On the other hand, these results demonstrate that plasma tends to maintain a rather steady vitamin A alcohol level. Although this mechanism is still unknown, one possible explanation is that the plasma concentration is dependent upon the degree of association of vitamin A alcohol with a protein carrier. Variations in this degree of association may explain the temporary increase in plasma vitamin A alcohol immediately following absorption of vitamin A. The sharp increase in the ester form of vitamin A during active absorption may likewise be due to its association with another protein.

SUMMARY

1. The rate of appearance of vitamin A ester and alcohol in plasma and liver, respectively, of vitamin A-depleted male rats following a single dose of vitamin A was studied. In plasma, the ester form

presented a typical sharp peak, and the free form showed relatively less increase. In liver, the ester form continued to be deposited up to 18 hr. after dosing, whereas the alcohol form increased for only 5 hr. and was then maintained at a fairly constant level.

2. In separate experiments, normal stock and vitamin A-depleted male rats were used either as such or after vitamin A dosage, to determine the vitamin A content in plasma and in liver. A direct relationship between the plasma and the liver vitamin A alcohol could not be confirmed, inasmuch as, despite wide variations in the concentrations of vitamin A alcohol in the liver, the plasma level remained relatively constant.

This study was partly supported by a grant from The Nutrition Foundation, Inc. of New York.

We are grateful to Prof. H. J. Deuel, jun., and to Prof. J. W. Mehl, for helpful criticism and suggestions.

REFERENCES

- Booth, V. H. (1952). *J. Nutr.* **48**, 13.
Brenner, S., Brookes, M. C. H. & Roberts, L. J. (1942). *J. Nutr.* **23**, 459.
Ganguly, J., Krinsky, N. I. & Deuel, H. J., jun. (1952a). *Fed. Proc.* **11**, 218.
Ganguly, J., Krinsky, N. I., Mehl, J. W. & Deuel, H. J., jun. (1952b). *Arch. Biochem. Biophys.* **38**, 275.
Glover, J., Goodwin, T. W. & Morton, R. A. (1947). *Biochem. J.* **41**, 97.
Horton, P. B., Murrill, W. A. & Curtis, A. C. (1941). *J. clin. Invest.* **20**, 387.
Josephs, H. W. (1942). *Bull. Johns Hopk. Univ.* **71**, 253.
Kimble, M. S. (1939). *J. Lab. clin. Med.* **24**, 1055.
Lewis, J. M., Bodansky, O., Falk, K. G. & McGuire, G. (1942). *J. Nutr.* **23**, 351.
Moore, T., Sharman, I. M. & Ward, R. J. (1951). *Biochem. J.* **49**, xxxix.
Ralli, E. P., Baumann, E. & Roberts, L. B. (1942). *J. clin. Invest.* **20**, 709.
Thompson, S. Y., Ganguly, J. & Kon, S. K. (1949). *Brit. J. Nutr.* **3**, 50.
United States Pharmacopoeia (1950). **14**, 789.

Acetylation of Collagen

By R. W. GREEN, K. P. ANG AND L. C. LAM
Chemistry Department, University of Malaya, Singapore

(Received 22 September 1952)

In recent years it has been possible to account for almost the whole nitrogen content of collagen in terms of its constituent amino-acids (Bowes & Kenten, 1948a). From the known composition of collagen it is evident that many of its side chains should be distinguished by polar groups, of which the most important are carboxyl, amino, guanidino and aliphatic hydroxyl groups. Before asking which of these groups may be responsible for such complicated changes as occur in reaction with tanning agents, it seems desirable to inquire whether they are free to react quantitatively with simple chemical reagents. It has already been shown by Bowes & Kenten (1949) that the carboxyl groups can be quantitatively esterified, and one of us (Green,

1951) has demonstrated a stoichiometric relationship when dry hydrogen chloride reacts with the protein. We report here a detailed study of the reaction between collagen and acetic anhydride.

This substance might be expected to react with some or all of the amino, guanidino and hydroxyl groups. Olcott & Fraenkel-Conrat (1947) found that several proteins in aqueous alkaline solution reacted with acetic anhydride in an amount equivalent to their free amino groups. They recommended this reaction as a specific means for the *N*-acetylation of proteins. Several workers have found that, when the conditions are changed and the solid protein is treated with acetic anhydride, both amino and hydroxyl groups are substituted. This was the case

when solid insulin reacted with acetic anhydride, either in the presence or absence of pyridine (Freudenberg & Dirscherl, 1928; Jensen & De Lawder, 1930). Hendrix & Paquin (1938) obtained similar results with casein, egg albumin and edestan. Other investigators have used boiling acetic anhydride under conditions which might be expected to induce undesirable side reactions. It will be shown here that, by modifying the experimental conditions, it is possible to introduce into collagen a number of *N*-acetyl groups exactly equivalent to the reported free amino groups, or to effect a higher degree of acetylation in which about 80% of the hydroxyamino-acid residues are also involved.

MATERIALS

The collagen used was standard hide powder. Denatured collagen was prepared by immersing hide powder in water at 72° for 5 min., filtering, washing with cold water, dehydrating with acetone, drying in the air of the laboratory and grinding to pass a 40 mesh/in. sieve. Acetic acid and acetic anhydride were of A.R. grade. Anhydrous acetic acid was made by refluxing glacial acetic acid with the calculated quantity of acetic anhydride and then distilling with the usual precautions to exclude moisture. The distillate was used in the preparation of water-free mixtures of acetic acid and acetic anhydride, which were then analysed by the iodometric method of Orton & Bradfield (1927). The acetic anhydride itself was found to contain about 6% acetic acid.

METHODS

Acetylation in aqueous suspension

A method similar to that of Olcott & Fraenkel-Conrat (1947) was used. Collagen was soaked for 24 hr. in half-saturated sodium acetate solution below 10°. It was then stirred vigorously for 1 hr. while 10% by weight of acetic anhydride was added in small portions. The pH was held near 8.0 by adding NaOH solution, and the temperature was kept below 10°. The product was then filtered and freed from sodium acetate by washing with frequent changes of distilled water for 1 week. Thorough washing was essential, as the method described below for the determination of total combined acetyl groups would give a high result if any sodium acetate were still held by the collagen fibres. That washing for 1 week was sufficient was proved by analysing the product, washing for 1 week more and analysing again. The results of the two analyses were found to be identical and were therefore taken to represent combined acetyl. The washed product was dehydrated with several changes of acetone and then exposed to the air of the laboratory. It was found that prolonging the reaction beyond 1 hr. did not increase the degree of acetylation. However, re-acetylation of the washed and dried product did introduce more acetyl groups. After two acetylations there was no further increase. A few experiments in other aqueous media are reported in Table 1 (see Results).

Acetylation of collagen in non-aqueous media

It had already been observed (Green, 1951) that completely dry collagen could not be acetylated with cold

acetic anhydride. Hendrix & Paquin (1938), however, report having acetylated several proteins containing not less than 7% water. This indicated that the water content of the collagen might be important. We therefore used the isopiestic method of Robinson & Sinclair (1934), as previously described (Green, 1948), to prepare a range of samples of collagen extending from the perfectly dry material to a water content of 100% of the dry weight.

(a) *Acetylation medium free from water.* The samples were covered with 6 parts of acetic anhydride, calculated on the dry protein weight, and kept at laboratory temperature (30°) for periods ranging from a few hours to 9 days. They were then filtered, washed with several changes of acetone and extracted with acetone in a Soxhlet apparatus for 48 hr. Preliminary experiments had shown that, after the first day's extractions, no further change in the analytical figures occurred even after several days in the extraction apparatus. Prolonged washing with cold acetone led to the same result.

(b) *Acetylation medium and collagen both free from water.* Since the action of water in promoting acetylation might be through the medium of acetic acid, a series of experiments was carried out in the absence of water. Collagen was dried at 100° *in vacuo* over CaO and treated with water-free mixtures of acetic anhydride and acetic acid. After acetylation the products were washed and extracted with acetone as before. As a test of the reliability of the method of preparation for analysis, samples of dry collagen were soaked for 3 days in either pure acetic acid or pure acetic anhydride. Each, after extraction and analysis by the methods described below, gave a negligible value for combined acetyl groups. Any possibility that the acetyl figures reported in the next section might be due, even in part, to adsorbed acetic acid is thus excluded.

In another series, to demonstrate the relationship between the two principal methods, collagen was acetylated in alkaline suspension, dried and subjected to the action of a 1:1 mixture of acetic anhydride and acetic acid.

Finally, a series of experiments was made in which collagen of varying water content was acetylated with the same 1:1 mixture.

In all these experiments the weight of the acetylating mixture was six times the protein dry weight.

Analysis of products

Total acetyl. Acetylated collagen (0.5 g.) after drying in the air oven at 103°, was heated under reflux for 2 hr. with 50 ml. 2*N*-H₂SO₄. The mixture was then distilled in a current of steam until its volume had been reduced to about one-half, and about 500 ml. had been collected in the receiver. The distillate was titrated with 0.05*N*-NaOH, and from the result was subtracted a small blank obtained by a similar hydrolysis and distillation with unacetylated collagen.

N-Acetyl. Acetyl groups in proteins may be attached to either oxygen or nitrogen, and it is generally believed that the two forms of combination can be differentiated by their stability to alkali (Hendrix & Paquin, 1938). All those acetyl groups which can be hydrolysed by cold dilute NaOH are believed to be attached to oxygen, whilst *N*-acetyl groups can be removed only by boiling in dilute acid solution.

This principle, which was adopted by Blackburn & Phillips (1944), is supported by the observations of Syngé (1939) on amino-acids and of Wolfrom, Konigsberg & Soltzberg (1936) on sugars; but the means of its experimental

application to proteins must be chosen with care. Blackburn & Phillips, in their work on wool, immersed acetylated derivatives in excess alkali and then back-titrated in the absence of protein to pH 7-8. The alkali used up, corrected by the result of a similar titration with the original protein, was regarded as an approximate measure of the extent of *O*-acetylation. However, this use of a blank may be subject to some uncertainty, since *N*-acetylation will displace the pH titration curve of the protein, making it difficult to define the state of the ionizable groups before the addition of alkali. A sharp distinction between *N*- and *O*-acetyl groups is not easily achieved.

A more reliable application of the principle consists in saponification of the *O*-acetyl groups with excess dilute alkali, followed by removal of the soluble acetate by dialysis or, for insoluble proteins, by simple washing. The acid hydrolysis and distillation procedure applied to the residue then gives an accurate figure for the degree of *N*-acetylation. This method suffers from the very long period required for washing or dialysis of swollen or gelatinous proteins, and is unsuitable for the examination of large numbers of samples. It was used here only for confirmation of the method finally adopted, in which the *N*- and *O*-acetyl groups were differentiated by measurements of free amino N.

Free amino N. This was determined by a modification (Doherty & Ogg, 1943) of the manometric Van Slyke procedure, using samples which passed a 40 mesh/in. sieve.

Total N. This was determined by a Kjeldahl method.

RESULTS

Acetylation in aqueous suspension

Collagen was treated with acetic anhydride after it had been soaked in solutions of various degrees of acidity or alkalinity. In only one case was any *O*-acetylation detected. Table 1 shows that the most

Table 1. *Action of acetic anhydride on collagen in aqueous suspension*

(Composition of derivatives expressed as m-moles/g. original dry collagen.)

Nature of suspension	<i>O</i> -Acetyl	<i>N</i> -Acetyl	Amino N (Van Slyke)*
<i>N</i> -Acetic acid	Nil	0.05	0.31
Water	0.15	0.05	0.31
Sodium acetate (pH 8)			
First acetylation	Nil	0.24	0.12
Second acetylation	Nil	0.36	0.01
Third acetylation	Nil	0.38	0.00
Fourth acetylation	Nil	0.38	0.00
0.02 <i>N</i> -Sodium hydroxide	Nil	0.18	0.17

* Amino N for dry collagen = 0.35 m-mole/g.

successful method of *N*-acetylation entailed the use of sodium acetate solution at pH 8, as recommended by Olcott & Fraenkel-Conrat (1947) for soluble proteins. To attain the highest degree of *N*-acetylation, it was always necessary to repeat the treatment. Prolonging the time or increasing the amount of acetic anhydride added in a single acetylation

was not enough. Table 2 shows that the maximum degree of *N*-acetylation attainable with heat-denatured collagen is the same as that for the original protein, namely 0.40 m-mole/g. protein.

Fig. 1. Effect of initial water content of collagen on degree of total acetylation by: A, acetic anhydride for 1 day; B, acetic anhydride for 3 days; C, acetic anhydride for 5 days; D, mixture of equal parts acetic anhydride and acetic acid for 3 days.

Fig. 2. Acetylation of collagen: ●, action of acetic anhydride on collagen containing 16% water; ○, acetic anhydride on collagen containing 36% water.

Acetylation in non-aqueous media

Method (a). The action of excess acetic anhydride on collagen containing relatively small quantities of sorbed water introduced a much greater number of acetyl groups, many of which could be removed by dilute sodium hydroxide; these, however, were split off only very slowly by treatment with milder alkalis at pH 8-9. The more extensively acetylated specimens swelled noticeably during the reaction and after drying resembled heat-denatured collagen in appearance. Apart from this, the acetylated products retained their fibrous nature and their colour was unchanged.

Fig. 1 (curves *A*, *B*, *C*) shows the effect of initial water content of the collagen on the total degree of acetylation for various reaction times. In Fig. 2 are curves showing the rate of total acetylation of collagen of two different water contents. At 36% water, corresponding to the maxima of Fig. 1, acetylation appears to reach saturation at 1.7 m-moles/g. collagen, but the curve for 16% water rises only slowly.

Fig. 3. Degree of total acetylation of collagen by mixtures of acetic anhydride and acetic acid: *A*, oven-dry collagen for 3 days; *B*, calculated from curve *B* of Fig. 1 on the assumption that the water contained in the collagen reacts rapidly with the acetic anhydride and has no other influence on the course of reaction; *C*, calculated from curve *D* of Fig. 1 on the same assumption.

Method (b). Two other series of experiments were made to elucidate the function of the sorbed water in promoting acetylation. It was found that, although pure acetic anhydride did not react with dry collagen, acetylation did take place if acetic acid was present; curve *A* of Fig. 3 shows the total degree of acetylation achieved in 3 days by mixtures of acetic anhydride and acetic acid. The broad, flat maximum near 50% acetic acid indicates that the most effective acetylating agent for dry collagen is a mixture of approx. equal parts of the anhydride and the acid. When the acetylating mixture already has this optimum composition, curve *D* of Fig. 1 shows that any sorbed water in the collagen retards the reaction.

A more detailed picture of the reaction is presented in Figs. 4 and 5, setting out the rates of *N*- and *O*-acetylation for different reaction mixtures. A few experiments with denatured collagen in the 1:1 acetic anhydride-acetic acid mixture gave results, identical with those for native collagen, which have been omitted from the figures for the sake of clarity. For the same reason we have omitted from Fig. 5 the experimental points derived from the action of the same acetylating mixture on

collagen previously acetylated at pH 8. The composition of the end products of these two series appears in Table 2.

Fig. 4. Degree of *N*-acetylation of dry collagen by mixtures of acetic anhydride and acetic acid (w/w): $\circ$, 10% acetic acid; $\bullet$, 30% acetic acid; $\circ$, 50% acetic acid; $\bullet$, 70% acetic acid and 85% acetic acid (experimental points for these two mixtures were indistinguishable); $\circ$, 95% acetic acid.

Fig. 5. Degree of *O*-acetylation of dry collagen by mixtures of acetic anhydride and acetic acid (w/w): $\circ$, 10% acetic acid; $\bullet$, 30% acetic acid; $\circ$, 50% acetic acid; $\bullet$, 70% acetic acid; $\bullet$, 85% acetic acid; $\circ$, 95% acetic acid.

In Figs. 4 and 5, the degree of *N*-acetylation was obtained from a comparison of the Van Slyke amino-nitrogen values of the acetylated product and the original protein. Several determinations on collagen and on denatured collagen gave the value of 0.35 m-mole free $\text{NH}_2/\text{g.}$ protein, in good agree-

Table 2. *Composition of collagen derivatives containing the maximum proportion of acetyl groups related to the amino-acid composition of collagen (after Bowes & Kenten, 1948a)*

Starting material	Method of acetylation	Total N (%)	N-Acetyl (m-moles/g. collagen)	O-Acetyl (m-moles/g. collagen)
Collagen	None	18.0	0	0
	Alkaline suspension (I)	17.6	0.40	0
	Mixture of acetic acid and acetic anhydride (II)	16.6	0.40	1.33
	(I) followed by (II)	16.5	0.40	1.36
Denatured collagen	None	17.8	0	0
	I	17.3	0.40	0
	II	16.6	0.40	1.36

Composition of collagen

Amino-acid	m-Moles/g. collagen	Amino-acid	m-Moles/g. collagen
Lysine	0.31	Serine	0.33
Hydroxylysine	0.08	Threonine	0.20
Arginine	0.51	Hydroxyproline	1.07
Histidine	0.05	Tyrosine	0.08

ment with that reported by Bowes & Kenten (1948a). However, Table 1 shows that acetylation of collagen at pH 8 introduces 0.40 m-mole acetyl/g., and none of these groups is split off by dilute sodium hydroxide. They may therefore be regarded as being attached to amino nitrogen, suggesting as a more accurate value for the free amino groups of collagen, 0.40 m-mole/g. This is supported by the known proportions of lysine and hydroxylysine in the protein. The use of the empirical extrapolation procedure of Doherty & Ogg (1943) thus apparently leads to a figure for collagen which is too low by about 12%. The results for *N*-acetylation reported here have been derived by multiplying the observed Van Slyke figures by a factor of 1.14 and subtracting from 0.40. The *O*-acetyl values are then taken as the difference between total and *N*-acetyl. This method of calculation leads to good agreement with the more time-consuming method of alkaline hydrolysis described above.

It will be observed that the total degree of acetylation at saturation is the same, whether the starting material is collagen or *N*-acetylcollagen, and whether the acetylating agent is acetic anhydride in the presence of water or mixed with anhydrous acetic acid. Table 2 presents the composition of collagen derivatives containing the maximum proportions of *N*- and *O*-acetyl groups.

DISCUSSION

The collagen preparation used here had been made from limed hide and was therefore not the same as the material, prepared with a minimum of chemical treatment, whose composition has been summarized by Bowes & Kenten (1948a). However,

our collagen corresponded almost exactly in both total nitrogen and amino nitrogen to an alkali-treated collagen which the above authors showed, in a second paper (Bowes & Kenten, 1948b), to differ materially from the original only in the number of amide groups. We shall assume, therefore, that the basic and hydroxyamino-acid content of our collagen is the same as that reported by Bowes & Kenten, part of whose data we reproduce here. In Table 2 are shown the amounts of those amino-acids whose side chains might be expected to react with acetic anhydride.

Although free amino-acids are easily acetylated at the α -amino group, the distal groups of the basic amino-acids are more resistant to attack. Neuberger (1938) found that arginine and histidine in mixtures of acetic anhydride and pyridine reacted at the α -amino group only. Bergmann & Köster (1926) found that acetic anhydride with arginine gave the α -acetyl derivative in the cold and a 'triacetyl anhydroarginine' only at 100°. There appears to be no record of simple acetylation of arginine at the guanidino group or of histidine at the glyoxaline group. However, Synge (1939) prepared $\alpha\epsilon$ -diacetyllysine by the action of acetic anhydride in alkaline solution, so that, of the basic amino-acids, it would seem that only lysine and hydroxylysine are likely to be easily acetylated in the side chain under mild conditions; this may apply to their residues in the protein, as well as to the free amino-acids.

We have already pointed out that acetylation of collagen in alkaline suspension quickly introduces alkali-stable acetyl groups to the extent of 0.40 m-mole/g. protein, unchanged by repetition of this treatment. Simultaneously, the amino-nitrogen figure falls to zero. Since the number of groups intro-

duced is exactly equivalent to the sum of lysine and hydroxylysine residues shown in Table 2, it is clear that acetic anhydride in slightly alkaline suspension must react quantitatively with the free amino groups of collagen and with no others.

The reaction of dry collagen with acetic anhydride-acetic acid mixtures is more complex. Figs. 4 and 5 may be summarized by saying that the rate of *N*-acetylation is highest and almost constant in mixtures containing from 5 to 50 % acetic anhydride, and under these conditions *N*-acetylation is complete in a few hours; *O*-acetylation becomes appreciable only when the proportions of acid and anhydride are of the same order, and requires more than 4 days for completion. The combination of these two effects accounts for the shape of curve *A* in Fig. 3. The close agreement of the results for collagen and for previously *N*-acetylated collagen shows that the same nitrogen atoms are taking part in the reaction with acetic anhydride whether the medium is aqueous alkali or anhydrous acetic acid.

It may be pointed out that, in a 5 % solution of acetic anhydride in acetic acid, *N*-acetylation is complete in 8 hr., while *O*-acetylation has hardly begun (0.02 m-mole/g.). This provides an alternative to the method of Olcott & Fraenkel-Conrat (1947) for preparing *N*-acetylated proteins, and has the advantage that the degree of acetylation is more easily controlled.

The maximum number of alkali-labile acetyl groups introduced is 1.33 m-moles/g., a figure which has been confirmed in several independent experiments. It is unaffected by previous heat denaturation of the protein. It is most improbable that these groups have been introduced by reaction between acetic anhydride and the free carboxyl groups of the aspartic and glutamic acid residues. Not only is the number of acetyl groups far too great to be accounted for in this way, but any mixed anhydrides resulting from a hypothetical reaction of this kind would be expected to resemble the simpler acid anhydrides and to be easily hydrolysed by water and mild alkalis. On the other hand, the easy removal of the acetyl groups by 0.05*N*-sodium hydroxide makes it almost certain that they are present as esters of alcoholic hydroxyl groups.

Sakami & Toennies (1942) prepared the *O*-acetyl derivatives of serine, threonine, tyrosine, and hydroxyproline, and report that all were rapidly hydrolysed by dilute alkali. Syngé (1939) prepared *ON*-diacetyltyrosine and found that the *O*-acetyl group was completely split off by excess 0.005*N*-sodium hydroxide, while the *N*-acetyl group was unaffected. These considerations, taken with the fact that the only other possibility, arginine, is difficult to acetylate at the guanidino group and, in any case, is not present in sufficient quantity, lead us to the con-

clusion that the alkali-labile acetyl groups introduced into collagen are attached to the oxygen of the hydroxyamino-acids. Their number, 1.33 m-moles/g., is less than the total number, 1.73 m-moles/g. of these residues, indicating that about one in four of the hydroxyl groups of collagen is not free to react. This ratio is maintained even after denaturation, and emphasizes the fact that, whatever changes may occur during denaturation, they do not involve the availability of amino or hydroxyl groups.

The shape of the curves in Fig. 1 shows that sorbed water must play a double role in acetylation by acetic anhydride. Oven-dry collagen does not react with the anhydride, but small quantities of water have a catalytic effect, which is masked by dilution or hydrolysis of the anhydride when larger quantities of water are present. Since in the absence of water the curve for acetic anhydride-acetic acid mixtures in Fig. 3 also shows a maximum, it is relevant to inquire whether the water has any direct catalytic action or whether the only catalyst is acetic acid.

If the acetylating agent is a mixture of equal parts of acetic acid and acetic anhydride, corresponding to the maximum of Fig. 3, the presence of any quantity of water in the collagen diminishes the rate of reaction. This suggests that the only function of the water is to form acetic acid, which acts as a catalyst when it is present in small amount, but as a diluent when present in large amount, as in the experiments of Fig. 1*D*.

This presumption can be tested further by comparing the data of Fig. 1, curve *B*, with Fig. 3, curve *A*. The reaction of acetic anhydride with liquid water is almost complete in a few hours, and it is conceivable that, with water adsorbed on a regular surface which also has an affinity for the anhydride, hydrolysis may be much more rapid. We shall assume, then, that the collagen-bound water in the experiments of Fig. 1, *B* and *D*, reacts with acetic anhydride much more rapidly than does the protein. Any point from these experiments with collagen + acetic anhydride + water can then be recalculated as a mixture of collagen + acetic anhydride + acetic acid, and as such plotted in Fig. 3. The two broken curves are derived in this way. It is evident that they follow the principal curve of Fig. 3 over almost the whole range. The comparison fails only when the collagen contains quantities of water almost equivalent to the total anhydride used. Then the competition between protein and water for the anhydride becomes apparent and the curves diverge. The close agreement between the two methods of acetylation when reduced to a common basis, even by this approximate method, makes it clear that the catalyst is acetic acid and that the water is only an intermediate.

SUMMARY

1. In mildly alkaline suspension collagen is acetylated by acetic anhydride at the free amino groups. The number of *N*-acetyl groups introduced is equal to the total number of lysine and hydroxylysine residues found by analysis.

2. Dry collagen does not react with cold acetic anhydride, but in the presence of acetic acid or of small amounts of water both *N*- and *O*-acetylation take place.

3. The reaction is a slow one and is catalysed by acetic acid or, indirectly, by water. At its completion all the ϵ -amino groups and about 77% of the hydroxyamino-acid residues of the protein have reacted.

One of us (L.C.L.) was the holder of a Shell Research Fellowship during the session 1950-51, and grateful acknowledgement is made to the Shell Company of Singapore, Ltd., for their assistance. We also thank Prof. R. A. Robinson for valuable advice.

REFERENCES

- Bergmann, M. & Köster, H. (1926). *Hoppe-Seyl. Z.* **159**, 179.
 Blackburn, S. & Phillips, H. (1944). *Biochem. J.* **38**, 171.
 Bowes, J. H. & Kenten, R. H. (1948*a*). *Biochem. J.* **43**, 358.
 Bowes, J. H. & Kenten, R. H. (1948*b*). *Biochem. J.* **43**, 365.
 Bowes, J. H. & Kenten, R. H. (1949). *Biochem. J.* **44**, 142.
 Doherty, D. G. & Ogg, C. L. (1943). *Industr. Engng Chem. (Anal.)*, **15**, 751.
 Freudenberg, K. & Dirscherl, W. (1928). *Hoppe-Seyl. Z.* **175**, 1.
 Green, R. W. (1948). *Trans. Roy. Soc. N.Z.* **77**, 24.
 Green, R. W. (1951). *Trans. Roy. Soc. N.Z.* **79**, 485.
 Hendrix, B. M. & Paquin, F. (1938). *J. biol. Chem.* **124**, 135.
 Jensen, H. & De Lawder, A. (1930). *Hoppe-Seyl. Z.* **190**, 262.
 Neuberger, A. (1938). *Biochem. J.* **32**, 1452.
 Olcott, H. S. & Fraenkel-Conrat, H. (1947). *Chem. Rev.* **41**, 151.
 Orton, K. J. P. & Bradfield, A. E. (1927). *J. chem. Soc.* p. 983.
 Robinson, R. A. & Sinclair, D. A. (1934). *J. Amer. chem. Soc.* **56**, 1830.
 Sakami, W. & Toennies, G. (1942). *J. biol. Chem.* **144**, 203.
 Synge, R. L. M. (1939). *Biochem. J.* **33**, 1913.
 Wolfson, M. L., Konigsberg, M. & Soltzberg, S. (1936). *J. Amer. chem. Soc.* **58**, 490.

The Role of the Amino and Hydroxyl Groups of Collagen in Chrome Tanning

By R. W. GREEN

Chemistry Department, University of Malaya, Singapore

(Received 22 September 1952)

The reaction between collagen and chromic salts, whereby small quantities of the latter bring about profound changes in the physical and chemical properties of the former, is of great practical importance in the manufacture of leather and of theoretical interest as an example of the combination of proteins with metals. It has been the subject of many researches, which have been discussed in a recent review by Gustavson (1949). Both reactants are highly complex systems and, while many investigators have studied the mechanism of the reaction by modifying the chromic solution, the complementary method of attack, modification of the protein, has usually been restricted to changes which arise directly from the processes of manufacture. Attempts to measure the effect of simple chemical changes in the collagen, controlled in extent and limited to a specific group in the protein molecule, have been less numerous.

An outstanding example of the latter approach is the work of Bowes & Kenten (1949), who esterified

the free carboxyl radicals of collagen and showed that these groups are of primary importance in the reaction with chromium. However, simple salt formation between chromium and carboxyl is not sufficient to explain the marked increase in stability accompanying chrome tanning, particularly when we remember the ease of hydrolysis of chromic salts. It is now firmly established that the chromic salt must form, by means of its polynuclear co-ordination complexes, a bridge or network of covalent and co-ordinate links between reactive points on the same or neighbouring protein chains.

The data presented by Bowes & Kenten (1949) show that carboxyl and amino groups constitute many of these reactive points. This is in conformity with the common belief that the reactions of chrome tanning are almost exclusively associated with acidic and basic protein groups. No evidence has been published to suggest that the numerous hydroxyl groups of collagen might also be involved. In this paper we describe the use of acetylated