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.

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

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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

collagen to confirm the findings of Bowes & Kenten for the amino group and to show that aliphatic hydroxyl groups also play a major role in the fixation of chromium.

METHODS

Collagen and denatured collagen were the same as those used in the previous paper (Green, Ang & Lam, 1953).

Acetylation of collagen. This was carried out by the methods also described in the previous paper. *N*-Acetylcollagen resulted from the action of acetic anhydride on an alkaline suspension of the protein; and a complete range of *N*-acetylated samples was made by the action of a 5% (w/w) solution of acetic anhydride in acetic acid on dry collagen for periods varying from 30 min. to 8 hr. None of these preparations contained any measurable quantity of *O*-acetyl groups.

A further series of derivatives was made by treating fully *N*-acetylated collagen with a mixture of equal parts acetic anhydride and acetic acid in the absence of water, resulting in the acetylation of up to 80% of the hydroxyamino-acid residues.

One of these mixed acetyl derivatives was immersed overnight in excess 0.1*N*-NaOH below 10° to remove *O*-acetyl groups. It was then brought to equilibrium at pH 4 by means of acetic acid and washed for 1 week with distilled water. The product was still fully *N*-acetylated but contained no *O*-acetyl groups.

Cationic chromium solutions. Normal chromic sulphate was dissolved in cold water and made basic by slow addition, over a period of 2 days, of the calculated quantity of aqueous NaOH. The solutions were then diluted to 0.167*M*, i.e. to 25.3 g. Cr₂O₃/l. and allowed to age for 2 months. In this way basic chromic sulphate solutions were made, ranging from the normal salt to one in which one-half of the sulphate radicals had been replaced by basic groups. The basic solutions, of course, contained Na₂SO₄ in amount increasing with the basicity. We believe that the use of aged basic chromic solutions is to be preferred to the practice of adding alkali to the experimental solution while tanning is in progress, since this may result in precipitation, particularly when the pH is raised above 3.5. The solutions used here, with pH values from 1.8 to 3.3, covered the whole range of basicities employed in commercial tanning.

Anionic chromium solution. A solution of sodium oxalatochromate was prepared by reducing hot sodium dichromate solution with the theoretical quantity of oxalic acid, and the pH of the product was adjusted to 5.0 by the gradual addition of NaOH. It was diluted to a concentration of 25.3 g. Cr₂O₃/l., allowed to age for 3 months and filtered before use.

Chrome tanning. Collagen or acetylated collagen was dried in the air oven at 103°, and 1 g. was then soaked in 10 ml. distilled water for 24 hr. at room temperature (30°). Chromium solution (25 ml.) was added and the mixture was allowed to stand, with frequent agitation, for periods varying from 4 hr. to 4 days. The pH of the cationic chromic solution rose by 0.1–0.2 during the experiment. At the end of the reaction period the chromed collagen was filtered, washed for 2 days to free it from uncombined chromium, dried in the oven at 103° and analysed.

Analysis. Collagen and its derivatives before reaction with chromium were analysed for *N*- and *O*-acetyl as

described in the previous paper. No attempt was made to measure the Van Slyke amino N after reaction with chromium, but the total acetyl figure was determined as before.

Chromium was estimated by drying at 103°, ashing, heating the ash with a mixture of Na₂CO₃ and MgO, and estimating the chromate by titration against ferrous ammonium sulphate in the presence of *N*-phenylanthranilic acid. The chromium content of all specimens is expressed as mg. atoms Cr/g. moisture-free collagen.

Total N was determined by a Kjeldahl method.

RESULTS

Preliminary experiments demonstrated that a considerable diminution in chromium fixation resulted from acetylation of collagen. The measurements illustrated in Fig. 1 were made to determine whether this difference arose from a diminished combining capacity or from a slower rate of reaction. Collagen and two acetylated derivatives were allowed to react with 30%-basic chromic sulphate. The results show that the reaction is almost complete after 3 days and that acetylation does diminish the capacity of the collagen to fix chromium. In all subsequent experiments a standard reaction period of 72 hr. was adopted.

Fig. 1. Effect of time on fixation of chromium from a solution of the 30%-basic sulphate: ○, collagen; ◐, *N*-acetylcollagen (0.40 m-mole acetyl/g.); ●, fully acetylated collagen (1.73 m-moles acetyl/g.).

In Table 1 the analytical figures for several derivatives show that the loss of either *N*- or *O*-acetyl groups from acetylated collagen during chrome tanning is negligible. The behaviour of a number of collagen derivatives in chromic sulphate solutions of different basicities is shown in Fig. 2.

Fig. 3 illustrates how chromium fixation from solutions of both cationic and anionic chromium is influenced by the degree of acetylation of the collagen. Both *N*- and *O*-acetyl derivatives have been included in this figure, since chromium fixation seems to be linearly related to the total degree of acetylation, irrespective of whether amino or

Table 1. Composition of collagen derivatives before and after reaction with 30 %-basic chromic sulphate for 3 days

	Total N (%)	Cr		Acetyl	
		mg. Atoms/g. final product	mg. Atoms/g. collagen	m-Moles/g. final product	m-Moles/g. collagen
Collagen	18.0	—	—	—	—
Chromed collagen	16.0	1.10	1.24	—	—
N-Acetylcollagen	17.6	—	—	0.39	0.40
Chromed N-acetylcollagen	15.8	0.98	1.12	0.35	0.40
Fully acetylated collagen	16.6	—	—	1.60	1.73
Chromed fully acetylated collagen	15.4	0.67	0.78	1.39	1.63

hydroxyl groups are involved. Experimental points corresponding to not more than 0.40 m-mole total acetyl/g. collagen represent samples containing N-acetyl groups only; other points represent samples which are fully N-acetylated and contain some O-acetyl groups in addition. The scatter of the experimental points in this diagram is due in part to slow changes in the chromium solutions even after 2-3 months' ageing. The data of Fig. 3 were not all derived at the same time, and there appeared to be a very slow trend towards higher chromium fixation with age.

Fig. 2. Effect of basicity of chromic sulphate solution on chromium fixation by collagen and its derivatives: ○, collagen; ◐, denatured collagen; ◑, N-acetylcollagen; ●, fully acetylated collagen (1.73 m-moles acetyl/g.); ●◐, fully acetylated denatured collagen (1.76 m-moles acetyl/g.).

The approximately linear relations demonstrated in Fig. 3 imply that the decrease in chromium fixation is a direct result of acetylation and is not a consequence of secondary physical changes in the substrate. This conclusion is strengthened by the data of Table 2. It is shown there that collagen containing no acetyl groups takes up a constant

amount of chromium, whether it is the original material or whether it has been exposed to either acetic anhydride or acetic acid alone in the complete absence of water. Fully acetylated collagen, from which all O-acetyl groups have been removed by

Fig. 3. Effect of degree of acetylation on fixation of chromium from 30 %-basic chromic sulphate (upper curve) and sodium oxalatochromiate (lower curve): ●, N-acetylated collagen; ○, collagen containing 0.40 m-mole N-acetyl/g. and further O-acetyl groups.

Table 2. Chromium fixation by collagen containing no combined O-acetyl groups

Pretreatment	Acetyl (m-moles/g. collagen)	Cr (mg. atoms/g. collagen)
None	—	1.24
Dried at 103°, soaked in acetic anhydride 3 days, extracted with acetone	—	1.26
Dried, soaked in acetic acid 3 days, extracted with acetone	—	1.20
Soaked in 0.1N-NaOH, brought to pH 4 with acetic acid, washed 1 week	—	1.22
Alkaline de-acetylation fully acetylated collagen, thorough washing	0.41	1.18

treatment with alkali and thorough washing, still contains its full complement of *N*-acetyl radicals. Its chromium-binding capacity lies between that of the original collagen and that of *N*-acetylcollagen prepared by the standard method.

DISCUSSION

***N*-Acetylation.** That the free amino groups of collagen might be involved in the chrome-tanning reaction was suggested by Thomas & Kelly (1926), who found that deaminated hide powder showed a greatly diminished chromium fixation from a 52 % basic solution. Proceeding by a different method, Bowes & Kenten (1949) have reported similar findings, so that the effect of deamination is well established. Nevertheless, it is well known that side reactions occur when collagen is treated with nitrous acid, and some confirmatory evidence of the role of the amino groups is desirable. This is provided by the results given here for *N*-acetylated collagen.

It was found that *N*-acetylcollagen, prepared by the alkaline method, bound almost as much chromium as the original protein. However, this result was misleading, since raw collagen treated with a strong solution of sodium acetate and thoroughly washed was found to have enhanced chromium-binding capacity. On the other hand, as is shown in Fig. 2 and Table 2, treatment of collagen with either acetic anhydride or acetic acid in the absence of water, followed by extraction with acetone, leaves its capacity for chromium fixation unchanged. For that reason, all the *N*-acetyl derivatives used here were prepared by the action of a 5 % solution of acetic anhydride in acetic acid on the dry protein. The data reported in Table 1 and the slope of the upper curve in Fig. 3 both show that blocking free amino groups by acetylation decreases the amount of chromium fixed from a moderately basic chromic sulphate solution to the extent of about one atom Cr/3NH₂ groups. Only at 50 % basicity (Fig. 2) does the ratio depart widely from this figure. It is much less than the diminution reported by Thomas & Kelly (1926), suggesting that the effect of nitrous acid treatment on chromium fixation may not be confined to simple removal of amino groups. Indeed, in the light of our observations on *O*-acetylation, it would appear that simple deamination, in which amino groups are replaced by an equivalent number of hydroxyl radicals, should have no effect on chromium fixation. Bowes & Kenten (1949) found decreases in chromium uptake of as much as two atoms Cr/NH₂ group, but only at pH values above 4, where the chromium complex is in a very high state of aggregation and tends to be precipitated.

***O*-Acetylation.** Since we have at present no method of acetylating the hydroxyl groups of a

protein without simultaneously substituting the amino groups, the effect of blocking the former is best studied on collagen which is already completely *N*-acetylated. The presentation is simplified by the observation, already mentioned, that there is no significant difference between *N*- and *O*-acetylation in their effect on chromium fixation.

It is clear from the results shown in Figs. 1 and 3 that introduction of *O*-acetyl groups into collagen also diminishes its power to fix chromium, no matter how long the derivative is left in contact with the chromium solution. Denatured collagen, which takes up rather more chromium than native collagen under the same conditions, exhibits a similar loss of affinity upon complete acetylation. The full combining capacity of collagen is almost entirely restored if the *O*-acetyl groups are removed by dilute alkali followed by prolonged washing with water. Moreover, collagen samples which have been exposed, in the complete absence of water, to the action of either acetic anhydride or acetic acid alone, followed by thorough washing with acetone, and which contain no combined acetyl groups, have their combining capacity unimpaired. All these observations, together with the regular form of Fig. 3, leave little doubt that the decrease in chromium fixation is directly related to the blocking of hydroxyl groups in the protein.

The regression line drawn through the upper set of experimental points in Fig. 3 is represented by the equation:

$$y = 1.19 - 0.29x,$$

where x = m-moles acetyl/g. collagen and y = mg. atoms Cr/g. collagen. This means that, when the tanning agent is 30 % basic chromic sulphate, between three and four amino or hydroxyl groups must be acetylated to block the entry of one chromium atom. Reference to Fig. 2 shows that this value is dependent on basicity and lies between one and four groups/Cr atom, increasing fairly regularly throughout the range 0–50 % basicity.

The course of the curves in Fig. 2 can be explained in terms of amino-acid content of collagen (Bowes & Kenten, 1948) by assuming that the primary reaction is between the polynuclear chromic complex and carboxyl, but that the product is stable only after ring formation has been achieved by the co-ordination of a second protein group at some point of the complex. The groups available for co-ordination are amino, guanidino, carboxyl or hydroxyl, whose relative abundance in collagen is approximately 1:1.3:1.9:4.4. In a highly acetylated collagen, with half of these groups inactivated, some carboxyl radicals will be too far distant from another group to permit co-ordination, and these carboxyl radicals will be unable to form stable compounds with chromium. Thus, at low basicities, we find acetylated collagen taking up far less than one

atom Cr/carboxyl group. With increasing basicity the size of the chromic complex increases, so that the carboxyl groups which can form stable compounds will now be associated with several chromium atoms. The more isolated groups remain ineffective, and we see that the ratio of fixed chromium to total carboxyl approaches unity. The absence of an upward inflexion from the curve for highly acetylated collagen may imply that the largest chromic complexes cannot be firmly held without the help of more than one stabilizing group. When all the amino and nearly all the hydroxyl groups have been acetylated, stabilization of the bound chromium is dependent entirely on the carboxyl and, perhaps, guanidino groups.

Normal collagen, on the other hand, contains about 2.8 amino or hydroxyl groups for every free carboxyl, and there is a much higher probability that every chromic complex reacting with carboxyl will also be able to co-ordinate with one or other of the former. More chromium will be fixed even from solutions of low basicity, and at high basicities the rapidly growing polynuclear complexes will come within the range of additional co-ordinating groups and will be firmly held. This double effect of large particle size in the chromic salt coupled with greater availability of amino and hydroxyl groups can be expected to cause the sharp upward sweep of the curves in Fig. 2.

The fact that several groups must be acetylated to block the entry of one chromium atom does not, of course, imply that all are attached to that atom. It is purely a probability effect resulting from the fact

that all groups can be expected to be equally susceptible to acetylation but that only some of them are placed near carboxyl groups. If it is assumed that the amino and hydroxyl groups of collagen are randomly distributed, that all are equally effective in co-ordinating chromium and that all groups of the same kind are equally easily acetylated, then, at least at moderate basicities, the fixation of chromium should be linearly related to the degree of acetylation, as in Fig. 3. The lower curve of this figure shows that similar considerations apply to reactions with anionic chromium complexes.

SUMMARY

1. Acetylation of either free amino or free hydroxyl groups of collagen causes a marked diminution of its ability to take up either cationic or anionic chromium complexes from solution.

2. For chromic sulphate solutions, this effect has been studied over a wide range of basicities and for differing degrees of acetylation. It is most pronounced at high basicity and increases almost linearly with the degree of acetylation.

3. The experimental results suggest that, while the initial reaction between the chromic complex and collagen occurs at the carboxyl groups of the latter, the co-ordination of amino or hydroxyl radicals is essential to the formation of a stable compound.

4. In the basicity range normally employed in industrial chrome tanning, approximately one collagen amino or hydroxyl group in every three appears to be involved in the reaction.

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The Influence of Energy Intake on Protein Metabolism

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Although it is well known that protein metabolism is influenced by energy intake, the underlying biochemical mechanism remains obscure. We thought that it might be profitable to study animals under circumstances in which changes in energy intake fail to affect protein metabolism and to compare them with animals under conditions in which the normal relationship holds. Examination

of the literature suggested that rats receiving low and high levels of protein intake provide the necessary contrast. Several reports indicate that reduction of the intake of a protein-free diet may not alter the nitrogen balance of rats (Mitchell, 1924; Treichler & Mitchell, 1941; Vars & Gurd, 1947), at least until intake represents less than half the voluntary consumption (Swanson, 1951). On the