ADDENDUM

The Presence in Commercial Sulphonated-Polystyrene Resins of some Amino Acids

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Samples of Dowex 50 and Zeo-Karb 225 obtained fresh from the manufacturers were shown to contain trace amounts of amino acids which could be displaced by distilled N-ammonia. The quantities of these amino acids in the resin would have little influence on the total amino acid analysis of a protein hydrolysate, but their presence was sufficient to cause errors in the micro-analysis of non-protein-nitrogen fractions which were desalted on the resins before their analysis by paper chromatography.

Dowex 50, which had previously been brought to the state when no amino acids were eluted with ammonia (as shown by paper chromatography), yielded small but significant amounts of amino compounds after regeneration by the method of Hirs, Moore & Stein (1952). This procedure involves heating the resin at 70° in 2N-NaOH for 5 hr. and complete removal of the amino acids was effected only on prolonged washing with water immediately after digestion with 2N-sodium hydroxide; the normal method of washing to neutrality was insufficient to remove all the amino acid.

Trace amounts of the following amino acids, listed so far as possible in decreasing order of concentration, were detected after elution of a resin column with ammonia: glycine, serine, alanine, aspartic acid, glutamic acid, threonine, leucine.

All batches of resin used to desalt the non-protein-nitrogen fractions were first shown to be

free of amino acids. The results presented in Table 2 of the main paper for the non-protein-nitrogen fraction (estimated as dinitrophenyl derivatives) which had not been desalted and the free amino acids in the non-protein-nitrogen fraction which had been desalted on the resin were quantitatively in good agreement with the major components of the non-protein-nitrogen fraction. This gave further evidence that the resin did not contribute amino acids to the analysis of the non-protein-nitrogen fractions.

It has also been found that water prepared by de-ionization (Deminrolit, The Permutit Co. Ltd.) contained significant amounts of free amino acids. Accordingly glass-distilled water was used in the present investigation.

R. L. M. Synge and Mary A. Youngson (personal communication) have observed the liberation of the same amino acids, under similar conditions, from Dowex 50, Zeo-Karb 225 and Amberlite CG-120, amounting to about 1 µg. of α-amino nitrogen/ml. of resin bed.

Lindlar (1960) has also observed the liberation of ninhydrin-staining substances from an anion-exchange resin.

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The Origin of N-Terminal Residues in Acetic Acid-Soluble Calf-Skin Collagen

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Insoluble collagen is usually considered to have no N-terminal residue, although Bowes, Elliott & Moss (1957) detected traces of aspartic acid and alanine as N-terminal groups in citrate-soluble

calf-skin collagen. Evidence is presented below for the presence of detectable quantities of 13 amino acids in N-terminal positions in acetic acid-soluble collagen. Steven & Tristram (1962) described the

Table 1. *N*-Terminal residues of acetic acid-soluble collagen, gelatin prepared from this soluble collagen, and acetone-precipitated collagen

N-Terminal residues are shown both as determined (a) and corrected for overall loss (b). Values are expressed as moles/10⁶ g. of protein.

Dinitrophenylated amino acid	Overall recovery (%)	Acetic acid-soluble collagen (A)		Gelatin		Acetone residue (B)		Acetone residue (C)	
		(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Gly	16	0.02	0.13	0.02	0.13	0.03	0.19	0.02	0.13
Glu	60	0.10	0.17	0.07	0.12	0.07	0.12	0.07	0.12
Asp	60	0.10	0.17	0.09	0.15	0.09	0.15	0.09	0.15
Ser	35	0.13	0.37	0.22	0.63	0.10	0.28	0.12	0.34
Thr	21	0.04	0.19	0.05	0.23	0.02	0.10	0.02	0.10
Ala	50	0.05	0.10	0.11	0.22	0.05	0.10	0.06	0.12
Val	60	0.06	0.10	0.05	0.08	0.03	0.05	0.03	0.05
Leu } Ileu }	63	0.12	0.19	0.11	0.18	0.04	0.06	0.04	0.06
Phe	67	0.02	0.03	0.05	0.07	0.01	0.01	0.01	0.01
Lys	80	0.02	0.03	0.02	0.03	0.02	0.03	0.02	0.03
Tyr	—	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Pro	—	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Hypro	—	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Total	—	0.66	1.48	0.79	1.84	0.46	1.09	0.48	1.11

presence of non-protein nitrogen in collagen; the *N*-terminal analysis of this collagen and the residual protein material remaining after removal of the acetone-soluble non-protein-nitrogen fraction is described here. Evidence is also presented that the thermal conversion of collagen into gelatin at neutral pH is accompanied by only a fractional increase in the number of *N*-terminal groups.

MATERIALS

The acetic acid-soluble collagen and acetone residues described as (A), (B) and (C) in Scheme 1 of the preceding paper (Steven & Tristram, 1962) have been examined. The collagen solution was brought to pH 7 and heated for 1 hr. at 100° to convert it into a gelatin [as defined by Ward (1960)]. The gelatin solution became a rigid gel on cooling, and was rapidly degraded by trypsin and pepsin at 20°, whereas both these enzymes were inactive on the original collagen.

METHODS

Dinitrophenylation of proteins was carried out as described by Sanger (1945), except that the reaction was continued for 2 days with stirring in the presence of excess of sodium hydrogen carbonate and 66% (v/v) ethanol. The whole reaction mixture was acidified and concentrated by vacuum distillation before hydrolysis in 5.7*N*-hydrochloric acid for 18 hr. under reflux in the dark. The mixture was examined as a whole to avoid losses of dinitrophenyl-peptides during acid precipitation and preparation of the dinitrophenyl-protein. This method was successfully employed with commercial gelatins by Heyns & Legler (1957, 1958). The ether-soluble dinitrophenyl-amino acids were examined by a combination of column and paper chromatography described in detail by Steven & Tristram (1962). Although arginine was not found as a *N*-terminal amino acid in collagen [it was found as *N*-terminal in a

peptide fraction (Steven & Tristram, 1962)], its occurrence cannot be excluded entirely since the presence of excessive quantities of ϵ -dinitrophenyl-lysine makes the analysis of small amounts of dinitrophenylarginine extremely difficult.

Control hydrolysates were prepared in duplicate in which 1 g. of collagen was hydrolysed with known quantities of standard dinitrophenyl derivatives, and the combined losses incurred in hydrolysis, extraction, column and paper chromatography were determined. Recovery values varied from 16 to 80% for different dinitrophenyl-amino acids; derivatives of glycine, serine and threonine are well known to be rapidly destroyed during hydrolysis. Dinitrophenyl-proline and dinitrophenylhydroxyproline are very unstable (Phillips, 1955) and for this reason trace amounts of these derivatives were not determined in the hydrolysed dinitrophenyl-proteins examined here. Fraenkel-Conrat & Singer (1954) considered the dinitrophenylation technique to be unsuitable for the analysis of *N*-terminal proline residues.

RESULTS

The rate of hydrolysis of a terminal dinitrophenyl-amino acid in a protein will be influenced by the lability of the adjacent peptide bond and thus the hydrolytic destruction of a control-free dinitrophenyl-amino acid can give only an approximation of the loss involved in the hydrolysis of the same derivative present in a dinitrophenyl-protein. The results are presented in Table 1.

DISCUSSION

The acetone-insoluble residues (B) and (C) were almost identical in their distribution of *N*-terminal residues. Comparison of these values with those obtained in the original collagen (A) indicates a

small loss in all cases with the exception of glycine (perhaps the most unreliable dinitrophenyl-amino acid to estimate accurately owing to the very low recovery after hydrolysis). These losses might be expected since all these amino acids were found in the acetone-soluble non-protein-nitrogen fraction which was examined by Steven & Tristram (1962). Lysine values remained unaltered; this amino acid was absent from the non-protein-nitrogen fraction.

Table 1 shows that the loss in protein-bound α -amino groups reacting with fluorodinitrobenzene in the acetone-precipitated materials (B) and (C) is approximately 0.39 mole/10⁶ g. of protein. The combined dinitrophenyl-amino acids in fractions (A) and (B) of the non-protein nitrogen account for approximately 1.6 moles of α -amino groups/10⁶ g. This unexpected result can only be accounted for if part of the free amino acids liberated from the collagen as acetone-soluble non-protein nitrogen is unable to react with fluorodinitrobenzene when the non-protein nitrogen is still bound to the protein before acetone treatment. This situation might well explain why part of the non-protein nitrogen was diffusible at pH 3.5 though the other part required acetone treatment for its liberation (Steven & Tristram, 1962). This is a possible explanation for the fact that, although the acetone-treated non-protein-nitrogen fraction was chromatographically identical with the diffusible fraction, the former could not be removed from collagen by dialysis yet was readily diffusible in the absence of collagen.

The corrected values for the combined *N*-terminal residues reacting with fluorodinitrobenzene in the acetone-precipitated collagens (B) and (C) indicate approximately one α -amino group/10⁶ g. of protein. The possibility that the light-sensitive dinitrophenyl-derivatives of proline and hydroxyproline may be major terminal residues in dinitrophenyl-collagen can be disregarded since only trace amounts of these derivatives were detected when all possible precautions had been taken to prevent decomposition. It is suggested that the *N*-terminal residues determined in this work are derived from non-protein nitrogen firmly linked to collagen and to the acetone-precipitated residues (B) and (C). The reasons for this suggestion are as follows: (a) The combined *N*-terminal residues of acetone-precipitated collagen amounted to less than one per molecule of a procollagen of molecular weight 700 000 determined by physical means (Orekhovitch & Shpikiter, 1957). (b) The molecular weight proposed by Orekhovitch & Shpikiter (1957) means that at least one *N*-terminal residue of a single amino acid should be present in 700 kg. of collagen, and if this molecule includes the units of the triple helix there should be up to

three such *N*-terminal groups. In the present work, a total of one molar proportion of *N*-terminal groups (involving a number of amino acids, cf. Table 1) has been shown to be present in 10⁶ g. of acetone-precipitated collagen. This value may be in excess of the actual amount of collagen *N*-terminal groups since not all the non-protein nitrogen may have been removed by acetone treatment. Such a finding is inconsistent with collagen having a simple repeating unit of molecular weight 700 000. If indeed collagen is composed of such sub-units, the present findings may only be explained by assuming that the *N*-terminal groups of the protein sub-units are masked and that those characterized in the present work derive from firmly bound non-protein amino compounds. So far we have not carried out measurements of physical molecular weight and it is therefore possible that the soluble collagen prepared under our conditions may differ from that reported by Orekhovitch & Shpikiter (1957). (c) The acetone-soluble non-protein-nitrogen fraction has been shown to contain just those amino acids that have been found to be present as dinitrophenyl derivatives in collagen.

Although acetone precipitation reduced the availability of α -amino groups towards fluorodinitrobenzene, removed as non-protein-nitrogen fraction, it is suggested that part of the total non-protein-nitrogen remained firmly attached to the protein even after acetone precipitation.

Table 1 shows that the thermal conversion of collagen into gelatin at neutral pH is accompanied by very small changes in the *N*-terminal pattern, serine, threonine and phenylalanine being slightly increased and the other terminal amino acids remaining unaltered. The results suggest that gelatin was formed by thermal depolymerization of collagen with minimal rupture of peptide bonds. Heyns & Legler (1957, 1958) detected these same *N*-terminal residues, with the exception of tyrosine, in commercial gelatins when the whole reaction mixture was hydrolysed. Courts (1954, 1959) found that the first six amino acids in Table 1 contributed practically all the *N*-terminal groups in most classes of commercial gelatins. Both groups of authors found a much greater frequency of *N*-terminal residues in commercial gelatins than in the gelatin studied in this work. This would confirm the generally accepted statement that the formation of commercial gelatin from collagen is dependent upon the rupture of peptide bonds during acid or alkali pretreatment followed by the thermal depolymerization of the partially degraded collagen (Ward, 1960). Heating at neutral pH is sufficient to cause the depolymerization of and gelatin formation from collagen without serious degradation of the peptide bonds of the collagen triple-helix structure.

SUMMARY

1. Compounds containing *N*-terminal residues have been shown to be present in preparations of soluble collagen which account for most if not all the reaction of fluorodinitrobenzene with α -amino groups.

2. The significance of the residues has been discussed in relation to the known molecular weight of collagen as determined by physical means. The terminal residues are thought to be due to non-protein nitrogen firmly attached to collagen and not to belong to collagen as true *N*-terminal residues in the usually accepted meaning of the term '*N*-terminal residue'.

3. The thermal conversion of collagen into gelatin has been shown to take place as a physical dissociation with the minimal rupture of peptide bonds.

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The Degradative Metabolism of L-Cysteine and L-Cystine *in vitro* by Liver in Cystinosis

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Since Abderhalden (1903) first detected widespread storage of crystalline cystine in the tissues of an infant at autopsy, and noted the familial incidence of the disorder, an increasing number of cases have been reported in which cystinosis has been diagnosed by post- and ante-mortem examination (Lignac, 1924; McCune, Mason & Clarke, 1943; Worthen & Good, 1958). The associated clinical features of this disease include vitamin D-resistant rickets, glucosuria, generalized hyper-amino aciduria, and acidosis, symptoms which characterize a group of diseases based on the failure of reabsorptive and base-conserving function of the renal tubules, and often referred to as the Fanconi syndrome (Fanconi, 1936). Several hypotheses seeking to explain cystine storage and to relate this to the Fanconi syndrome have been put forward. Bickel *et al.* (1953) reported increased concentrations of a number of plasma amino acids in cystinosis, and suggested that these resulted

from a generalized failure of their normal incorporation into proteins. The intracellular deposition of the poorly soluble cystine and the renal anomalies were considered to be manifestations of this primary defect. However, essentially normal plasma amino acid concentrations have been found by several authors using chromatographic methods (Dent, 1947; Evered, 1956; Brigham, Stein & Moore, 1960), and these are now generally accepted as proof that the amino aciduria is of renal origin.

A second suggestion proposes a specific enzymic defect in cysteine-cystine metabolism, leading to storage of the resultant excess of cystine in the tissues and consequent nephrotoxic effects. The reduction of cystine to cysteine by cystine reductase has been considered the most probable site of such a defect (see Worthen & Good, 1958). Since no investigation of these latter proposals had been reported, a comparison of cystinotic- and normal-