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Formation by Formaldehyde of a Cross-link between Lysine and Tyrosine Residues in Wool

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The reaction of formaldehyde with proteins has been extensively investigated (French & Edsall, 1945), although the general picture is not yet clear since the nature of the reaction is dependent on the conditions employed. The reaction with wool keratin has been studied by a number of workers. Stoves (1943) used a 2% (w/v) solution of formaldehyde at 100° and, from measurements on the mechanical properties of treated fibres, deduced that the disulphide bond had been attacked with formation of $-S-CH_2-NH-$ and $-S-CH_2-S-$ cross-links. At high pH values, hydrolysis of the disulphide bond occurs, and Ratner & Clarke (1937) showed that formaldehyde then reacts with the cysteine residue produced to form a residue of thiazolidine-4-carboxylic acid. This reaction, however, appears to be reversible, since

Middlebrook & Phillips (1942) found that the formaldehyde bound by the cystine residues at high temperatures was removed by dilute phosphoric acid at 100°. Middlebrook (1949) showed that at pH 1 and 100° part of the formaldehyde which combines is not removed by boiling acid, and he suggested that the irreversibly combined formaldehyde has reacted with the asparagine amide groups to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid, which is stable to boiling acid.

It is well known that formaldehyde reacts with amino groups to form a methylol derivative with a different pK value from that of the original base. Such compounds are, however, very unstable, and after removal of the formaldehyde they immediately dissociate. In the case of wool, all the ϵ -amino

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groups of lysine react in this way with a 10% (w/v) formaldehyde solution almost instantaneously at room temperature (Steinhardt, Fugitt & Harris, 1946). This reaction is completely reversed on washing the wool with water, and would therefore not have been detected by Stoves (1943) or Middlebrook & Phillips (1942). Fraenkel-Conrat & Olcott (summarized, 1948) in a detailed series of investigations found that under mild conditions the basic groups of proteins combine irreversibly with formaldehyde, forming cross-links between the following pairs of groups: amine and amide; amine and phenol; amine and indole. Formaldehyde cannot, in their opinion, form a stable link between two amine or two amide groups.

We have found that some of the properties of wool are greatly altered by treatment with formaldehyde under very mild conditions. For example, the susceptibility to degradation by alkali is greatly diminished on treatment with a solution of formaldehyde as weak as 0.003M at 60° and pH 7. The formaldehyde bound by the wool under these conditions is almost entirely irreversibly combined, and a study of the nature of this reaction forms the subject of this paper.

METHODS

Lincoln fibres of average diameter 24.2 μ ., fully extracted with ether and ethanol, were used for load/extension experiments and supercontraction measurements. In other experiments a fully extracted knitted fabric was used, consisting of 60's Merino wool which had received no previous treatment other than cleaning with soap and NH_3 to remove dirt and wool grease.

Treatment with formaldehyde. Formaldehyde solution (40%, w/v) was diluted to the desired M concentration. Of this solution 50 ml./g. of wool were used. The reaction was found to be independent of the pH of the solution in the range 3–8, and treatments were therefore carried out without buffering, since the pH was found to stay between 6 and 7 throughout the reaction. All treatments were carried out at 60° for 30 min. with gentle stirring. The wool was then withdrawn, rinsed in running tap water (pH approx. 8) for 2 hr. and dried at 110° to constant weight. It was found that weight increases of the order of 0.1% could be reliably and reproducibly determined. Direct weighing has been used for following other reactions in the same way (Alexander, Bailey & Carter, 1950).

Acetylation of wool. The NH_2 groups were acetylated by the I. G. Farben process (Alexander & Whewell, 1947). From the acid-combining figures it was found that 75% of the NH_2 groups were blocked.

Esterification of wool with methanol. The carboxyl groups of the wool were esterified to the extent of 70% by treatment with 0.2N-HCl in anhydrous methanol (20 ml./g. of wool) under reflux at the boiling point for 2 hr., after which the wool was washed free of HCl in running tap water. Alexander, Carter, Ford & Earland (1951) showed that after hydrolysis of the ester groups formed in this way with dilute alkali the titration curve was identical with that of untreated wool, indicating that no amide hydrolysis had occurred.

Oxidation of cystine in wool. The treatment with peracetic acid, prepared by the method of Greenspan (1947), and chlorine in 0.1N-HCl was carried out as described by Alexander, Hudson & Fox (1950).

Susceptibility to degradation by alkali. Wool fabric, previously dried to constant weight at 110°, was cut into small pieces and placed in 0.1N-NaOH (100 ml./g. of wool) at 65° for 1 hr. The amount dissolving, expressed as g./100 g. of original wool, was taken as a criterion for the susceptibility of the wool to degradation by alkali (Harris & Smith, 1936).

Determination of reversibly combined formaldehyde. Wool (1 g.) was immersed in 100 ml. of 0.3M- H_2PO_4 , which was concentrated by distillation until about 5 ml. were left. The H_2PO_4 removed the reversibly combined formaldehyde, which came over in the distillate and was absorbed by passing directly into a solution of 5:5-dimethyldihydroresorcinol (dimedon) in aqueous ethanol (Weinberger, 1931). The methylene-bis-dimedon precipitate was filtered off, washed free from acid, dissolved in ethanol and titrated as a monobasic acid with 0.01N-NaOH (Vorlander, 1929).

Supercontraction of wool fibres. Single fibres were treated for 30 min. at 100° in (a) a 5% (w/v) solution of NaHSO_3 (Speakman, 1936), and (b) a 100% (w/v) solution of LiBr (Alexander, 1949), and the contraction in length expressed as a percentage of the original length.

Work necessary to stretch fibres by 30%. Fibres were stressed at a constant rate of loading, either in pH 7 buffer or in 0.1N-HCl, before and after treatment, and from the areas under the stress/strain curve the change in work corresponding to 30% increase in length was determined (Speakman, 1947).

Acid and alkali titration curves. Weighed treated and untreated samples were placed in HCl and NaOH solutions of known concentrations at 20° and allowed to reach equilibrium (Speakman & Hirst, 1933). The final pH value was taken and the quantity of acid or alkali combined expressed as mmol. of HCl or NaOH/100 g. of dry wool.

Estimation of tyrosine. The wool was hydrolysed by heating with 5N- H_2SO_4 in a sealed tube for 5 hr. at 120°, and the tyrosine was determined colorimetrically using Millon's reagent (Lugg, 1937). The reagent fails to give a colour when the tyrosine is substituted in the 3:5-positions.

RESULTS

Increase in resistance to degradation by alkali. The alkali solubility test of Harris & Smith (1936) was used for determining the resistance to alkali degradation of the wool brought about by formaldehyde. This resistance is progressively increased as the concentration of formaldehyde in the solution is increased (see Fig. 1) until, under the conditions of the reaction, a limit is reached with a 0.15M-solution. The increased resistance to alkali is lost when the treated wool is heated at 100° with buffer solutions at pH < 4, but not with solutions at pH > 6 (see Fig. 2). After boiling in solutions of pH lower than 3, the amount of wool dissolving in the alkali under the test conditions was the same for untreated as for formaldehyde-treated wool.

Formaldehyde bound by wool. The percentage weight increase was determined by drying to constant weight at 110° after treatment with different amounts of formaldehyde. The quantity of formaldehyde which reacted was very small, and the concentration of the solution was not appreciably

neutral solution, but in acid solution this was considerably increased. This increase in strength was only slightly reduced on boiling in water for 10 min., but was lost on heating at 100° for 10 min. in 0.01N-hydrochloric acid (see Table 2).

Fig. 1. Changes in susceptibility of wool to degradation by alkali (as determined by the alkali-solubility test of Harris & Smith (1936) following treatment of wool with solutions of formaldehyde of various concentrations.

diminished. The extent of the reaction appears to be determined not by the quantity of formaldehyde available, but by its concentration. The amount combined could not be determined from changes in concentrations, since some is adsorbed and very loosely bound so that it comes off during the washing of the wool. By making the very reasonable assumption that cross-links are formed (see Discussion), the amount combined was calculated from the weight increase of the wool, by assuming that a $-\text{CH}_2-$ group is introduced for every molecule of formaldehyde reacting. From this value and the quantity liberated by phosphoric acid distillation the proportion of formaldehyde reversibly combined was calculated (see Table 1).

Mechanical properties of the fibres. Fibres were calibrated by stretching either in pH 7 buffer or in 0.1N-hydrochloric acid, treated with 0.033M-formaldehyde at 60° for different times and then restretched in the same solution. After formaldehyde treatment there was no significant change in work necessary for stretching the fibres 30% in

Fig. 2. Changes in susceptibility of wool to degradation by alkali (see Fig. 1) after heating for 1 hr. at 100° in buffer solutions at different pH values. ○, Untreated wool; ●, formaldehyde-treated wool.

Treatment with increasing quantities of formaldehyde progressively decreases the degree of supercontraction of wool fibres in boiling bisulphite solution, but the contraction in lithium bromide is not significantly changed (see Table 3). The effect on the supercontraction behaviour of heating at 100° in buffer solutions of various pH values subsequent to the formaldehyde treatment is shown in Fig. 3. The formaldehyde-treated fibres boiled in acid solutions for 1 hr. supercontract to the same extent as untreated fibres below pH 4. In the region pH 4–6, the treated fibres contract less than control fibres, and all fibres heated in buffer solutions above pH 8 fail to supercontract owing to lanthionine formation.

Table 1. *Formaldehyde bound by wool at 60° in neutral solution and proportion liberated on distilling with 0.3M-phosphoric acid*

Concentration of formaldehyde (M)	Formaldehyde bound by wool (g./g.)	Formaldehyde liberated on distillation (g./g.)	Reversibly bound formaldehyde (% of total bound)
0.0033	0.00326	0.00057	15.9
0.0067	0.00629	0.00093	14.8
0.0133	0.00740	0.00129	17.4
0.033	0.00920	0.00197	21.4

Table 2. *Change in work necessary to stretch fibres 30% in 0.1N-HCl after treatment with 0.033M-formaldehyde solution at 60° for different times*

Time of treatment (min.)	Change in work to stretch 30% after		
	Formaldehyde treatment (%)	Formaldehyde treatment followed by 10 min. at 100° in buffer at pH 7 (%)	Formaldehyde treatment followed by 10 min. at 100° in buffer at pH 2 (%)
10	+15.3	+7.8	-10.2
15	+21.7	+20.6	-1.8
30	+20.4	+15.6	-3.7

Table 3. *Effect of formaldehyde treatment on supercontraction of wool fibres at 100°*

Concentration of formaldehyde solution (M)	Supercontraction	
	In 5% NaHSO ₃ (%)	In 100% LiBr (%)
Nil	22	15
0.0033	20	14
0.0067	10	14
0.0133	3	13
0.067	6	11
0.667	4	10

Acid and alkali titration curves. The acid titration curve is unchanged by formaldehyde treatment, but the alkali curve is shifted to lower pH values in the range of pH 9–11.5 (see Fig. 4), and as a rough estimate it would appear that the pK of the lysine groups is lowered by 1–1.5 units.

Fig 3 Supercontraction in NaHSO₃ at 100° of (○) untreated and (●) formaldehyde-treated fibres after heating for 1 hr. at 100° in buffer solutions of different pH values.

Tyrosine content of treated wool. The diminution in tyrosine content of the wool after various formalde-

hyde treatments is shown in Table 4. After distillation with 0.3M-phosphoric acid the tyrosine content increases, showing that some of the reversibly combined formaldehyde is held by the tyrosine. In

Fig. 4. Alkali titration curves of (○) untreated wool and (●) formaldehyde-treated wool.

agreement with Middlebrook's (1949) finding that most of the formaldehyde combined at pH 1 and 100° is bound reversibly, we have found that after distillation with phosphoric acid the free tyrosine content of wool thus treated is only slightly diminished. When the formaldehyde is applied at neutrality and at a lower temperature, most of it is combined irreversibly with tyrosine (Table 4).

Table 4. *Diminution in tyrosine content of wool treated with formaldehyde solutions of various concentrations*

Concentration of formaldehyde solution (M)	Loss of tyrosine* (% of original content)
0.0067	Nil
0.0133	18.0
0.133	30.8
0.333	36.0
0.667	53.0
0.333†	26.8

* Untreated wool gave 5.40% tyrosine, and from this figure the percentage reduction was calculated.

† Treatment with formaldehyde followed by distillation in presence of H₃PO₄.

The cystine content of wool determined colorimetrically (Shinohara, 1935) is unchanged by formaldehyde treatment.

Table 5. *Effect of pretreatment with formaldehyde on susceptibility to degradation by alkali (see text), supercontraction and tyrosine content of acetylated wool*

Concentration of formaldehyde (M)	Wool dissolving in alkali (g./100 g. of wool)	Supercontraction in boiling NaHSO ₃ solution (%)	Loss of tyrosine (% of original content)
Nil	45.3	16	*
0.0067	—	—	4.8
0.0133	—	—	9.8
0.067	—	—	3.4
0.133	44.4	20	6.1

* The tyrosine content of acetylated wool was 4.98 %.

Table 6. *Effect of treatment of oxidized wool with 0.133M-formaldehyde on the susceptibility to degradation by alkali (see text)*

Initial treatment	Cystine oxidized (%)	Wool dissolving in alkali (g./100 g. of wool)	
		Before formaldehyde treatment	After formaldehyde treatment
Acid chlorination	16	17.4	9.91
Peracetic acid (30 min.)	70	86.5	34.6
Peracetic acid (24 hr.)	90	91.5	29.0

Examination of modified wools. Formaldehyde treatment does not affect the resistance to degradation by alkali, supercontraction behaviour and tyrosine content of acetylated wool (Table 5). Esterification of the carboxyl groups does not influence the effectiveness of the formaldehyde treatment, and with a 0.133M-solution of formaldehyde the alkali solubility, as measured by the standard test, is lowered from 13.1 g./100 g. to 8.3 g./100 g. Oxidized wool in which the cystine links have been broken still reacts with formaldehyde, and the resistance to degradation by alkali is considerably increased (Table 6).

DISCUSSION

Under very mild conditions of treatment, wool combines with as much as 1 % of formaldehyde with a resultant weight increase of approximately 0.5 %. The reaction increases very substantially the resistance of the wool to degradation by alkali (Fig. 1), diminishes the supercontraction (Table 3) of the fibres in bisulphite and improves the tensile properties (Table 2). After treatment with dilute solutions of acid at 100°, but not with alkaline solutions, these effects are no longer apparent (Figs. 2 and 3; Table 2), although only a small proportion of the combined formaldehyde is liberated by reaction with acid (Table 1). It is apparent, therefore, that the formaldehyde has reacted to form a new cross-link (shown by Table 3), which is stable to alkali but broken by boiling acid, without, however, liberating more than a small fraction of the combined formaldehyde.

The previous researches of Fraenkel-Conrat & Olcott (1946), Highberger & O'Flaherty (1939), Gustavson (1943), Sirain, Kokes, Hipp, Wood & Jackson (1948) and French & Edsall (1945) have shown that formaldehyde reacts with protein amino groups. The elastic properties of formaldehyde-treated wool suggest that the ϵ -amino groups from lysine are involved in the cross-link formed. It was found that the work required to stretch formaldehyde-treated fibres in 0.1N-hydrochloric acid (see Table 2) is increased, whereas the tensile properties are unchanged in pH 7 buffer. Speakman & Hirst (1933) and Speakman (1947) consider that the salt links between the free amino and carboxyl groups in wool contribute to the strength of the fibre and that the diminution in the work necessary for stretching fibres by 30 % in 0.1N-hydrochloric acid as compared with stretching in water is due to the breaking of these salt links. A covalent cross-link involving the amino group would not be expected to affect the tensile properties in pH 7 buffer, since it merely replaces the salt link. The new link would, however, increase the tensile strength in 0.1N-acid when salt links are broken. The alkali-titration curves of treated and untreated wool (Fig. 4) are different in the pH range 9–11.5 in which lysine titrates, and this suggests that the amino groups which have reacted can be identified with those of lysine.

Since the breaking of disulphide links by oxidation and the esterification of the carboxyl groups do not interfere with the reaction of formaldehyde, it can be assumed that they take no part in the reaction. Analysis of the free tyrosine content of treated wools (see Table 4) indicates that there is a methylol sub-

stitution in the nucleus, preventing the reaction with Millon's reagent. Reaction of formaldehyde at the 3:5-positions of tyrosine is a typical Mannich reaction (Blicke, 1942), and has been found in proteins by Fraenkel-Conrat & Olcott (1948). The compound formed is stable to acid, and this explains why the reaction is not reversed either by distillation with phosphoric acid or in the hydrolysis of the wool prior to tyrosine analysis.

The failure of formaldehyde to bring about cross-linking in acetylated wool can be the result of either the blocking of the amino groups or of the hydroxyl group in tyrosine. It is impossible to establish if the latter is acetylated; any *O*-acetyl groups formed would be removed by hydrolysis during the analysis for tyrosine. The change in alkali titration curve shows that formaldehyde reacts with the amino groups. It is clear, however, that formaldehyde does not combine with the tyrosine in acetylated wool (Table 5). Consideration of the facts made it highly probable that the new cross-link formed is between the ϵ -amino group of lysine and the 3-position in the phenol radical of tyrosine:

Methylolamines in proteins though stable to alkali are hydrolysed by acids (Fraenkel-Conrat, Cooper & Olcott, 1945) and the cross-link is probably broken at the $-\text{CH}_2-\text{NH}-$ bond. The formaldehyde remains in the protein as a methylol tyrosine. It is of interest to note that the irreversible weight increases obtained on treating wool with formalde-

hyde are of the order theoretically required for the postulated link. Complete reaction with tyrosine would require a 0.45% weight increase, with lysine 0.35%, and those obtained were in the region of 0.4%.

It is also interesting that the formaldehyde reaction decreases supercontraction brought about by bisulphite, but does not influence contraction in lithium bromide solution. Alexander (1950*a, b*) has shown that the physical properties of wool fibres suggest a structure of polypeptide chains held together by hydrogen bonds in micelles which are linked to one another by disulphide bonds. Supercontraction can then be brought about either by breaking intermicellar disulphide bonds (e.g. by bisulphite) or intramicellar hydrogen bonds (e.g. with lithium bromide solution). The new cross-link appears therefore to be formed between micelles and not within the micelle.

SUMMARY

1. Wool combines irreversibly with formaldehyde at low concentrations in neutral solution at 60°. It is suggested that new cross-links are formed which increase the resistance to alkaline degradation, prevent supercontraction and increase the tensile strength of the fibres. These effects are reversed by hot solutions of pH < 3 without, however, liberating the formaldehyde. The links are stable to alkali.

2. From analytical data and from the effects of formaldehyde treatment of chemically modified wools, it seems probable that formaldehyde forms cross-links between phenyl groups from tyrosine and ϵ -amino groups from lysine. These are broken by acid without liberation of the formaldehyde, which remains in the wool as methylol tyrosine.

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The Virulence-Enhancing Factor of Mucins

3. FURTHER FRACTIONATION OF HOG GASTRIC MUCIN; AN ADDITIONAL FACTOR IN THE ADJUVANT ACTION

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The first two papers in this series (Smith, 1950*a, b*) described a biological assay of the virulence-enhancing activity of mucins for bacteria, and the use of this assay in preliminary fractionation of hog gastric mucin for a factor responsible for such activity. A fraction was obtained which was only sparingly soluble in water at pH 7, and which, although active alone, showed a far greater activity when suspended in an inert viscous solution such as gum tragacanth, agar or semi-pure blood-group substance. It was suggested that the viscosity of the medium probably delays the absorption and inactivation of the active factor by the host at the site of injection.

The present paper describes further purification of the virulence-enhancing factor to a stage where some insight into its chemical nature has been obtained, although it is still not homogeneous. This purification has led to the recognition of a further factor having an adjuvant action. It is becoming increasingly evident, therefore, that the virulence-enhancing action of hog gastric mucin is due to the specific active factor in association with other non-specific physical factors which are necessary for the full manifestation of its activity.

The difficulty in maintaining the factors having an adjuvant action at a constant value in the biological assay, while studying fractions in the purification of the virulence-enhancing factor proper, is one of the major problems of this work.

EXPERIMENTAL AND RESULTS

Activities are given in 'Wilson units' by direct comparison with a standard mucin, with fiducial limits in brackets for $P = 0.95$.

Modification of the biological assay of virulence-enhancing activity

The evidence that the semi-pure blood-group substance, which retains the viscosity of the original mucin, exerts its adjuvant action on the virulence-enhancing factor by virtue of this viscosity is further strengthened by the following experiments.

Semi-pure blood-group substance (40 g.), prepared as described before, was further fractionated with ethanol from 90% phenol solution as described by Morgan & King (1943). The following fractions were obtained: (1) insoluble in 90% phenol, 15 g.; (2) precipitated by 10% (v/v) ethanol, 9.4 g.; (3) precipitated by 25% (v/v) ethanol, 2.9 g.; (4) precipitated by pouring the mother liquor into excess ether, 2.2 g.

The same concentration of a virulence-enhancing fraction was suspended in solutions of the original semi-pure blood-group substance, and of the first three fractions: the viscosities of the suspensions were maintained constant by varying the concentrations of the different fractions. Table 1 shows that the activity of the virulence-enhancing fraction is the same, irrespective of the concentration and nature of the fraction, as long as the viscosity is the same. It was impossible to use fraction 4 because a large proportion of it was insoluble, and a 5% (w/v) suspension had a viscosity scarcely greater than water, and it was toxic to mice.

In view of the adjuvant action on the virulence-enhancing factor of the viscosity of the original mucin, it was essential in future assays that this adjuvant action should remain constant. The viscosity of all test solutions for injection has therefore been maintained at an approximate standard value by adding very dilute tragacanth mucilage. Viscosities were determined at 37° in an Ostwald viscometer. Since only approximate values are needed, the small differences in density of various dilute aqueous solutions were neglected, and viscosities were quoted as times in seconds to flow through the same viscometer. The viscosity chosen as the fixed figure was the mean of those of the 1.75% (w/v) (140 sec.) and 1.25% (w/v) (75 sec.) 'standard mucin'. Workable limits on this figure were 90–125 sec. and the viscosity is about seven