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The *N*-Terminal Amino Acid Residues of Gelatin

1. INTACT GELATINS

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During the past five or six years there has been a considerable revival of interest in the biochemistry and physical chemistry of collagen and gelatin (Randall, 1953; Pouradier & Venet, 1950, 1952; also others). Gelatin may be defined as the protein derived from collagen by irreversible procedures which render the collagen water-soluble. The irreversibility differentiates gelatin from 'soluble collagen'. Gelatin is not homogeneous within any one sample, and different samples differ in important properties and structural characteristics (e.g. molecular weights, molecular-weight distribution, isoelectric point, viscosity of solutions, rigidity of gels). The possibility arises that these differences may be due in part at least to the method of manufacture, of which there are two main procedures, the acid process and the alkali process (Ward, 1951; Ames, 1952) or to the type of collagenous tissue used. There are three principal sources of collagen used commercially: skin, bone and sinew. This fact was not always appreciated in the past by those unfamiliar with the industrial process and has often led to difficulties in correlating results of different workers engaged on similar research.

The nature of the bonds broken when collagen is converted into gelatin is still a matter of speculation, and the problem is evidently connected with theories of the forces which bind individual protein chains into collagen fibrils. Recent views put forward include hydrogen bonding (Gustavson, 1950), the presence of chondroitin sulphuric acid (Jackson, 1953) and a —CO—NH—CO— linkage (Ames, 1952). Bowes & Moss (1951), using Sanger's 1-fluoro-2:4-dinitrobenzene (FDNB) technique, have shown that, while native collagen appears to have no *N*-terminal end groups, gelatin gives small amounts of *N*-terminal aspartic acid, glutamic acid, glycine and threonine. It appears, therefore, that the rupture of peptide bonds may be involved in the conversion of collagen into gelatin.

The purpose of the present investigation was to ascertain any gross differences in *N*-terminal residues between gelatins with different collagenous precursors or with the process of manufacture used.

The *N*-terminal-residue method gives useful approximations for the number-average molecular weights of gelatin samples, based on the assumption that the protein comprises single polypeptide chains each with one *N*-terminal residue.

As Sanger's end-group technique has been applied to an increasing number of proteins, certain workers have reported that not all of the ϵ-amino groups of lysine and hydroxylysine residues are available to react with FDNB, although in some instances these groups are available to nitrous acid (Salo, 1950) or keten (Porter, 1948). Porter found that more than one-third of ϵ-amino groups of β-lactoglobulin and of several serum globulins failed to react with FDNB when these proteins were in the native state. On denaturation all the groups became available to the reagent. It was suggested, therefore, that the configuration of the polypeptide chain in the native protein could not accommodate the bulky 2:4-dinitrophenyl (DNP) group. A similar phenomenon has been reported with collagen and gelatin, although there is disagreement over the extent of availability of ϵ-amino groups; Bowes & Moss (1951) found about 50% availability with native collagen and gelatin, while Sykes (1952) reported about 75–80% availability with hide powder. It was therefore of interest to examine DNP-gelatins for their content of N^{ϵ}-DNP-lysine.

MATERIALS AND METHODS

Gelatins. Eleven high-grade gelatins were chosen to give a wide cross-section of types, and these are described in Table 1. Eight of these were commercial samples; *F* and *J* were laboratory preparations kindly given by Dr W. M. Ames; *L* was prepared from isinglass, made from Beluga sturgeon (*Huso huso*) swim bladder which was kindly supplied by James Vickers and Co.

Reaction with FDNB. The method used was essentially that of Sanger (1945). It was found, however, that gelatin was sensitive to the rapid substitution which occurred in the presence of ethanol, yielding a viscous liquid identical in appearance with the DNP-derivative of a highly degraded gelatin. Ethanol was therefore excluded from the reaction mixture. A solution of gelatin (2 g.) in water (100 ml.) and 8% (w/v) $NaHCO_3$ (100 ml.) was shaken with excess FDNB (4 ml., 5·7 g.) for 24 hr. The solution was initially at 35°, decreasing to room temp. after about an hour. Neutralization with dilute hydrochloric acid deposited a yellow gel, which was dried over P_2O_5 and NaOH, crushed in a mechanical disintegrator and washed with acetone until no further yellow colour was removed. The powder was dried at 105° and allowed to equilibrate with the atmosphere. Samples were then taken for moisture, ash and end-group determinations. The mother liquors contained only a negligible quantity of soluble DNP-peptides and so were ignored. DNP-gelatin was similar to the unsubstituted gelatin in having a moisture content which was very sensitive to atmospheric humidity. The ash content was largely NaCl formed during neutralization. It was found that this salt had no deleterious effect on the stability of DNP-amino acids during the hydrolysis of DNP-gelatin and a similar lack of effect was found with NaF and $MgSO_4$. Consequently, it was unnecessary to remove the salts by prolonged washing with water and hence the risk of removing any soluble DNP-peptides was eliminated. This precaution was particularly important when dealing with acid-processed gelatins whose DNP derivatives were markedly water-soluble.

Moisture. About 100 mg. of material was heated at 105° for 16 hr. in a micro moisture dish.

Ash. Samples (about 200 mg.) were ashed in Pt dishes at 550° for 16 hr.

Hydrolysis. DNP-gelatin (about 500 mg.) was heated with 5·5 N-HCl for 16 hr. in sealed tubes at 100°. These conditions lead to destruction of free DNP-proline and DNP-hydroxyproline (Porter & Sanger, 1948). It was therefore necessary to choose alternative hydrolysis conditions when attempting to detect these amino acids as *N*-terminal residues. Porter & Sanger showed that the complete destruction of the DNP-derivatives may be avoided by employing 12 N-HCl at 105° for 16 hr., giving a recovery of 50%. Bowes & Moss (1953) recovered 20% of DNP-proline using similar conditions in the presence of collagen. The present author could only recover 15% of DNP-proline using these hydrolysis conditions in the presence of gelatin. Consequently, in attempts to detect DNP-proline and DNP-hydroxyproline in DNP-gelatin hydrolysates, about 4 g. of DNP-gelatin were subjected to the conditions of Porter & Sanger, to make it possible to detect them with certainty. The DNP derivatives of acidic and neutral amino acids were removed from the hydrolysate by extraction with ether.

Identification. The DNP-amino acids were separated chromatographically on buffered Celite columns (Celite 545, Johns-Manville Co., London) using essentially the method described by Perrone (1951).

Two columns were required. The DNP-amino acid mixture was introduced firstly to the top of a column of 4·5 g. Celite (0·5 ml. stationary phase/g. Celite) buffered at pH 4 with phosphate–citric buffer (54 ml. 0·2 M-Na_2HPO_4 + 46 ml. 0·2 M citric acid), the solvent being $CHCl_3$–ether (90:10 by vol.). All artifacts and DNP-amino acids, with the exception of DNP-glutamic acid (*R*, 0·4), DNP-threonine (*R*, 0·2), DNP-serine (*R*, 0·1), DNP-aspartic acid (*R*, 0·05) run fast on this column. These four derivatives separate well and their elution may be speeded up by the following procedure. When DNP-glutamic acid has been run off, the solvent is changed to $CHCl_3$–ether (50:50 by vol.); when DNP-threonine has been run off, the solvent is changed to $CHCl_3$–ether (20:80 by vol.); when DNP-serine has been run off the solvent is changed to $CHCl_3$–ether (5:95 by vol.). When DNP-aspartic acid was removed, the column was washed through with about 10 ml. of $CHCl_3$ in preparation for the

Table 1. *Origin and properties of gelatin specimens*

Key	Process	Origin	Jelly strength* g.	Viscosity† cp.	Reduced viscosity‡
A	Alkali	Ox bone	217	7·05	0·59
B	Alkali	Ox bone	186	5·33	0·45
C	Alkali	Calfskin	208	7·65	0·60
D	Alkali	Calfskin	250	7·40	—
E	Alkali	Oxhide	191	7·79	0·56
F	Alkali	Ox sinew	274	7·75	—
G	Acid	Pigskin	235	7·31	0·43
H	Acid	Pigskin	221	5·62	0·39
J	Acid	Ox sinew	276	4·60	—
K	Acid	Ox bone	242	4·15	0·34
L	Acid	Swim bladder	—	—	—

* Essentially a measure of gel rigidity. Determined according to British Standards Institution (1944) 757 at 6·67% (w/v).

† Determined according to British Standards Institution (1944) 757 at 6·67% (w/v) and 40°.

‡ The reduced viscosity, as defined by Stainsby, Saunders & Ward (1954) and expressed as $1/c \ln \eta_{rel.}$, was determined at pH 7 in M-NaCl at 35°, the gelatin concentration, *c*, being 0·5% (w/v). Under these conditions, small changes in pH or ionic strength produce only very small changes in the value of $1/c \ln \eta_{rel.}$. The author would like to thank Dr G. Stainsby for these determinations.

Table 2. *N-terminal amino acid residues of various gelatins*

	moles/100000 g. gelatin (moisture and ash free)										
	Alkali-processed						Acid-processed				
	A Ossein	*B* Ossein	*C* Calf	*D* Calf	*E* Oxhide	*F* Sinew	*G* Pigskin	*H* Pigskin	*J* Sinew	*K* Ossein	*L* Isinglass
Glycine	0·95	0·98	0·83	0·80	1·27	1·00	0·74	0·59	0·96	0·72	0·25
Serine	0·24	0·23	0·19	0·19	0·24	0·23	0·10	0·05	0·04	0·06	0·05
Threonine	0·11	0·10	0·11	0·11	0·14	0·14	0·08	0·03	0·03	0·05	0·03
Alanine	0·13	0·17	0·11	0·10	0·20	0·17	0·24	0·18	0·23	0·16	Nil
Aspartic acid	0·10	0·12	0·11	0·08	0·14	0·15	0·13	0·06	0·02	0·04	0·03
Glutamic acid	0·14	0·13	0·10	0·10	0·15	0·12	0·07	0·04	0·04	0·03	0·04
Others	0·01	0·14	0·12	0·12	0·20	0·08	0·19	0·19	0·11	0·05	Nil
Total	1·68	1·87	1·57	1·50	2·34	1·89	1·55	1·14	1·43	1·11	0·40
Number-average mol.wt. $\times 10^{-3}$	60	53	64	67	43	53	65	87	70	88	250

Table 3. *Availability of ε-amino groups to FDNB in various gelatins*

Key	ε-Amino group availability (%)
A	100
B	97
C	97
E	96
G	97
H	88
K	90

following procedure. The fast band previously obtained from this column was evaporated to dryness and taken up with 0·5 ml. $CHCl_3$. When reintroduced to the top of the column and eluted with $CHCl_3$, DNP-glycine (*R*, 0·5) separated well from the faster-moving material. This last, after evaporation to dryness, was dissolved in 0·5 ml. $CHCl_3$ and run on a 3 g. Celite column buffered at pH 7·1 (83 ml. 0·2M-Na_2HPO_4 + 17 ml. 0·2M citric acid), using $CHCl_3$ initially as the moving phase, until the artifacts (2:4-dinitroaniline and 2:4-dinitrophenol) were removed. The eluent was then changed to ether, enabling the separation of DNP-phenylalanine (*R*, 0·6), DNP-valine (*R*, 0·3) and DNP-alanine (*R*, 0·07). When only DNP-alanine remains on the column, its elution may be speeded up by adding 2 drops of glacial acetic acid to 10 ml. of the solvent. The proportion of aqueous and organic-solvent mixtures used in the preparation of these two-phase liquid mixtures was not found to be critical for the separations of the DNP-amino acids associated with gelatin. The various bands were compared with authentic samples of DNP-amino acids under identical conditions.

Many workers, and Mills (1952) in particular, have noted the adverse effects of daylight on the stability of DNP-amino acids. An important feature of this method of separation is the speed of operation, and the eight *N*-terminal residues associated with gelatin may be isolated within 2 hr. Each DNP-amino acid solution collected was taken to dryness on the rotary evaporator (Partridge, 1951) and estimated colorimetrically in 1% (w/v) $NaHCO_3$ solution with the Spekker absorptiometer (Hilger and Watts Ltd., London) using Ilford 601 filters (Ilford Ltd., London) at 430 mμ. Confirmation of the identity of the DNP-amino acids was effected by de-arylation according to the method of Lowther (1951). The DNP-amino acid was heated in aqueous NH_3 (sp.gr. 0·880) in sealed tubes at 100°. The free amino acid thus obtained was identified on paper chromatograms alongside authentic samples.

ε-Amino groups of lysine. About 10 mg. of DNP-gelatin was hydrolysed with 5·5N-HCl for 16 hr. in sealed tubes at 100°. The hydrolysate was extracted with ether and the aqueous liquors were evaporated to dryness over P_2O_5 and NaOH. The residue was taken up with methyl ethyl ketone–ether (2:1 by vol.), which had been previously saturated with water, until no further yellow colour was extracted. The extract was introduced to the top of a Celite column buffered at pH 7·1 (83 ml. 0·2M-Na_2HPO_4 + 17 ml. 0·2M citric acid) using methyl ethyl ketone–ether (2:1 v/v) as the organic-phase eluent.

The column showed 3 bands: a fast artifact (*R*, 1·2), N^{ϵ}-DNP-lysine (*R*, 0·2) and a slow band (*R*, 0·1), presumed to be N^{ϵ}-DNP-hydroxylysine. This last band has not been confirmed but occurs in such proportions as to give a colour density relative to that of the N^{ϵ}-DNP-lysine as 1:4·5, whereas the proportion of hydroxylysine to lysine in gelatin is reported as 1:4·2 (Chibnall, 1946). Calculations of the recovery of N^{ϵ}-DNP-lysine from DNP-gelatin (after allowing for 30% decomposition during hydrolysis) are based on a lysine content of 4·3 g./100 g. gelatin (Chibnall, 1946). This value is in good agreement with a value of 4·3 g. for gelatin using the technique of Moore & Stein (1951) (Dr Eastoe, private communication) and with a value of 4·18 g. for hide collagen (Salo, 1950).

RESULTS

In all samples of gelatins examined, the principal *N*-terminal residues have been found to be glycine, serine, threonine, aspartic acid, glutamic acid and alanine. Small amounts of valine and phenylalanine and possible traces of others are also found and are listed collectively as 'others' in Table 2.

It was found that, for DNP-amino acids occurring in small amounts, agreement between duplicates was within 5–15%. Agreement for DNP-glycine was generally to within 5%. It is probable, therefore, that the figure for total *N*-terminal residues, and consequently for the number-average molecular

weights calculated from this total, is of similar reproducibility.

ε-Amino-group availability: Values, shown in Table 3 correspond to almost complete reaction between the ε-amino groups situated on lysine residues and FDNB.

DISCUSSION

Glycine is the predominant *N*-terminal residue of gelatin and accounts for at least half of the total residues. No gelatin has been found which is an exception to this rule. Again, all gelatins so far examined, whatever their origin or process of manufacture, clearly show the presence of *N*-terminal serine, threonine, aspartic and glutamic acids and alanine residues as well as traces of others. Differences in the proportions of these subsidiary residues, however, are detected. These differences depend upon whether the gelatin was obtained by acid or alkali pretreatment. The hydroxyamino acids, serine and threonine, and the dicarboxylic amino acids, aspartic acid and glutamic acid, all occur in much larger proportions in alkali-processed materials. On the other hand, the proportion of alanine tends to be larger in acid-processed gelatin. The alkali process hydrolyses a number of side-chain amide groups to free carboxyl groups. The proportion of altered groups will depend upon the intensity of pretreatment. Thus a collagen which has received prolonged alkali treatment will yield a gelatin of isoelectric point about pH 4·8. Less intensive treatment yields gelatins of isoelectric point between this value and about pH 5·5. Gelatins obtained by the acid process generally have the isoelectric point in the region of pH 9. Hence, the two processes yield gelatins of slightly different chemical constitution, differences which could possibly modify the lability of different links of the peptide chain over and above the inherent differences in lability to acid and alkali of the various peptide bonds. No significant differences of *N*-terminal residues have been found which are attributable to the origin of the collagenous precursor. There is evidence from X-ray diffraction studies on collagens of different origins (Astbury, 1940) for a fundamental structural identity. Amino acid analysis (Neuman, 1949; Neuman & Logan, 1950) also shows that gelatins derived from hide, bone and sinew by the same process are of similar chemical composition. Table 2 indicates, however, that in the transformation of collagen to gelatin certain peptide bonds are broken and the nature of some of these bonds will depend upon the process employed. The high-grade alkali-processed gelatins examined showed one mole of *N*-terminal residue in approx. 60000 g. of gelatin the range (excluding sample *E*) being 53000 to 67000. These are number-average values of the molecular weight of a molecularly heterogeneous system and the samples may well contain fractions ranging from one-quarter to 4 times this value (Pouradier & Venet, 1950; Stainsby *et al.* 1954). The mean values are in reasonable agreement with those of Pouradier & Venet (1950), who, using similar types of gelatin, obtained values of 61000 to 67000 by osmotic-pressure measurements. The agreement supports the single-chain structure for gelatin.

The molecular weights calculated for the acid-processed gelatins are somewhat higher than for alkali-processed, corresponding to the detection of fewer *N*-terminal residues. Further considerations, however, suggest a cautious approach to these values (Table 2). A discrepancy with these samples is found in the reduced viscosity values (Table 1), these being substantially lower than might be expected from the values for alkali-processed gelatins. It will, however, be necessary to await physical measurements of the molecular weights, before these results can be explained.

Note added in Proof. The recent paper of Grassmann, W. & Hörmann, H. (1953), *Hoppe-Seyl. Z.* **292**, 24, reports both the *N*-terminal and *C*-terminal groups of gelatin. The *N*-terminal groups correspond closely with those observed in the present work.

SUMMARY

1. The *N*-terminal residues of a number of gelatins have been determined using Sanger's fluorodinitrobenzene technique. Glycine was found to be the predominant end group. Serine, threonine, alanine, aspartic acid and glutamic acid residues also occurred.

2. The number-average molecular weight of these samples was calculated assuming one *N*-terminal residue in each molecule. Average values of 60000 were in agreement with those obtained by osmotic-pressure methods and support the view of a non-branching peptide chain.

3. The method has been used to determine the lysine residues of gelatin. Generally, over 90% of the ε-amino groups of lysine were available to the reagent.

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The *N*-Terminal Amino Acid Residues of Gelatin

2. THERMAL DEGRADATION

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Gelatin in solution undergoes changes in the presence of proteinases or at elevated temperatures, especially at acid or alkaline pH values, to which the general term 'degradation' is given. The effect of degradation in reducing the solution viscosity, and in impairing the gel-forming power, is so readily demonstrated for gelatin, as compared to other proteins, that it has received considerable study. The change in physical properties has frequently been used as a measure of the extent of the degradation reaction (Sheppard & Houck, 1930, 1932; Pouradier & Venet, 1952 and previous papers; Gerngross & Brecht, 1922; Ames, 1947). In view of the uncertain relationships between such properties and the rate of bond breaking the results obtained must be looked on as only empirical, although of considerable practical value.

The degradation of gelatin gives rise to an increase of α-amino groups and this property has been used for assessing the degree of hydrolysis. Northrop (1921*a*, *b*) has used the formol-titration method. Greenberg & Burk (1927) have employed the Van Slyke nitrous acid method. The predominance in gelatin of ε-amino groups of lysine and hydroxylysine residues would make small changes in α-amino groups difficult to detect by these techniques (the proportion of α- to ε-amino groups in material of molecular weight 50000 is 1:20). In addition, neither method will distinguish between α-amino groups formed by rupture of peptide bonds and any other amino groups such as would be involved in the possible conversion of an arginine residue into ornithine. These methods have been mainly of value in studying extensive degradation where the number of α-amino groups released has become large.

The *N*-terminal residue method as described in the preceding paper (Courts, 1954) is likely to be of value in following the course of protein degradation, especially in the early stages where preferential breaking of labile bonds may occur. Thus the identification of the *N*α-2:4-dinitrophenylamino acids would give some information as to the nature of the weaker peptide bonds and also a measure of the extent of hydrolysis. This paper reports some experiments on the controlled thermal degradation of gelatin using the *N*-terminal residue method. The theoretical considerations arising from the results are discussed and related to the physical properties of the gelatins obtained.

MATERIALS AND METHODS

Gelatin. A de-ionized ossein gelatin of isoelectric point pH 5·1 was used. This was prepared from bone collagen and characterized by its Bloom jelly strength (6·67% concentration), 217 g., and viscosity (40° and 6·67% concentration) 7·05 centipoise.

Bone collagen. This was prepared by the method of Eastoe & Eastoe (1954). I am indebted to Dr J. E. Eastoe for a gift of this material.

Technique of degradation. The extent of protein hydrolysis is determined by time and temperature of heating and by pH of solution. Each of the three was varied in turn while the other two were kept constant. Degradations were carried out in sealed tubes using 5% (w/v) solutions.

The pH of gelatin solutions shifts slightly during degradation (Ames, 1947) towards the isoelectric pH, the shift becoming very large at high pH values. This effect is minimized when sealed tubes are used, but no attempt was made to maintain the pH values constant during degradation with buffers since this was likely to introduce unwanted complications. All pH measurements were made with a Marconi pH meter (Marconi Instruments, London) using