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17. Aldehyde Tannage

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The mechanisms developed for explaining the aldehyde tanning of proteins are fundamental to the understanding of the mechanisms of all types of tanning and the general reactions of proteins. The importance of aldehydes in the stabilization of synthetic protein fibers has led to a tremendous increase, since 1940, in the volume of research on the reaction of aldehydes with proteins. The impact of this mass of accumulated data upon the theory of the reactions of aldehydes with proteins has not been fully realized. The results to be presented will show that many of the early hypotheses were based upon unsound generalization, therefore a full development of the early hypotheses will be of little value in understanding the complex nature of this reaction. Most of the early history of the reaction can be traced through recent review articles.^{28, 31, 39, 77}

The emphasis in recent years has been on improved analytical techniques, more rigidly controlled experiments, and the use of model structures to prove some of the more elusive characteristics of the reaction. The true significance of many of the former researches has been realized; this has revealed the complexity of the reaction, and many of the anomalous results previously obtained can now be assigned to clearly defined phases of the reaction.

The reaction of aldehydes with proteins can be conveniently separated into three types: the equilibrium type, the nonequilibrium but chemically reversible type, and the truly irreversible type. The complexity of the reaction, however, arises from the fact that the product from one type may become involved in further reactions of a different type. The existence of these three types of reaction has been responsible for much of the confusion in the early studies. This is shown clearly in the discussion of the analytical problems.

Since formaldehyde has several distinct advantages over the other aldehydes both for industrial application and for ease of analysis, it is not surprising that formaldehyde has been used in most of the studies on aldehyde tanning. The discussion to follow will, therefore, be devoted mainly

to the formaldehyde reaction. The use of other aldehydes will be covered in a separate section.

PROBLEMS OF PROTEIN-FORMALDEHYDE REACTIONS

Removal of Excess Formaldehyde from Proteins. Most formaldehyde-hardened proteins are prepared by treating the protein in a large excess of formaldehyde solution. A protein so treated will contain not only combined formaldehyde but also formaldehyde held mechanically in the solution wetting the solid. Nitschmann and Hadorn⁶⁴ have shown that if the mechanically held formaldehyde solution is removed by washing (the generally accepted practice), the reversibly bound formaldehyde will also be removed. The rate of removal of formaldehyde from treated hide powder by washing with water was shown by Green³² to be fairly uniform even after 9 days of continuous washing. Bowes and Kenten⁴ found that formaldehyde-treated collagen which had been washed for 24 hr in a Wilson-Kern extractor lost an additional 10 per cent of its formaldehyde content when it was washed for another 24 hr.

Since the reported duration of washing used in different laboratories varies from a few hours to several days, the total amount of formaldehyde bound by treatment with solutions of the same formaldehyde concentration may be found not to be identical. If, however, adequate control experiments have been run, the conclusions on the amounts and locations of the nonequilibrium and irreversibly bound formaldehyde should be valid. It is these two types of reaction which contribute most of the desirable effects produced by formaldehyde hardening of proteins.

A pressing technique devised by Cameron *et al.*⁹ to remove free water and the electrolyte from protein samples was employed by Theis⁷⁴ to remove the solution and unbound formaldehyde from hide preparations. The preparations obtained by this pressing technique should theoretically contain the reversibly as well as the irreversibly bound formaldehyde. Gustavson,³⁷ however, showed that the pressing technique can yield high results because some of the solution may remain within the pressed mass. The pressing technique, therefore, appears to be of little value in aiding the study of the reversibly bound formaldehyde, although it could, perhaps, be used to approximate the maximum amount of total formaldehyde bound by a protein. The error would be small for very dilute formaldehyde solutions but might become appreciable where higher concentrations of formaldehyde are used.

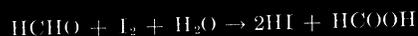
Determination of Dissolved Formaldehyde. Several methods exist for determining the amount of formaldehyde in solution; however these do not always give unequivocal results when used to determine the unreacted formaldehyde left in solution. In reactions of formaldehyde with in-

soluble proteins, the unreacted formaldehyde left in solution can be easily determined. However, the probable presence of nonsolvent water bound to the protein creates an uncertainty in the total solution volume and a corresponding uncertainty in the values for bound formaldehyde obtained by this method.

With soluble proteins and model compounds the methods for determining formaldehyde in solution fail to distinguish between free and easily reversibly bound formaldehyde. The Vorländer⁷⁸ dimedone (5,5-dimethyl-1,2-cyclohexanedione) reaction was developed into a very accurate gravimetric determination for formaldehyde by Yoe and Reid,⁸⁶ who worked out a specific set of conditions for the determination. However, the required 12-hr period of standing permits the transfer of some reversibly bound formaldehyde from the protein to the reagent if the formaldehyde-treated protein is present with the reagent.

MacFadyen⁵⁶ studied the dimedone reaction with the aid of a spectrophotometric modification of the chromotropic acid (4,5-dihydroxy-2,7-naphthalene disulfonic acid) reaction. He discovered that a combination of the dimedone and chromotropic acid reactions was a valuable method for studying the formaldehyde content of fairly labile formaldehyde derivatives. The dimedone reaction carried out at pH 7 or 8 for 30 min at 37°C will precipitate the "free" formaldehyde. The methylene bis dimedone derivative formed in this reaction is stable in the boiling 10M sulfuric acid used in the chromotropic acid procedure. The boiling acid, however, hydrolyzes all reversibly bound formaldehyde from the treated protein. The "free" formaldehyde can thus be determined from the difference in value for formaldehyde obtained by the chromotropic acid method on the original mixture and that found in a sample of this mixture after treatment with dimedone. This method will not distinguish between free formaldehyde and formaldehyde bound in an easily reversible manner.

Where formaldehyde solutions do not contain other materials which will react with iodine, the formaldehyde is probably best determined by an iodometric method.



This reaction occurs rapidly in alkaline media. The amount of iodine required can be measured either by direct titration with standard iodine or by back titration of a known amount of iodine with standard thiosulfate. The iodometric method is usually used to determine the formaldehyde liberated by acid distillation in the determination of combined formaldehyde.

Determination of Combined Formaldehyde. The significance of the acid-distillation method for the determination of formaldehyde in treated proteins has also been reinterpreted. The method of Highberger and

Retzsch⁴⁶ has been shown to be incapable of determining all of the formaldehyde bound by some proteins and, under some conditions, may actually result in an irreversible binding of some of the previously reversibly bound formaldehyde. Nitschmann and Hadorn⁶² were able to recover only 85 per cent of the formaldehyde added to a sample of casein when they analyzed it according to the method of Highburger and Retzsch. When the 2*N* sulfuric acid of Highburger's method was replaced with 0.1*N* phosphoric acid, the recoveries were raised to 100 per cent. Later, however, with Lauener,⁶³ they found it necessary to use multiple distillations and even a subsequent addition of acid to obtain complete discharge of formaldehyde from samples of formaldehyde-tanned casein that had been heat-treated. Swain *et al.*⁷² did not find a difference between the results when either 2*N* sulfuric acid or 0.1*N* phosphoric acid was used, provided that the sample was distilled an adequate number of times, with the addition of water between each distillation.

Nonrecoverable Formaldehyde. In the meantime, Baudouy² had shown that distillation with sulfuric acid did not give full recoveries of formaldehyde from proteins that contained appreciable quantities of histidine and tryptophane. Proteins containing little or none of these amino acids released their formaldehyde completely. Nitschmann and Lauener⁶⁶ believed that this difference between proteins must be due to a reaction of formaldehyde with the tryptophane and histidine liberated by the hydrolytic action of the strong acid. Free tryptophane has been shown⁸¹ to react with formaldehyde at high temperatures and in strong acid to produce 1,2,5,6-tetrahydro-4-carboline-5-carboxylic acid. This compound will not yield formaldehyde on acid hydrolysis. Histidine also reacts with formaldehyde under the same conditions to produce 1,2,5,6-tetrahydro-pyrido-3, 4-iminazole-6-carboxylic acid which also fails to produce formaldehyde on acid hydrolysis.

This, however, does not appear to be the complete story, for Nitschmann and Lauener⁶⁶ have demonstrated that acetylation of casein prevents the irreversible binding of formaldehyde during the distillation. Since the acetyl groups are expected to hydrolyze during the acid distillation, they should have little effect on the irreversible binding of formaldehyde if the action occurs only during the distillation and only on free tryptophane and histidine.

The occurrence of a reaction of formaldehyde with tryptophane in polypeptides was shown by Fraenkel-Conrat, Brandon, and Oleott.²⁵ They noted a close agreement between the equivalents of tryptophane in gramicidin with the amount of formaldehyde introduced, and the number of hydroxyl groups formed when formaldehyde reacts with gramicidin. The usual color tests for the indole nucleus do not produce color with either the hydrolyzed

or unhydrolyzed formaldehyde-treated gramicidin. This gramicidin derivative will liberate formaldehyde in strong alkali, but it will only partially liberate the formaldehyde in acid solution. The formaldehyde, therefore, appears to be bound to the tryptophane as a methylol group before the analytical distillation. This preliminary binding may be essential to the formation of the irreversibly bound formaldehyde which occurs during the analysis.

Another example of irreversibly bound formaldehyde was reported by Alexander, Carter, and Johnson,¹ who found that the reaction of formaldehyde on wool caused a reduction in the amount of tyrosine recovered after hydrolysis. The formation of a methylene cross bond between an epsilon amino group of lysine and the 3 position of the tyrosine ring was postulated. This bond is believed to break during the acid distillation to yield a substituted tyrosine and result in the apparent disappearance of the formaldehyde involved in the linkage.

In the early part of this discussion, it was emphasized that there was a degree of uncertainty in the values for formaldehyde held by easily reversible binding. In the latter part, it was shown that some formaldehyde was also bound to proteins in such a fashion that the usual analytical procedure would not detect it. These limitations of the analytical data should be borne in mind through the following discussions, for there will be numerous instances where the accumulated data still are not sufficiently comprehensive to resolve completely the many complexities of the formaldehyde reaction.

These difficulties with the analytical techniques have been minimized to a large extent by limiting the scope of the reaction through the use of simpler systems. Much of the recent work has been done on model systems containing only one or two reactive groups. These studies were then extended to proteins or polypeptides rich in the particular groups under investigation. The effect of a single group or the interaction between two groups could thus be evaluated even though the analytical techniques were not sufficiently reliable to give an accurate over-all picture of the reaction.

Similar results were also obtained by removing some of the reactive groups from a protein by chemical means such as deaminization or deamidation or by masking the groups chemically by acetylation or esterification. In these cases the effect of the altered group is obtained by difference, and the analytical methods are sufficient to give reliable data in many instances. The results of these studies will be presented later under the discussion of the effects of the various individual groups.

FACTORS INFLUENCING FORMALDEHYDE FIXATION

Effect of Formaldehyde Concentration. Although the analytical methods available do not give a complete picture of the extent of the formal-

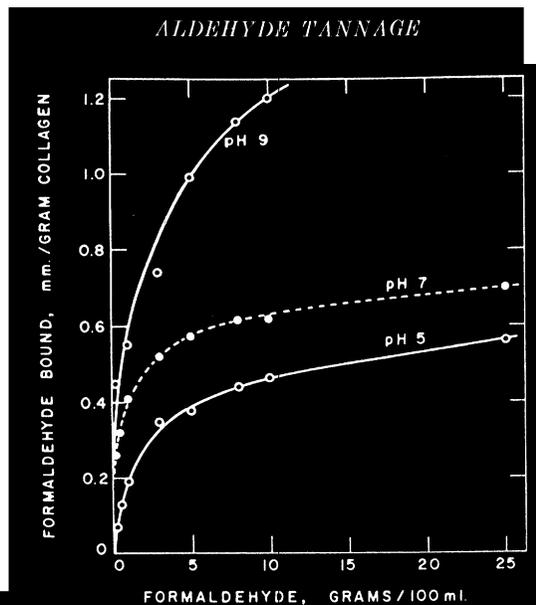


Figure 17.1. Effect of concentration on the amount of formaldehyde bound by hide powder.

dehyde reaction, the studies already made are of value to indicate the gross aspects of the reaction. The data of Highberger *et al.*^{46, 47, 48} on the reaction of formaldehyde with a purified collagen give the most complete picture of the effects of concentration and pH upon the reaction. When the data of these papers are interpolated along a smooth curve to give values at a constant pH, we can construct a family of curves such as those in Figure 1 where the amount of formaldehyde bound in 24 hr is plotted against the concentration of formaldehyde solution in which the collagen is treated. The shape of the curve shows that there is an appreciable binding of formaldehyde from dilute solutions and that the amount of bound formaldehyde increases with increasing formaldehyde concentration even up to about 25 per cent.

Since most of the experimental work has been performed with low or moderate concentrations of formaldehyde, the groups or reactions responsible for the additional binding at high concentrations have not been definitely worked out. It is doubtful, however, that it can be held by a primary linkage to any of the reactive groups, because these appear to be completely utilized at much lower concentrations of formaldehyde. Perhaps the reactions in this region of concentration are those of polymer formation initiated on formaldehyde already bound to the protein.

Highberger and Salcedo⁴⁸ suggested that the limit of formaldehyde binding at pH 7 appears to be twice the amino concentration. This is probably a coincidence since it neglects the binding by other groups which is now

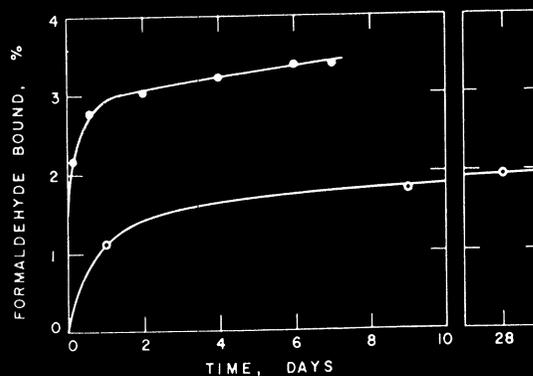


Figure 17.2. Rate of formaldehyde binding on casein.

○ Data of Nitschmann and Hadorn⁶⁴ pH 7, 17°C, 2 per cent formaldehyde.

● Data of Wormell⁵⁵ pH 4, 35°C, 4 per cent formaldehyde in saturated sodium sulfate.

known to occur in this pH region also. Another explanation is the possible increase in crosslinking reactions due to the dehydrating effects of the more concentrated formaldehyde solutions.

Formaldehyde Fixation Rate. Highberger and Retzsch⁴⁷ determined the rate of binding of formaldehyde on collagen in neutral solutions of 1 per cent formaldehyde, and showed that there was a very rapid initial reaction which gradually slowed down and approached completion within 12 to 18 hr. Nitschmann and Hadorn,⁶⁴ however, found that, in addition to the rapid initial reaction, casein in neutral solutions also showed a very slow reaction which continued for days. The long time required to obtain equilibrium between casein and formaldehyde was also shown by Carpenter and Lovelace.⁴⁴ The lower curve of Figure 2 shows a set of data obtained by Nitschmann for the formaldehyde bound at neutrality from a 2 per cent solution of formaldehyde. The upper curve is from the data of Wormell and Kaye⁵⁵ and shows a similar type of reaction obtained under more drastic conditions—4 per cent formaldehyde in saturated sodium sulfate solution at pH 4.0 and at 35°C. The initial rapid fixation, followed by a slower fixation requiring several days, is again evident.

Even with the simple urea and formaldehyde model system, Smythe⁶⁵ showed that the reaction starts with a rate about fourteen times that of the subsequent slower reaction.

Evidence for a multiple-reaction rate system was also presented by Waugh and Livingstone,⁸⁰ who noticed that formaldehyde inactivates fibrinogen by a rapid reversible reaction and a slower irreversible reaction. In 0.38 per cent formaldehyde, complete inactivation will occur within 60 min, but within this time the reaction can be completely reversed by rapid

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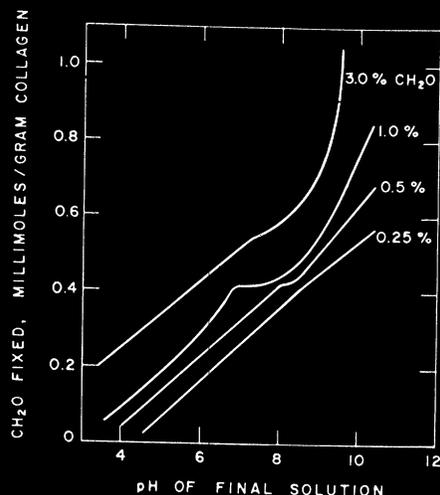


Figure 17.3. Effect of pH upon the formaldehyde binding of collagen powder according to Highberger and Retzsch.⁴⁷

dialysis. If the reaction proceeds longer than 60 min before the dialysis occurs, irreversibly inactivated fibrinogen is obtained in amounts which increase with the time of reaction.

Effect of pH. The effect of the pH of the solution upon the binding of formaldehyde by proteins appears to be quite similar for all proteins. At normal temperatures the binding is usually rather low under moderately acid conditions, and it increases to quite high values under moderately alkaline conditions. Figure 3, from the work of Highberger and Retzsch⁴⁷ on the formaldehyde binding of a purified collagen powder, shows this trend at several formaldehyde concentrations. The presence of a plateau in the curves at some concentrations has been ascribed on a speculative basis to various causes. The amino group probably plays no great role in this phenomenon, since the plateau also occurs in completely deaminized collagen. This is shown in Figure 6. It is also unlikely that the phenomenon can be explained by the saturation of any particular group with formaldehyde at these pH values, because the plateau does not occur in the solutions of higher or lower formaldehyde content.

Under highly acid conditions there may be an increased binding of formaldehyde if certain dehydrating conditions are present. The combination of high formaldehyde concentration, high temperature, high salt concentration, and high acidity enables the amide groups to react with formaldehyde. Proteins high in amide content thus can show marked increases in formaldehyde binding in the very low pH region. This reaction will be discussed further in the section on the reaction of the amide groups.

FUNCTIONAL GROUPS INVOLVED IN THE REACTION

Reactions of the Amino Group. The amino group ($-NH_2$) is perhaps the most important reactive group in the proteins. Because of its basic nature, it plays a major role in all the phenomena related to the saltlike linkages in proteins. Any reaction which involves the amino groups can be expected to cause profound changes in the properties of the protein. It is, therefore, not surprising that the amino groups played the most important role in the early studies of the action of formaldehyde on proteins. These early studies are adequately reported in previous reviews and will not be described here.

There are two types of amino groups in proteins: the alpha amino groups of the terminal amino acid of the protein chain and the epsilon amino group of the lysine side chain. It is impossible at present to determine any differences in the manner of their reaction with formaldehyde.

The reactions of the alpha amino group with formaldehyde cannot be studied conveniently in the proteins themselves, but many studies have been made on the alpha amino groups of the amino acids and their derivatives. One of the most promising techniques involves the use of the optical activity of the amino acids. When a reaction occurs on a group which is attached to an optically active center of a molecule, the optical rotation of the system will change. The relationship of this change to the concentration of the reagent causing the change will usually show some of the characteristics of the reaction.

Figure 4, obtained by Carpenter and Lovelace,¹² shows that when asparagine is treated with formaldehyde there is an abrupt rise in the levoro

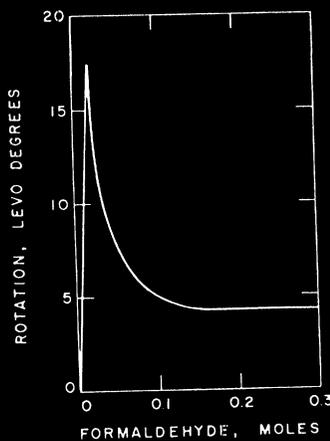


Figure 17.4. Effect of added formaldehyde on the angular rotation of 0.02 mole of sodium L-asparaginate according to Carpenter and Lovelace.¹²

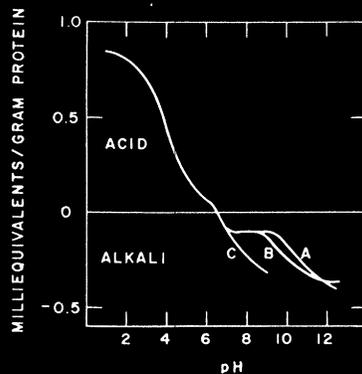
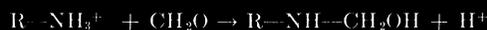


Figure 17.5. Titration curves for collagen and formaldehyde-treated collagen according to Theis.⁷¹ A, collagen; B, collagen treated with 1 per cent formaldehyde; C, collagen treated with 5 per cent formaldehyde.

tatory power of the mixture until 1 mole of formaldehyde has been added for each mole of asparagine present. Beyond this point there is a rapid decrease in the optical rotation as a secondary reaction occurs. No excess of formaldehyde is found by Vorländer's reagent until the first molecule of formaldehyde is completely added. The second molecule to be bound is so lightly held that it is removed by Vorländer's reagent. With the addition of the first molecule of formaldehyde, there was a rapid increase in the acidic properties of the asparagine. This indicates that the first molecule of formaldehyde reacts with the amino group.

Theis⁷¹ has shown very clearly (Figure 5) that formaldehyde produces a marked change in the titration curve of collagen only in the region attributed to the amino groups. Many of the older papers expressed the belief that the amino groups could react with formaldehyde only in the uncharged state (-NH_2), even though Svehla⁷¹ was able to show in 1923, by the use of freezing-point measurements, that compounds were formed between amino acids and formaldehyde without the addition of alkali. Recently Fraenkel-Conrat *et al.*^{21, 23} and Gustavson³³ have shown that formaldehyde reacts with amino groups in the acid region, causing the discharge of hydrogen ions from the NH_3^+ groups which exist under these acid conditions:



This is, in principle, the mechanism involved in the formol titration of amino acids.

The reaction of formaldehyde with aspartic and glutamic acids was shown by Carpenter and Lovelace¹³ to form definite monomethylol compounds which would react with additional formaldehyde to form unstable compounds that could not be isolated. Glutamic acid required 2.2 times the con-

centration of formaldehyde used by aspartic acid to reach the same ratio of mono- to disubstituted derivatives. This shows that proximity of the second carboxyl group in these compounds has a pronounced effect upon the secondary reaction.

Frieden, Dunn, and Coryell³⁰ were able to show by polarimetric measurements that anionic lysine adds 1 formaldehyde molecule to each amino group in the first stage of the reaction. Since the optical rotation changed less for the first equivalent of formaldehyde added than for the second, one might claim that the epsilon amino group reacts before the alpha amino group. The difference in degree of reaction is slight, however, and for the remainder of the discussion no distinction will be made between the alpha and epsilon amino groups.

The number of reactive groups in proteins makes it difficult to ascribe a portion of the total formaldehyde adsorption to any specific group. Many studies have, therefore, been made with modified proteins wherein one or more of the groups have been changed. This creates a difference in composition which may be compared with a change in formaldehyde binding.

The limited data of Meunier and Schweikert,³⁷ showing that the formaldehyde-binding capacity of deaminated collagen is lower than that of native collagen, were supplemented by the more rigorous data of Highberger and Retzsch³⁷ which are shown in Figure 6. These data, over a wide pH range, show very clearly that the effect of deamination on formaldehyde binding occurs even at low pH, and that there is practically no additional effect above pH 8. Above this pH the curves have about the same slope and are roughly parallel to each other. These results indicate that the amino group reacts with 1 molecule of formaldehyde.

Nitschmann and Hadorn³⁵ found a similar difference between casein and deaminated casein treated with 10 per cent formaldehyde solutions

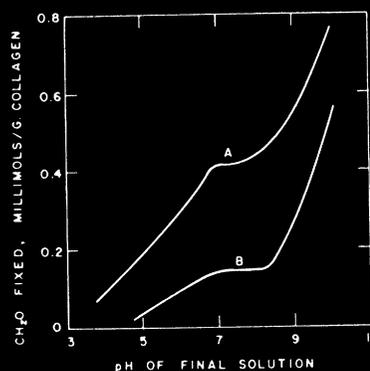


Figure 17.6. Comparison of the amount of formaldehyde fixed by collagen (A) and deaminated collagen (B) according to Highberger and Retzsch.³⁷

for 24 hr. However, when the treatment was continued for 30 days, the difference in formaldehyde uptake between the two materials was much less than the amount obtained during the short-time treatment, even though the total formaldehyde uptake had more than doubled. They also determined that there was a loss of water during the condensation of gaseous formaldehyde on casein and concluded that the formaldehyde which first binds to the amino group as a methylol group must undergo a subsequent reaction to form a methylene bridge:



When the casein was acetylated,⁶⁶ there was no condensation reaction, and the formaldehyde was believed to be bound by addition only. Nitschmann hypothesized that the formation of this methylenic bridge was the true tanning action of formaldehyde on proteins.

Wormell and Kaye⁸⁵ studied the effect of deamination of casein on the binding of formaldehyde in the presence of strong acid and salt. Under these conditions formaldehyde reacts much more rapidly than at neutrality and in the absence of salt. Fixation was complete in 24 to 30 hr at 35°C. When neutral hardened casein was rehardened in the presence of acid and salt, there was a large increase in bound formaldehyde, while under the same conditions very little additional formaldehyde was bound on the deaminated casein.

The marked change in physical properties which occurs when a neutral hardened protein is rehardened under the acid-salt conditions with the addition of only a trace of formaldehyde may indicate the formation of crosslinkages by the formaldehyde which is already bound. No attempt to produce this change without the additional trace of formaldehyde has been reported. Therefore, the possibility that only a few crosslinkages are formed by the additional formaldehyde may account for the change in properties. That this effect is not due entirely to the amino group is shown by the fact that acid-salt hardening does not give the same strength properties to a neutral hardened deamidized protein that it gives to the native protein. This type of linkage to the amide group will be discussed more fully in the section on the reaction of the amide group. It would seem, however, that the acid-salt hardening of casein occurs largely through the formation of crosslinkages between formaldehyde already bound on the amino groups and the amide groups:



The formation of amino to amide crosslinkages by formaldehyde has been shown to occur at room temperature and over a wide pH range by the model experiments of Fraenkel-Conrat and Oleott.²³ The guanidino group may

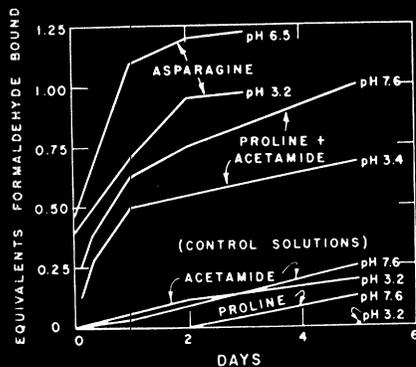


Figure 17.7. Rate of fixation of formaldehyde by various compounds at 23°C according to Fraenkel-Conrat and Olecott.²¹

take the place of the primary amide group, but the peptide group which Nitschmann postulated to form such a linkage does not appear to do so.

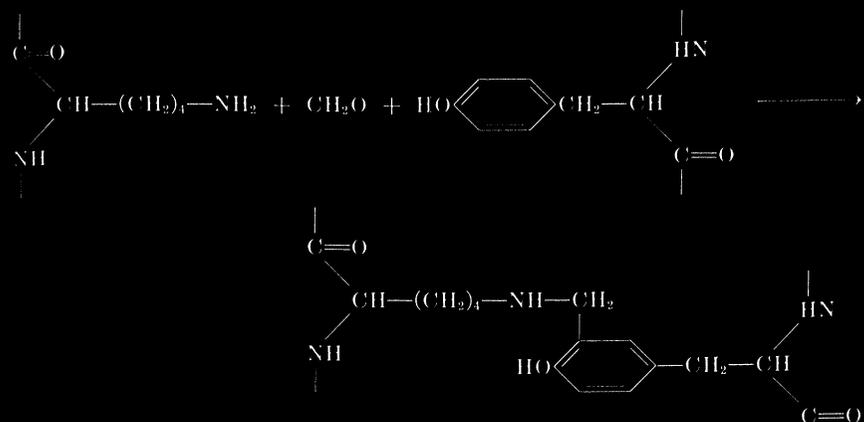
The formaldehyde-binding curves for proline and acetamide obtained by Fraenkel-Conrat²¹ are shown in Figure 7 along with the binding curves of a mixture of the two materials. The increased binding by the mixture is due to the formation of crosslinkages between the imino group of proline and the amide group. Although one can visualize the first step of this reaction as the binding of formaldehyde by either the imino group or the amide group, the binding on the imino group seems to be the only possibility supported by experimental evidence.

The reaction of formaldehyde with the amino group even at pH 3.6 is shown by the lowering of the pH of the reaction mixture and the change produced in the optical rotation of the solutions when optically active amino acids are used as the source of the amino groups. Furthermore, the methylolamines formed separately will react with amide groups to form crosslinkages, while an amidomethylol group formed separately will not react with an amino group to form the crosslinkage.

This model reaction was performed on proteins rich in amino groups. In the presence of formaldehyde at room temperature and from pH 2 to 8, these proteins would bind both acetamide and methylguanidine by the formation of methylene crosslinkages. This condensation of the methylol-amino groups with the low-molecular-weight amide seems to occur in preference to the condensation with the amide groups of the protein itself. Concentration and steric factors may be the main directive influences in causing this preferential reaction.

The formaldehyde bond formed between the amino groups and the amide or guanidino groups resembles the bond formed by the Mannich reaction,³ and Fraenkel-Conrat and Olecott²³ tried the reaction with such active hy-

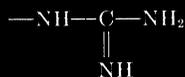
drogen donors as phenolic, imidazole, and indole groups. The reactions were completed within a few days, and the methylene bonds formed were resistant to acid hydrolysis. The Mannich type of phenol methylene derivatives were even stable to alkaline hydrolysis:



The effect of this Mannich type of crosslinking between protein molecules was demonstrated by Fraenkel-Conrat and Mecham²⁷ with bovine serum and egg albumins. The importance of the amino group was shown when the acetylation of the amino groups of these proteins prevented the reaction. In a like manner, Alexander, Carter, and Johnson¹ showed that the acetylation of wool prevented a Mannich-type crosslinking between the amino and tyrosine groups of wool. This reaction will be discussed more fully under the reaction of the tyrosyl group.

In contrast with earlier theories which could not permit the amino groups to bind formaldehyde under any but neutral or alkaline conditions, the more recent experiments reported here show that situations have been found under which formaldehyde will bind with amino groups even under quite acid conditions. The amino group can form a relatively stable methylol derivative. This methylol derivative can bind additional formaldehyde in loose combination, or it can react with the amide, guanidyl, imidazole, indole, or phenolic groups. The latter reactions, which appear to be Mannich-type reactions, give rise to methylene crosslinkages which produce the main tanning effects of the bound formaldehyde. This same reaction can be used to add substituents to the amino groups of a protein and thereby change its properties.

Reactions of the Guanidino Group. The arginine residues in proteins contain the guanidino group



which is more basic than the amino group and might, therefore, be expected to play as important a part in the formaldehyde binding of proteins as the amino group. Wadsworth and Pangborn⁷⁹ found that arginine would combine with 2 molecules of formaldehyde while guanidine would combine with only 1. Both molecules of formaldehyde bound by arginine are so reversibly bound that they can be removed by materials, such as alanyl-glycine, which bind formaldehyde more strongly.

The polarimetric measurements of Frieden, Dunn, and Coryell³⁰ show that arginine reacts instantaneously with the first molecule of formaldehyde and slowly with the second. A Sakaguchi test for the guanidino group shows that the slow reaction involves the guanidino group.

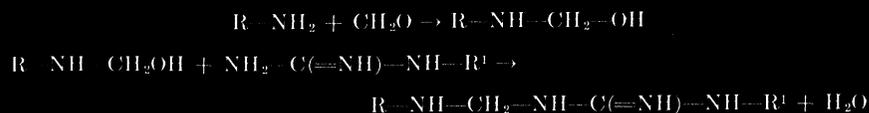
Higberger and Saleedo^{38, 49} removed the guanidino groups from collagen with hypochlorite and found that the greatest reduction in the formaldehyde binding caused by this change was in the alkaline region. The sharp rise in the formaldehyde uptake curve at pH 8, Figure 3, was, therefore, believed to be due to binding by the guanidino groups. Theis,⁷⁴ however, believed that the guanidino group of arginine did not react with formaldehyde to any appreciable extent up to pH 11.5. Steinhardt, Fugitt, and Harris⁶⁹ demonstrated a pH effect caused by the action of formaldehyde on the guanidino groups of wool and showed that the titration curves of most proteins have not been carried to sufficiently high alkalinity to show the effect.

In contrast to these questionable indications that the guanidino group reacts with formaldehyde under alkaline conditions, there are very definite indications that the guanidino groups react with formaldehyde under neutral or acid conditions. Fraenkel-Conrat and Olcott²² showed that at 70°C both arginine and methyl guanidine bound up to 2 molecules of formaldehyde, and guanidine combined with almost 3. At room temperature only about half of this formaldehyde was introduced, and then only after a considerably longer time.

The protamine, salmine, which contains 0.22 mole of guanidino group per mole of nitrogen, combined with 0.29 to 0.42 mole of formaldehyde per mole of nitrogen, depending upon the concentration of the formaldehyde. Since there are few other reactive groups present, there may be as many as two formaldehydes bound to each guanidino group. The formaldehyde-treated protamine was no longer completely dialyzable, and the molecular weight had increased from 4,000 to 11,000. Conditions and reagents which dissociate secondary valences had no effect on the high-molecular-weight derivative. Therefore, Fraenkel-Conrat and Olcott concluded that intermolecular methylene bridges must be formed.

In a subsequent paper²⁴ they showed that more formaldehyde could be

bound by a mixture of amino and guanidino compounds than could be bound by the individual compounds under the same condition. A methylene bond formation was suggested. It was also shown that an aminomethylol group would react with a guanidino group to form a methylene bond between the amino and guanidino groups. The possibility of a guanidino-methylol reacting with an amino group was believed unlikely because of the slow rate of formation of the guanidinomethylol. Therefore, the most probable path for this reaction is the formation of a methylol on the amino group and a subsequent reaction of this methylol with the guanidino group to form the methylene bond:



This reaction has been used to bind small molecules containing guanidino groups to proteins rich in amino groups. This was discussed in more detail as a reaction of the amino group.

Gustavson²⁸ obtained an increase in the shrinkage temperature of normal collagen by treating it with formaldehyde in the presence of salt to prevent swelling. A similar treatment on deaminated collagen produced no change in shrinkage temperature. Gustavson, therefore, concluded that formaldehyde bound on guanidino groups did not form stabilizing crosslinkages. In a later paper¹⁹ he showed that there still remained the possibility of crosslinking by an aminomethylol group reacting with a guanidino

group. Difficulty in restricting the reactivity of the guanidino group prevents a clear-cut demonstration of the regions in which the guanidino group reacts. The majority of data available have been interpreted to show that the guanidino group acts as a primary binding site for formaldehyde mainly in highly alkaline solutions. The maximum binding capacity of the guanidino group has probably never been attained. It is not probable that the primary binding of formaldehyde on the guanidino group has much influence on the structural stabilization produced by the reaction. However, the reaction of guanidino groups with the aminomethylol groups formed by the primary binding of formaldehyde on the amino groups is probably a very important reaction which contributes to the tanning effect of formaldehyde on proteins.

Reactions of the Amide Group. The normal amide groups of proteins are the amide derivatives of the side-chain carboxyl groups of the aspartic and glutamic acid residues present in the proteins. As early as 1905, Einhorn¹⁶ showed that, in acid solution, amides would form methyl-

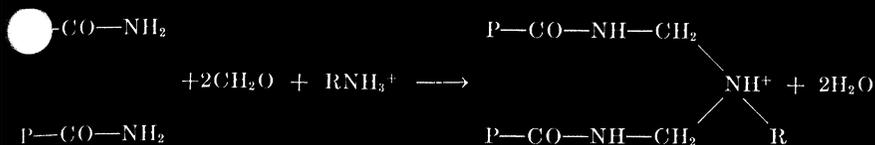
enebisamides in the presence of formaldehyde. The polarimetric data of Carpenter and Lovelace¹² show that, if the amide group of L-asparagine reacts with formaldehyde, the binding is very weak. This is reasonable, for all the methylol amides reported in the literature are unstable and easily give off formaldehyde.

Theis and Lams⁷⁵ noted that the formaldehyde-binding values for wool obtained in acid solutions were much higher than expected, and they attributed the increased amount to binding by the amide groups. The employment of the pressing technique to remove the excess formaldehyde solution from these wool samples may have prevented the very labile formaldehyde held by the amide groups from being washed out of the samples.

Ferretti¹⁸ patented a process for formaldehyde-hardening protein fibers in acid-salt baths. The theoretical aspects of this condition for the reaction were studied by Wormell and Kaye.⁸⁴ Deamidated casein and normal casein reacted with the same amount of formaldehyde under neutral conditions. However, when these neutral hardened materials were rehardened under acid-salt conditions, the deamidated casein did not react with additional formaldehyde, while the normal casein almost doubled its formaldehyde content.

The reaction of zein—a protein with very low amino nitrogen and very high amide content—under these acid-salt conditions is very startling. Zein, which will react with only 0.4 per cent of its weight of formaldehyde under neutral conditions, will react with 4.1 per cent of its weight of formaldehyde under the acid-salt conditions. There is little doubt that the amide groups are involved in the reaction, but the binding is too stable to be a simple methylol formation. Since Wormell and Kaye⁸⁵ were able to show some marked changes in physical properties when neutral hardened casein was rehardened under the acid-salt conditions with a minimum of added formaldehyde, a crosslinking reaction was postulated to occur between the aminomethylol groups and the amide groups. This still does not explain the reaction of the amides when no amino groups are present.

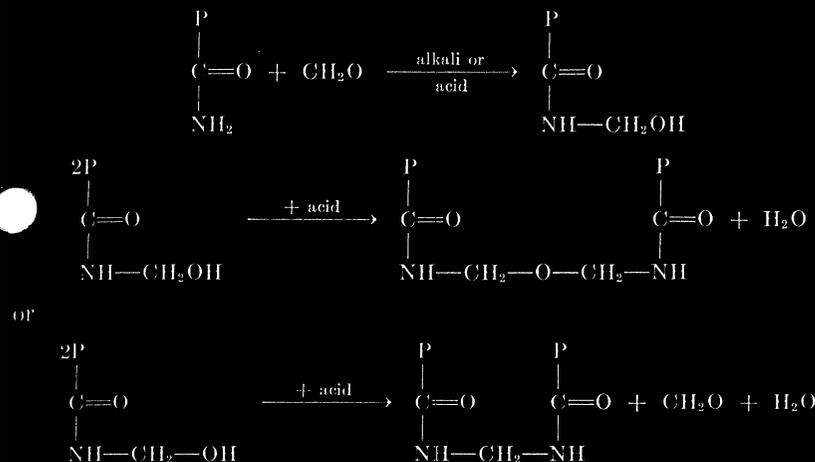
Fraenkel-Conrat and Mecham²⁷ noted that although 1 per cent solutions of egg albumin showed little evidence of increased molecular weight, 10 per cent solutions did show an increased molecular weight at formaldehyde concentrations ranging from 0.4 per cent to 10 per cent at both pH 3.5 and pH 7.5. Gliadin—a protein rich in amide but poor in amino groups—did not show much change in molecular weight under similar conditions, but, when some ammonium salts (2 eq. per 10,000 gm) were incorporated into the reaction media, a marked increase in molecular weight occurred. Polyglutamine and zein showed a similar behavior. The following reaction was suggested.



In their model studies, Fraenkel-Conrat and Olcott²⁴ showed that amidomethylol compounds will not condense with primary amines. This may explain why zein and gliadin, which have been reacted with formaldehyde so that all the amide groups are saturated as methylols, will not gel when amines are added to them.

Middlebrook⁶⁰ has shown that the stability to supercontraction which formaldehyde confers on wool may involve an amide crosslinkage, for formaldehyde treatment of deamidated wool produced no stability.

Evans and Croston,¹⁷ who studied the increase of tensile strength and elasticity of zein fibers treated with formaldehyde under highly acid conditions, believed that the stabilizing crosslinkages could be formed only by the dehydration of methylolamides to form oxymethylene or methylene linkages between the adjacent protein chains. The lack of amino groups in zein makes these reactions more plausible. With *P* to indicate the protein residue, the reactions have the following formulation:



A useful method for detecting the presence of methylolamides in proteins was developed by Haworth, MacGillivray, and Peacock.⁴² These authors found that methylolamides condense with β -naphthol in cold alcoholic hydrochloric acid to form 1-acylamidomethyl-2-naphthols. These derivatives hydrolyze with warm alcoholic hydrochloric acid to yield 1-amino-methyl-2-naphthols. Brominated β -naphthols also undergo this condensation and introduce a bromine atom which can be detected in the products

more easily than the aminomethyl-naphthol can be isolated from the same mixture.

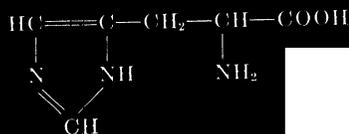
When silk fibroin, which contains negligible amounts of amide groups, is treated with formaldehyde, it will condense with a negligible amount of 6-bromo-2 naphthol. Arachin, gliadin, and gluten, which contain appreciable amounts of amide groups, react with formaldehyde in 1 per cent potassium carbonate (pH 10) to form derivatives which will condense with 6-bromo-2 naphthol. Thirteen per cent of the amide groups are shown to be present as methylolamide derivatives. If these same proteins are treated with formaldehyde in very strong acid (pH 0) only a very small amount of condensation with the naphthol occurs. This would tend to confirm the results of Evans and Croston,¹⁷ which show that the amides form very stable crosslinkages in this acid region.

Formaldehyde-treated peanut protein that had been freed of loosely bound formaldehyde by boiling in water was subsequently partially digested with enzymes, and the fragments were chromatographed by Harworth, MacGillivray, and Peacock.⁴³ Most of the formaldehyde-containing fragments were also shown to contain either glutamic or aspartic acids or both acids together. The presence of *N*-methylenebisamide linkages between aspartic and glutamic acids in the original formaldehyde-treated protein is strongly suggested.

The action of formaldehyde on the amide groups of proteins appears to result in several possible end products. In alkaline or acid solution at room temperature, methylolamides are formed. These are quite unstable and can usually be completely decomposed by boiling water. Under the effects of strong acid and dehydrating conditions, the amide groups react with formaldehyde to produce methylenebisamides. The formation of these crosslinkages produces a marked change in the physical properties of the proteins.

The relative rapidity with which amide groups react with formaldehyde when amino groups or ammonium salts are present would lead one to suspect that the formation of bisamides is important only where there is a lack of amino groups. Formaldehyde which is bound to an amino group as a methylol readily condenses with an amide group to form a stable crosslinkage which alters the physical properties of the protein. This reaction is one of the important reactions in formaldehyde tanning.

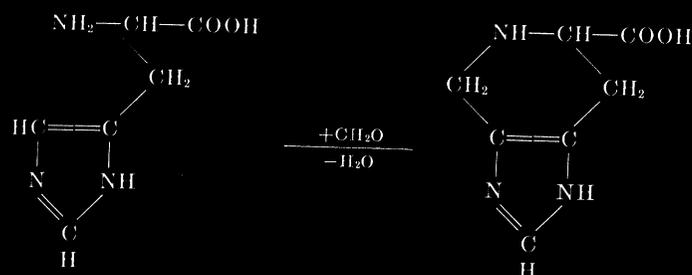
Reactions of the Imidazole Group. The structural formula for histidine shows that this



amino acid contains the imidazole group. Although this five-membered ring, containing two nitrogens and a conjugated double bond, possesses some basic properties, it is considerably less basic than the amino groups of lysine. However, it was basic enough to make it of interest to the earlier workers studying the reaction of formaldehyde with proteins.

Theis and Lams⁷⁵ compared the formaldehyde-binding and the acid-binding properties of the same wool and showed that the titration value for the imidazole group between pH 6.1 and 8.0 agreed almost identically with the amount of formaldehyde bound in this region. They concluded that the imidazole group must bind 1 molecule of formaldehyde in this region. Gustavson,³⁶ however, believed that these conclusions were purely speculative and did not reflect the true range of reactivity of the basic groups because the amino groups have also been shown to be active in this region, as indicated by the decrease in formaldehyde binding following deamination.

Polarimetric measurements on the formaldehyde-binding reactions of histidine by Frieden, Dunn, and Coryell²⁹ and Carpenter¹⁰ do not yield the same results, and a more detailed study of the reaction may be necessary. Neither group of workers postulated a reaction which was shown to occur by Neuberger.⁶¹ When 1 mole of formaldehyde was incubated with 1 mole of histidine, a stable compound was formed according to the indicated reaction.



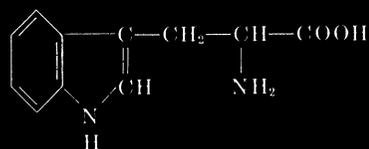
This compound contains only one bound formaldehyde, gives a negative "Ninhydrin" reaction, does not give off nitrogen when reacted with nitrous acid, and is stable to acid and alkali. This is the same compound which Nitschmann and Lauener⁶⁶ believe accounts for part of the formaldehyde lost during the analytical distillation. Since acylation of the amino group of histidine prevented the condensation, the reaction is of little value in interpreting the interaction of formaldehyde with proteins unless some of the histidine is terminal in the protein chains.

Fraenkel-Conrat and Olcott²³ obtained a condensation reaction between threonine, formaldehyde, and α -N-acetyl-L-histidine at 40°C but not at room temperature. Since the acetyl histidine would not bind formaldehyde at any pH studied, this reaction appears to be the same Mannich-type re-

action discussed under the reactions of the amino group. Fraenkel-Conrat also showed that, in the presence of formaldehyde, the amino groups of bovine serum albumin and gramicidin would condense with acetyl histidine by the same type of reaction.

The histidine groups of a protein were shown to react with aminomethylol groups of the same protein to form methylene crosslinkages which are resistant to acid hydrolysis. Proteins rich in histidine and amino groups bound much more formaldehyde irreversibly than did proteins rich in histidine but poor in amino groups. Therefore, the chief reaction of the imidazole group during the formaldehyde tanning of proteins is the donation of active hydrogens for the Mannich reaction which forms methylene crosslinkages between amino groups and the imidazole group of histidine.

Reactions of the Indole Group. The structure given below for the amino acid tryptophane, which is present in small amounts in most proteins, shows that it contains the double-ring structure known as the "indole group."



Fraenkel-Conrat, Brandon, and Olecott²⁶ used model substances to show that 3-alkyl indole residues similar to those in tryptophane would react with 1 mole of formaldehyde under alkaline conditions to form a methylol. The same reaction will occur in acid solutions if high temperature and high concentration of formaldehyde are used. The reaction is reversible in strong alkali, but acid solutions will liberate only part of the formaldehyde. The chromogenic activity of tryptophane disappears when proteins are treated with formaldehyde and reappears when the treated protein is put into strong alkali. This indicates that the reaction has formed a methylol on the indole nitrogen.

Baudouy² showed that proteins containing tryptophane appeared to form complexes with formaldehyde from which the formaldehyde could not be recovered. Nitschmann and Lauener⁶⁶ and Swain *et al.*⁷² also supported this view. This irreversible combination of indole groups with formaldehyde was shown to occur under the conditions of the acid distillation used for the recovery of bound formaldehyde. The failure of acetylated proteins to show this irreversible binding of formaldehyde may mean that a previous binding of formaldehyde to the amino groups of the protein may be involved. A reaction of the Mannich type previously described under the amino group reactions could combine a formaldehyde between an amino group and the indole group. On acid distillation the bridging methylene

group attached to the indole group may switch amino groups and become attached to the amino group of the same tryptophane residue to which the indole group belongs. If this happens, a stable ring structure will be formed, and the formaldehyde will become nonrecoverable. Fraenkel-Conrat and Oleott²³ showed that the required Mannich-type reaction did occur between the indole groups of gramicidin and various amines in the presence of formaldehyde. In many cases the interaction was manifested by changes in the physical properties of the mixture. However, an actual demonstration of this crosslinking reaction between amino and indole groups of a protein has not been presented.

Reactions of the Tyrosyl Group. The reaction of phenols with formaldehyde has been very important in the manufacture of plastics, and it would be surprising if the phenoliclike tyrosyl group of proteins did not also bind formaldehyde. A model experiment by Fraenkel-Conrat and Oleott²³ showed that at room temperature and pH 4 to 8 where neither threonine nor *p*-cresol would react with formaldehyde, an equimolar mixture of the two would bind approximately one equivalent of formaldehyde in an acid-resistant linkage. Amino groups appeared to be required for the reaction. By the same reaction, serum albumin bound phenol groups equivalent to about half of its amino groups.

Middlebrook⁶⁰ and Brown *et al.*⁷ showed that wool did not possess any formaldehyde irreversibly bound on its tyrosine residues either at pH 1.0 and 100°C or at pH 6 and 50°C, respectively. However, at room temperature and at neutrality, Alexander *et al.*¹ were able to show that formaldehyde was bound to tyrosine both reversibly and irreversibly. They postulated an intermolecular methylene linkage of the Mannich type between amino groups and tyrosine rings. This increases the alkali stability of the wool, but the stability can be lost by heating the derivative in acid. The crosslinkage is destroyed, but the formaldehyde is not liberated because it remains attached to the tyrosine by a carbon-carbon bond. This substituted tyrosine will not give the Millon test. Johnson,⁵² after a detailed study of the conditions for the binding of phenols to wool, decided that a 0.133 molar solution of both formaldehyde and resorcinol at 60°C for 30 min at pH 7 to 8 was the optimum condition for the reaction. These are close to the conditions found desirable by Alexander for the formation of crosslinkages between the amino and tyrosine residues of wool.

Reactions of the Sulfhydryl Group. The reaction of formaldehyde with cysteine was studied quite thoroughly by Ratner and Clarke.⁶⁷ Since *N*-acetyl cysteine reacts rapidly with formaldehyde and *S*-ethyl cysteine reacts slowly, the reaction of cysteine with formaldehyde was believed to be a two-step process in which the formaldehyde became attached first to the sulfhydryl group and then underwent a secondary reaction with the adja-

cent amino group. This forms the ring compound, thiazolidine-4-carboxylic acid.

Bowes and Pleass⁵ had suggested that a reaction of formaldehyde with sulfhydryl groups might account for some of the formaldehyde bound in excess of the amount required by the basic groups of wool. Hegman⁴⁹ recognized a type of reaction with feather keratin in which the rate depended upon the concentration of formaldehyde and the concentration of some group which appeared to increase in concentration with increasing pH. The sulfhydryl group which is formed by the splitting of the disulfide group at high alkalinity could meet this requirement. Middlebrook and Phillips⁵⁸ showed by disulfide determination that formaldehyde at room temperature did not react with the disulfide groups of wool below pH 7.0. At 70°C, however, a slow reaction occurred involving about one-third of the total sulfur. Only 1 sulfur atom from each disulfide group reacted. Wool treated in this manner does not give stable *S*-cysteine sulfonate groups when treated with sodium bisulfite, and, therefore, this reaction of formaldehyde at elevated temperature appears to be on the same fraction of the disulfide groups of wool which react with sodium bisulfite. Middlebrook and Phillips⁵⁹ showed by means of paper chromatography that, when wool was treated with boiling 1 per cent formaldehyde in either acid or neutral media, thiazolidine-4-carboxylic acid could be found in the hydrolyzate of the treated wool. When samples of this treated wool were distilled with 0.1M phosphoric acid, the formaldehyde could be recovered from the distillate, and the residual wool showed the same number of disulfide groups as the wool before the formaldehyde treatment. This would indicate that under these conditions the formaldehyde is combined as a methylol on the sulfhydryl groups and that, on hydrolysis of the wool, the thiazolidine-4-carboxylic acid can be formed as the amino groups of the cysteine are liberated.

The disulfide groups of wool can also be converted to sulfhydryl groups by treatment with reducing agents. Stoves⁷⁰ has shown that the resistance of reduced wool fibers to extension could be restored by treatment with formaldehyde at pH 8.0. This would indicate that a crosslinkage was formed between the sulfhydryl groups of the main polypeptide chains of the fiber. The presence of such a crosslinkage was confirmed by Conden, Gordon, and Martin,¹⁵ who detected djenkolic acid,



in hydrolyzates of formaldehyde-treated reduced wool. Middlebrook and Phillips⁵⁹ reduced wool with thioacetic acid and reacted all the sulfhydryl groups with formaldehyde at pH 1.0. About one-half of the sulfhydryl groups were reacted so that they would yield thiazoline-4-carboxylic acid

on hydrolysis, and the remaining sulfhydryl groups were combined to form djenkolic acid which is stable to boiling phosphoric acid.

When the sulfhydryl groups of reduced wool were reacted with methylene iodide, only djenkolic acid was formed, even though some of the sulfhydryl groups remained unreacted. These free sulfhydryl groups could be reacted with formaldehyde and would eventually yield thiazolidine-4-carboxylic acid on hydrolysis. These sulfhydryl groups are believed to belong to the beta fraction of the sulfhydryl groups of wool.

Brown and Harris⁶ used sodium sulfoxylate formaldehyde



to reduce wool and simultaneously establish the methylene crosslinkages between the sulfhydryl groups. The crosslinkages were formed so rapidly that the peptide chains did not have sufficient time to become disarranged and, therefore, stronger and more stable fibers were produced.

Reactions of the Peptide Group. Although there have been many speculations^{34, 36, 65, 74} that the peptide groups of proteins must be involved in the crosslinking or stabilization of proteins by formaldehyde, all attempts to demonstrate this type of reaction^{11, 24, 44} have led to negative results. Carpenter¹¹ showed that the length of a methylene bridge between two nitrogens, two carbons, or a carbon and a nitrogen was about 2.5 \AA , whereas the distance between two peptide chains in most proteins is 4.5 \AA . Therefore, there could not be a direct crosslinkage between the peptide groups of two adjacent chains. A special case, however, can be found in the reaction of formaldehyde with nylon.^{8, 41, 55} Methylol groups can be introduced into undrawn nylon by treatment with formaldehyde. These methylol groups increase the solubility of the treated nylon in many solvents, but, when exposed to heat, they react further to form crosslinkages which make the resulting nylon more rigid, less extensible, and of higher melting point. Since *N*-methyl nylon does not react⁸ with formaldehyde and since infrared absorption spectra show that there is reduction of the N-H bond intensities when nylon is treated with formaldehyde, it is believed that the methylol groups are on the peptide nitrogens and that the crosslinkage formed by heating is between two peptide nitrogens.

SHRINKAGE TEMPERATURE AND CROSSLINKAGE FORMATION

The previous discussion of the individual reactive groups of proteins has shown that some of these groups bind formaldehyde in simple combination while others show a more complex type of binding which apparently results in the formation of crosslinkages between two segments of the protein chains. The presence of these crosslinkages in a protein can be expected

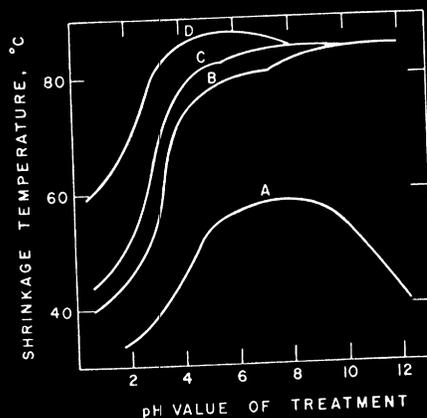


Figure 17.8. Shrinkage temperature of collagen and formaldehyde-treated collagen according to Theis.⁷⁴ A, untreated collagen; B, C, and D, collagen treated with 0.5, 1.0, and 5.0 per cent formaldehyde, respectively.

to restrict the movement of the individual protein chains and thus cause changes in the physical properties of the material.

The previous chapter shows that collagen fibers have a definite temperature at which they contract owing to increased thermal activity. This temperature is increased as a result of the tanning process, and some connection between this shrinkage phenomenon and the formation of crosslinkages during tanning has been claimed by many investigators.

It has been shown quite definitely^{1, 31, 49, 74} that formaldehyde, even in small amounts, will raise the shrinkage temperature of collagen significantly at all pH values. A comparison of the shrinkage temperature of native and formaldehyde-treated collagen made by Theis⁷⁴ is shown in Figure 8. An effect due to formaldehyde concentration occurs only in acid solutions as shown by curves B, C, and D which were made for 0.5, 1.0, and 5.0 per cent formaldehyde solutions, respectively. These three concentrations give only one curve in the alkaline region.

Highberger and Salcedo⁴⁹ found no correlation between shrinkage temperature and the amount of bound formaldehyde, but they did find that at any given pH there was a decrease in the per cent of shrinkage with an increase in bound formaldehyde. The shrinkage temperature, however, appears to reach a maximum value with the binding of a very small amount of formaldehyde, and there is no relationship between the shrinkage temperature and the degree of shrinkage for these formaldehyde-tanned collagens.

Gustavson^{31, 38} showed that deaminated collagen had the same shrinkage temperature as normal collagen, and this temperature did not increase when the deaminated collagen was treated with formaldehyde, even at high pH. This experiment indicated that the amino groups of collagen are actively

involved in the reaction resulting in increased shrinkage temperature, while the guanidino groups had no effect on the reaction. Gustavson then showed⁴⁰ that even though formaldehyde was bound to collagen in the presence of methylguanidine, urea, and ammonia, no increase in shrinkage temperature occurred. These reagents are believed to react with the amino methylol groups produced on the collagen to form methylene-linked derivatives and thus prevent the formation of crosslinkages within or between the collagen molecules. The possibility of such linkages originating from an amino group and terminating with an amino, guanidino, amide, tyrosyl, imidazole, or indole group has been amply demonstrated by Fraenkel-Conrat and Olcott.^{22, 24}

Highberger and Salcedo,^{49, 50} however, did not believe that the crosslinkage theory answered all the questions raised by the shrinkage phenomenon and proposed an electrostatic theory to account for all the phenomena, including the reversal of the shrinkage of formaldehyde-tanned collagen on cooling. This spontaneous re-extension of the fibers on cooling after thermal shrinkage has also been shown to occur with collagen modified with epoxy resins²⁰ and with tetrakis (hydroxymethyl) phosphonium chloride.¹⁹

Wiederhorn, Reardon, and Browne⁸¹ applied the theory of stress-strain behavior of rubberlike materials to determine the molecular weight between points of crosslinking in thermally contracted formaldehyde-tanned collagen. They decided that a maximum of 3 crosslinkages per 55,000 molecular weight were introduced into collagen by formaldehyde treatment. This implied that only about 1 in 7 or 8 amino groups was in a favorable position for the formation of crosslinkages. These crosslinking amino groups, however, appeared to react preferentially, and the crosslinking was completed before the other amino groups acquired formaldehyde in the form of methylol groups. If the collagen was shrunk before the formaldehyde treatment, many more crosslinkages were introduced. The change in structure caused by shrinkage apparently brings more amino groups into positions favorable to crosslinkage formation. The apparently small number of crosslinkages required to produce the maximum shrinkage temperature would indicate that change in shrinkage temperature is not an adequate measure of all the changes produced by formaldehyde tannage.

COMMERCIAL TANNAGES

Combination Tannages. Very little has been written on the use of formaldehyde in combination with other types of tanning materials, and these studies will be discussed in this chapter so that they will not be lost in the discussion of other types of combination tannage in Chapter 29. Their

and Blum⁷³ have shown that, when a vegetable-tanned leather was retanned with formaldehyde, there was an increase of almost 18°C in the shrinkage temperature of the leather. Vegetable retanning of a formaldehyde-tanned leather produced a negligible change in the shrinkage temperature. Gustavson³⁴ reported similar results, and Kremen⁵³ prepared sufficient formaldehyde-retanned vegetable-tanned leather to show that it had increased resistance to deterioration as an insole leather but that it did not have an increased wear resistance over normal outsole leather. When the retanning was carried out under alkaline conditions, a higher shrinkage temperature was obtained than when it was carried out under acid conditions. A shrinkage resistance for more than 5 min in boiling water was obtained in some cases. Either some active sites still remain in vegetable-tanned leather or the formaldehyde reacts with the bound vegetable-tanning material as it does with the free tannin. In support of the latter supposition, vegetable tannin appears to be more firmly bound after the formaldehyde treatment.

Gustavson³⁴ showed that chrome-tanned leather also reacted with formaldehyde and thereby became more resistant to swelling. Theis and Kleppinger⁷⁶ studied this reaction and noted that the formaldehyde content of the formaldehyde-retanned chrome-tanned leather was higher than the formaldehyde content of straight formaldehyde-tanned leather. A chromium complex involving the formaldehyde was postulated. The same workers also studied the effect of chrome retanning on formaldehyde-tanned leather and discovered an interesting salt effect. If no salt was present during the formaldehyde pretanning, the final shrinkage temperature after chrome tanning was lower than for straight chrome-tanned leather; if salt was present, however, the shrinkage temperature was higher than for straight chrome-tanned leather.

Tannage of White Glove Leather. Probably the only commercial use of straight formaldehyde tanning existing today is the production of fine-quality light-colored glove leathers. For this purpose kidskins and the skins of hair sheep are the chief raw materials, although few kidskins are now being used in the United States for glove manufacture.

The process for converting these dried skins into white glove leather includes soaking to restore their moisture, and liming for 4 to 8 days to loosen the hair, followed by unhairing and fleshing. The unhaired skins are then degreased with Stoddard solvent or kerosene, pickled in salt and acid to a pH of from 1 to 2, and sorted and graded for final tanning. The time lapse between pickling and tanning is about 10 days, and all the tanning reagents are calculated as a percentage of this 10-day drained pickle weight. The tanning is usually performed in a drum with a load of about 1,200 lb of hides and about an equal weight of water. Four per cent (on pickle weight) of Glauber's salt is usually added to prevent swelling, and from 6 to 12 per

ent formalin (37 per cent formaldehyde) based on the pickle weight is usually used. The starting pH is usually about 2 or 3, and the skins are drummed for 4 hr. During this time the batch is partially neutralized. Borax is sometimes used for the neutralization because it does not produce as much plumping as do other bases. The partially neutralized batch is allowed to stand overnight. In the morning the temperature and pH are raised gradually. The temperature is raised by introducing live steam, and the final volume may be as great as 250 per cent of the starting volume. The final temperature is usually around 95°F but may go as high as 135°F. The final pH is usually about 6.8 to 7.0.

After the tanning is completed, the excess formaldehyde is removed by washing with running water in a slatted drum. About 12 min per 100 lb of pickle weight is customarily used. If the excess formaldehyde is not removed, the leather is tight and stiffened or hardened when finished. The shrinkage temperature of the finished leather is about 180°F.

Some generalizations about the process and its effect on the final product can be made. At lower formaldehyde concentrations a higher starting pH is necessary to obtain the same degree of tanning. Reaction at lower pH does not give as much plumpness to the hide but does give greater area. Any excess of base used has to be neutralized to produce a good grain and to obtain a good penetration of fatliquor. The optimum pH for fat liquoring appears to be about 5.5. One of the disadvantages of formaldehyde as a tanning agent is the reduction in the fastness of dyestuffs shown by most formaldehyde-tanned leathers.

Darker-colored glove leathers are usually produced by a combination of chromium and formaldehyde tannage. This combination tannage is most efficiently carried out and produces finer-grain leathers if the chromium tanning is accomplished prior to the formaldehyde tanning, although the reverse process can be used. The chromium tanning is started at a very low pH, and it is convenient to use the skins directly out of the pickle because little acidification is required. When prechromed, the chromium oxide (Cr_2O_3) content of the dry leather will be about 2 to 6 per cent. The leather will be thinner at lower chromium contents and plumper at the higher chromium contents. A formaldehyde-tanned skin can be shaved thinner than a chrome-tanned skin. This is important because the finest glove leathers are only about 0.018 in. thick. The chrome-tanned leather has an advantage in its increased washability; with the combination tannage the washability decreases greatly as the formaldehyde content increases.

A much simplified tanning procedure, called soap tanning, is now being used for tanning poor or defective skins for use as cheap work-glove leathers. The raw skin without fleshing is drummed with about $\frac{1}{2}$ per cent soap or detergent, 8 per cent Glauber's salt, 1 per cent bicarbonate, and 8 per cent

formaldehyde for about 2½ hr. The skins are washed and then fatliquored with soap and sulfonated oil. The fat liquor is exhausted by treatments with salts and acids, and the skins are then fleshed and dried slowly to make them as mellow as possible.

THE REACTION OF OTHER ALDEHYDES

In contrast with the volume of research reported on the formaldehyde tanning of proteins, there is little published information on the reaction of other aldehydes with proteins. This may be a reflection of the low reactivity of the other aldehydes. A summary by Gustavson³⁹ of the early work with other aldehydes showed that whereas formaldehyde markedly increased the shrinkage temperature of collagen, the action of acetaldehyde was very feeble, and the higher aliphatic aldehydes had practically no action on proteins. Some of the unsaturated aldehydes, such as acrolein and crotonaldehyde, showed a significant effect on the shrinkage temperature. Glyoxal, a dialdehyde, was known to have a stabilizing action, but benzaldehyde and related aromatic aldehydes, although they reacted with proteins, produced no stabilizing effect upon their structure.

Glyoxal has probably been studied more than the other aldehydes. Gustavson³⁹ has shown that, although glyoxal condenses and polymerizes, its tanning action is due mainly to the monomer. This was also shown to be true for pyruvic aldehyde which can be considered to be a methyl derivative of glyoxal. Brown and Harris⁶ demonstrated that glyoxal will form a crosslinkage between the sulfhydryl groups of reduced wool, but they did not indicate whether one or both aldehyde groups were involved. Winheim and Doherty³³ believe that only one aldehyde group of the glyoxal reacts with collagen and that the other is free to react with urea to form a stabilizing resin tannage. This reaction will be discussed in a later chapter. In another resin tannage described by Windus,³² formaldehyde, acetaldehyde, crotonaldehyde, furfural, and benzaldehyde react with collagen in the presence of polyhydric phenols to form stabilizing tannages. This tannage will also be discussed in a later chapter.

Acrolein, which is an unsaturated aldehyde, has been isolated from the auto-oxidation products of chamois-tanning oils by Küntzel and Nungesser.⁵¹ This aldehyde was formed exclusively from the highly unsaturated fatty acids. No acrolein was formed from glycerin under these reaction conditions. The acrolein formed by this auto-oxidation of the oil is believed to be the active tanning agent in the oil tannage of chamois leather.

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