penicillin, and it is carried out by measuring the rate of decolorization of the blue starch–iodide complex when enzyme and substrate react in the presence of starch–iodide.

2. The method was developed for the study of penicillinase reaction kinetics; with it, reaction rates were measured for penicillin concentrations between 1 and 800 μM, and for enzyme activities between 0·001 and 5 μmoles/ml./hr.

3. The micro-assay is 1000 times as sensitive as existing chemical methods, and despite non-specific iodine absorption was useful for the detection and measurement of small amounts of penicillinase in complex mixtures such as broth.

4. Staphylococcal penicillinase was apparently not affected by starch or by iodine under the conditions of the assay, and the method was used for the measurement of the Michaelis constants (K_m) of the enzyme with various substrates.

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The Presence of Non-Protein Nitrogen in Acetic Acid-Soluble Calf-Skin Collagen

BY F. S. STEVEN AND G. R. TRISTRAM
Department of Physiology and Biochemistry, St Salvator's College, University of St Andrews

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During investigations on the *N*-terminal groups in soluble collagen it was observed that small but significant amounts of non-protein nitrogen were present in the soluble collagen prepared from calf skin. These nitrogenous compounds, now known to be a mixture of amino acids and peptides, donate *N*-terminal groups previously attributed to the protein molecule. The material has been examined by paper chromatography of both the free amino acids and the dinitrophenyl derivatives. It may be separated from the bulk of the protein by acetone precipitation or dialysis at pH 3·5, or by acetone precipitation at pH 11·0 (Steven, Tristram & Tyson, 1961).

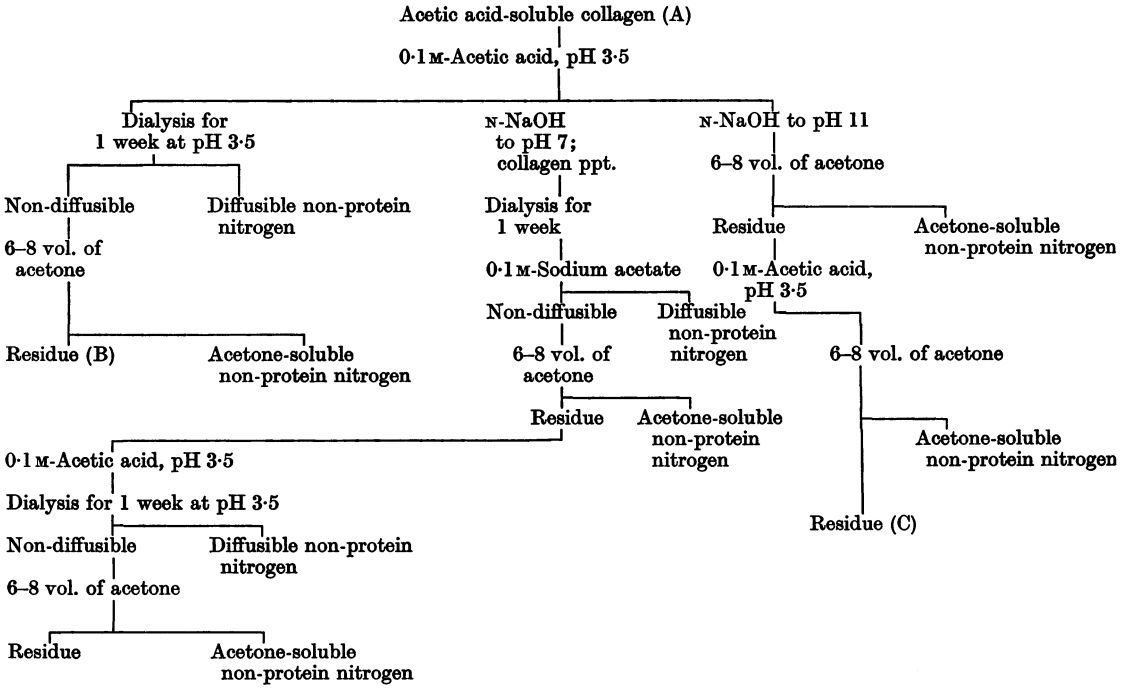
Similar compounds have also been found in: (*a*) the collagenous residues remaining after extraction of soluble collagen from calf skin with 0·1 M-citric acid; (*b*) several commercial gelatins [as defined by Ward (1960)].

Non-protein constituents have also been reported in wool (Zahn & Meienhofer, 1955*a*, *b*) and Synge (1953) predicted their occurrence generally in protein systems.

EXPERIMENTAL

Preparation of soluble collagen

Hair was removed from calf skin, which was then minced and suspended in 0·2 M-disodium hydrogen phosphate at room temperature overnight; toluene was added to prevent bacterial contamination. The insoluble residues were separated by centrifuging and further reduced in size by disintegration in a modified hammer-mill or in a commercial-size Waring Blendor. The extraction with phosphate was then repeated five times or until little or no protein was extracted. Collagen was extracted with 0·1 M-acetic acid and precipitated selectively from the pooled acetic acid extracts by the dropwise addition of 30% (w/v) sodium chloride solution with continuous stirring, precipitation of the protein being complete, as a fine fibrous mass, at a final concentration of 7% sodium chloride. After centrifuging, the precipitate was redissolved in 0·1 M-acetic acid, and the protein was again precipitated by the addition of sodium chloride. This procedure was repeated five times to attenuate the non-collagen contaminants. The dry, ash-free protein contained 18% of total nitrogen [determined by the method of Chibnall, Rees & Williams (1943)] and 14% of hydroxyproline [determined by the method of Neuman & Logan as modified by Leach (1960)].



Scheme 1. Isolation of collagen non-protein-nitrogen fractions. Non-protein-nitrogen fractions isolated at pH 3·5, 7·0 and 11·0 from acetic acid-soluble collagen. [Preparations (A), (B) and (C) are referred to in the next paper (Steven & Tristram, 1962).]

Preparation of non-protein-nitrogen fractions (Scheme 1)

Dialysis and acetone precipitation at pH 3·5. Collagen, dissolved in 0·1 M-acetic acid, was dialysed against this solvent for 1 week at room temperature with constant stirring.

The diffusates were concentrated *in vacuo* (external temperature 60°) and the concentrates desalted by passing through either Dowex 50 (200–400 mesh) or Zeo-Karb (40–80 mesh), the non-protein fractions being eluted with aq. N-ammonia (prepared by dilution of glass-distilled concentrated aq. ammonia) and the eluates concentrated *in vacuo*.

Collagen was precipitated from the non-diffusible fraction by the addition, with stirring, of 6–8 vol. of acetone, a further quantity of the non-protein fraction being recovered from the aq. acetone. This non-protein material was shown to diffuse freely in the absence of collagen. The acetone residue (B) was recycled through 0·1 M-acetic acid and acetone precipitation, and the non-protein-nitrogen fraction obtained contained 0·1% of the total protein nitrogen. This demonstrated the binding power of collagen for non-protein nitrogen and that a single acetone precipitation was insufficient to remove all protein-bound non-protein nitrogen.

Extraction at pH 7. The above procedure was repeated at pH 7, the collagen solution being adjusted to this pH by the addition of N-sodium hydroxide. After dialysis and treatment with acetone at pH 7, the precipitate was redissolved in 0·1 M-acetic acid and reprecipitated by the addition of 6–8 vol. of acetone.

Table 1. *Extraction of non-protein material from calf-skin collagen*

The yield is given as a percentage of the original total nitrogen.

pH	Diffusible fraction	Acetone-soluble fraction	Total
3·5	1·0	1·2	2·2
7	0	0	0
7 followed by 3·5	1·3	1·0	2·3
11 followed by 3·5	—	1·9	2·2
	—	0·3	

Extraction at pH 11·0. To avoid hydrolysis at pH 11, dialysis was not attempted. After precipitation with acetone the protein was redissolved at pH 3·5 and again precipitated with acetone. The various procedures are outlined in Scheme 1 and the yields of non-protein nitrogen are given in Table 1.

The diffusible and acetone-soluble fractions (cf. Scheme 1 and Table 1) were analysed by two-dimensional paper chromatography (ascending) with the following solvent pairs: system I (with Whatman no. 4 paper), (*a*) ethanol–water–ammonia (80:20:1, by vol.); (*b*) butan-1-ol–acetic acid–water (3:1:1, by vol.); system II [with Whatman no. 20 paper, 20 cm. × 20 cm., in multi-sheet frame tanks (Shandon and Co. Ltd.) (Datta, Dent & Harris, 1950)],

(*a*) methanol–water–pyridine (20:5:1, by vol.); (*b*) 2-methylpropan-2-ol–butan-2-one–water–diethylamine (10:10:5:1, by vol.) (cf. Redfield, 1953).

With system I the material was shown to contain 11 ninhydrin-staining components, and with system II 9 free amino acids and two other components, thought to be peptides, were detected (Fig. 1). Synge (1953) suggested that not all the peptides present in tissues react with ninhydrin, and further work is needed with the starch–iodide technique of Rydon & Smith (1952).

The relative concentrations of the major components were determined semi-quantitatively by cutting out the spots and measuring the extinction at 575 mμ after elution with 75% (v/v) ethanol, the concentration being assessed by comparison with four sets of standard concentrations of each acid. The results of this analysis confirmed those presented in Table 2 for free dinitrophenyl-amino acids, with the exception of proline and phenylalanine, which were not detected as the free amino acids (Fig. 1). This type of analysis can give only approximate values for the major constituents in a complex mixture and is unsuitable for the quantitative estimation of minor constituents present in concentrations less than 10–15% of those of the largest components.

Analysis of the non-protein nitrogen by the separation of dinitrophenyl derivatives

The amino acid–peptide mixture was treated with fluorodinitrobenzene by the method of Sanger (1945) and the resultant mixture extracted as indicated in Scheme 2.

Extracts (A) and (B) were freed from excess of fluorodinitrobenzene and artifacts (2,4-dinitrophenol and 2,4-dinitroaniline) by a modification of the method of Li & Ash (1953). Columns were packed with 5 g. of silicic acid (Mallinckrodt 100 mesh) which had been ground with 2·5 ml. of 0·067 M-disodium hydrogen phosphate and sufficient chloroform to make a slurry. The mixture of dinitrophenyl compounds, dissolved in chloroform, was applied to

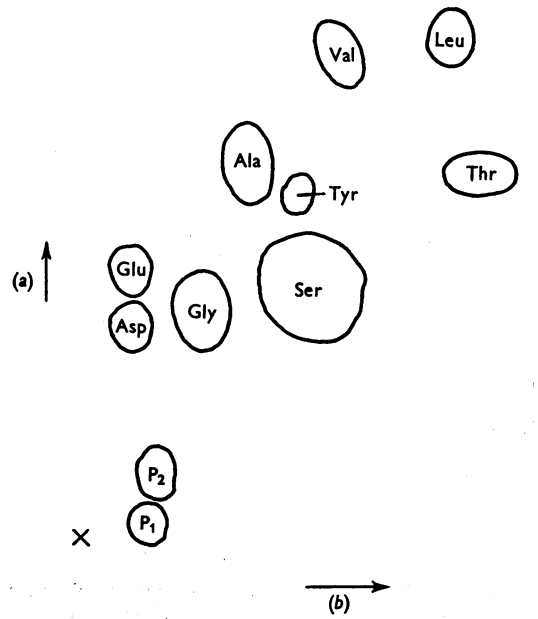
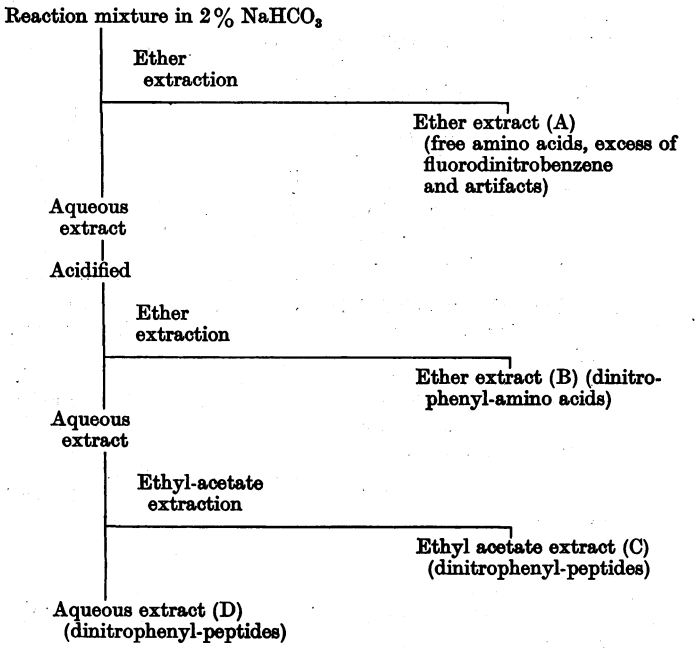


Fig. 1. Ninhydrin-staining constituents of collagen non-protein-nitrogen fraction after chromatography in system II (see text).



Scheme 2. Fractionation of dinitrophenyl derivatives of non-protein-nitrogen material.

Table 2. *Molar composition of free amino acids in the non-protein-nitrogen fraction*

The values are expressed as percentage of the total free amino acid reacting to form dinitrophenyl derivatives; total value equivalent to 1·6 moles/10^6 g. of collagen. The acetic acid-soluble collagen was precipitated by the addition of 6–8 vol. of acetone to the collagen solution. The free amino acids were determined as the dinitrophenyl derivatives isolated in fractions (A) and (B) of Scheme 2.

Glycine	9	Valine	4
Glutamic acid	2	Leucine + Isoleucine	10
Aspartic acid	8		
Serine	35	Phenylalanine	3
Threonine	7	Tyrosine	8
Alanine	10	Proline	4
		Hydroxyproline	Trace

Table 3. *Analysis of dinitrophenyl-peptides obtained from fractions (C) and (D)*

The values quoted are relative molar concentrations.

Free amino acids	(C)	(D_2-insol.)	(D_2-sol.)
Aspartic acid	2	1	.
Glutamic acid	2	2	.
Glycine	4	5	1
Serine	3	4	1
Alanine	3	1	.
Valine	1	1	.
Leucine + Isoleucine	2	1	.
Phenylalanine	.	1	.
ε-Dinitrophenyl-lysine	1	1	1
Dinitrophenylarginine	1	1	1

the column, which was then developed with chloroform equilibrated against 0·067 M-phosphate. Dinitrophenyl-amino acids remained near the top of the column but the artifacts were displaced. Finally the dinitrophenyl-amino acids were eluted with chloroform–acetic acid (99:1, v/v).

Fraction (A) contained a small amount of dinitrophenyl-amino acids and this was combined with the major fraction (B). The dinitrophenyl-amino acids were then separated by paper chromatography with the method of Biserte & Osteux (1951) (toluene–1·5 M-phosphate). The individual spots of dinitrophenyl-amino acid were cut out, eluted with aq. 1% sodium hydrogen carbonate and estimated at 360 mμ, from the molecular extinction coefficient reported by Fraenkel-Conrat, Harris & Levy (1955). The results are given in Table 2.

Fraction (C) was evaporated to dryness, extracted with acetone to free the dinitrophenyl-peptide from traces of salts, and hydrolysed by refluxing in 5·7 N-hydrochloric acid, and the ethereal and aqueous extracts were examined by the chromatographic methods described above. Dinitrophenylarginine and ε-dinitrophenyl-lysine were conveniently separated during the analysis of the aqueous fraction by the Redfield (1953) system. The amino acid composition was determined within the limits of accuracy permitted by quantitative paper chromatography (Table 3).

The aqueous residue (D) was adsorbed on a talc column equilibrated with N-hydrochloric acid and fractionated into three coloured zones: (D_1), a pink component which remained at the top of the column; (D_2), a yellow component which moved slowly down the column; (D_3), a fast yellow component which was an artifact, with a magenta colour in N-sodium hydroxide, with absorption maxima at 370 and 520 mμ. Fraction (D_2) was eluted with ethanol–N-hydrochloric acid (4:1, v/v) by adaptation of the method of Bailey & Bettelheim (1955), evaporated to dryness and extracted with acetone to yield a minor acetone-soluble fraction (D_2-sol.) and a major insoluble fraction (D_2-insol.). Both these fractions were examined after hydrolysis and neither contained an ether-soluble dinitrophenyl derivative. The aqueous extracts were examined by the methods used for the other fractions.

RESULTS

The non-protein-nitrogen components are separated at pH 3·5 or pH 11 but not at pH 7 by either dialysis or acetone precipitation. Table 1 shows that the yield is constant. Each fraction so far examined contains the same components. The same type of non-protein-nitrogen material has been observed in two commercial gelatins (lime- and acid-processed materials) and in the insoluble residues remaining after exhaustive citrate extraction of calf skin (I. R. Tyson, personal communication). More detailed chromatography (cf. Fig. 1) shows each to contain nine free amino acids and two peptide components.

Analysis of the dinitrophenyl-amino acids suggests that the free amino acids are present in the proportions given in Table 2. Analysis of the free amino acids by the method of Redfield (1953) has confirmed the values given in Table 2. The composition of the dinitrophenyl-peptides is presented in Table 3.

DISCUSSION

The free amino acids and peptides found in collagen and related proteins might originate from a number of sources or be derived by several means.

Co-precipitation of non-protein nitrogen with collagen during preparation. This would seem unlikely since the quantity of non-protein nitrogen was identical for collagen precipitated two and five times.

Chemical hydrolysis at pH 3·5 *or* 11 *at room temperature.* The number of different free amino acids and peptides found in the non-protein-nitrogen fraction make this seem unlikely. With more vigorous conditions Partridge & Davis (1950) found that the acidic amino acids were preferentially liberated in boiling dilute acetic acid and oxalic acid solutions of gelatin. Evidence has now been obtained that no increase in acetone-extracted non-protein nitrogen occurred after prolonged treatment of soluble collagen with acetic acid at pH 3·5. In the present work the predominant free amino acid was serine and the dicarboxylic acids were present to the extent of 10% of the total number of residues. 'Ester-like' linkages have

been reported in gelatin by Gallop, Seifter & Meilman (1959); such weak bonds might conceivably give rise to non-protein-nitrogen compounds over the pH range studied.

Enzymic hydrolysis. The extremes of pH at which the non-protein-nitrogen fractions were obtained and the known resistance of collagen to enzymic hydrolysis suggest that proteolysis may be discounted.

Liberation of non-protein nitrogen physically attached to the intact collagen molecule by ionic links. The binding of the non-protein-nitrogen components might be weakest at extremes of pH and greatest near neutrality. Zahn & Meienhofer (1955*a*, *b*) demonstrated the binding of free amino acids and peptides to wool; the amino acid compositions of these peptides were very similar to those reported in this paper. Similar findings have been reported by Schneider, Bishop & Shaw (1960) and by Schneider, Bishop, Shaw & Frazer (1960) for wheat gluten. C. B. Coulson (personal communication) obtained evidence for the presence of free aspartic acid, glutamic acid, serine, glycine, threonine, alanine, methionine, valine and the leucine isomers, as well as traces of the basic amino acids and proline.

The removal of non-protein nitrogen is incomplete after acetone treatment and it is possible that reagents other than acetone may remove more and varied non-protein-nitrogen fractions. Preliminary experiments suggest that ethanol has a similar action to that of acetone.

The amino acid compositions of the two long peptide chains show a common feature in each case, namely $Arg(Asp_1,Glu_2,Gly_4,Ser_3,Ala_1,Val_1,Leu_1,Lys_1)$, which suggests a common sequence in the peptides. Although we have no proof that the two major peptides are homogeneous, chromatographic evidence suggests that they may well be discrete peptides. If this were so the peptides could well have an important physiological function in the intact collagen macromolecule.

Synge (1953) presented data obtained for 28 different tissue juices and he commented on the frequency with which certain amino acids [glutamic acid (22), aspartic acid (17), glycine (21), alanine (15), valine (9), leucine (14) and proline (8)] have been encountered in bound non-protein form. These amino acids, together with serine and threonine, predominate in the non-protein-nitrogen fraction isolated from soluble collagen.

SUMMARY

1. Soluble calf-skin collagen and other collagenous proteins have been shown to contain a small quantity of non-protein-nitrogen material.

2. The non-protein-nitrogen fraction contained 11 free amino acids which have been estimated quantitatively by the dinitrophenylation technique.

3. Three peptides have been isolated as dinitrophenyl derivatives from the non-protein-nitrogen fraction and their amino acid compositions have been determined.

4. The origin of the non-protein-nitrogen fraction is briefly discussed.

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ADDENDUM

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

By F. S. STEVEN and G. R. TRISTRAM
Department of Physiology and Biochemistry, St Salvator's College, University of St Andrews

(*Received* 11 *September* 1961)

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 chromatrography.

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

By F. S. STEVEN and G. R. TRISTRAM
Department of Physiology and Biochemistry, St Salvator's College, University of St Andrews

(*Received* 11 *September* 1961)

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