appreciable extent. Some *o*-tolylurea is excreted unchanged.

4. *m*-Tolylurea is oxidized to *m*-ureidobenzoic acid (45%), 4% of which is excreted as ester glucuronide, and hydroxylated to *N*-(4-hydroxy-3-methylphenyl)urea (24%), of which 10% is excreted as ethereal sulphate and 14% as ether glucuronide. Some *m*-tolylurea is excreted unchanged.

5. *p*-Tolylurea is almost completely oxidized to *p*-ureidobenzoic acid (86%), of which only 6% is conjugated as ester glucuronide, the remainder being excreted free.

6. *N*-Phenylurea is partly hydroxylated to *N*-(*p*-hydroxyphenyl)urea (46%) which is conjugated almost equally with sulphuric acid (20%) and glucuronic acid (26%). Some *N*-phenylurea is excreted unchanged. 4-Ureidophenylglucuronide was isolated from *N*-phenylurea urine as its crystalline potassium salt.

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The Effect of Deamination and Esterification on the Reactivity of Collagen

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The most important reactive groups in collagen are amino, guanidino and carboxyl, all of which are present in comparatively large numbers; few phenolic, hydroxyl and imidazole groups are present and indole groups are absent (Bowes & Kenten, 1948*a*). A study has now been made of the effect of modification of the amino, guanidino and carboxyl groups on the acid- and base-binding capacity, and on the swelling of collagen. The combination of the modified collagens with vegetable tannins, basic chromium salts and formaldehyde has also been investigated primarily because of the importance of these three classes of compound in the conversion of collagen to leather. Moreover, the fixation of metals by proteins is of general interest, and information concerning the factors affecting the fixation of tannins may help to throw light on the association of tannins and proteins in nature.

The amino groups of proteins can readily be converted to hydroxyl groups by the action of nitrous acid. The reaction is not entirely specific, however, since at low pH values, or if the reaction is prolonged, modification of the guanidino, imidazole, tyrosine and glutamide groups may also take place (for review of literature see Olcott & Fraenkel-Conrat, 1947). With soluble proteins, reaction with the amino groups is rapid and other reactions are probably negligible, but with fibrous proteins, such

as collagen, the reaction must be prolonged to allow time for the nitrous acid to diffuse into the interior of the fibres, and some modification of other groups probably occurs. Previous workers who have used this method with collagen did not determine the extent of specificity of the reaction (Thomas & Foster, 1926; Thomas & Kelly, 1926; Gustavson, 1926; Meunier & Schweikert, 1935; Atkin, 1937; Bowes & Pleass, 1939; Highberger & Retzsch, 1939; Wilson & Yu, 1941; Gustavson, 1943; Lollar, 1943; Chang, Yen & Chen, 1944; Theis, 1945).

Modification of the guanidino groups has received little attention and no satisfactory method appears to be available. Treatment with hypochlorite, as in the Sakaguchi reaction, has been found to destroy all the arginine present in soluble proteins, but histidine, tyrosine, tryptophan and lysine are also affected, though to a lesser extent (Sakaguchi, 1925). Only about 43% of the arginine in collagen is affected by the reaction (Highberger & Salcedo, 1940). In the present investigation, it was again found that only 40–50% of the arginine in collagen was destroyed by this treatment. Roche & Morgue (1946) have shown that the reactivity, in the Sakaguchi reaction, of the guanidino group in amino-acid derivatives is influenced by the presence of other groups, some of which, notably aliphatic hydroxyl, prevent over 90% of the guanidino groups from reacting. It is possible that the difficulty of removing more than about half the guanidino groups from the collagen is related to the same cause. Since the reaction was obviously not specific to the guanidino groups, and was accompanied by extensive general breakdown of the collagen, it was not studied in detail.

A number of the reagents which react with the carboxyl groups of proteins, e.g. diazomethane, acetic anhydride, keten, methanol, are not specific to carboxyl groups (Herriott, 1947; Olcott & Fraenkel-Conrat, 1947), and with some the conditions necessary for reaction cause breakdown of the protein structure (Fodor & Epstein, 1937). Blackburn, Carter & Phillips (1941) and Blackburn & Phillips (1944) have studied the methylation of wool with methyl sulphate and methyl halides, and conclude that the main reaction is esterification of the carboxyl groups. It appeared, therefore, that these methods were likely to be satisfactory for modifying the carboxyl groups of collagen without affecting other groups to any appreciable extent, and with minimum risk of degradation.

EXPERIMENTAL METHODS

Material

The collagen was prepared from sheepskins which had been dewooled with $Ca(OH)_2$ and Na_2S ('limed'), treated with pancreatic trypsin ('bated'), and preserved with H_2SO_4 and NaCl ('pickled'). The skins were washed free of acid and salt, dehydrated with acetone, cut into strips 1 × 5 cm. (the edges of the skins being discarded), brought to equilibrium with distilled water (pH *c.* 5), and finally dehydrated with acetone. The titration curves of three batches of collagen prepared as above (*A*, *B* and *C*) are given in a previous paper (Bowes & Kenten, 1948*b*); they are referred to in text and tables as collagen *A*, *B* and *C*, with the prefixes *D* and *M* to denote deaminated and methylated, respectively.

Treatment of collagen

Deamination. The method used was based on that of Thomas & Foster (1926). Collagen (100 g.) was soaked in distilled water overnight, 100 g. $NaNO_2$ then added, followed by 100 ml. glacial acetic acid. A stream of CO_2 was passed through the solution to minimize oxidation of nitrous to nitric acid and to agitate the solution. After 24 hr., a further 100 g. $NaNO_2$ and 100 ml. acetic acid were added and the reaction allowed to proceed for another 24 hr. The collagen was rinsed with water, washed to remove acid in several changes of water containing 10% (w/v) NaCl to minimize swelling, brought to equilibrium with distilled water (pH 5·0), and dehydrated with acetone. Two batches of collagen *C* (*DC*$_1$ and *DC*$_2$) and one of collagen *A* (*DA*) were treated in this way.

Methylation. Methods used were similar to those of Blackburn *et al.* (1941): (1) Dimethyl sulphate. Collagen (50 g.) was shaken with 500 ml. M-Na acetate buffer (pH 7·5) and 25 ml. methyl sulphate for 1 hr. This procedure was repeated fourteen or more times. To reduce the extreme swelling of the methylated collagen it was placed in a 10% (w/v) solution of NaCl for 20 hr., then washed in distilled water until free from Cl^-, and dehydrated with acetone. (2) Methyl bromide. The collagen was brought to equilibrium with a borate buffer at pH 9·0, dehydrated with acetone, air dried, and immersed in methyl bromide for 14 days. The latter was then allowed to evaporate, and the collagen washed as before and dehydrated with acetone. In some cases the collagen was rebuffered and treated for a further 14 days with methyl bromide. Several batches of collagen *A* (*MA*$_1$ to *MA*$_4$) and of collagen *C* (*MC*$_1$ to *MC*$_6$) were methylated; also the oxhide collagen prepared as previously described (Bowes & Kenten, 1948*a*).

Combination of modified collagen with tannic acid, mimosa tannins, chromium and formaldehyde. The modified collagen (2 g.) in the form of pieces 1 cm. square, and a piece 1 × 5 cm. for shrinkage-temperature determinations were soaked in water overnight and then placed in 100 ml. of the following tanning solutions adjusted to different pH values for the times stated. Approximate equilibrium should have been reached in these times:

(1) 10% (w/v) tannic acid (British Drug Houses, Ltd.) for 7 days.
(2) 10% (w/v) tannic acid + 0·5 M-NaCl for 7 days.
(3) 10% (w/v) commercial mimosa tannin extract for 7 days.
(4) 3·4% (w/v) chromic sulphate for 4 days.
(5) 0·36% (w/v) formaldehyde for 3 days.

All pH values were attained with HCl or NaOH. The tannin solutions were initially adjusted to the required pH and then subsequently twice daily during the treatment. The pH of the $Cr_2(SO_4)_3$ solutions was adjusted after 24 hr., and maintained at this pH value by frequent further additions

during the following 48 hr.; no additions were made in the last 24 hr. and the pH fell slightly during this time. The pH's of the formaldehyde solutions were initially adjusted, and then allowed to fall during the treatment.

On the completion of the treatment the pieces were drained, washed in Wilson-Kern extractors (Wilson & Kern, 1921) with 9 l. distilled water for 20 hr. and air dried.

Analysis of products

Total N, *amide* N, *amino* N *and arginine.* The methods have been described previously (Bowes & Kenten, 1948*a*, *b*).

Lysine. This was determined using a specific decarboxylase (Gale, 1945). Though this method may also determine an unknown fraction of the hydroxylysine it gives an indication of the extent to which ϵ-amino groups are modified. Recent evidence (Heathcote, 1948) suggests that the apparent reaction of hydroxylysine with lysine decarboxylase may in fact be due to contamination of the hydroxylysine with lysine.

O-*Methyl groups.* These were determined as methyl iodide using a semimicroalkoxyl apparatus (Clark, 1932, 1939; Viebock & Schwappach, 1930). Methionine also yields methyl iodide by this method (Baernstein, 1932; 1936): the oxhide and sheepskin collagens gave values of 0·08 and 0·05% of methyl, respectively.

N-*Methyl groups.* The method is described by Pregl (1930). *O*-Methyl and *N*-methyl contents are expressed as a percentage of methyl on moisture- and ash-free collagen and are corrected for the methionine content of the collagen.

Chloride and sulphate. The former was determined as described by Highberger & Moore (1929). In the determination of $SO_4^=$, methylated collagen (3 g.) was heated for 3 hr. on a steam bath with 30 ml. 2N-HCl. The solution was evaporated to dryness, the residue dissolved in 100 ml. hot water and boiled with charcoal (Norite) for 15 min. The solution was filtered, the charcoal extracted with boiling water, and the combined filtrates neutralized with 2 ml. 5N-HCl, and the $SO_4^=$ determined as $BaSO_4$. Collagen gave no $SO_4^=$ by this method, and recovery tests showed an accuracy of $\pm 5\%$.

Tannin in combination with collagen. This was determined by drying in a vacuum oven at 100–102° for 6 hr. and weighing. The amount of tannin fixed/100 g. moisture- and ash-free collagen was then calculated from the increase in weight of the collagen.

Chromium. The method used is described by Davies & Innes (1944). Results are expressed as g. Cr_2O_3/100 ml. and mmol. Cr/g. moisture- and ash-free collagen.

Formaldehyde. The treated collagen (2 g.) was steam-distilled with 40 ml. 2N-H_2SO_4, 500 ml. distillate being collected. The formaldehyde was precipitated from suitable samples of distillate with dimedone (Yoe & Reid, 1941).

Shrinkage temperature. The temperature at which shrinkage occurs in water is a property characteristic of both collagen and treated collagens. It has been related to cross linking in the protein structure (Theis, 1946). A modified form of the apparatus described by Küntzel (1943) was used, in which a stream of air was blown continuously round the side arm to aid circulation. When the shrinkage temperature exceeded 100° the tanned collagens were wetted by immersion in water under reduced pressure and the determination then made in liquid paraffin. The shrinkage temperature so determined is the same as that determined in water under pressure.

Titration and swelling curves. These were determined as previously described (Bowes & Kenten, 1948*a*, *b*).

RESULTS

Except where otherwise stated, results are expressed on a moisture- and ash-free basis. Amino-N contents, etc., are expressed as mmol./g. rather than as a percentage of total N, since variations in the N content of the modified collagens render the latter method of expression unsuitable for comparative purposes.

Analysis

Deamination. The deaminated collagens had the characteristic orange-yellow colour associated with proteins which have been treated with nitrous acid (cf. Philpot & Small, 1938). Reaction of the amino groups was almost complete in all the samples examined (Table 1). As with other proteins (Steudel & Schumann, 1929; Wiley & Lewis, 1930) there was some loss of arginine (*c.* 20%).

The amide N was higher than that of the original collagen, and this additional NH_3 may have come from —NH.CN groups formed by the action of the nitrous acid on guanidino groups (see p. 149). The increase in amide N was approximately equal to the decrease in arginine content.

Methylation. The methylated collagens varied from brown to grey in colour and in two cases were almost black. They had a smell of methylamines. The methoxyl content of the different collagens treated with methyl sulphate varied from 0·94 to 1·57% (as methyl), the maximum methoxyl content being attained in 14 methylations (see oxhide collagen, Table 2). Variations in methoxyl content are probably due to slow hydrolysis of these groups during washing (see below). Methylation with methyl bromide introduced rather fewer methoxyl groups. Deamination of the collagen did not affect the extent of methylation with methyl sulphate, but increased the methoxyl groups introduced by methyl bromide to the same value as that obtained with methyl sulphate. This suggests that the lower values obtained with methyl bromide on untreated collagen are due, not to inability to introduce the same number of methoxyl groups as methyl sulphate, but to the difficulty of keeping the collagen at a suitable pH for the reaction to proceed to completion; diminution in the number of basic groups will reduce the change in pH of the collagen which occurs as a result of methylation of the carboxyl groups, and hence makes it easier to keep the collagen at the required pH.

Methylated collagen fixes $SO_4^=$ and Cl^- approximately equivalent to the methoxyl groups introduced. Blackburn *et al.* (1941) found that when wool was methylated with

Table 1. *Analyses of collagens and deaminated collagens*

(Results expressed on moisture- and ash-free basis except where otherwise stated.)

	Untreated	Treated with HNO_2		Untreated	Treated with HNO_2
	C	DC_1	DC_2	*A*	DA_1
Moisture (%) air dry	16·2	6·3*	16·0	18·1	14·1
Ash (%) air dry	0·23	1·78	0·23	0·14	0·58
Total N (%)	17·2	16·9	—	17·3	17·3
Amide N (mmol./g.)	0·25	0·38	0·39	0·30	0·38
Amino N (mmol./g.)	0·36	0·05	—	—	0·04
Lysine† (mmol./g.)	0·30	—	Nil	0·31	0·01
Arginine (mmol./g.)	0·44	—	0·35	0·43	0·37

* The low value is due to the collagen having been dried in a desiccator over anhydrous $CaCl_2$ for some days; other samples air-dry.

† May include some hydroxylysine.

Table 2. *The methylation of collagen and deaminated collagen with methyl sulphate and with methyl bromide*

Sample	OCH_3 groups introduced (as CH_3) (%)	OCH_3 groups introduced (as CH_3) (mmol./g.)	Anions (m-equiv./g. collagen) Chloride*	Sulphate	Total
Methylated with methyl sulphate					
Oxhide collagen (free carboxyl groups, 0·87 mmol./g.):					
Methylated 14 times MN_1	1·19	0·79	Nil	0·64	0·64
Methylated 14 times MN_2	0·94	0·63	Nil	0·52	0·52
Methylated 16 times MN_3	1·37	0·91	Nil	0·78	0·78
Methylated 16 times MN_4	1·22	0·81	Nil	0·70	0·70
Methylated 18 times MN_5	1·15	0·77	Nil	0·65	0·65
Methylated 18 times MN_6	1·24	0·83	Nil	0·75	0·75
Collagen *A* (free carboxyl groups 0·96 mmol./g.):					
MA_1	1·25	0·83	0·61	—	—
MA_2	1·32	0·88	0·73	0·07	0·80
MA_3	1·49	0·99	0·58	0·40	0·98
MA_4	1·32	0·88	0·50	0·34	0·84
Collagen *C* (free carboxyl groups, 1·01 mmol./g.):					
MC_1	1·32	0·88	—	—	—
MC_2	1·57	1·05	0·52	0·52	1·04
MC_3	1·18	0·79	0·65	—	—
MC_4	1·04	0·69	—	—	—
Collagen *A* after treatment with nitrous acid:					
MDA_1	1·32	0·88	0·42	0·05	0·47
Methylated with methyl bromide					
Collagen *C*:					
Methylated 14 days MC_5	0·35	0·23	0·11	—	0·11
Methylated 28 days MC_6	0·85	0·57	0·48	—	0·48
Collagen *C* after treatment with nitrous acid:					
Methylated 28 days MDC_1	1·22	0·82	0·30	—	0·30

* Arises from displacement of SO_4^- by Cl^- during immersion of methylated collagen in NaCl.

methyl iodide and bromide, I^- and Br^- were held in excess of the methoxyl groups, and attributed this to *N*-methylation. Attempts to determine the percentage of *N*-methyl groups were unsuccessful. Under the conditions of Pregl's method proteins and amino-acids yield appreciable amounts of volatile iodides, and large and variable values for the apparent *N*-methyl are obtained (Burns, 1914; Geake & Nierenstein, 1914; Lindley & Phillips, 1947). Determination of *N*-methyl groups in methylated collagen gave values of the same order as those obtained with the original collagen. Though it may be unwise to conclude that no *N*-methylation takes place, it appears that it only occurs to a small extent, if at all. The amino N of the collagen methylated with methyl bromide (MC_5) and with methyl sulphate (MC_4) was 0·24 and 0·31 mmol./g., respectively, compared with a value of 0·36 mmol./g. for the original collagen. The amino N of collagen MA_4 methylated with methyl sulphate was 0·30 mmol./g.

Stability. Methoxyl groups were only slowly split by hydrolysis when the methylated collagens were immersed in buffer solutions between pH 2 and 8, but outside this range the stability decreased rapidly with rise or fall in pH (see Fig. 1). A similar stability curve for methylated wool constructed from the data of Blackburn *et al.* (1941) is given for comparison. Soaking in water at pH 5·0 for 20 days

reduced the methoxyl content from 1·38 to 1·12 % (as methyl).

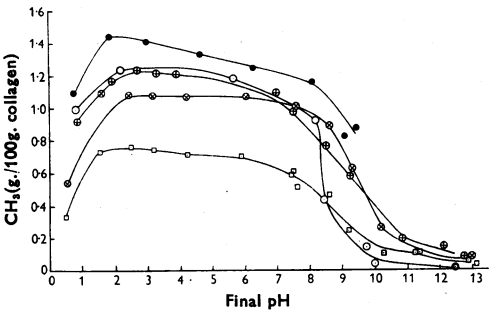


Fig. 1. Stability of OCH_3 groups introduced into collagen, deaminated collagen and wool in pH range 0·5–13·0. Percentage CH_3 after soaking in universal buffer solution for 2·5 days. ⊙—⊙, collagen esterified with methyl sulphate; ⊡—⊡, collagen esterified with methyl bromide; ⊕—⊕, collagen deaminated and esterified with methyl sulphate; ⊗—⊗, collagen deaminated and esterified with methyl bromide; •—•, wool esterified with methyl sulphate (from Blackburn *et al.* 1941).

Table 3. *Soluble nitrogen liberated from methylated collagen after 3 days' exposure to alkaline solutions*

Sample	pH of solution	Soluble N (mg./g.)	OCH_3 groups removed (g. CH_3/100 g. collagen)
	Untreated		
C	10·10	2·4	—
C	12·08	3·0	—
C	12·27	3·4	—
C	12·32	3·7	—
C	12·69	4·8	—
C	13·00	6·6	—
A	12·08	3·2	—
	After methylation with dimethyl sulphate		
MC_4	7·12	2·6	0·28
MC_4	7·42	4·9	0·29
MC_4	8·60	4·9	0·33
MC_4	8·79	10·4	0·59
MC_4	10·73	15·9	0·98
MC_4	11·80	21·5	1·01
MA_2	12·09	4·9	—
MA_3	12·12	6·3	—
MA_4	12·10	8·3	—
MDA	12·16	5·7	—
	After methylation with methyl bromide		
MC_5	8·76	1·1	0·32
MC_5	9·28	1·1	0·27
MC_5	10·20	1·1	0·33
MC_5	11·61	1·6	0·20
MC_5	12·01	1·5	0·34
MC_5	12·24	2·1	0·33

At high pH values loss of methoxyl groups from collagen methylated with methyl sulphate is accompanied by the production of an appreciable amount of nitrogen in the solution (see Table 3). Collagen methylated with methyl bromide, on the other hand, gave less soluble nitrogen than the untreated collagen. The solutions smelt of methylamines and gave a positive test for dimethylamine (Dowden, 1938). This smell was also noted by Blackburn *et al.* (1941) during the methylation of wool.

It was considered possible that the N in solution was related to the methylation of one of the basic groups of the collagen and specific degradation of this group on treatment in alkaline solution. The arginine content of the collagen as determined by Vickery's method, however, was unchanged by methylation and treatment with alkali, suggesting that the soluble N was not derived from guanidino groups. (There is some evidence, however, that some methylation of guanidino groups does take place. Lindley & Phillips (1947) found no decrease in the arginine content of methylated wool as determined by Vickery's method, but tests on the flavaniate precipitate indicated that some change had occurred.) The solution gave only a slight positive reaction for protein, and the results as a whole suggest that the soluble nitrogen is derived from general hydrolysis of the collagen to polypeptides and amino-acids.

Titration curves

Deaminated collagen. The titration curve of deaminated collagen (DC_2) is given in Fig. 2. Deamination has caused a shift in the isoelectric point from 5·5 to 4·5, a decrease in the acid-binding capacity, an increase in the base-binding capacity immediately on the alkaline side of the isoelectric point, and a slight increase in the total base bound at pH 12·5. The decrease in acid binding can be attributed mainly to loss of amino groups, but consideration of the free amino N of the original collagen (0·38 mmol./g.) shows that at least 0·07 mmol./g. of some other basic group has also been affected. The increase in base-binding from the isoelectric point to pH 7·0 corresponds to the decrease in acid-binding capacity, and may be attributed to titration of carboxyl groups which have reverted to the uncharged form on the removal of the basic groups. The increase in base-binding capacity at pH 12·5 indicates that about 0·18 mmol./g. of some group or groups not titrating in the original collagen is now binding base between pH 7 and 12·5.

Methylated collagen. The titration curves of collagens MC_2 and MC_4 (methylated with dimethyl sulphate) and MC_5 (methylated with methyl bromide) are shown in Fig. 2. In agreement with the hypothesis that methylation involves esterification of ionized carboxyl groups which are the hydrogen ion-fixing groups in the original protein, whilst the basic groups remain charged, the acid-binding capacities of the methylated collagen are decreased by an amount approximately corresponding to their methoxyl contents. The decrease in acid-binding capacity, however, is slightly less than the methoxyl content of the collagen, possibly owing to

loss of methyl groups by hydrolysis during contact with the solution. Also, since the isoelectric point of the methylated collagen is about one pH unit higher than that of the original collagen, some of the imidazole groups of histidine which were previously charged, and, therefore, titrated with alkali, will have lost their charge and now contribute to the acid-binding capacity.

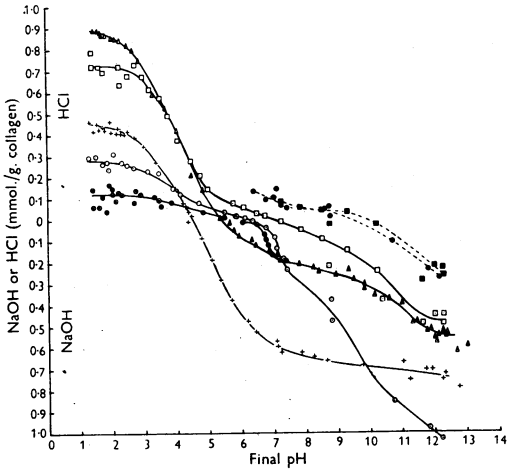


Fig. 2. Titration curves of collagen and modified collagens. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate, CH_3 content 1·04%; •—•, esterified with methyl sulphate, CH_3 content 1·04%; corrected for hydrolysis of OCH_3 groups; ⊗—⊗, esterified with methyl sulphate, CH_3 content 1·57%; ⊡—⊡, esterified with methyl bromide, CH_3 content 0·35%; ■—■, esterified with methyl bromide, corrected for OCH_3 hydrolysis; ⊕—⊕, deaminated and esterified with methyl sulphate, CH_3 content 1·29%.

The alkaline section of the curve is more difficult to interpret for, except over a comparatively small pH range, hydrolysis of methoxyl groups takes place with the consumption of alkali. The methoxyl content of the collagens, after contact with the solution, was determined and a correction applied for the alkali consumed; this is only approximate, but the corrected results suggest that appreciably less alkali is bound by the methylated than by the original collagen. In the case of collagen methylated with methyl bromide, the titration curve shows that less alkali is bound even before the correction is applied.

Swelling curves

Deaminated collagen. The uptake of water by the deaminated collagen (see Fig. 3) differs from that of the original collagen in a manner broadly corresponding to the differences in the titration curves (Fig. 2), but at all pH values the water uptake is rather greater than would be expected from consideration of the changes in the reactive groups. This suggests that deamination has decreased the cohesion of the collagen so allowing it to take up more water.

Methylated collagen. In the pH range of stability of the methoxyl groups, i.e. 2–9, the water uptake of methylated collagen is constant, and, when the collagen is fully methylated, is approximately the same as the maximum water uptake of the untreated collagen at pH 2·0 (see Fig. 3). This high water uptake over such a wide pH range may be ascribed to the presence of anions in electrovalent association with the basic groups, and consequent setting up of a Donnan membrane equilibrium and a swelling pressure (Donnan, 1911, 1924; Bolam, 1932). In this respect, methylated collagen corresponds to collagen at the point of maximum combination with acid.

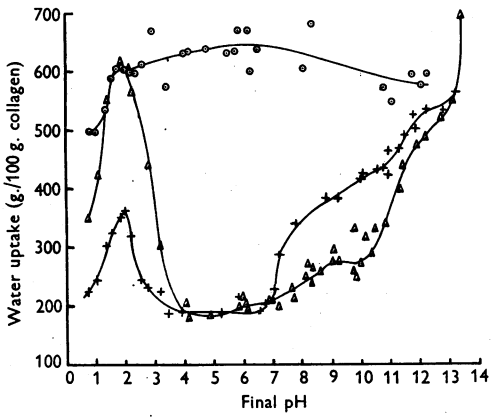


Fig. 3. The uptake of water by untreated △—△, deaminated +—+, and methylated collagen ⊙—⊙, at pH values from 0·5 to 13·5.

Combination of untreated and modified collagens with tannic acid, mimosa tannins, chromium and formaldehyde

Tannins. The conditions of treatment were the same for all the modified collagens, and it is reasonable, therefore, to assume that any differences in the amount of tannin bound are primarily due to modification of the collagen.

Modification of the amino and carboxyl groups of the collagen affects the combination of both tannic acid (hydrolyzable tannin) and mimosa (condensed tannin) with collagen in a similar manner (Figs. 4–6). Deamination decreased the amount of tannin bound at pH values below 4·0, but had little effect at higher pH values, and esterification of the carboxyl groups caused maximum combination of tannin to take place at much higher pH values, and to remain constant at this maximum over a wide pH range; this was especially marked with mimosa tannin (Fig. 6).

The addition of 0·5 M-NaCl decreased the amount of tannic acid bound by untreated and deaminated

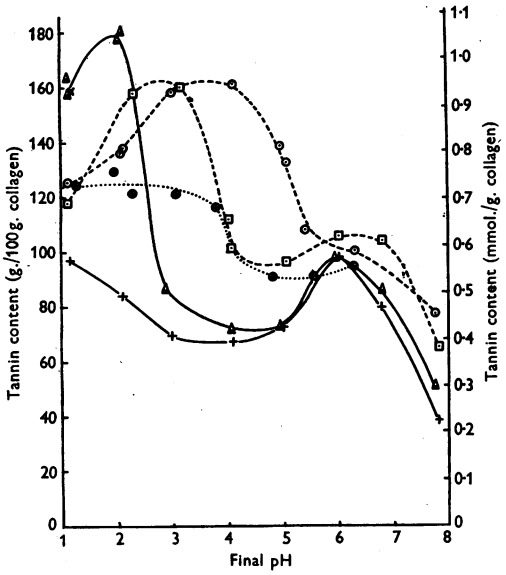


Fig. 4. Combination of tannic acid with collagen and modified collagen in the absence of added NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide; ⊕—⊕, deaminated and esterified with methyl sulphate.

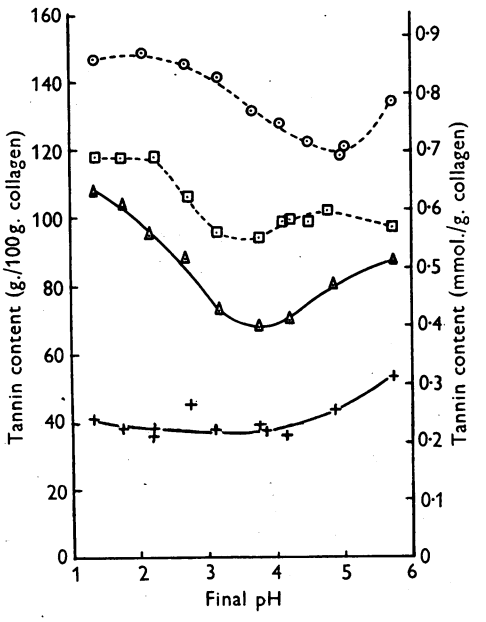


Fig. 5. Combination of tannic acid with collagen and modified collagen in the presence of 0·5 M-NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide.

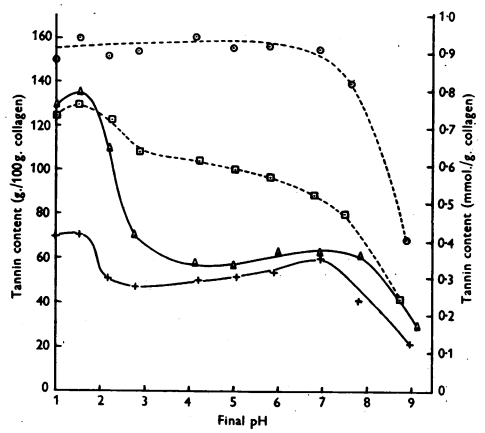


Fig. 6. Combination of mimosa tannins with collagen and modified collagen in the absence of added NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide.

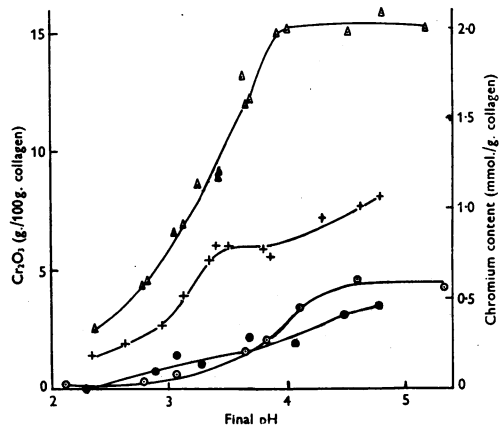


Fig. 7. Combination of chromium with collagen and modified collagen. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊕—⊕, deaminated and esterified with methyl sulphate.

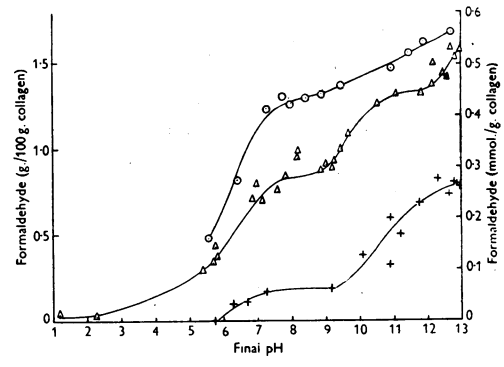


Fig. 8. Combination of formaldehyde with collagen and modified collagen. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate.

collagen, and by collagen methylated with methyl bromide, especially at low pH values. The tannic acid bound by the collagen methylated with methyl sulphate was only slightly decreased at pH values below 5·0, and at higher pH values was appreciably increased by the presence of NaCl.

The shrinkage temperatures of the collagen and modified collagen were increased from about 60 to between 66 and 84° by combination with tannic acid or mimosa tannin, maximum values in general being obtained when the pH of the tannin solution was between 3·0 and 4·0. The increase in shrinkage temperature was rather greater with mimosa tannin than with tannic acid, possibly because of the lower acidity of mimosa tannin (Hobbs, 1940). The deaminated and methylated collagens tended to have lower shrinkage temperatures than the original collagen treated under the same conditions, and variations of shrinkage temperature with the pH of the tannin solutions were less.

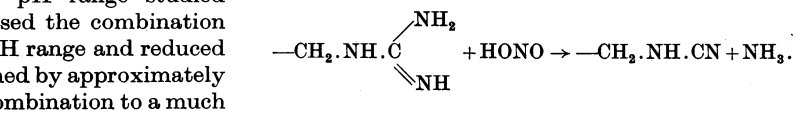
Chromium. The chromium bound by the original collagen increased sharply with increase in pH of the chromium sulphate solution up to 4·0, and then remained constant over the pH range studied (Fig. 7). Deamination decreased the combination of chromium over the whole pH range and reduced the maximum amounts combined by approximately half. Methylation decreased combination to a much greater extent. Chromium hydroxide was precipitated on the surface of the methylated collagens at the higher pH values and was difficult to remove; this, coupled with the possibility that some groups are displaced by chromium during the treatment, makes it not unreasonable to assume that in the absence of carboxyl groups no chromium would be bound by collagen. Deamination of the methylated collagen caused only a further small decrease in the chromium bound. Although the amount of chromium bound by the deaminated collagen was less than that bound by the original collagen, the maximum shrinkage temperature obtained was the same. Treatment with chromium sulphate caused no change in the shrinkage temperature of methylated colagen.

Formaldehyde. At pH values below 2·0, negligible amounts of formaldehyde were bound by both the untreated and modified collagens: from pH 2 to 8 the amount bound increased, remained constant between pH 8 and 9, and then increased again at higher pH values (see Fig. 8). Deamination greatly reduced combination between pH 2 and 9. The amount of formaldehyde bound by the untreated collagen up to pH 9·0, and the decrease in combination following deamination, correspond to considerably less than the combination of one formaldehyde molecule by each amino group; similarly, the amount bound from pH 9·0 to 13·0, the upper limit of pH covered, only corresponds to the combination of one molecule of formaldehyde by each of half the total number of guanidino groups.

With untreated and methylated collagen the combination of as little as 0·4 g. formaldehyde/100 g. raised the shrinkage temperature to its maximum value (78–80°), whereas the combination of 0·8 g. formaldehyde/100 g. left the shrinkage temparature of the deaminated collagen unchanged.

DISCUSSION

Deamination. Under the experimental conditions employed, treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by about 20 %. The course of the reaction with the amino groups is well known, but little attention appears to have been given to the reaction with the guanidino groups. Bancroft & Belden (1931) and Bancroft & Ridgway (1931) have shown that guanidine reacts with nitrous acid to give cyanamide and ammonia, and it is possible that a similar reaction takes place with the guanidino groups in proteins, namely,



$$—CH_2.NH.C(=NH)(NH_2) + HONO \rightarrow —CH_2.NH.CN + NH_3.$$

In agreement with this hypothesis, Kanagy & Harris (1935) have shown that ammonia is formed when arginine is treated with nitrous acid; this ammonia will react with nitrous acid to give the excess nitrogen observed in the Van Slyke determination of amino N (Plimmer, 1924; Hunter, 1929; Kanagy & Harris, 1935; Lieben & Loo, 1942; Van Slyke, Hiller & Dillon, 1942). This hypothesis would also explain the higher amide N of the deaminated collagen compared with the original collagen since, under the conditions of the determination, the —NH.CN group would probably yield ammonia. This increase in amide N is of the same order as the decrease in the arginine content of the collagen.

The reaction postulated above is also consistent with those changes in the titration curve which cannot be attributed to loss of amino N. Loss of the strongly basic guanidino groups accounts for the decrease in acid-binding capacity; carboxyl groups equivalent to the guanidino groups lost will revert from the zwitterion state ($—COO^-$) to the uncharged state, and will titrate between the isoelectric point and pH 7·0, and the —NH.CN groups, being feebly acidic, will titrate between pH 7 and 12, thus accounting for the increased base-binding capacities in these ranges. Since guanidino groups do not titrate with base up to pH 12·5 (Bowes & Kenten, 1948*a*), the total base-binding capacity should be increased by an amount equal to twice the —NH.CN groups formed, i.e. base bound by the additional

un-ionized carboxyl groups plus that bound by —NH.CN groups. On this basis, and assuming that 0·05 mmol./g. amino groups remain (see Table 1), the curve indicates that about 0·10 mmol./g. guanidino groups are converted to cyanide groups. (Decrease in acid binding in excess of that due to amino groups, 0·12 mmol./g.; base bound between 7 and 12, 0·10 mmol./g., and total increase in base-binding capacity, 0·20 mmol./g.) This figure is in good agreement with decrease in arginine content found by analysis, 0·09 mmol./g.

Esterification. Like the carboxyl groups of amino-acids, those of wool, silk, gelatin, and collagen can be esterified with methyl sulphate and methyl bromide (Blackburn *et al.* 1941; Blackburn & Phillips, 1944).

The evidence obtained in the present investigation confirms this view with respect to collagen. The reaction would appear to necessitate the presence of basic groups equivalent to carboxyl groups; it was found, however, that deamination did not decrease the extent of methylation. Blackburn & Phillips (1944) considered that the lysine content of wool is so low that its removal does not materially affect the number of basic groups. This explanation is not applicable to collagen since amino groups form about one third of the total basic groups, and a more likely explanation is that as ionized carboxyl groups are esterified, further carboxyl groups ionize under the influence of the guanidino groups, so that eventually all carboxyl groups are esterified.

Consideration of the values for the free carboxyl groups in the untreated collagens, as indicated by analysis and by their titration curves (Bowes & Kenten, 1948*b*), shows that all the methoxyl groups introduced can be accounted for on the basis of esterification of carboxyl groups. In three experiments, the number of methoxyl groups was slightly in excess of the number of free carboxyl groups but this excess was within the experimental error. Blackburn *et al.* (1941) and Blackburn & Phillips (1944) found that the methoxyl groups introduced by methyl sulphate exceeded the number of free carboxyl groups believed to be present in wool, silk, gelatin, and collagen, and suggested that methylation also occurred at certain 'activated' peptide links. It is now known (Bowes & Kenten, 1948*a*) that the values taken for the number of carboxyl groups in gelatin and collagen were low, and the present work shows that with collagen there is no necessity to postulate any form of *O*-methylation other than that of carboxyl groups. It is possible that future determinations of the dicarboxylic acids in wool and silk will obviate the necessity of assuming that these proteins undergo peptide methylation.

The low amino-nitrogen values of the methylated collagens suggest that some *N*-methylation has occurred, especially with methyl bromide. The lower base-binding capacity of the methylated collagens as compared with that of untreated collagen may also be due to *N*-methylation, for although the introduction of a methyl group will not eliminate the basic characteristics of the amino group, it will, by analogy with the methylamines, increase the pK of these groups and hence cause a shift in the titration curve to higher pH values. It is possible that the titration curves of the methylated collagen would show the same maximum base-binding capacity as the original collagen if carried to higher pH values. It is also possible that the reduction in base-binding capacity of the collagen methylated with methyl sulphate is related to the presence of nitrogen in the solutions after contact with the collagen; if this nitrogen were present as a base (for instance, as methylamine) it would titrate and so reduce the apparent fixation of base by the collagen.

Combination of collagen and modified collagens with tannins, chromium and formaldehyde. Although tannic acid, in contrast to the tannins of mimosa, contains acidic groups (Sunthankar & Jatker, 1938; Abichandani & Jatker, 1938; Cheshire, Brown & Holmes, 1941) which might be expected to form salts with the basic groups of the collagen, the mode of combination with collagen appears to be the same with both materials. The results as a whole are consistent with the supposition that combination of tannin is related to the positive charge carried by the protein. With the untreated collagen, combination is greatest between pH 1·5 and 2·0 when the collagen carries its maximum net positive charge, decreases as the pH increases and the net positive charge decreases, and eventually is reduced to negligible proportions at pH 8·0–9·0 when the basic groups begin to lose their positive charge and the protein carries a net negative charge. Deamination which decreases the positive charge on the collagen, decreases combination of tannin, and esterification, which causes the collagen to carry its maximum positive charge over the whole pH stability range of the methoxyl groups, causes combination of tannin also to be at a maximum over this range. Experiments indicate that the molecular weight of tannic acid and mimosa tannin is 1700 (Brintzinger & Brintzinger, 1931; Humphreys & Douglas, 1937), and the equivalent weight of tannic acid is of the same order (Cheshire *et al.* 1941). Using these figures, an interesting comparison can be made between the amounts of tannin bound and the acid-binding capacity of the collagens (see Table 4). The molecular amounts of tannin combined with the untreated and deaminated collagens between pH 1·5 and 2·0 are of the same order as their acid-binding capacities; deamination halves the acid-binding capacity and also halves the amount of tannic acid and mimosa tannin bound. This suggests that one molecule of tannin is associated with each basic

group. Although the amounts of tannin bound are dependent on the concentration of tannin in the solution, the time of treatment and method of washing, it would seem reasonable to assume that there is some significance in these stoicheiometric relationships.

Table 4. *Acid and tannin bound by collagen and deaminated collagen*

(Results in mmol./g.)

Collagen	Acid-binding capacity	Tannin bound between pH 1·5 and 2·0	
		Tannic acid	Mimosa tannin
Untreated collagen *A*	0·88	1·0	0·80
Deaminated collagen DA_1	0·44	0·50–0·55	0·41

The present results emphasize the essential part played by the carboxyl groups in the binding of chromium, and strengthen the current view that the fixation of metals involves complex formation with the carboxyl groups of the protein in a manner analogous to the complex formation which occurs with the carboxyl groups of organic acids (for review of literature see Bowes, 1948). The amino groups also appear to be involved since deamination decreased the amounts bound by the untreated collagen, though not by the methylated collagen. These findings are consistent with the hypothesis that combination of chromium involves co-ordination of both carboxyl and amino (or other basic groups) with the same chromium complex. A similar hypothesis has been put forward to account for the fixation of calcium by proteins (Greenberg, 1944), and for the high thermal stability of chrome-tanned leather (Küntzel & Riess, 1936).

Although the validity of stoicheiometric relationships between the amounts of chromium bound and the reactive groups of the collagen may, in view of the complexity of the system, be open to question, it is of interest to note that the amount of chromium bound between pH 4 and 5 approximately corresponds to the fixation of two chromium atoms by each carboxyl group, and the decrease in combination caused by treatment with nitrous acid corresponds approximately to two chromium atoms for each basic group lost. It is probable that the chromium aggregates in the tanning solutions used contained, on the average, two chromium atoms (Bowes, 1948).

The evidence suggests that, in collagen, the amino and guanidino groups are the main centres involved in the binding of formaldehyde, and there is no evidence that the amide groups are concerned under the conditions employed. The importance of the amino groups raising the shrinkage temperature (Gustavson, 1943) is confirmed. Free amino groups also play an essential part in the hardening of casein with formaldehyde (Nitschmann & Hadorn, 1944; Nitschmann & Lauener, 1946), and it is probable that a similar mechanism is involved, namely, the formation of cross links between adjacent polypeptide chains. Nitschmann & Hadorn (1944) and Nitschmann & Lauener (1946) discuss the various ways in which such cross links may be formed and consider that a cross link between ϵ-NH_2 groups and —NH— groups of the peptide link is the most probable. (For further discussion on this point see Fraenkel-Conrat, Cooper & Olcott, 1945; French & Edsall, 1945; Bowes, 1948.)

SUMMARY

1. Collagen has been deaminated with nitrous acid and esterified with methyl sulphate and methyl bromide, and the effect of these treatments on the reactivity of collagen towards acids, bases, tannins, chromium and formaldehyde has been determined. Modification of the guanidino groups by treatment with hypochlorite (Sakaguchi (1925) reaction) was only partially successful; only 40–50% of the arginine was destroyed and there was extensive general breakdown of the collagen.

2. Treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by approximately 20%. Evidence deduced from analysis and titration curves suggests that the guanidino groups are converted into cyanamide groups.

3. All the *O*-methyl groups introduced into collagen by methyl sulphate and methyl bromide can be accounted for on the basis of esterification of carboxyl groups. There is an indication that some *N*-methylation occurs, especially with methyl bromide.

4. From a study of the combination of tannins, chromium and formaldehyde with untreated, deaminated and methylated collagen, it is suggested that combination of tannins is related to the positive charge carried by the collagen, combination of chromium involves co-ordination of both amino and carboxyl groups of the collagen with the same chromium complex, and combination with formaldehyde occurs mainly with the amino and guanidino groups. Increase in thermal stability results only from combination of formaldehyde with amino groups.

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