols of formaldehyde.

## 2. The Role of Polymerization on the Collagen-Formaldehyde Reaction

It is well known from various physico-chemical investigations of aqueous formaldehyde solutions that the proportion of hydrated polymers of formaldehyde present in such solutions increases markedly as the total concentration of formaldehyde

increases. In the previous paper (8) it was suggested that only monomeric formaldehyde reacted with the collagen since the increase in combined formaldehyde was too low compared to the expected relative increase in polymerized formaldehyde. It was decided to study whether collagen is permeable to polymerized formaldehyde or not. The experimental procedure adopted was as follows:

One-gram samples of dry ash-free collagen were tanned with 1 per cent formaldehyde in buffered solutions in the usual way. Duplicate samples were used for each given pH value. After the tanning period of 1 day each tanned sample is transferred to a tared ashless filter paper (Whatman No. 40, 12 cm. in diam.) in a Buchner funnel, rinsed with two 50 ml. portions of distilled water using suction and weighed in a large weighing bottle. Blanks to determine the water take-up by the filter paper were made. After subtracting the weight of the filter paper and the water blank the weight obtained gives the weight of the swollen formaldehyde tanned collagen. The tanned sample was then analyzed for its formaldehyde content. This value is a measure of the total amount of formaldehyde, combined and uncombined, that diffused into the collagen fibers. The amount of the uncombined formaldehyde is obtained by subtracting the value of the uncombined formaldehyde as obtained from Fig. II. The results are shown in Fig. I. The values obtained for rather low pH values are not so accurate due to the difficulty in filtering

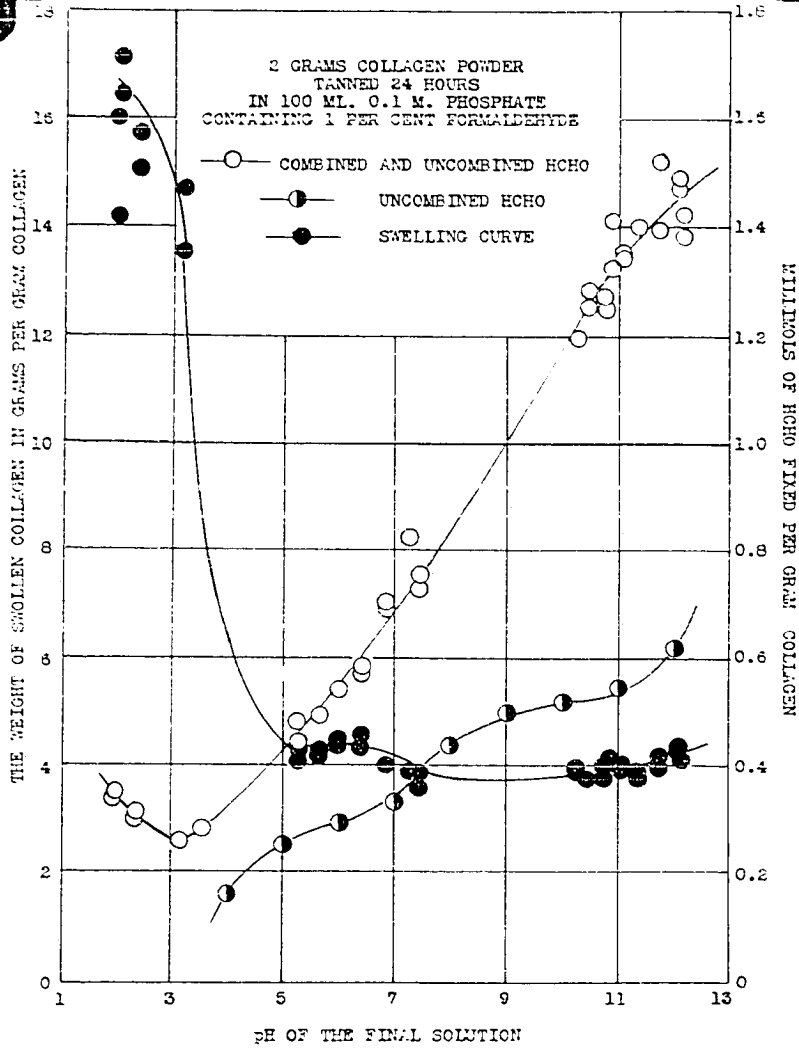


FIGURE I

the swollen samples. It will be noticed that the amount of formaldehyde that was able to diffuse through the collagen fibers increased with the pH value. It is of interest too, to note that the degree of swelling has very little to do with the formaldehyde take-up. This is very well shown on the more acid pH values where the swelling is at a maximum and the formaldehyde content is at a minimum. If the collagen fibers are permeable to polymerized formaldehyde then it would be expected that the formaldehyde take-up will be a function of the swelling and the concentration of the formaldehyde used.

In acid and neutral solutions formaldehyde exists mostly in the polymerized form (14) but is depolymerized by changing to an alkaline reaction. Jahoda (9) using the polarographic method has shown that formaldehyde depolymerizes with increase of hydroxyl ions and rise of temperature. In Fig. I, the marked decrease in formaldehyde take-up on the acid side and the gradual increase with pH value can be very well explained on the basis that collagen fibers are not permeable to polymerized formaldehyde.

Since polymerized formaldehyde can not diffuse into the collagen fibers, then any polymerized formaldehyde formed inside the fibrous structure due to a change in pH from alkaline to acid values, will not be able to diffuse out. Moreover, it has also been shown that the rate of attainment of polymerization equilibrium depends on pH. Using interferometer readings as a measure of rates, Madano, Trogus,

and Hess (15), found a minimum rate between pH 2 and 4, and a rapid rate of pH 6 and above. This explains why distilled water and acid buffered solutions are poor washing agents.

From Fig. I it will be noticed that there is not much uncombined formaldehyde to be removed on the acid side and the selection of the proper washing agent is not so essential as on the alkaline side where the amount of uncombined formaldehyde increases with pH value. It was also observed during the washing operation that the collagen-formaldehyde combination below pH 3 is more stable than that taking place in higher pH values.

The apparent high formaldehyde fixation when collagen samples tanned at alkaline pH values were washed either with distilled water (pH usually less than 6) or with acid buffered solutions can be attributed to a polymerization of the uncombined formaldehyde inside the fibers. The collagen-formaldehyde reaction is actually being reversed as the reaction medium is made more acid as will be discussed later. To verify this conclusion the following experiment was carried out:

Nine 2-gram samples of dry ash-free collagen were tanned with 1 per cent formaldehyde at a pH value of 11.50 in the usual way. After the usual sodium sulfite washing the samples were transferred to 100 ml. portions of buffered solutions and placed in the shaking machine for 1 day. Samples 1, 4, and 7 were then washed with two 50 ml. portions of distilled water

TABLE I

2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml.  
0.1 M Phosphate Buffer Containing 1.0 Grams Formaldehyde

Sample No.	Final pH of Buffered Solutions	Millimols Formaldehyde Fixed per Gram Collagen
1	2.48	0.55
2	2.48	0.52
3	2.48	0.31
4	5.14	0.60
5	5.14	0.58
6	5.14	0.41
7	10.88	0.62
8	10.88	0.57
9	10.88	0.60

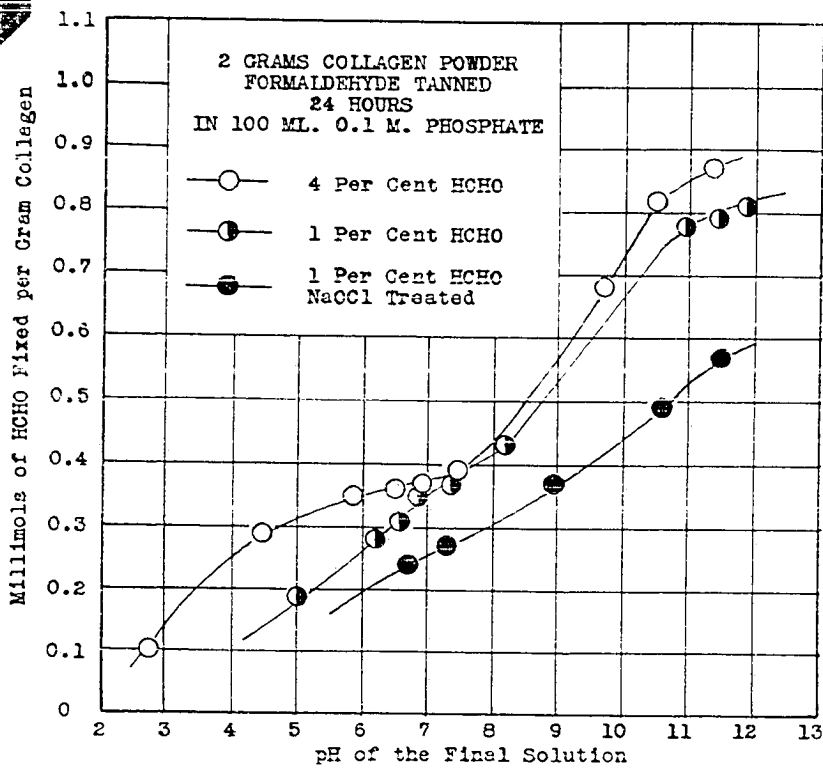


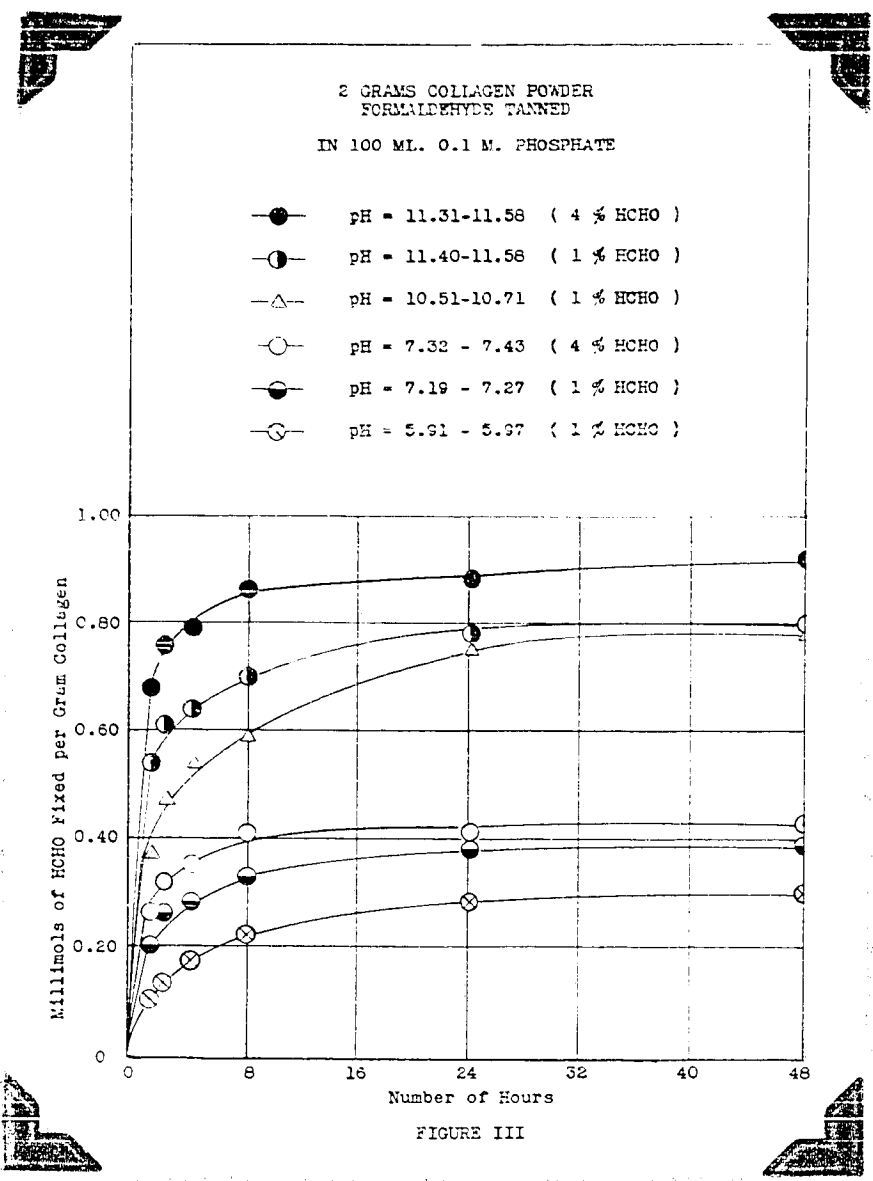
FIGURE II

and analyzed for formaldehyde. Samples 2, 5, and 8 were given three 2-hour washes with sodium sulfite, and one 2-hour and one overnight wash with distilled water. Samples 3, 6, and 9 were dried in the vacuum oven at 100°C for a day. The results are shown in Table I.

By this procedure all the samples have the same amount of combined formaldehyde (0.79 millimols, see Fig. II) before being placed into the buffered solutions. It will be observed from Table I that more the combined formaldehyde was removed by the more acid buffered solutions showing a greater reversal in the reaction. It will also be noticed that of the samples dried in the oven the sample at pH 2.40 showed a marked decrease in combined formaldehyde showing that the polymerized formaldehyde which must have been formed appreciably at such a pH value, had been depolymerized by heat and thereby expelled.

### 3. The Nature and Stability of the Collagen-Formaldehyde Reaction

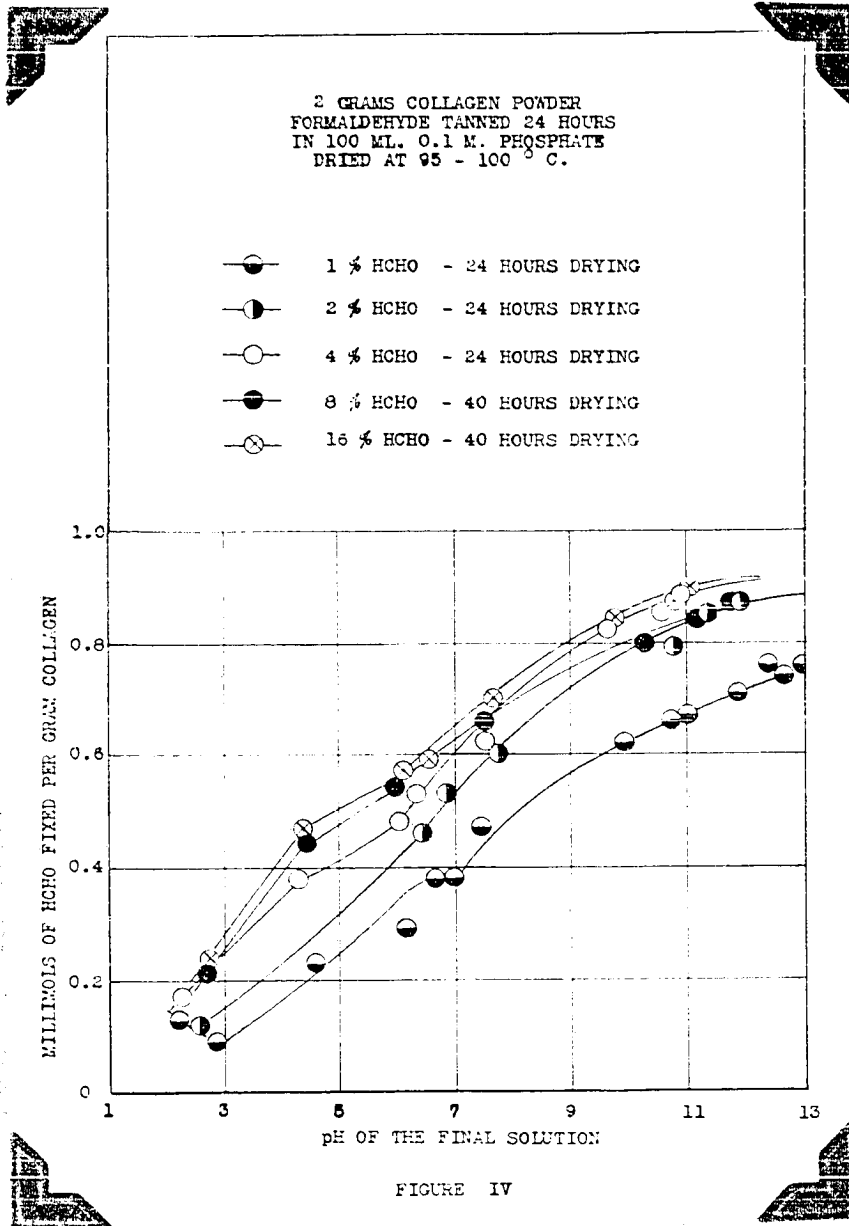
Several 2-gram samples of collagen on the dry ash-free basis were tanned with 1 per cent and 4 per cent formaldehyde in the usual way, covering the pH range from 4 to 12. After the usual sodium sulfite washing the combined formaldehyde was analyzed. The results are shown in Fig. II. It will be observed that there is a flattening in the region between pH 7 and pH 7.5 corresponding to about 0.57 millimols of com-



bined formaldehyde and another flattening at a pH greater than 11.5 corresponding to a combined formaldehyde content of from 0.82 to 0.88 millimols. These values are in good agreement with values of 0.38 millimols of lysine per gram (5.49% lysine, corrected value (5) ) and 0.45 millimols per gram of arginine (7.84% arginine, corrected value(5) ) or a total of 0.83 millimols assuming that one molecule of formaldehyde reacted with each free amino group.

Five 2-gram samples of dearginised collagen (8) were likewise tanned with 1 per cent formaldehyde, given the usual sodium sulfite washing, and the combined formaldehyde determined. From the results shown in Fig. II it can readily be observed that there is a marked decrease in combined formaldehyde on the alkaline side comparable to the amount of arginine destroyed (8).

Fig. III shows the results which were obtained when the time of tanning was varied from 1 hour to 48 hours, the experimental conditions being otherwise as described above. Here again, the combined formaldehyde shows a limiting value of 0.39 to 0.42 millimols for a pH value of approximately 7.50 and 0.81 to 0.89 for a pH value around 11.50. The higher limits are for samples tanned with 4 per cent formaldehyde. The slight increase of combined formaldehyde in higher concentrations of formaldehyde will be discussed later. It is obvious from Fig. III that an increase in formaldehyde concentration increases the rate of fixation markedly and so



does an increase in the pH value of the tanning solution.

Fig. IV shows the results when the collagen samples tanned as described above with different concentrations of formaldehyde were dried in the vacuum oven at 100°C. instead of being washed with sodium sulfite solution. Forty hours of drying were used for the 8 per cent and 16 per cent formaldehyde tanned samples for it was observed that there was still some formaldehyde being driven off after the first day of drying of samples tanned with 4 per cent formaldehyde. Checks were made for the effect of longer duration of drying for each formaldehyde concentration. The results are shown in Table II.

From Fig. IV the total formaldehyde combination ranges from 0.76 to 0.89 millimols. Here again, we have evidence of a collagen-formaldehyde reaction corresponding to a ratio of one molecule of formaldehyde for every free amino group. This shows too the stability of the reaction to dry heat. It will also be noted that the collagen samples tanned with 1 per cent and 4 per cent respectively and given a drying period of more than 2 days seem to indicate the formation of a more stable compound. The formation of a methylene compound is not unlikely as was pointed out in a previous paper (7).

Twelve samples were tanned in the usual way at different pH values with 1 per cent formaldehyde. Samples 1, 4, 7, and 10 were washed with two 50 ml. portions of distilled

TABLE II

2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml.  
0.1 M Phosphate Buffer

Formaldehyde Concentration	Final pH	Drying Period	Millimols Formaldehyde Fixed per Gram Collagen
1%	6.46	24 hours	0.43
1%	6.46	48 "	0.41
1%	6.46	72 "	0.40
1%	6.46	96 "	0.40
1%	6.46	120 "	0.40
2%	6.42	24 "	0.46
2%	6.42	48 "	0.59
2%	11.33	24 "	0.82
2%	11.33	48 "	0.80
4%	6.02	24 "	0.48
4%	6.02	48 "	0.44
4%	10.55	24 "	0.85
4%	10.55	48 "	0.80
4%	11.74	24 "	0.81
4%	11.74	48 "	0.79
4%	11.74	72 "	0.78
4%	11.74	96 "	0.78
4%	11.74	120 "	0.78
8%	5.92	40 "	0.54
8%	5.92	90 "	0.52
8%	11.17	40 "	0.64
8%	11.17	90 "	0.81
16%	6.10	40 "	0.57
16%	6.10	90 "	0.55
16%	10.78	40 "	0.87
16%	10.78	90 "	0.85

water and analyzed for the formaldehyde take-up. The other samples were washed in the same manner and replaced into buffered solutions of the same pH as the solutions in which they were tanned. Samples 2, 5, 8, and 11 were shaken for 1 day and samples 3, 6, 9, and 12 for 2 days. They were then washed with two 50 ml. portions of distilled water and analyzed. The results are shown in Table III.

Six collagen samples were tanned in the usual way with 2 per cent, 4 per cent, and 8 per cent formaldehyde. Samples 1, 3, and 5 were washed with two 50-ml. portions of distilled water and analyzed for formaldehyde content. Samples 2, 4, and 6, after washing with two 50 ml. portions, were transferred into their original bottles and shaken for 1 day with 100 ml. of a buffered solution of pH 12.05. The results are shown in Table IV.

The results in Tables III and IV again show the 1:1 collagen reaction. The uncombined formaldehyde diffuses out of the collagen fibers easily. The values of combined formaldehyde are a little higher in higher concentrations of formaldehyde since the uncombined formaldehyde left in the collagen fibers must be in equilibrium with the concentration of formaldehyde which diffused out.

All the data presented above point to a stable chemical combination of formaldehyde with the free amino groups of lysine and arginine in the ratio of 1:1. When the formaldehyde tanned collagen fibers were washed with distilled water

TABLE III

2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml.  
0.1 M Phosphate Buffer Containing 1.0 Grams Formaldehyde

Sample No.	Final pH of Buffered Solutions	Millimols Formaldehyde Fixed per Gram Collagen
1	5.52	0.54
2	5.52	0.30
3	5.52	0.26
4	7.14	0.78
5	7.14	0.42
6	7.14	0.40
7	10.72	1.30
8	10.72	0.75
9	10.72	0.73
10	11.40	1.45
11	11.40	0.74
12	11.40	0.73

TABLE IV

2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml.  
0.1 M Phosphate Buffer

Sample No.	Formaldehyde Concentration	Final pH	Millimols Formaldehyde Fixed per Gram Collagen
1	2%	11.54	1.74
2	2%	11.54	0.79
3	4%	11.13	2.24
4	4%	11.13	0.83
5	3%	10.85	2.66
6	3%	10.85	0.95

or acid buffered solutions there seemed to be a higher formaldehyde take-up. As pointed out earlier in this work the high results so obtained may be only apparent rather than real due to the role of polymerization. The possibility, however, of the chemical combination of two molecules of formaldehyde for every free amino group should not be overlooked. This combination, if it does take place, is so unstable as not to manifest itself in the collagen-formaldehyde curves. There is only one break in the curves in Fig. II showing that only two combinations (with the free amino groups of lysine and arginine) having different pK values are possible.

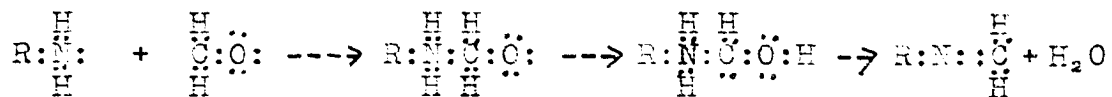
The widespread inference that in formaldehyde tanning more than one molecule of formaldehyde combined with each free amino group has been drawn from work carried out on the compounds of various amino acids with formaldehyde. The conclusions drawn from these results have been carried over by analogy to the proteins. It should be pointed out that the analogy is not so valid since the reacting groups in the protein, the  $\epsilon$ -amino group of lysine and the  $\delta$ -guanidino group of arginine, are less reactive than the  $\alpha$ -amino group of the amino acid. It has been shown that formaldehyde polymers can not diffuse into the collagen fibers and hence could not react with the free amino groups of lysine and arginine. Bergman's (2) triformal glycine ester which contains three molecules of formaldehyde in combination with the amino group of glycine ethyl ester, changed to the monoformyl compound

on the addition of alkali. As the possibility of the reaction of two molecules of formaldehyde for every reactive amino group can only take place at high pH values, it is not unlikely that the only combination taking place is the 1:1 collagen-formaldehyde reaction.

As to the nature of the combination of the free amino group with the formaldehyde, it is reasonable to believe that it is not necessarily a removal of hydrogen through condensation with the subsequent removal of a molecule of water. This type of reaction does not seem likely to occur in an aqueous medium. Tomiyama (14) cited the work of Heyrovsky (8) on the polarographic study of the glycine-formaldehyde combination. Heyrovsky noted that the diffusion current of formaldehyde which corresponds to the concentration of formaldehyde did not change whether or not glycine was added. If formaldehyde should condense with the glycine, forming the methylene compound, it might be expected that the observed reduction potential should be appreciably different from that of formaldehyde. This was not the case and he concluded that formaldehyde combines with the amino acid without any substantial change in its constitution.

As pointed out in a previous paper (7) it seems probable that the first stage of the reaction is a coordination of one molecule of formaldehyde with each amino group, the nitrogen atom donating its unshared pair of electrons to the carbon atom of the formaldehyde. After the formation of

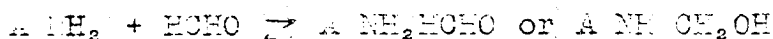
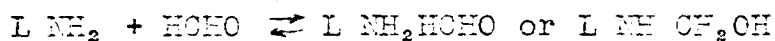
the coordination compound, it seems not unlikely that a series of rearrangements occurs, probably resulting in the formation of methylol, and even of methylene compounds thus:



The methylene compound may be formed in the absence of water as in the oven-dried formaldehyde tanned collagen samples.

#### 4. The Application of the Law of Mass Action to the Collagen-Formaldehyde Reaction

Assuming that only one molecule of formaldehyde reacts with each free amino group, the reactions involved may be written thus:



where L NH<sub>2</sub> denotes the free uncharged amino group of lysine and A NH<sub>2</sub> the free uncharged amino group of the guanidino radical of arginine. At equilibrium the dissociation equilibrium constants can therefore be expressed as follows:

$$K = \frac{[\text{L NH}_2][\text{HCHO}]}{[\text{L NH}_2\text{HCHO}]} \quad (1)$$

$$K = \frac{[\text{A NH}_2][\text{HCHO}]}{[\text{A NH}_2\text{HCHO}]} \quad (2)$$

where the brackets [ ] denote molal concentrations. The molal concentration of the free uncharged amino group of lysine  $[L NH_2]$  and of the free uncharged amino group of arginine  $[A NH_2]$  can be calculated from their pH values assuming that these values obtained from the pure amino acid are not much changed in the structured proteins. Using the pK values given by Schmidt (11) we have the following equations:

$$K_L NH_3^+ = \frac{[L NH_2][H^+]}{[L NH_3^+]} = 10^{-10.55} \quad (3)$$

$$K_A NH_3^+ = \frac{[A NH_2][H^+]}{[A NH_3^+]} = 10^{-12.48} \quad (4)$$

It has been shown that only monomeric formaldehyde takes part in the reaction since polymerized formaldehyde can not diffuse into the collagen fibers. The collagen-formaldehyde reaction can then be visualized as taking place only between the collagen and the formaldehyde present in solution in the swollen collagen fibers. In the light of the modern theory of structured proteins the free amino groups of lysine and that of the guanidino groups of arginine are uniformly distributed throughout the swollen sample and being polar in character, are really in solution in the aqueous medium of the swollen collagen sample. With this in mind the collagen-formaldehyde reaction can be treated as a homogeneous system and the homogeneous equilibria equations given above are applicable.

At any point in the graphs in Fig. II the concentration of the combined formaldehyde is always in equilibrium with the concentration of the uncombined formaldehyde and that of the free uncharged amino groups of lysine or arginine as the case may be. From Figs. I and II the following quantities can be calculated:

1. The weight in grams of the solution per gram of collagen in the dry ash-free basis in the swollen sample. All other concentrations are then calculated to moles in 1000<sup>1</sup> grams of this solution.

2.  $[HCHO]$ , the molal concentration of the uncombined formaldehyde.

3.  $[L-NH_2HCHO]$  or  $[A-NH_2HCHO]$ , the molal concentration of the combined formaldehyde.

4.  $[L-NH_3^+] + [L-NH_2]$  or  $[A-NH_3^+] + [A-NH_2]$ , the molal concentration of the uncombined free amino groups of lysine or that of arginine using 0.58 millimols per gram collagen for the lysine content and 0.45 millimols for that of arginine.

If  $[L-NH_3^+] + [L-NH_2]$  be denoted by  $[R_L]$  and  $[A-NH_3^+] + [A-NH_2]$  by  $R_A$  equations (3) and (4) can be written as follows:

$$K_{L-NH_3^+} = \frac{[L-NH_2][H^+]}{[R_L][L-NH_2]} = 10^{-10.53}$$

$$K_{A-NH_3^+} = \frac{[A-NH_2][H^+]}{[R_A][A-NH_2]} = 10^{-12.48}$$

$[L-NH_2]$  and  $[A-NH_2]$  can then be calculated since  $[H^+]$  is known.

5. Knowing  $[L NH_2]$  ,  $[HCHO]$  , and  $[L NH_2HCHO]$  ,  $K$   $L NH_2HCHO$  can be calculated. In a similar manner  $K$   $A NH_2HCHO$  can be computed. Table V gives the  $K$  constants as calculated from experimental data.

It will be noticed that the  $K$  values do not vary much from pH 6.58 to pH 7.42 and from pH 10.24 to 11.05. The rather low values obtained at pH values lower than 6.58 may be attributed to an increase in formaldehyde combination due to an increase in pH since the sodium sulfite washing solution has a pH of 8.90. Thus the denominator in equation (1) is actually higher than the real value and hence a smaller  $K$  constant will result. The rather high  $K$  values at a pH greater than 11.05 can be explained in a similar manner. The decrease in pH due to the sodium sulfite washing causes a slight reversal of the reaction thus decreasing the denominator in equation (2) below its actual value.

Too much stress should not be laid on the accuracy of the values obtained but it is clear that they support the idea that the collagen-formaldehyde combination is a chemical reaction where one molecule of formaldehyde combines with each free amino group. The application of the mass-action law also explains why the addition of acid causes a reversal of the reaction. A low hydrogen ion concentration,  $[H^+]$ , will decrease the concentration of the free uncharged amino group,  $[L NH_2]$  or  $[A NH_2]$  . This will disturb the equilibrium in equations (1) and (2) and more  $L NH_2HCHO$  or

TABLE V

Final pH	$L_{NH_2}$	$K_{L_{NH_2}HCHO}$
5.29	$2.95 \times 10^{-7}$	$3.58 \times 10^{-7}$
5.67	$5.80 \times 10^{-7}$	$6.52 \times 10^{-7}$
5.98	$9.98 \times 10^{-7}$	$1.11 \times 10^{-6}$
6.38	$1.70 \times 10^{-6}$	$1.70 \times 10^{-6}$
6.62	$2.49 \times 10^{-6}$	$2.55 \times 10^{-6}$
7.25	$1.84 \times 10^{-6}$	$1.79 \times 10^{-6}$
7.42	$2.87 \times 10^{-6}$	$2.95 \times 10^{-6}$

Final pH	$L_{NH_2}$	$K_{A_{NH_2}HCHO}$
10.24	$2.64 \times 10^{-4}$	$4.28 \times 10^{-4}$
10.44	$3.13 \times 10^{-4}$	$4.69 \times 10^{-4}$
10.75	$5.66 \times 10^{-4}$	$4.88 \times 10^{-4}$
10.87	$4.11 \times 10^{-4}$	$5.44 \times 10^{-4}$
11.05	$4.82 \times 10^{-4}$	$6.35 \times 10^{-4}$
11.35	$7.03 \times 10^{-4}$	$9.54 \times 10^{-4}$
11.71	$9.74 \times 10^{-4}$	$1.36 \times 10^{-3}$
12.07	$8.93 \times 10^{-4}$	$1.28 \times 10^{-3}$
12.12	$9.59 \times 10^{-4}$	$1.36 \times 10^{-3}$

A  $\text{NH}_2\text{CHO}$  will dissociate in order to restore the K constant. This is also the reason why washing with a buffered solution of the same pH value as that of the tanning solution was not adopted. The reversal of the reaction increases with decreasing pH values and the problem of when to stop washing the formaldehyde tanned collagen samples becomes complicated. The sodium sulfite washing eliminates this difficulty for it is rapid and can be carried out to the same end-point for the different samples.

#### 5. The Effect of Aging

To study the effect of aging on the collagen-formaldehyde reaction the following experiments were conducted. Thirty-two 2-gram samples were tanned with 1 per cent formaldehyde in the usual manner, samples 1 to 12 at a pH = 8.10, samples 13 to 24 at a pH = 7.47, and samples 25 to 32 at a pH = 11.90. The method of washing, the procedure employed and the duration of aging were varied for different samples. The results are shown in Table VI.

All the samples were given the usual sodium sulfite washing after the aging period except samples 6, 10, 12, and 26 which were analyzed without washing. These experiments were primarily designed to verify whether some other type of a stable collagen-formaldehyde reaction takes place on aging under varied conditions. The treatments given

T.ELLE VI

2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml.  
0.1 M. Phosphate Buffer Containing 1.0 Grams Formaldehyde

Sample	Method of Washing	Procedure Employed	Duration	Millimols Combined HCHO per Gram Collagen
1	Usual 2 day washing	air dried	1 week	0.34
2	using distilled H <sub>2</sub> O	Dried at 100°C. in vacuum oven	1 day	0.29
3	Usual 2 day washing with distilled H <sub>2</sub> O;	analyzed		
4	dehydrated in C <sub>2</sub> H <sub>5</sub> OH and ether	air dried	1 week	0.34
5	Usual 2 day Na <sub>2</sub> SO <sub>3</sub> washing;	analyzed		
6	dehydrated in C <sub>2</sub> H <sub>5</sub> OH and ether	air dried	1 week	0.37
7		" "	1 week	0.33
8	Usual Na <sub>2</sub> SO <sub>3</sub> washing	analyzed		0.33
9	" "	air dried	1 week	0.39
10	" "	" "	1 week	0.33
11	" "	Dried at 100°C. in vacuum oven	1 week	0.36
12	" "	" "	1 week	0.30
13	" "	" "	1 week	0.33
14	" "	analyzed		0.25
15	" "	air dried	8 weeks	0.29
16	" "	" "	8 weeks	0.30
17	" "	" "	16 weeks	0.28
18	" "	" "	15 weeks	0.28
19		analyzed		0.35
20	Usual Na <sub>2</sub> SO <sub>3</sub> washing;	air dried	4 weeks	0.30
21	dehydrated in ethyl	" "	4 weeks	0.30
22	alcohol and ether	" "	8 weeks	0.29
23		" "	8 weeks	0.28
24		" "	16 weeks	0.28
25		" "	16 weeks	0.28
26		Dried at 100°C. in vacuum oven	1 day	0.64
27		" "	1 day	0.77
28	washed well with	air dried	4 weeks	0.82
29	distilled water without	" "	4 weeks	0.82
30	being allowed to stand	" "	8 weeks	0.72
31		" "	6 weeks	0.73
32		" "	16 weeks	0.78
		" "	16 weeks	0.78

1 2 7 1

Samples 1 to 24 were contemplated to show whether a more stable combination in which a molecule of formaldehyde unites with 2 free uncharged amino groups forming a methylene bridge can take place. Thus dehydration and drying in the vacuum oven were tried, the removal of water favoring such a reaction. If such a reaction took place then the combined formaldehyde would be halved. The decrease in combined formaldehyde is just what one should expect to be removed by the sodium sulfite washing. It is therefore unlikely that such a reaction takes place.

Samples 25 to 32 were just given two rinses with 50 ml. of distilled water in order to retain some uncombined formaldehyde inside the fibers. Aging may result in a stable combination of more than one molecule of formaldehyde for every free uncharged amino group. Here again the experimental results show that such a possibility is unlikely.

The aging experiments, therefore, also give credence to the stability of the 1:1 collagen-formaldehyde reaction.

#### 6. The Relation of Shrinkage Temperature and Degree of Shrinkage to the Formaldehyde Tannage

In this phase of the work, an electric heating stage (after Walton) procured from E. Leitz, Inc. N.Y., was used. A sliding rheostat was connected in parallel to the instruments' regular rheostat and the current so adjusted that a temperature of 100°C. was attained in twenty to twenty-

five minutes. A few fibers were taken from the formaldehyde tanned collagen samples, placed in water or 50 per cent glycerol solution in the concavity of a hanging drop slide and covered with a cover glass. The micro slides used were 1.25 to 1.50 mm. in thickness with concavities having 12 mm. diameter and 0.8 mm. deep. Petrolatum was used around the edge of the concavity to prevent rapid drying of the water and the formation of bubbles under the coverslip. This was not necessary for determinations in 50 per cent glycerol solution. A small cardboard box was fitted on top of the heating stage to prevent air diffusion currents which may change the temperature readings due to displacement of the heated air in the heating compartment.

A straight fiber was selected for measurement and the slide adjusted so that it was in the center of the field. The shrinkage temperature was taken when the fiber under examination began to shrink and the degree of shrinkage was determined by measuring the initial uncontracted length and that of the contracted fiber using a filar micrometer eyepiece for the purpose. The per cent shrinkage is calculated as the difference in the two lengths divided by the initial uncontracted length multiplied by 100. The fibers were heated to 95°C. in the water medium and 100°C. in the 50 per cent glycerol solution. The results are shown in Tables VII and VIII and Fig. V.

The following observations were noted:

TABLE VII

2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml.  
0.1 M Phosphate Buffer Containing 1.0 Gram Formaldehyde

Sample No.	Final pH	Millimols Formaldehyde Fixed per Gram Collagen	Shrinkage Temperature °C	Percentage Shrinkage
1	4.45	0.11	73.0	62.8
2+	4.47	0.29	78.5	54.4
3	4.99	0.16	76.3	53.2
4	5.89	0.29	78.0	51.4
5	5.91	0.31	78.5	52.6
6	5.92	0.23	74.0	55.2
7	5.97	0.11	67.0	59.9
8	6.56	0.27	78.5	56.3
9+	6.92	0.37	81.0	46.2
10	7.22	0.27	74.0	53.4
11	7.22	0.29	79.5	52.7
12	7.27	0.21	73.5	66.9
13+	7.32	0.44	80.5	46.7
14+	7.41	0.53	79.5	53.2
15+	7.43	0.27	78.5	55.1
16	9.02	0.45	78.5	44.6
17	9.40	0.42	77.6	45.4
18	10.53	0.47	74.0	50.3
19	10.71	0.36	76.5	54.8
20	11.02	0.51	75.0	56.2
21	11.40	0.79	80.5	46.5
22	11.45	0.71	76.5	44.8
23	11.45	0.65	74.3	49.5
24	11.45	0.62	72.0	57.3
25	11.53	0.55	73.5	43.9
26+	11.51	0.93	75.5	47.4
27+	11.45	0.89	74.0	47.3
28+	11.46	0.97	76.0	50.4
29+	11.52	0.80	75.0	46.6
30+	11.62	0.77	74.5	45.7
31+	11.58	0.69	73.0	49.5
32	12.42	0.57	74.0	43.8
33	4.11	Blank	50.5	64.4
34	5.02	Blank	51.6	63.7
35	6.44	Blank	52.8	65.4
36	12.20	Blank	57.0	66.4
37	12.26	Blank	54.0	72.6
38	Water	Blank	57.0	66.4
50% Glycerol Solution				
39+	4.47	0.29	82.0	60.4
40	5.89	0.29	84.0	61.3
41	5.91	0.31	86.0	55.2
42+	6.92	0.37	84.0	51.3
43	7.15	0.40	85.0	37.7
44	10.05	0.63	84.5	56.4
45	10.51	0.79	88.5	54.9
46	11.18	0.73	82.0	32.3
47	11.70	0.81	90.0	40.6

+ Tanned in 4% Formaldehyde

TABLE VIII

2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml.  
0.1 M. Phosphate Buffer Containing 1.0 Gram Formaldehyde

Sample No.	Final pH	Combined HCHO	Shrinkage Temperature	Percentage Shrinkage	1 hour	4 hours	1 day
40	5.89	0.29	84.0	61.3			51.0
41	5.91	0.31	86.0	52.6			62.1
48+	7.25	0.21	79.0	52.2	83.1	88.2	91.6
43	7.15	0.40	85.0	37.7	94.7		95.8
44	10.05	0.63	84.5	36.4	90.2	92.3	92.8
45	10.51	0.79	88.5	54.9			83.6
46	11.18	0.73	82.0	32.3	85.4		87.6
47	11.70	0.81	90.0	40.6			89.1

+ Shrinkage in distilled water

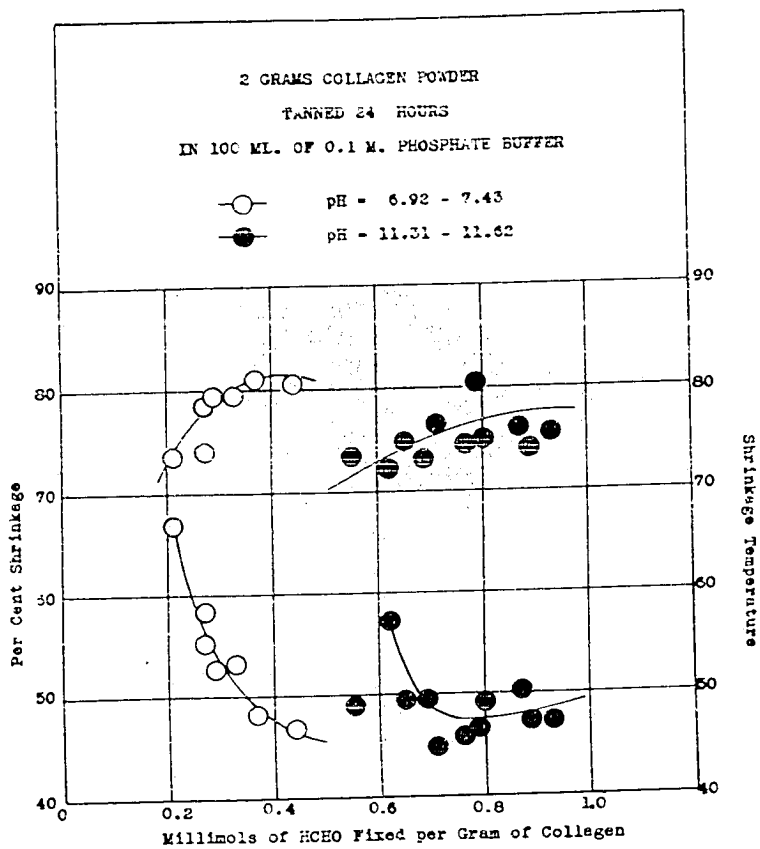


FIGURE 7

1. The formaldehyde tanned fiber when heated near its shrinkage temperature exhibited striking contortions before finally contracting into a short straight rod with a decided decrease in volume. On cooling it assumed its original shape again.

2. The shrinkage temperature became less sharp as the amount of combined formaldehyde increased.

3. There seemed to be no definite relation between shrinkage temperature and degree of shrinkage with the amount of combined formaldehyde in the pH range covered. However, for the same pH value (duration of tanning varied) the percent shrinkage decreased as the amount of combined formaldehyde increased whereas the shrinkage temperature increased with the amount of combined formaldehyde. This relation is not so marked in the alkaline range as it is on the acid side as shown in Fig. V.

4. The percentage recovery when the tanned collagen samples were allowed to stand for 1 day increased with the amount of combined formaldehyde for samples tanned at a pH value below 7.5. At higher pH values there seemed to be a decrease in percentage recovery with increasing pH values in spite of an increase in the amount of combined formaldehyde. The results are shown in Table VIII.

5. The shrinkage temperature and percentage recovery of previously contracted samples which were allowed to stand for 1 day were much lower than the original values. Thus

sample 3 in Table VIII began to shrink at 50°C. and gave 49.9 per cent shrinkage and a percentage recovery of 75.8 after the first hour, 78.9 after the fourth hour, and 83.6 after 1 day. Sample 6 exhibited a much more gradual shrinkage, 56.6 per cent shrinkage and 85.0 per cent recovery in 1 day.

Undoubtedly there are other factors that affect the shrinkage temperature and degree of shrinkage aside from the formaldehyde tannage. Swelling will necessarily affect the degree of shrinkage and the per cent recovery. Collagen fibers are more extended when in the moist condition and should exhibit a higher degree of shrinkage. Of course, there is a limit to the extensibility of the fiber due to swelling for when maximum swelling is approached, the bulging out of the fibers will counteract any further increase in length. Such factors as length and thickness of the fibers will have to be considered.

A slight modification of Astbury's view (1) on the mechanism of shrinkage has been adopted to explain the observations noted above. Astbury has pointed out that if the molecules of two reacting substances come together the force of the valency bonds will tend to hold them together, and the vibrational forces due to the kinetic energy of the individual molecules will tend to shake them apart. "When the thermal agitation is sufficient to overcome the inter-chain attractions, the chain bundles may be said to "melt" and the chains collapse on themselves. It follows that

anything that interferes with the solvation and interactions of the side chains that form the rungs of the polypeptide grid, or with any inter-chain linkage for that matter, must inevitably influence the thermal transformation temperature."

The mechanism of shrinkage of formaldehyde tanned collagen fibers can be explained in the following manner:

The thermal agitation required to overcome the attraction between the combined formaldehyde molecules and the free amino groups of the collagen side-chain must be greater than the interchain attraction due to the salt linkage since formaldehyde combination takes place in the presence of the latter. Thus the shrinkage temperature will increase with the amount of combined formaldehyde. The shrinkage itself can be explained as the result of the electrostatic attraction between neighboring rungs of the polypeptide grid.

Such a mechanism of electrostatic attraction and repulsion has been expounded by K. H. Meyer and H. Mark (10) to explain the swelling in acid, neutral, and alkaline solutions. This electrostatic attraction will be less the higher the pH value of the solution inside the fibers since the concentration of the charged amino groups decreases as the pH value increases. This explains why the shrinkage temperature of formaldehyde tanned collagen fibers become less sharp and the shrinkage less the greater the amount of combined formaldehyde.

Swelling is the major factor affecting the percentage recovery. Collagen fibers swollen in acid and alkaline media

during tanning will not be able to recover the original uncontracted length when allowed to stand in water with a pH in the isoelectric zone. The difference in the degree of swelling at the two pH values will more or less determine the per cent recovery. When the collagen fibers contract much of the swell water will be expelled since there is a decided decrease in the volume of the contracted fiber. At the same time part of the combined formaldehyde will be lost by diffusion.

The rate of recovery will be more or less controlled by the electrostatic repulsion set up when the formaldehyde recombines with the collagen reactive groups as the temperature goes down. Since the concentration of the positively charged reactive amino groups decreases as the pH value increases the force of repulsion created by the neutralization of the positive charges due to the formaldehyde combination will necessarily be less at higher pH values. From these considerations the recovery will be more or less a maximum around pH 7 and will be attained at a much faster rate. At low pH values while the concentration of the positively charged amino groups is great the number neutralized by formaldehyde combination will be relatively small.

The shrinkage temperature, shrinkage, and per cent recovery of previously contracted samples which were much lower than the original values can be attributed to the decrease in swelling and loss of formaldehyde during the

previous treatment.

## 7. The Collagen-Formaldehyde Reaction in Organic Solvents

The usual method of washing with sodium sulfite can not be used for formaldehyde tannage in organic solvents. Drying in the vacuum oven and shaking 1 day in 100 ml. of the solvent used for tanning, were resorted to. The organic solvents tried were ethyl alcohol, methyl alcohol, chloroform, and benzene. Solutions of formaldehyde in chloroform and benzene were prepared by heating solid paraformaldehyde and bubbling the depolymerized formaldehyde gas into the respective solvents. They are then allowed to stand overnight and filtered from the polymerized formaldehyde separating out from solution. Pellets of potassium hydroxide were added to the benzene and chloroform while being saturated with formaldehyde gas for the solutions used for samples 18 to 29. The alkali was added to inhibit polymerization. The results are shown in Table IX.

The amount of combined formaldehyde in ethyl alcohol and methyl alcohol at pH values of 8.04 and 7.87 respectively is about the same as that in aqueous solutions of the same pH. The formaldehyde combination in the two solvents at alkaline pH values is much less than that occurring in aqueous solutions of the same pH. The explanation of the results obtained may be attributed to the change in the pK values of

TABLE IX

The Collagen-Formaldehyde reaction in Organic Solvents  
2.000 Grams Collagen Powder Tanned 24 Hours in 100 ml. Solution

Sample No.	Final pH	Solution Used	Millimols HCHO Fixed per Gram	Method Employed to Remove Uncombined Formaldehyde
1	11.92	0.1 M Phosphate Buffer containing	1.20	Rinsed with 2 50-ml. portions of H <sub>2</sub> O
2	11.92	50% C <sub>2</sub> H <sub>5</sub> OH and 1 gram HCHO	0.68	Dried in vacuum oven at 100°C
3	8.04	90% C <sub>2</sub> H <sub>5</sub> OH solution containing 1 g.	0.58	Rinsed with 2 50-ml. portions of C <sub>2</sub> H <sub>5</sub> OH
4	8.04	HCHO, pH adjusted by adding 1 N KOH	0.43	Shaken 1 day in 100 ml. of C <sub>2</sub> H <sub>5</sub> OH of the same pH
5	12.56	"	1.18	Rinsed with 2 50-ml portions of C <sub>2</sub> H <sub>5</sub> OH
6	12.56	"	0.68	Shaken 1 day in 100 ml. of C <sub>2</sub> H <sub>5</sub> OH of the same pH
7	11.85	0.1 Phosphate Buffer containing	1.03	Rinsed with 2 50-ml. portions of water
8	11.85	30% CH <sub>3</sub> OH and 1 gram HCHO	0.65	Dried in vacuum oven at 100°C
9	7.87	95% CH <sub>3</sub> OH solution containing 1 g.	0.58	Rinsed with 2 50-ml. portions of CH <sub>3</sub> OH
10	7.87	HCHO, pH adjusted by adding 1 N KOH	9.43	Shaken 1 day in 100 ml of CH <sub>3</sub> OH of the same pH
11	12.05	"	0.53	the same pH
12	12.05	"	0.87	Rinsed with 2 50-ml. portions of CH <sub>3</sub> OH
13		Chloroform solution containing	2.40	Rinsed with 2 50-ml. portions of CHCl <sub>3</sub>
14		1 gram HCHO	0.37	Shaken 1 day in 100 ml. of CHCl <sub>3</sub>
15		"	0.14	Dried in vacuum oven at 100°C for 1 day
16		"	0.13	Dried in vacuum oven at 100°C for 2 days
17	CHCl <sub>3</sub>	solution containing 0.5 g. HCHO	0.58	Shaken 1 day in 100 ml. of CHCl <sub>3</sub>
18	CHCl <sub>3</sub>	solution containing 0.95 g. HCHO	1.89	Rinsed with 2 50-ml portions of CHCl <sub>3</sub>
19		"	0.67	Shaken 1 day in 100 ml. of CHCl <sub>3</sub>
20		"	0.61	Dried in vacuum oven at 100°C for 1 day
21	CHCl <sub>3</sub>	solution containing 0.59 g. HCHO	1.50	Rinsed with 2 50-ml. portions of CHCl <sub>3</sub>
22		"	0.62	Shaken 1 day in 100 ml. of CHCl <sub>3</sub>
23	C <sub>6</sub> H <sub>6</sub>	solution containing 0.017 g HCHO	0.28	Rinsed with 2 50-ml. portions of C <sub>6</sub> H <sub>6</sub>
24		"	0.27	Shaken 1 day in 100 ml. of C <sub>6</sub> H <sub>6</sub>
25	C <sub>6</sub> H <sub>6</sub>	Solution containing 0.19 g. HCHO	1.57	Rinsed with 2 50-ml. portions of C <sub>6</sub> H <sub>6</sub>
26		"	1.30	Shaken 1 day in 100 ml. of C <sub>6</sub> H <sub>6</sub>
27		"	0.61	Dried in vacuum oven at 100°C for 1 day
28	C <sub>6</sub> H <sub>6</sub>	solution containing 0.14 g. HCHO	1.04	Shaken 1 day in 100 ml. of C <sub>6</sub> H <sub>6</sub>
29	C <sub>6</sub> H <sub>6</sub>	solution containing 0.14 g. HCHO	0.66	Shaken 1 day in 100 ml. of C <sub>6</sub> H <sub>6</sub> and then rinsed with 2 50-ml. portions of water

the reactive amino groups in alcoholic solutions. Amino acids and their peptides exist predominantly in the zwitter ion form in alcoholic as well as in aqueous solution. Jukes and Schmidt (11) have shown that the pK value of the  $\alpha$ -amino group of lysine does not change at all in alcoholic solutions whereas that of the  $\epsilon$ -guanidino group showed a marked increase from 12.48 to 14.1 in 72 per cent ethyl alcohol solution.

The tanning results in chloroform showed that the uncombined formaldehyde was easily removed by shaking the tanned samples in 100 ml. of chloroform. On drying in the vacuum oven sample 20 tanned in chloroform where potassium hydroxide pellets were added retained more of the combined formaldehyde than sample 14. This seems to indicate that some alkali is needed to make the collagen-formaldehyde combination stable to heating.

It will be noticed that the amount of combined formaldehyde is high in chloroform and benzene in spite of the fact that no alkali was present in solution. This is very marked in benzene where the concentration of formaldehyde is comparatively lower. It may be that the protein zwitter ion passes over to its uncharged tautomer in non-polar solvents. Another probable contributing factor is the polymerization of formaldehyde in such solvents. Absorption spectra measurements have shown that the characteristic absorption due to the double bond of the carbonyl grouping is almost completely absent in aqueous solutions although it is easily

observed in solutions such as chloroform or benzene (16). That formaldehyde is present in the monomeric form to a greater extent in benzene and chloroform than in ethyl alcohol and methyl alcohol is not unlikely.

Formaldehyde is only very slightly soluble in benzene and tanning experiments have to be limited to dilute solutions. Some quite interesting results were obtained, however. In very dilute solutions (samples 23 and 24) all of the formaldehyde in solution was taken up by the collagen sample (0.28 millimols = 0.0168 g.). The results for samples 25 and 26 show that very little of the formaldehyde take-up was removed by shaking the tanned sample 1 day in 100 ml. of benzene. To verify whether all of the formaldehyde take-up is in combined form sample 29 was rinsed with two 50 ml. of water instead of benzene. This brought down the formaldehyde take-up from 1.04 to 0.66 millimols showing that a fraction of the formaldehyde take-up was just adsorbed on the surface of the collagen fibers.

An explanation of the results obtained in organic solvents may be traced to their dielectric constants and dipole moments (12) which are given in Table X.

Table X

Substance	Dielectric Constant at 20° C.	Dipole Moment $\times 10^{-18}$ e.s.u.
Benzene	2.28	0
Chloroform	5.05	1.15
Ethyl alcohol	24.0	1.7
Methyl alcohol	33.0	1.7
Water	80.0	1.9

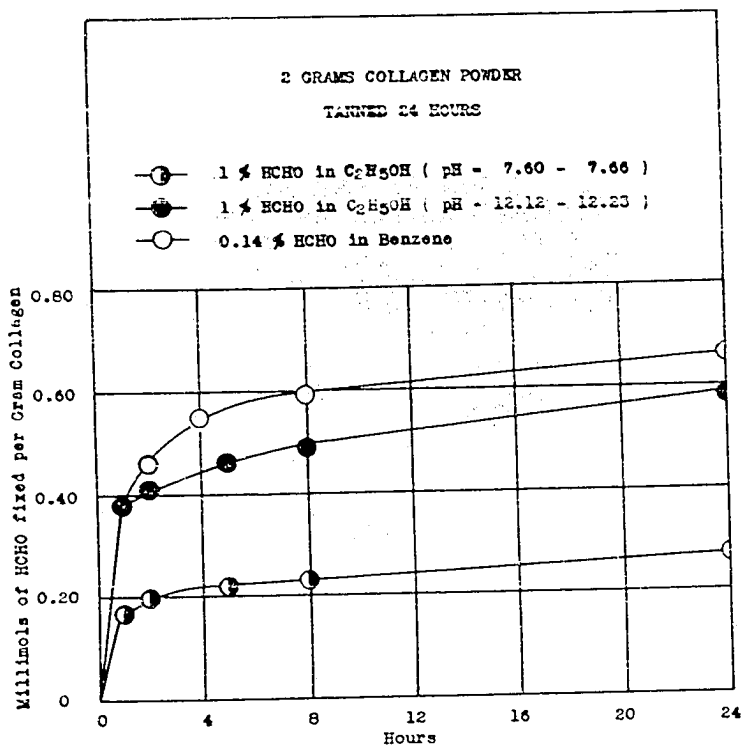


FIGURE VI

The stability of the collagen-formaldehyde combination, i.e., the attraction between the reactive amino group and the formaldehyde molecule will be greater in the solvent with a lower dielectric constant.

The adsorption of formaldehyde by the collagen samples in benzene solutions may be attributed to the attraction of two dipolar compounds in a non-polar solvent. It may also be stated that a substance is adsorbed the better from liquids in which it is least soluble.

To study the comparative reaction rates in organic solvents a non-polar organic solvent, benzene, and a polar organic solvent, ethyl alcohol, were chosen. The alcohol solutions used were 90 per cent ethyl alcohol solutions containing 1 gram formaldehyde per 100 ml. the pH being adjusted by adding 1 N KOH. The benzene solution contains 0.14 g. formaldehyde per 100 ml. The results are shown in Fig. VI. It will be noticed that the reaction rate curve in benzene is above those in alcohol in spite of the lower formaldehyde concentration.

The data obtained are not sufficient to draw definite conclusions. However, it brings out the fact that such factors as polarity and dielectric of the solvent, the state of polymerization of the formaldehyde, and the effect of the solvent on the dipolar character of the collagen may have to be considered, and therefore necessitate further detailed investigations. It is interesting to note too, that a stable combination approximating the 1:1 collagen-formaldehyde

hyde reaction occurs even in organic solvents.

Summary and Conclusions:

1. A rapid and effective method of removing uncombined formaldehyde was developed.
2. It was shown that formaldehyde polymers could not diffuse through the collagen fibers and therefore could not take part in the reaction. The role of polymerization in the process of washing was also presented.
3. The reaction between the collagen and formaldehyde was shown to be a combination of one molecule of formaldehyde for each reactive amino group.
4. A discussion as to how the law of mass action could be applied to the collagen-formaldehyde reaction was presented.
5. Aging experiments showed that the 1:1 collagen-formaldehyde reaction is stable.
6. It was shown that other factors affected the shrinkage temperature and percentage shrinkage of formaldehyde tanned collagen fibers aside from the formaldehyde tannage.
7. The 1:1 collagen-formaldehyde reaction was found to take place in organic solvents. The nature of the solvent affected the rate of the reaction to a great extent.

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