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N-TERMINAL RESIDUES IN ACETONE-PRECIPITATED COMMERCIAL GELATIN CHANGES IN THE APPARENT AVERAGE CHAIN WEIGHT

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SUMMARY

Physically associated non-protein nitrogen constituents were removed from a sample of commercial gelatin by serial acetone precipitation of the protein from dilute acetic acid solution. The average chain weight of the gelatin, 55 000, (measured by N-terminal analysis) increased after each precipitation until a limiting value of 104000 was obtained which was in good agreement with the values quoted in the literature for thermally degraded ichthyocol gelatin. The significance of these observations has been discussed in relation to the structure of the collagen fold and the possible multi-chain structure of gelatin. Attention has been drawn to the fact that the determination of the average chain weight of a high-molecular-weight protein is meaningless unless physically associated non-protein nitrogen constitutents are carefully removed prior to the N-terminal analysis.

INTRODUCTION

Commercial gelatins (as defined by WARD[1]) are well known to be mixtures of partial-degradation products obtained during the thermal denaturation of acid- or alkali-pretreated collagenous tissues. STAINSBY[2] claimed that fractionation of commercial gelatin by alcohol coacervation was more satisfactory than the method of alcohol precipitation used by SINGER AND MOSIMANN[3].

COURTS AND STAINSBY[4] prepared fractions of a lime processed hide gelatin by alcohol coacervation and determined the weight average molecular weight (M_w) by the method of light scattering and the average chain weight (C_n) by N-terminal analysis[5]. They found that M_w values of the fractions showed a wide variation but that the values of C_n were virtually constant. The weight average molecular weights of certain fractions were found to be five or six times greater than the corresponding average chain weights. This observation suggested that these fractions were made up of molecules with an average of five or six associated (or cross-linked) polypeptide chains. Support for the concept that gelatin might exist as a multi-chain molecule (or molecules) was provided by the suggestion that collagen contained interchain ester linkages[6,7] and by the finding that thermally denatured soluble collagen contained β- and γ-components

which were composed of two and three associated polypeptide chains respectively[8].

Soluble collagen has been shown to contain free amino acids and peptides physically associated with the protein[9] whilst a similar observation was reported for commercial gelatins[16]. These physically associated non-protein constituents may be separated by acetone precipitation of the protein from acid (pH 3.5) or alkaline (pH 11) solution and it was shown that the C_n value of the precipitated protein was thereby increased[11].

The alcohol coacervation technique used by COURTS AND STAINSBY[4] was carried out at neutral pH and this is significant since near neutrality the non-protein nitrogen components were most strongly held to the collagen[9]. Ionic attraction between oppositely charged polar groups of the protein and associated molecules would be expected to be at a maximum under these conditions. A theoretical graph is presented in Fig. 1 to represent the change in apparent C_n value of a mixture of gelatin (limiting C_n approx. 100000) and a variable amount of amino acids (average molecular weight taken as 100 for ease in calculation). This graph emphasises the importance of

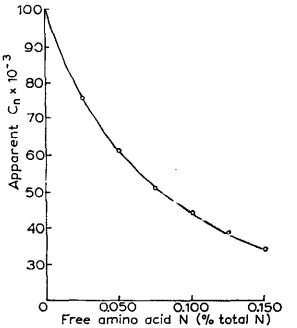

Fig. 1. Theoretical graph of the changes in the apparent average chain weight of a mixture of gelatin (mol. wt. 100000) and free amino acids (mol. wt. 100).

the removal of non-protein constituents from a high-molecular-weight protein before the average chain weight of the protein can be determined by analysis of terminal amino groups.

The present work describes the influence of successive acetone precipitations on the apparent average chain weight (C_n) of the commercial gelatin referred to as "X" by COURTS AND STAINSBY[4].

MATERIALS

The sample of commercial lime processed calf hide gelatin used in this study has been extensively examined by other workers and was generously supplied by the British Gelatine and Glue Research Association, London. This material has been variously described as Gelatin "X", "X-127" or simply "127" (see refs. 1, 4, 12–14). COURTS[15] referred to this same material as lime processed gelatin "C" (personal communication).

METHODS

Serial acetone precipitation of the gelatin from 0.1 M acetic acid was found to be incomplete after the addition of 6–8 vol. of acetone, part of the protein remained as a colloidal suspension which could be precipitated by the addition of a few drops per litre of saturated NaCl solution. The precipitated protein was redissolved in 0.1 M acetic acid and samples removed for total nitrogen and duplicate N-terminal analyses. The remaining solution was recyclised twice through the acetone-precipitation process.

A separate sample of the gelatin was taken through ten cycles of the acetone-precipitation process in order to determine whether a limiting value of C_n was obtained after exhaustive precipitation.

Dinitrophenylation of the gelatin was carried out by a modification of the technique of SANGER[5] and the DNP-protein was hydrolysed by the resin method[16]. The chromatographic isolation and estimation of the individual DNP-amino acids was carried out as already described[17]. The presence of two brown artifacts on paper chromatograms in the neighbourhood of DNP-serine and DNP-alanine interfered with the estimation of these derivatives dissolved in 1 % (w/v) $NaHCO_3$. This difficulty was overcome by adding 0.2 ml glacial acetic acid to the 10 ml $NaHCO_3$ which had been used to extract the DNP-derivatives. Under these conditions the artifacts were decolourised and caused no interference in the estimation.

RESULTS

Two samples of gelatin were taken through three precipitation stages and their N-terminal analyses were found to be almost identical after the third precipitation, although slight variation was observed after the first and second precipitation. This situation

TABLE I

N-TERMINAL RESIDUES IN ACETONE-PRECIPITATED GELATIN X-127

Values expressed as moles per 10^6 g gelatin.

Residue	*Number of precipitations*				
	0	*1*	*2*	*3*	*10*
Gly	10.30	8.40	6.90	6.00	6.00
Glu	1.04	0.98	0.90	0.63	0.62
Asp	0.70	0.59	0.48	0.38	0.39
Ser	2.19	2.18	2.00	1.90	1.66
Thr	1.02	0.72	0.51	0.35	0.34
Ala	1.05	1.00	0.69	0.60	0.59
Val	0.23	0.10	Trace	Trace	Trace
Leu/Ileu	0.66	Trace	Trace	Trace	Trace
Phe	0.34	Trace	—	Trace	—
Lys	Trace	Trace	—	—	—
Tyr	Trace	—	—	—	—
Pro	0.24	—	—	—	—
Hypro	0.57	0.17	—	—	—
Total	18.34	14.14	11.48	9.86	9.60
$C_n \times 10^{-2}$	55	71	89	101	104

might be expected since it is clear that the removal of non-protein nitrogen is incomplete after a single acetone precipitation of gelatin. A typical series of results is presented in Table I. Exhaustive acetone precipitation yielded a pattern of N-terminal residues similar to that found after three precipitations.

DISCUSSION

Acetone precipitation of commercial gelatin dissolved in 0.1 M acetic acid released physically associated non-protein nitrogen from the protein. Although only a very small quantity of non-protein nitrogen was released (approx. 2% of original total nitrogen) the effect was to double the apparent C_n value of the gelatin. Only a fraction of the non-protein nitrogen was found to represent free α-HN_2 groups on the original gelatin (*viz.* equivalent to 0.07% original total nitrogen) when calculated by difference between the original and limiting values of C_n. This increase in C_n was virtually complete after three precipitations and reached a limiting value of approx. 104000 after ten precipitations. This value of the chain weight derived from chemical analysis is in fair agreement with the value of 135000 obtained from physical determinations by BOEDTKER AND DOTY[18] for ichthyocol "Parent gelatin". The C_n value reported here for gelatin stripped of physically bound non-protein nitrogen is compatible with single-chain molecules of this molecular weight (*cf.* BOEDTKER AND DOTY[18]) and/or the presence of triple-chain units of molecular weight comparable with the figure quoted by GROSS[19] for the tropocollagen molecule of 340000. The variation in the weight average molecular weights (*e.g.* 60000–300000) obtained from light-scattering studies[4] for the fractions of gelatin X-127 could be explained if both single and cross-linked chains, with a maximum of three polypeptide chains per molecule, were present. The lower values of C_n quoted by COURTS[15] for gelatin "C" of 64000 and for fractions of X-127, *e.g.* 55000–70000, quoted by COURTS AND STAINSBY[4] suggested an average of five or six chains per molecule in certain fractions with weight average molecular weight of 300000. It is suggested from the present work, if multi-chain gelatin molecules are present in X-127, that a maximum of three chains per molecule is possible. This suggestion would be in agreement with the isolation of triple-chain peptides from the tryptic digestion of heat-denatured soluble collagen[20].

ZAHN AND MEIENHOFER[21,22] demonstrated the physical binding of amino acids and peptides to wool keratin and the presence of these compounds might account for the large variety of N-terminal amino acids detected in wool[23,24]. It is of interest that all the species of amino acid N-terminal residues found in wool also occur as physically associated amino acids in collagen and gelatin and, as such, in the latter instances contribute to the N-terminal residues when examined by the DNP-technique. SCHNEIDER *et al.*[25,26] have demonstrated a similar binding of non-protein nitrogen by gluten and it may be that the binding of small quantities of non-protein nitrogen by proteins is a more wide-spread phenomenon than has hitherto been recognised (*cf.* SYNGE[27]). The amphoteric nature of amino acids would appear to offer excellent sites for ionic linkages with oppositely charged ionic groups on proteins. This type of binding might be expected to be strongest near neutral pH for most proteins and weakest at extremes of pH. This situation was in fact found to take place in the binding of non-protein nitrogen to collagen[11].

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