competing ΔH and ΔS factors as the ring size is increased from three to higher members. A semi-quantitative treatment of this hypothesis is being subjected to experimental test.

It is also of interest to compare complexes of ammonia with the corresponding methylamine compounds. If their thermodynamic functions are comparable, the more abundant data for ammonia complexes can be used to compare entropy effects in cases where the data on the complexes of methylamine are unobtainable. Although Frank and Evans,[10] on the basis of unpublished work of Calvin, reported large differences between the thermodynamic functions of $Ni(NH_3)_6^{++}$ and $Ni(NH_2CH_3)_6^{++}$, the data for $Cd(NH_3)_2^{++}$ and $Cd(NH_2CH_3)_2^{++}$ shown in Table IV reveal almost negligible differences in the thermodynamic functions of the two cadmium complexes.

	ΔH, kcal./mole	ΔS, cal./deg. × mole
$Cd(NH_3)_2^{++}$	7.12	1.2
$Cd(NH_2CH_3)_2^{++}$	7.02	1.5
$Cd(NH_3)_4^{++}$	12.7	8.5
$Cd(NH_2CH_3)_4^{++}$	13.7	16.0

Although data for the complexes containing 4 ligands are much less precise than the data for ions containing 2 ligands, it is interesting to note that differences between the tetra-coördinated species (*i.e.*, $Cd(NH_3)_4^{++}$ as compared to $Cd(NH_2CH_3)_4^{++}$) are perhaps significant. The data of Basolo and Merman[11] for the copper(II) complexes of ethylenediamine and N-methylethylenediamine indicate a similar phenomenon. The placing of the methyl group on the nitrogen of the ethylenediamine may be considered analogous to the placing of the methyl group on the nitrogen of the ammonia. Their data, summarized below, indicate that the ΔH values for the $Cu(en)^{++}$ complex and the $Cu(N\text{-}Me\text{-}en)^{++}$ system are essentially the same, whereas ΔH values for bis complexes, $Cu(en)_2^{++}$ and $Cu(N\text{-}Me\text{-}en)_2^{++}$ differ somewhat.

	$\Delta H(0°)$	$\Delta S(0°)$[11]
[Cu en]$^{++}$	8.6	−21
[Cu(N-Me-en)]$^{++}$	8.5	−20
[Cu(en)$_2$]$^{++}$	17.2	−35
[Cu(N-Me-en)$_2$]$^{++}$	15.5	−36

Such a trend might be of importance in comparing the $Ni(NH_3)_6^{+6}$ and $Ni(NH_2Me)_6^{++}$ data of Calvin with data of other investigations.

(10) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 531 (1945).

(11) F. Basolo and R. Kent Murmann, THIS JOURNAL, **74**, 5243 (1952).

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The Roles of the Amino and Hydroxyl Groups of Collagen in its Reactions with Formaldehyde, Tannic Acid and Quinone

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The controlled partial acetylation of collagen at its amino and hydroxyl radicals has been used to investigate the functions of these groups in some reactions with common tanning agents. The binding of formaldehyde, tannic acid and quinone is examined as a function of the degree of acetylation, and possible interpretations of the experimental data are discussed.

Introduction

The reactions of proteins with formaldehyde have given rise to an extensive literature which has recently been reviewed by French and Edsall[1] and by Gustavson.[2] Although a wide variety of protein groups can be involved, evidence from titration curves,[3] deamination,[4] acetylation[5] and from experiments with model substances[6] indicates that in neutral or weakly alkaline solution the most important reaction occurs at the ϵ-amino groups of lysine residues. The marked change in physical properties accompanying the binding of formaldehyde near *p*H 8 has led to the general opinion that covalent cross-linkages are introduced into the molecule in the form of methylene bridges.

In the particular case of collagen, the importance of free amino groups in formaldehyde tanning has been inferred from experiments[4,7] on deamination with nitrous acid, a reagent which is well known[8] to cause more than one side reaction. Controlled acetylation of specific side-chain groups[9] offers a less equivocal means of modifying the formaldehyde reaction and we describe here experiments with a range of collagen derivatives containing varying proportions of N- and O-acetyl groups. The effect of acetylation on the fixation of two other tanning agents, tannic acid and quinone, whose action is believed to be associated with amino groups[7,2] is also described.

Experimental

Acetylation.—Collagen, in the form of the standard hide powder of the International Society of Leather Trades' Chemists, was oven-dried and acetylated with anhydrous mixtures of acetic anhydride and acetic acid, as described by Green, Ang and Lam.[9] The derivatives, after thorough extraction with acetone, were exposed to the air of the laboratory and then again dried *in vacuo* at 100° over freshly-

(1) D. French and J. T. Edsall, *Advances in Protein Chemistry*, **2**, 277 (1945).

(2) K. H. Gustavson, *ibid.*, **5**, 353 (1949).

(3) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 235 (1936).

(4) J. H. Highberger and E. W. Retzsch, *J. Am. Leather Chem. Assoc.*, **34**, 131 (1939).

(5) H. Nitschmann and H. Lauener, *Helv. Chim. Acta*, **29**, 184 (1946).

(6) H. Fraenkel-Conrat and H. S. Olcott, THIS JOURNAL, **70**, 2673 (1948).

(7) J. H. Bowes and R. H. Kenten, *Biochem. J.*, **44**, 142 (1949).

(8) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947).

(9) R. W. Green, K. P. Ang and L. C. Lam, *Biochem. J.*, in press.

prepared calcium oxide. The dry samples were analyzed for N- and O-acetyl[9] and for total nitrogen by the Kjeldahl method.

Since various pretreatments of collagen, notably soaking in 3 *M* acetic acid,[2] can affect its ability to bind some tanning agents, it was necessary to prepare control specimens by treating the dry protein with either acetic acid or acetic anhydride and to compare them with the acetylated collagen. After extraction with acetone, the control specimens contained no acetyl groups or adsorbed acetic acid.

Formaldehyde Reaction.—Two-gram samples of dry collagen or its derivatives were soaked overnight in 50 ml. of a 0.1 *M* phosphate buffer at *p*H 8.0. 50 ml. of a 6% solution of formaldehyde in 0.1 *M* phosphate was then added at the same *p*H, and the mixture was allowed to stand with frequent agitation for 24 hours.

The product of the reaction contains both combined formaldehyde and mechanically held formaldehyde solution. Removal of the latter by washing with water is inevitably attended by some loss of the former.[10] An experiment was therefore made to determine a suitable period for washing in the present instance. After a sample of collagen had been caused to react with the above solution, it was washed continuously with distilled water and portions were removed at intervals for analysis. After drying, first at room temperature *in vacuo*, and then at 100° in the air oven, formaldehyde was estimated by the method of Highberger and Retzsch.[4] The results, plotted in Fig. 1 against time of washing, show that even after nine days formaldehyde was still being lost at a fairly steady rate. Since substances of low molecular weight which do not combine with collagen can be washed out completely in much shorter periods, it seems that this prolonged treatment was removing combined formaldehyde. For the purpose of comparing formaldehyde fixation by different collagen derivatives, a knowledge of the exact state of combination is less important than accurate reproducibility of results, and the latter might be achieved by extrapolating the data to zero time of washing. The time and quantity of material consumed make this course unattractive, but reference to Fig. 1 shows that the formaldehyde content after 6 hours washing closely approximates to the extrapolated value and should provide a good basis for comparison. It also was found that, although samples dried *in vacuo* at room temperature still suffered a slight loss of formaldehyde during the early stages of oven drying, no further loss occurred after the first day in the oven. In this investigation, therefore, the standard procedure was adopted of washing for six hours, drying at room temperature *in vacuo*, and heating for 24 hours at 100° in the air oven. The aldehyde content of specimens prepared in this way was reproducible to within approximately 3% and must correspond closely to the firmly combined aldehyde responsible for the tanning process.

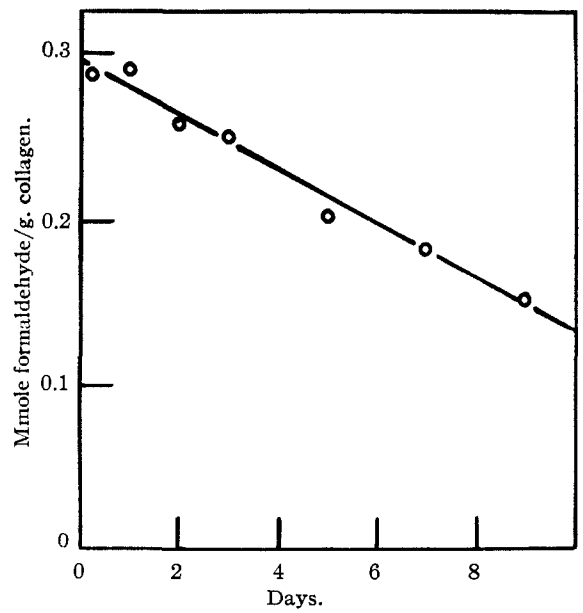

Fig. 1.—Removal of formaldehyde from collagen by continuous washing with water.

Quinone Reaction.—A solution of 14 g. of *p*-benzoquinone per liter was prepared in 0.1 *M* phosphate buffer at *p*H 8.0. Two grams of dry collagen or derivative was soaked overnight as before and 200 ml. of quinone solution was then added. After 48 hours, the product was thoroughly washed with distilled water, dried at 100° in the air oven, and analyzed for total Kjeldahl nitrogen. Estimation of combined acetyl is difficult in quinone-tanned collagen but it was found that two days soaking of acetylated collagen in phosphate buffers at *p*H 8 had no effect on the N-acetyl figure and only a very slight effect on the number of O-acetyl groups. The acetyl groups were therefore assumed to be equally stable during the quinone reaction and, in the absence of a specific method for the estimation of combined quinone, it was calculated from the acetyl value and from the nitrogen contents of the original protein, the appropriate acetyl derivative and the quinone-tanned product.

Tannic Acid Reaction.—Two grams of protein, after soaking overnight in water, was placed in a 10% tannic acid solution, previously made 0.5 *M* with respect to sodium chloride and adjusted to *p*H 3.5 with sodium hydroxide. The reaction was allowed to proceed for one week with frequent shaking, after which the product was exhaustively washed with distilled water, dried and analyzed as in the case of the quinone derivatives.

Results

The results of all three series have been plotted in Fig. 2. The amount of combined tanning agent, expressed as millimoles per gram of original dry collagen, is plotted against the total degree of acetylation expressed in the same way. The molecular weight of tannic acid has been taken as 1700,[11]

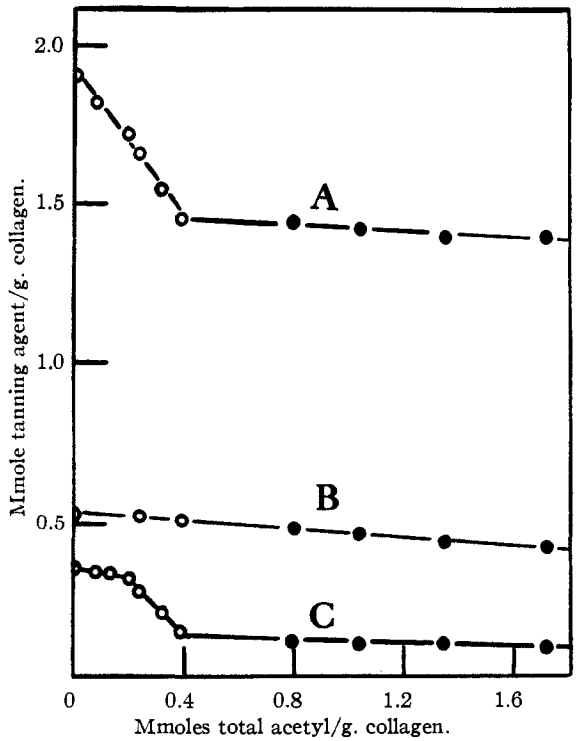

Fig. 2.—Effect of degree of acetylation of collagen on fixation of formaldehyde, tannic acid and quinone: O, N-acetyl only; ●, N-acetyl + O-acetyl; A, quinone; B, tannic acid; C, formaldehyde.

(10) H. Nitschmann and H. Hadorn, *Helv. Chim. Acta*, **26**, 1075 (1943).

(11) A. Cheshire, W. B. Brown and N. L. Holmes, *J. Intern. Soc. Leather Trades' Chem.*, **25**, 254 (1941).

and that of quinone has been taken for the monomeric form, $C_6H_4O_2$.

As no method is available for acetylating protein hydroxyl groups without simultaneous reaction at the amino groups, the total acetyl figures have been set out as abscissas. However, the samples were so chosen that all $-NH_2$ groups had been acetylated before -OH was attacked. All derivatives with not more than 0.40 mmole of total acetyl per g. contain N-acetyl groups only, while higher total acetyl values are composed of 0.4 mmole of N-acetyl per g. together with O-acetyl ranging from zero to 1.32 mmoles per g. By plotting all derivatives on the same scale we are able to distinguish any differences between the functions of amino and hydroxyl radicals.

Analysis of samples of acetylated collagen before and after reaction with formaldehyde showed that only a negligible proportion of acetyl groups were hydrolyzed by the buffer solution.

Collagen pretreated with glacial acetic acid as described above had the same affinity for all three tanning agents as did the native protein. The effect of 3 *M* acetic acid, referred to by Gustavson,[2] is evidently a property of the aqueous solution which does not extend to the anhydrous acid. However, pretreatment of dry collagen with acetic anhydride for 24 hours, although it introduced no acetyl groups,[9] reduced the fixation of tannic acid to an extent almost equal to that caused by complete acetylation.

Discussion

All three curves of Fig. 2 present unexpected features. While much more detailed work remains to be done, particularly in the direction of reconciling our results with those obtained by deamination, we advance here some possible interpretations as a basis for further inquiry.

The total number of lysine and hydroxylysine residues in collagen, reported by Bowes and Kenten[12] as 0.39 mmole/g., have been shown[9] to be capable of reaction at their ϵ-amino groups with acetic anhydride in weakly alkaline suspension. It is therefore reasonable to suppose that these same groups will be available for reaction with such substances as formaldehyde in that *p*H range. Many workers[2] have, indeed, pointed out that the amount of formaldehyde bound near *p*H 8 is very nearly 0.40 mmole per g. and have concluded that each methylene bridge introduced during formaldehyde tanning forms a link between a lysine amino group and some other radical, such as an amide or peptide group.

While there is ample evidence for the participation of amino groups in a condensation reaction with aldehydes, the supposition that the methylene bridge links two dissimilar radicals is to some extent dependent upon the numerical agreement between amino nitrogen and bound aldehyde. This is less firmly established. A cursory examination of the literature reveals figures for formaldehyde bound by collagen at *p*H 8 ranging from 0.28 mmole/g.[7] to 0.94 mmole/g.,[13] depending upon the concentration of the solution and the method employed for removing uncombined reagent. The mean certainly lies near 0.4, but it is the mean of a rather scattered population.

A more precise estimate of the effect of the $-NH_2$ group should be obtained by measuring the reduction in bound formaldehyde when the amino groups are removed or blocked. By this procedure, any errors arising from methods of washing will tend to be cancelled out. The data summarized in Table I show that the number of molecules of formaldehyde bound by the amino groups of collagen at *p*H 8 is only a little more than half the known number of those groups. When it is remembered that most of the data of Table I are derived by deamination methods well known to cause such side reactions as the removal of arginine,[7,8] it will be seen that the true ratio may not be far from 1:2.

In the absence of other evidence, this observation might be taken to support the earlier view that two amino groups are linked by one methylene bridge; but the middle portion of the curve in Fig. 2 appears finally to dispose of that possibility. The figure shows that, after a certain proportion of amino groups have been acetylated with little influence on formaldehyde binding, a break appears in the curve which then assumes a slope of approximately unity. Each subsequent N-acetyl group introduced blocks one molecule of formaldehyde, showing that, of the 0.39 mmole amino groups present in 1 g. of collagen, approximately 0.2 mmole bind formaldehyde in equimolecular ratio, while the remainder do not react. The unreactivity of the latter suggests that they are unfavorably placed with regard to suitable groups for fixing the other end of the methylene bridge, a situation which could hardly result if the second group were a peptide imino. This appears to support the view of Fraenkel-Conrat and Olcott[6] that primary amide radicals are involved, rather than the suggestion of Nitschmann and Hadorn[10] concerning the much more numerous polypeptide groups.

TABLE I

FORMALDEHYDE BOUND AT *p*H 8 BY COLLAGEN BEFORE AND AFTER INACTIVATION OF ITS AMINO GROUPS

Method of inactivation	Formaldehyde bound (mmole/g.) Before treatment	After treatment	Difference
Deamination[a]	0.28	0.06	0.22
Deamination[b]	.31	.02	.29
Deamination[c]	.44	.15	.29
Acetylation[d]	.34	.14	.20

[a] Bowes and Kenten (ref. 7). [b] K. H. Gustavson, *Kolloid-Z.*, **103**, 43 (1943). [c] Highberger and Retzsch (ref. 4). [d] This paper, Fig. 2.

The almost horizontal first portion of the curve in Fig. 2 cannot be explained by any model in which the collagen amino groups are uniformly distributed along the polypeptide chain. However, such a model, based on rigid adherence to the periodicity hypothesis of Bergmann and Niemann,[14] has little experimental support. The valuable contributions of Pauling and Corey[15] to the stereo-

(12) J. H. Bowes and R. H. Kenten, *Biochem. J.*, **43**, 358 (1948).

(13) E. R. Theis and M. M. Lams, unpublished data quoted in G. D. McLaughlin and E. R. Theis, "The Chemistry of Leather Manufacture," Reinhold Publ. Corp., New York, N. Y., 1945, p. 351.

(14) M. Bergmann and N. Niemann, *J. Biol. Chem.*, **115**, 77 (1936).

(15) L. Pauling and R. B. Corey, *Proc. Natl. Acad. Sci.*, **37**, 272 (1951).

chemistry of the proteins indicate that X-ray diffraction patterns of collagen and gelatin are best explained if every third residue in the peptide chain is a proline residue. Each proline is believed to be followed by a glycine residue, so that some regularity is established. However, these are the least polar and most abundant of the amino acids of collagen and statistical effects might be expected to predominate among the factors controlling their incorporation in the protein molecule. There is less reason for supposing that polyfunctional amino acids like lysine and glutamic acid are arranged in the chain in the simplest statistical manner. On the contrary, in the only proteins which have been studied in detail, the distribution of amino acids over the whole molecule is far from uniform. The work of Sanger[16] on the structure of insulin shows it to consist of two different types of polypeptide chain, one of which contains all the basic and hydroxyamino acid residues of the protein. There is thus a good precedent for postulating an unsymmetrical distribution of amino acid residues in collagen if it appears essential to an understanding of experimental results.

If we assume that the lysine and hydroxylysine residues of collagen are grouped in pairs, the two members of which compete for the same primary amide group, acetylation of almost half of the amino groups would have a very small effect on formaldehyde binding. Thereafter, every N-acetyl group introduced would be expected to prevent fixation of exactly one formaldehyde molecule. In this way the course of the experimental curve might be explained.

The last section of the formaldehyde fixation curve shows that the result of acetylating aliphatic hydroxyl groups when all the amino groups are already blocked is negligible. This section of the curve is similar for all three tanning agents, and we may conclude that the small negative slope of approximately 0.05 is a consequence, not of a specific hydroxyl effect, but of a general fall in reactivity, perhaps due to the severe dehydrating conditions obtaining during acetylation. In this connection it will be remembered that pretreatment of collagen with acetic anhydride alone, in the absence of any acetylating reaction, reduces tannic acid fixation to the same extent as does complete acetylation.

The curve for tannic acid fixation has this small negative slope over the whole range, with no change at the point where blocking of amino groups is complete. At this point, the amount of tannic acid fixed has been reduced by only about 6%, emphasizing the difference between this technique and the deamination method which, under the same conditions, causes a reduction of as much as 40%.[7] The results reported here indicate that neither the amino nor the hydroxyl groups of collagen take any important part in the reaction and point to the major role probably played by the polypeptide groups. A full review of other evidence on this point is given by Gustavson.[2]

The curve for quinone fixation also exhibits some unusual characteristics. It is divided into two portions by an abrupt change of slope when all the amino groups have been acetylated. The changes in physical properties accompanying quinone tanning may be associated with either or both of these reactions. The position and slope of the second show that a large amount of quinone is bound independently of the amino and hydroxyl groups. This may be attached to peptide groups, either by addition to single points or by bridge formation between pairs.

The first part of the curve indicates that each amino group binds rather more than one quinone molecule, and seems to require modification of the view held by earlier workers[2] that each quinone molecule formed a bridge between two lysine residues. If this quinone is engaged in cross-linking, then it must unite two dissimilar groups, the second probably being a peptide group. We realize that this interpretation of the experimental results requires a peptide to react with quinone at the same time as an N-acetyl amino group fails to react. Furthermore, we have not discussed the possibility of polymerization of the quinone. These are points which must be reserved for more detailed investigation.

(16) F. Sanger, *Biochem. J.*, **45**, 563 (1949).

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