The Determination of the Substitution Achieved at the α -Amino, ϵ -Amino and Imidazole Groups of Proteins with Special Reference to Derivatives of Gelatin

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1. The diminution of the quantity of pigment formed in the ninhydrin colorimetric reaction or the titre in two types of formol titration of a protein after substitution is used to determine the degree of substitution achieved at the various amino groups of a protein. 2. The determination of the substitution achieved at α -amino plus ϵ -amino groups by the ninhydrin colorimetric method as described by Cobbett, Gibbs & Leach (1964) has been modified to correct for the hydrolysis of the peptide chain which takes place during colour development. 3. The degree of substitution at the ϵ -amino groups was determined by the formol titration carried out at pH 9, essentially as described by the above authors. 4. Substitution at the α -amino plus ϵ -amino plus imidazole groups was determined from the diminution of the titre of a modified formol titration. This titration was carried out by adjusting the protein solution to pH 6.5 followed by the addition of formaldehyde and then titrating to pH 9.0. 5. The three methods have been applied to carbamoylated and benzenesulphonylated gelatin derivatives. The values for the degrees of substitution obtained by the ninhydrin (α -amino plus ϵ -amino) and the formol (pH 9.0, ϵ -amino) methods were shown to be almost identical. The values from the formol (pH 6.5–9.0, α -amino plus ϵ -amino plus imidazole) titration were consistently lower than the values obtained by the other methods and corresponded to no substitution having taken place at the imidazole groups by the preparative methods employed. 6. The application of the methods to other protein systems is discussed.

The study of the influence of substitution at the amino groups of a protein on its properties requires a knowledge of the level of substitution achieved in the sample being examined. Cobbett, Gibbs & Leach (1964) describe the application of three techniques for this purpose. The spectrophotometric method described by these authors depended on the increased u.v. absorption of the protein after substitution with a substituent containing a suitable chromophore. From this information together with a knowledge of the amino acid composition of the protein could be calculated the level of substitution achieved at the α -amino plus ϵ -amino, plus imidazole groups. The chromophoric requirement limits the application of the method and furthermore, as these authors point out, the method is subject to influence by other amino acids, particularly tyrosine, when they are present in significant amounts and are involved during the substitution reaction.

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These authors also describe the application of the formol titration, which under the conditions employed gives a measure of the substitution achieved at the ϵ -amino groups. The present paper describes a modification of this technique which enables the groups measured in the spectrophotometric method (α -amino plus ϵ -amino plus imidazole) to be examined.

The ninhydrin procedure, which is used for the determination of the substitution achieved at the α -amino plus ϵ -amino groups, was considered by Cobbett *et al.* (1964) to give low values, and these were attributed to the effects of hydrolysis which takes place during colour development. This method has now been modified to correct for these effects. Carbamoylated and benzenesulphonylated derivatives of gelatin are examined by this procedure together with the two types of formol titration and the results compared and discussed particularly with regard to the role of the imidazole groups in these derivatives.

METHODS

Carbamoylated gelatins. Stark, Stein & Moore (1960) have shown that cyanates react with the amino groups of proteins yielding carbamoyl derivatives. Whilst carbamoylation is not a simple substitution reaction, the product contains $\text{HN}\cdot\text{CO}-$ substituted for $\text{H}-$ and it is convenient to refer to it as substitution. In the present work gelatin solutions were reacted with potassium cyanate under the conditions described in Table 1. The resultant solutions were gelled, cut into pieces (approx. $\frac{1}{2}$ in. $\times$ $\frac{1}{2}$ in. $\times$ 3 in.) with a wire, and washed with flowing water in a beaker at room temperature for at least 24 hr., the current of water being sufficient to keep the contents of the beaker stirred. The products were dried in a current of air and ground. The parent gelatins which were used for comparative purposes in the analyses were treated in an identical manner throughout except that the cyanate was omitted.

Benzenesulphonylated gelatins. Gelatin solutions were caused to react with benzenesulphonyl chloride at pH 9 and 60°, the heating continued for 30 min., the solutions gelled, and then treated as the carbamoyl derivatives. The only difference between samples was the amount of reagent used; up to twice that theoretically required to give complete substitution was employed. The parent gelatins were treated in an identical manner except that the reagent was omitted.

Moisture and ash. All the calculations were carried out on an ash and moisture-free basis. About 1 g. of the material, accurately weighed, was melted with 10 ml. of water and dried as a thin film for 18 hr. at 105°. The loss in weight was recorded as the moisture content. A moisture dish with a lid was used to avoid gain of moisture in the desiccator. For the ash content about 1 g. of material in a platinum dish was moistened with conc. H_2SO_4 , charred over a Bunsen burner and heated at 650° in a muffle furnace for 2 hr. and the residue weighed as ash. These techniques can readily be scaled down should the need arise.

Ninhydrin colorimetric method. This was carried out essentially as described by Cobbett *et al.* (1964), and is described here for convenience along with the present modification for the hydrolysis correction. The ninhydrin reagent was prepared as described by Moore & Stein (1948). The solution was prepared just before use or it had been stored for not more than 3 days in an O_2 -free atmosphere in the dark.

Table 1. Reaction conditions employed in the preparation of carbamoylated gelatins

The weight of $x\%$ (w/w) solution of an alkali-processed gelatin used was 300 g.

Sample no.	x (% w/w)	KCNO (g.)	pH	Time (hr.)	Temp.
C1	10	4	7	1	50°
C2	10	4	8	1	50
C3	10	8	*	114	approx. 17
C4	20	16	7	5	50
C5	10	8	7	17	50
C6	20	16	*	114	approx. 17

* No pH adjustment; pH increases with time to about 9.

Recommended procedure. About 20–70 mg. of the gelatin or gelatin derivative (about 20 mg. for an unmodified gelatin to about 70 mg. for a gelatin completely substituted at its α -amino plus ϵ -amino groups) was accurately weighed from a scoop into a 50 ml. beaker. Water (25 ml.) was pipetted into the beaker, the sample allowed to swell for about 30 min. and then warmed and stirred at about 40° to bring it into solution. A portion (1 ml.) of this solution was pipetted into a test tube; 2 ml. of the ninhydrin reagent was added and the solutions were thoroughly mixed by swirling the tube. The tube was capped and placed in a bath of vigorously boiling water for 20 min. The solution was cooled and mixed thoroughly and the extinction measured at 570 $m\mu$ in a 0.5 cm. cell against water. If a larger cell is to be used the solution is diluted with 5 ml. of ethanol–water (1:1, v/v). Triplicate determinations were carried out for each sample and the reagent blank. The determinations on the parent and substituted gelatins were carried out simultaneously since the colour yield varied slightly with the age and batch of the reagent and to ensure that the development period was identical for both samples. An identical set of solutions prepared at the same time was heated for 40 min. in the boiling water and the measurements were carried out as above (Recommended procedure; method 1). These values were used to calculate the hydrolysis correction. Experiments were also carried out in which an initial period of 25 min., with correction periods of 25 min. (method 2), or 25 min. calculated from 15 min. (method 3), was used. The results of all these experiments are in Table 2. During the development of the correction procedure, samples of a gelatin, a highly substituted gelatin and a reagent blank were heated under the above conditions for a range of heating periods; results of these experiments are in Fig. 1.

If the solution of the protein or its derivative was markedly different from pH 5 so that it significantly changed the pH of the ninhydrin reagent, then either the solution was adjusted to pH 5 before making up to volume or 4 ml. of ninhydrin reagent was used. In the latter method, parent protein, derivative and blank solution were all treated in the same way. With gelatin or its derivative this procedure was required only when the material had both a low degree of substitution and a pH above 7 or below 4.5, i.e. its buffering capacity was large.

The degree of substitution, α , in the present work is defined as the proportion of the reacted amino groups to the total of the amino groups originally available, and is expressed as a percentage. The amino groups are those being measured by the particular analytical procedure employed, and thus the values obtained by different methods need not be the same. The significance of these differences is discussed later.

The calculation, which is as follows, is carried out in terms of extinction per unit weight of protein.

Parent gelatin:

$$\begin{aligned}
 \text{Wt. of parent gelatin in 25 ml.} &= M \text{ mg.} \\
 \text{Protein (\%)} &= 100 - (\% \text{ ash} + \% \text{ moisture}) = X \\
 \text{Extinction of test solution after 20 min.} &= D_{p20} \\
 \text{Extinction of test solution after 40 min.} &= D_{p40} \\
 \text{Extinction of blank solution after 20 min.} &= B_{20} \\
 \text{Extinction of blank solution after 40 min.} &= B_{40} \\
 \text{Hydrolysis correction} &= \\
 (D_{p40} - B_{40}) - (D_{p20} - B_{20}) &= H_p
 \end{aligned}$$

Hence the colour yield per mg. of parent protein

$$= \frac{2500}{MX} (Dp_{20} - B_{20} - Hp) = Cp$$

Similarly for the substituted gelatin we have the colour yield per mg. of substituted protein

$$= \frac{2500}{NY} (Ds_{20} - B_{20} - Hs) = Cs$$

The degree of substitution, α , is given approximately by

$$\frac{(Cp - Cs)}{Cp} \times 100$$

This value is only approximately correct because no allowance has been made for the amount of substituent which has been added on to the protein, so that the protein in a given weight of sample is less than that used in the calculation.

The correct value is given by (Cobbett *et al.* 1964):

$$\alpha = \frac{(Cp - Cs)}{(Cp + kCs)} \times 100$$

where k is the maximum theoretical gain in weight for complete substitution of 1g. of dry, ash-free protein.

The evidence provided below indicates that the imidazole groups are not substituted by the benzenesulphonyl chloride or carbamoylated by cyanate under the conditions employed. The complete substitution of the α -amino and ϵ -amino groups (calculated from the work of Eastoe, 1955) in 1g. of gelatin (dry, ash-free), by benzenesulphonyl chloride would therefore increase the weight by 0.053g. It has been shown by Cobbett *et al.* (1964) that this reagent also reacts with the tyrosine, so that a value for k of 0.06 would be more accurate. For carbamoylation k is 0.02 (0.016g.).

Formol (pH 6.5–9.0) titration. A sample (0.5–3g.) of a gelatin or its derivative (about 0.5g. for an unmodified gelatin to about 3g. for a fully substituted gelatin) was accurately weighed into a 250 ml. beaker containing 100 ml. of water. The gelatin was allowed to swell in the water, brought into solution by warming at 40° and the beaker placed in a water bath maintained at 30 ± 0.5°. The stirred solution was adjusted to pH 6.5 with a Radiometer automatic titrator. A portion (25 ml.) of an approx. 10% (w/v) formaldehyde solution which had been previously adjusted to below pH 9 (pH 8.7–8.9) was added and the volume of approx. 0.02N-NaOH required to adjust the solution to pH 9.0 determined. A large volume of formaldehyde solution was prepared each day and stored in a beaker covered with a closely fitting plastic sheet containing a small hole for the pipette. The titration was carried out as rapidly as the response of the electrodes would allow to minimize side reactions. Blank determinations were carried out about every 2 hr. with 100 ml. of water that had been stored under the same conditions as the test solutions. This reading was subtracted from the other values before they were used in the calculations. For the most accurate work it is preferable to carry out the titration under N₂, any CO₂ having been swept from the solution. The results (Tables 3 and 4) indicate that the errors from this source cannot have been large. The calculation is carried out as described by Cobbett *et al.* (1964) for the formol titration carried out entirely at pH 9.0, i.e. Tp = ml. of 0.02N-NaOH/g. of

protein (moisture and ash-free) of the parent gelatin. Ts = ml. of the corresponding titration for the gelatin derivative. The degree of substitution, α , is approximately

$$\frac{(Tp - Ts)}{Tp} \times 100$$

or correcting for the residues added to the protein as described for the ninhydrin method

$$\alpha = \frac{(Tp - Ts)}{(Tp + kTs)} \times 100$$

Formol (pH 9.0–9.0) titration. This was carried out essentially as described by Cobbett *et al.* (1964), and is determined as in the above method except that the initial pH of the solution before the addition of formaldehyde was 9.0. The calculation is carried out as above. The results are in Table 3.

RESULTS AND DISCUSSION

Ninhydrin colorimetric procedure. The method described by Cobbett *et al.* (1964) is based on the observations of Slobodian, Mechanic & Levy (1962), who showed that both the α -amino and ϵ -amino groups of a protein react quantitatively with ninhydrin. The quantity of pigment produced by the ϵ -amino groups is about 65–75% of that obtained

Fig. 1. Effect of the period of heating on the yield of pigment in the ninhydrin colorimetric procedure. Extinctions were measured in 0.5 cm. cells against water at 570 mμ. O, 0.0201 g. of parent gelatin in 25 ml. of water. ●, 0.0201 g. of parent gelatin in 25 ml. of water, extinction corrected for reagent blank. Δ, 0.0646 g. of carbamoylated gelatin in 25 ml. of water. ▲, 0.0646 g. of carbamoylated gelatin in 25 ml. of water, extinction corrected for reagent blank. ■, Reagent blank. Calculated degree of substitution at α -amino plus ϵ -amino groups: 95.8% uncorrected; 98.5% corrected.

Table 2. *Degree of substitution at the α -amino plus ϵ -amino groups of gelatin as determined by the ninhydrin colorimetric procedure, with and without hydrolysis correction*

Values on the same horizontal line were determined in the same experiment. Method 1, 20 min./20 min.; method 2, 25 min./25 min.; method 3, 25 min./15 min.

Sample no.	Type of derivative	Type of gelatin	Initial heating period (min.)	Hydrolysis correction period (min.)	Degree of substitution (%)	
					Uncorrected	Corrected
C1	Carbamoylated	Alkali, hide	20	20	38.9	40.3
			25	25	38.0	39.1
			25	15*	39.5	39.3
C2	Carbamoylated	Alkali, hide	20	20	40.6	41.8
			25	25	38.4	38.3
			25	15*	41.1	40.6
C3	Carbamoylated	Alkali, hide	20		88.1	
			20		88.1	
			20	20	87.8	90.5
			25	25	87.6	90.2
			25	15*	87.4	89.5
C4	Carbamoylated	Alkali, hide	20		95.2	
			20	20	95.2	97.7
			25	25	94.1	97.7
			25	15*	93.4	96.0
C5	Carbamoylated	Alkali, hide	20		96.2	
			20		96.1	
			20	20	95.9	99.0
			20	20	95.8	98.8
			20	20	95.8	98.5
			25	25	95.7	98.8
			25	15*	94.8	97.8
C6	Carbamoylated	Alkali, hide	20		95.3	
			20	20	95.2	97.8
			25	25	94.4	97.7
			25	15*	94.0	96.8
BSC1	Benzenesulphonylated	Acid, pigskin	20	20	47.1	49.8
			25	25	44.0	45.6
BSC2	Benzenesulphonylated	Acid, pigskin	20	20	63.2	64.6
			20	20	64.3	65.7
			20	20	62.9	64.3
			25	25	63.3	66.5
BSC3	Benzenesulphonylated	Acid, pigskin	20	20	69.7	71.7
BSC4	Benzenesulphonylated	Acid, pigskin	20	20	75.1	77.8
BSC5	Benzenesulphonylated	Alkali, hide	20	20	93.6	95.1
BSC6	Benzenesulphonylated	Acid, pigskin	20	20	94.6	96.5

* Used to calculate 25 min. period correction.

from an equivalent quantity of an α -amino group. If the ϵ -amino group is on *N*-terminal or free lysine the figure is 7–10%. The imidazole groups do not react, nor do the amino groups when substituted. The diminution of the quantity of pigment formed after substitution is used to calculate the degree of substitution at the α -amino plus ϵ -amino groups. In most proteins, particularly those of high molecular weight, nearly all the colour arises from ϵ -amino groups and therefore the ninhydrin method should give very similar or identical values

to those obtained by the formol titration carried out at pH 9. It was observed, however, by Cobbett *et al.* (1964) that the ninhydrin values tended to be lower and this was attributed to hydrolysis of the peptide chain during colour development. Although the method compares the protein with its derivative the hydrolysis effects are unlikely to cancel because the colour resulting from hydrolysis for a protein and a series of derivatives is not a fixed proportion of the total colour. The ratio of colour resulting from hydrolysis to that from

Table 3. *Degree of substitution at the ϵ -amino groups of gelatin determined by the formol (pH 9.0-9.0) titration, and at α -amino plus ϵ -amino plus imidazole groups determined by the formol (pH 6.5-9.0) titration*

Sample no.	Type of derivative	Type of gelatin	Degree of substitution (%)	
			Formol (pH 9.0-9.0)	Formol (pH 6.5-9.0)
C1	Carbamoylated	Alkali, hide	39.2	33.1
			39.2	31.2
			38.6	
			38.9	
C2	Carbamoylated	Alkali, hide	40.6	33.6
			41.3	34.5
C3	Carbamoylated	Alkali, hide	90.2	77.4
			90.9	77.3
C4	Carbamoylated	Alkali, hide	98.9	86.6
			98.9	85.6
C5	Carbamoylated	Alkali, hide	99.8	90.3
			100.0	90.6
			100.0	
			99.0	
C6	Carbamoylated	Alkali, hide	98.7	84.0
			98.7	84.1
BSC1	Benzenesulphonylated	Acid, pigskin	49.0	43.4
			49.0	43.7
				45.5
BSC3	Benzenesulphonylated	Acid, pigskin	72.3	62.3
			71.6	60.3
BSC4	Benzenesulphonylated	Acid, pigskin		61.3
			78.6	67.8
BSC5	Benzenesulphonylated	Alkali, hide	78.6	67.8
			97.3	84.7
BSC6	Benzenesulphonylated	Acid, pigskin	98.8	84.2
			98.3	

Table 4. *Comparison of the values for the degree of substitution determined by the ninhydrin colorimetric method (corrected for hydrolysis), the formol (pH 9.0-9.0) titration and the formol (pH 6.5-9.0) titration*

The average values from Tables 2 and 3 are employed.

Sample no.	Method	Groups determined ...	Degree of substitution (%)			
			Ninhydrin (A)	Formol (B) (pH 9-9)	Formol (pH 6.5-9)	Calc.* from (A)
			($\alpha + \epsilon$)-NH ₂	ϵ -NH ₂	($\alpha + \epsilon$)-NH ₂ + imidazole	($\alpha + \epsilon$)-NH ₂ + imidazole
C1			40.3	39.0	32.2	35.3
C2			41.8	41.0	34.0	36.7
C3			90.5	90.5	77.4	79.3
C4			97.7	98.9	86.1	85.7
C5			98.8	99.5	90.4	86.7
C6			97.8	98.7	84.1	85.8
BSC1			49.8	49.0	44.2	43.7
BSC3			71.7	72.0	61.3	62.9
BSC4			77.8	78.6	67.8	68.2
BSC5			95.1	97.3	84.7	83.4
BSC6			96.5	98.5	84.2	84.6

* For calculation see the Discussion section.

α -amino plus ϵ -amino groups increases with the degree of substitution. In addition the hydrolysis rates of a protein and its derivative need not be the same.

Moore & Stein (1948) have shown that amino acids and some peptides yield all the pigment possible in the ninhydrin reaction within 20 min. at 100°. Fig. 1 shows that with both gelatin and its derivative the quantity of blue pigment continues to increase after this period. The shape of the curve for an almost fully substituted gelatin derivative is almost a straight line, even over a total period of 40 min., and indicates that most of the colour arises from α -amino groups released by hydrolysis of the peptide chain during colour development. The parent gelatin follows a similar course after 20–25 min. when all the α -amino plus ϵ -amino groups already present in the material have completely reacted, leaving the α -amino groups resulting from hydrolysis as the sole source of increasing colour intensity.

In the present work the method of correcting for hydrolysis effects has been to heat a second set of colorimetric solutions for an equal additional period and to subtract the increase in extinction from that obtained during the first period. Such an approach had to be a compromise between the following: (i) the initial heating period must be sufficiently long to allow all the colour to develop from the α -amino plus ϵ -amino groups originally present in the sample; (ii) the additional heating period must be long enough to give a sufficient increase in extinction to allow accurate corrections to be applied; (iii) the total of (i) and (ii) must not be too great otherwise colour-fading effects become important. Three sets of initial/additional heating periods (methods 1, 2, 3, see the Methods section) were examined.

Method 1 gave values 1.2–3.1% higher (difference between actual values and not calculated as % of the original value) than the uncorrected values. The ratio of colour due to hydrolysis effects to the colour from groups already present increases with the degree of substitution and hence the errors increase from about 1.5% for half-substituted materials to about 3.0% for almost fully substituted derivatives. The values from method 2 (Table 2) were also close to the above two sets of values, whereas with method 3 the errors arising from the smaller increment in extinction were rather large. The presence of the opposing effects of colour fading and colour from hydrolysis effects is suggested by the similarities of the corrected and uncorrected values at low degrees of substitution with the 25 min. initial period.

The reproducibility of method 1 was good, although it decreased slightly with the degree of substitution.

Formol titrations. Kenchington & Ward (1954) in their studies of the titration curves of gelatin alone at 40° showed that pH 6.5 is the most accurate boundary between the carboxyl group titration and the α -amino plus imidazole titration. At pH 6.5 less than 1% of the carboxyl groups remain to be titrated above this pH and are almost equal to and offset by the titration of the α -amino and imidazole groups remaining below this pH. Thus by adjusting the initial pH of the protein solution in the formol titration to pH 6.5 and then adding formaldehyde and measuring the quantity of alkali required to adjust the solution to pH 9, one obtains a measure of the α -amino plus ϵ -amino plus imidazole groups. The diminution of this titre on substitution is used to calculate the degree of substitution at these groups. The small amount of carboxyl group titration above pH 6.5 which remains after substitution results in a slight underestimate of the degree of substitution. Although Kenchington & Ward (1954) carried out their studies at 40°, the temperature of the formol titration was reduced to 30° to minimize side reactions. The results obtained show that no large errors resulted from this temperature difference (Table 4).

The formol titration which is carried out entirely at pH 9 does not measure all the ϵ -amino groups as some of them are already titrated at pH 9.0 in the initial pH adjustment (Kenchington & Ward, 1954; Kenchington, 1960). As a fixed proportion is titrated at this pH and the present method is a comparative one, the values for the degree of substitution will be unaffected by the procedure. By the use of pH 9.0 possible participation by imidazole groups is avoided.

The reproducibility of the formol (pH 9.0–pH 9.0) titration was similar to that of the ninhydrin procedure, and that of the formol (pH 6.5–pH 9.0) titration slightly poorer (Table 3). Values obtained by the former titration gave the expected close agreement with those from the modified ninhydrin method (Table 4).

Examination of derivatives. The values for the degree of substitution of the carbamoylated and benzenesulphonylated gelatins obtained by the formol (pH 6.5–pH 9.0) titration, which gives a measure of α -amino plus ϵ -amino plus imidazole, were considerably lower than those obtained by the other two methods and corresponded to no substitution having taken place at the imidazole groups. No reaction of cyanate with the imidazole groups has been reported in the papers by Stark *et al.* (1960) and Stark & Smyth (1963). Staab & Wendel (1960) have shown that the benzenesulphonyl derivative of imidazole is very rapidly hydrolysed at an alkaline pH. With the benzenesulphonylated gelatins the derivative which may have been formed with the imidazole in the early

stages of the reaction was unlikely to survive the complete preparation procedure, which employed a reaction period of 30 min. at 60° and pH 9. It has also been shown by Gurin & Clarke (1934) that the imidazole groups were essentially unaltered during benzenesulphonylation, the derivative giving as intense a colour with the Pauly diazo reaction as the original gelatin.

From the work of Eastoe (1955, 1961) on the amino acid composition of gelatins and Courts (1954) on the *N*-terminal groups of gelatins, it can be calculated that imidazole represents 10.7–12.6% (on a residue basis) of the amino (α -amino plus ϵ -amino plus imidazole) groups of alkali-processed gelatins and 10.4–15.2% of those of acid-processed gelatins, with an average value for all the gelatins of 12.3%. By assuming the imidazole to be completely unsubstituted, the degree of substitution for the total amino groups has been calculated from the ninhydrin and formol (pH 9.0–pH 9.0) values by multiplying them by $(100 - 12.3)/100$. The results of these calculations, which are in Table 4, show very good agreement with the experimental values obtained by the formol (pH 6.5–9.0) titration. There is a tendency for the experimental values to be slightly lower than those of the calculated values, particularly when compared with the values from the alternative formol procedure. This can be attributed to the small amount of carboxyl titration which exists above pH 6.5 and which behaves in the procedure as unsubstituted amino groups.

Once it is established that the imidazole groups do not react in a particular preparative procedure, attention can be confined to the other amino groups and the ninhydrin technique employed, which requires only a few milligrams of the parent protein and its derivative. The additional advantage of the ninhydrin procedure is that a number of samples can be examined simultaneously. In cases where the imidazole groups do react the pH 6.5–pH 9.0 formol titration must be employed.

Application of methods to other proteins. The techniques described in the present work have been applied to gelatin derivatives and the following points would need to be considered before applying them to other proteins. If the number of amino groups per unit weight of protein is much greater than that for gelatin, then the quantity of protein used in the three techniques should be reduced, and vice versa. For the ninhydrin method curves

of the type shown in Fig. 1 would indicate whether other colour-development times and hydrolysis corrections might be more appropriate than those used with gelatin and its derivatives. With non-gelling proteins the temperatures at which the formol titrations are carried out can be reduced, thereby further reducing the side reactions between the formaldehyde and the protein.

The lower limit of pH 6.5 used in one of the formol titrations needs consideration with other proteins. Kenchington & Ward (1954), in their study of the titration curves of gelatins, point out that this limit may not apply to other proteins unless the ratio of histidine to dicarboxylic acid residues is similar to that in gelatin. A study of the titration curve of the protein in the manner of these authors would probably give the required information. It would not be sufficient merely to move the boundary to a lower pH so that the carboxyl and imidazole titrations cancel, because, as mentioned above, when the protein is substituted the increased carboxyl titration above the boundary would behave in the procedure as unsubstituted amino groups.

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