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The Chemistry of Connective Tissues

3. COMPOSITION OF THE SOLUBLE PROTEINS DERIVED FROM ELASTIN

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In Part 2 of this series (Partridge, Davis & Adair, 1955) the physical properties of the soluble degradation products produced by mild acid hydrolysis of elastin were described. It was further shown that when elastin from *ligamentum nuchae* of cattle was extracted with successive quantities of boiling dilute oxalic acid, a soluble protein of low molecular weight (the β -protein) was liberated early in the extraction; this was followed by the rapid dissolution of the remainder of the elastin with the liberation of large amounts of a soluble protein of high molecular weight and characteristic physical properties (the α -protein). This result could be taken to suggest that elastin is a heterogeneous substance composed of at least two elements. However, the chemical studies reported in this paper show that the derived fractions do not differ in amino acid composition.

MATERIALS

The preparation of the purified elastin powder and the two protein fractions derived from it by partial hydrolysis has already been described (Partridge *et al.* 1955). The α - and β -proteins were separated by repeated precipitation of the coacervate at 60°; osmotic-pressure determinations on the preparations gave a mean molecular weight of 67000 for the α -protein and 5500 for the β -protein.

The α -protein had ash 0.03%. Analysis by combustion

(Weiler and Strauss, Oxford) gave C, 52.9; H, 7.4; N, 16.5%. The β -protein had ash 0.28; C, 52.4; H, 7.75; N, 16.6% (analyses corrected for ash).

METHODS

Amino acid analysis of elastin and the two derived proteins

The procedure adopted for the separation and estimation of the amino acids was that of Moore & Stein (1951). The two samples of elastin powder, and the standard preparations of α - and β -protein were hydrolysed with acid and chromatographed under identical conditions. For hydrolysis, the protein (500 mg.) was mixed with 6N-HCl (50 ml.) and the mixture refluxed for 24 hr. Hydrochloric acid was then removed by repeated evaporation *in vacuo*, and the syrup made up to 50 ml. with distilled water. Portions were then taken for determination of N (Kjeldahl). Equal volumes of the hydrolysate and sodium citrate buffer (0.1M, pH 3.42) were then mixed, and 1 ml. of the buffered solution was applied to the columns. The weight of protein equivalent to the amino acid mixture applied to the column was calculated from the nitrogen content of the hydrolysate. The management of the columns exactly followed the procedure given by Moore & Stein (1951).

Arginine, lysine and histidine were estimated using the short (15 cm.) column of Moore & Stein. Hydroxyproline was estimated in separate experiments in which the 100 cm. column was first equilibrated with a sodium citrate buffer of slightly lower pH (pH 3.04). The column was then developed with a further quantity of pH 3.04

buffer. This caused the hydroxyproline peak to move ahead of aspartic acid (Moore & Stein, 1951). The peak was estimated by reference to a standard curve prepared with a sample of authentic hydroxyproline.

Analysis of N-terminal groups

The method adopted was substantially that originated by Sanger (1945) in which the protein, after reaction with 1-fluoro-2,4-dinitrobenzene (FDNB), is hydrolysed with acid and the resulting dinitrophenyl- (DNP-) amino acids are recovered by solvent extraction and separated chromatographically.

Preparation of the DNP-proteins. The standard preparation of elastin powder (dry wt., 2.13 g.) was suspended in 20 ml. of 10% (w/v) NaHCO_3 . FDNB (1.5 ml.) dissolved in 40 ml. of ethanol was then added, and the mixture was shaken for 2 hr. in the dark. The insoluble yellow product was washed, first with water and then with ethanol, followed by ether. The washings were collected and examined for DNP-amino acids; these were found to be absent.

For the α - and β -proteins, which contain a greater proportion of N-terminal residues, the preparation was carried out on a smaller scale. Preparations (dried from frozen state) of known moisture and ash content were used in each case. The protein (100 mg.) was dissolved in 10% (w/v) NaHCO_3 (4 ml.) and FDNB (1.2 ml.) dissolved in ethanol (8 ml.) was added. The mixture was shaken for 2 hr. in the dark and was then thoroughly extracted with ether to remove excess FDNB. The solution was finally neutralized with HCl, and the residual ether removed by evaporation. The solution was then ready for acid hydrolysis.

Hydrolysis of the DNP-proteins. In a number of preliminary experiments, pure specimens of DNP-amino acid were added to elastin powder and their recovery after different periods of hydrolysis in boiling HCl of various concentrations was investigated. The results led to the view that a short period of hydrolysis in concentrated acid solution would be favourable. Accordingly, the recovery of the DNP-amino acids from dinitrophenylated elastin and β -protein was investigated after refluxing for various lengths of time with 5.7N-HCl. With hydrolysis periods of 4 hr. or less, the presence of DNP-peptides in the reaction mixture was sometimes observed.

The best results were obtained by hydrolysing with 11.4N-HCl for 2 hr. under gentle reflux. The concentration of the acid diminished during the period and approached equilibrium after 2 hr., but recoveries of most amino acids were similar to those obtained after 8 hr. reflux with 5.7N-HCl. However, the ϵ -DNP-lysine and DNP-glycine recoveries were higher when using the shorter hydrolysis in more concentrated acid and this procedure was adopted for the final analyses.

After hydrolysis the reaction mixture was cooled, transferred to a separating funnel (with the washings) and extracted at least five times with 3 vol. of ether. The combined extracts were then evaporated on the vacuum pump. The residue of DNP-amino acids was then made up with a small volume of solvent in readiness for chromatographic analysis.

The aqueous phase from the ether extraction contained the mono-DNP derivatives of the dibasic amino acids, including ϵ -DNP lysine. The solution was reduced to dry-

ness by allowing it to stand in the dark in a vacuum desiccator over P_2O_5 and NaOH. The residue was then extracted with several (10–15) 10-ml. portions of a water-saturated mixture of methyl ethyl ketone (2 vol.) and ether (1 vol.). The combined extracts were then evaporated, leaving a residue suitable for chromatographic separation.

Chromatographic separation of the DNP-amino acids. The method adopted followed closely the procedure of Perrone (1951) who replaced the silica-gel columns of Sanger (1945) with a buffered system using Celite 545 (Johns-Manville Co., London) to support the stationary phase. In our experience results with such columns were more reproducible than those obtained by use of silica gel, and the separations were less disturbed by adsorption effects. If the columns were shielded from direct light the recovery of DNP-amino acids was substantially quantitative.

Colorimetric determinations

The fractions from the columns containing the DNP-amino acids in pure solution were evaporated to dryness, dissolved in 1% NaHCO_3 and made up to 25 ml. In the case of ϵ -DNP-lysine the residue was dissolved in N-HCl and made up to 50 ml. The optical densities of the solutions were then measured with the Hilger Uvispek spectrophotometer at 361 m μ . The optical densities were related to concentrations by calibration curves obtained for recrystallized preparations of the DNP-amino acids. The results so obtained were corrected for losses during hydrolysis by reference to recovery curves previously obtained by refluxing the pure DNP-amino acids for various times with 11.4N-HCl. (Correction factors: for DNP-alanine, DNP-valine, DNP-leucine, 1.05; for DNP-glycine, 1.25.)

EXPERIMENTAL AND RESULTS

Since purified elastin powder on partial hydrolysis is almost quantitatively converted into two protein fractions with different physical properties, an amino acid analysis carried out on the derived proteins should provide a good test of the chemical homogeneity of the starting material.

It seemed possible that the prolonged treatment of the fresh tissue by heating in an autoclave at 120° may itself have produced some change in the amino acid composition of the protein, and for this reason two samples of elastin powder were analysed. One of these was the standard preparation (preparation 1 in Table 1; N (Kjeldahl), 16.8%) which had been given five courses of treatment in the autoclave (Partridge *et al.* 1955), while the second preparation (preparation 2; N (Kjeldahl), 16.9%) had been autoclaved three times only. The results of the amino acid analysis are given in Table 1 and show that the amino acid compositions of the two samples of elastin and the α - and β -protein fractions derived from it are closely similar.

Estimation of cystine. Cystine in made-up mixtures can be estimated with a recovery of about 96% by the method of Moore & Stein but with proteins of small cystine content considerable losses occur during hydrolysis. Recoveries of cystine from the hydrolysis product of elastin yielded values in the range 0.05–0.1%. Since the sulphur content of elastin suggested that the true value was higher than this, an attempt was made to avoid losses of cystine during hydrolysis by first converting the cystine–cysteine residues of the whole protein into cysteic acid by oxidation with performic acid. Cysteic acid itself appears to be stable

under the conditions of hydrolysis used, and since this acid is readily separated on the short (15 cm.) column of Moore & Stein, it was felt that the procedure would offer an accurate and convenient method for estimating cystine-cysteine in the proteins.

The method of oxidation was that of Toennies & Homiller (1942) as modified by Sanger (1949*a*). Toennies & Homiller found that with short periods of treatment with performic acid (up to 1 hr. at room temp.) cystine consumes the theoretical 5 atoms of oxygen, but that, after 2 hr., a more general oxidation of a number of amino acids takes place. It is thus desirable to use a time of oxidation which is near the minimum to oxidize the cystine completely. Two-dimensional paper chromatograms carried out on oxidized and unoxidized elastin showed that the period required was about 15 min., i.e. it was about the same as for insulin (Sanger, 1949*a*). Accordingly, 0.25 g. of the standard preparation of elastin powder was mixed with 9 ml. formic acid, and 1 ml. 30% (w/w) H_2O_2 was added. The mixture was stirred gently for 15 min. at room temp. It was then diluted with an equal volume of water and dialysed against repeated changes of distilled water at 0° to remove formic acid. HCl was then added to the suspension of oxidized protein to a concentration of 6*N* and the mixture hydrolysed as already described for elastin powder. The experiments with the α -protein were carried out in a similar way; in this case the protein was soluble in formic acid but was recovered after the reaction by dialysis in the same way as for elastin powder.

Samples of hydrolysates of the oxidized proteins, after removal of HCl, were mixed with an equal volume of pH 3.4 buffer and were introduced on to the 15 cm. column described by Moore & Stein (1951). This had previously been equilibrated with 0.1*M* sodium citrate buffer of pH 3.42; the chromatogram was developed with the same buffer, the jacket temperature being held at 37°. The effluent solution was collected in fractions of 1 ml. A narrow peak consisting of cysteic acid in pure solution appeared between the third and tenth fractions. The quantitative estimation of cysteic acid in the peak was carried out by the photometric ninhydrin method by reference to a calibration curve determined with pure cysteic acid. The results are given in Table 1.

Since this work was completed a similar method for the determination of cystine as cysteic acid has been published by Schram, Moore & Bigwood (1954).

Estimation of glucosamine and galactosamine. Analysis of elastin powder for hexosamine by the method of Elson & Morgan (1933) gave a colour equivalent to 0.25% on the protein dry weight (glucosamine standard) but since the reliability of the colorimetric method is in doubt when applied to mixtures containing large amounts of certain proteins, an alternative procedure for estimating hexosamine was adopted.

It has been shown by Moore & Stein (1951) that glucosamine gives a symmetrical elution peak which appears between leucine and tyrosine during standard development of the 100 cm. column. Glucosamine in the peak was estimated by making use of the photometric ninhydrin procedure as for amino acids. In the present work, authentic glucosamine hydrochloride was added to the products of hydrolysis of a sample of gelatin in an amount representing about 1% of hexosamine calculated on the protein dry weight. The mixture was then applied to the 100 cm. column of Moore & Stein and the chromatogram

developed in the usual way. Each alternate fraction of the effluent was analysed for hexosamine by the method of Elson & Morgan, and the remainder were analysed by the ninhydrin reaction. Glucosamine appeared as a single fairly symmetrical peak between (and partly covering) the peaks due to isoleucine and leucine, and integration of the hexosamine peak showed that recovery was quantitative. The experiment was then repeated by mixing a sample of authentic galactosamine with the protein hydrolysate. Again a single peak was obtained, this time appearing immediately after phenylalanine. Finally a mixture of glucosamine and galactosamine was added to the protein hydrolysate and analysis of the resulting chromatogram showed two separate peaks in the same positions as before.

Analysis of the hydrolysed elastin powder by the same method showed the presence of two very small peaks giving the colour reaction with *p*-dimethylaminobenzaldehyde and occupying the positions of glucosamine and galactosamine, but in each case the amount present was less than 0.05% of the dry weight of protein taken. Several other very small peaks reacting with the Elson & Morgan reagents were also found, and in addition to these a larger peak was observed, falling between lysine and arginine on the 15 cm. column used for the separation of the basic amino acids. This peak gave no colour with ninhydrin but gave a colour with the Ehrlich reagent that only reached full intensity after 12 hr. standing at room temp. The result was checked by carrying out a displacement chromatogram on hydrolysed elastin following the method described by Partridge (1949). The fractions representing the hexosamine band were mixed and the whole solution estimated by the Elson & Morgan colorimetric procedure. The result again indicated about 0.09% total hexosamine.

It is clear therefore that the high result (0.25% total hexosamine) obtained by application of the colorimetric procedure directly to the products of hydrolysis was due to the prior existence, or production during hydrolysis, of other substances giving a positive Elson & Morgan reaction. Since this work was completed an excellent procedure for the separation of glucosamine and galactosamine has been published by Gardell (1953) in which the two hexosamines are eluted as separate peaks from a column of the cation-exchange resin Dowex-50 by means of 0.3*N*-HCl.

The analyses of the two preparations of elastin powder and the α - and β -proteins show little variation among themselves (Table 1), and on the whole accord well with the analyses of Newman (1949) and of Graham, Waitkoff & Hier (1949), which were based on microbiological methods. It may be noted that the α -protein appears to be lower in glycine, isoleucine and valine than the elastin powder, and the β -protein higher in glycine and valine. The results quoted in Table 1 for the α - and β -proteins are the average of duplicate experiments in which the internal agreement was good, and it is thought probable that the small variations in amino acid composition are real. However, such variations are to be expected in view of the fact that during the preparation of the soluble primary extraction product some 5% of the parent protein is lost as a fraction consisting of diffusible peptides. The amino acid composition of this peptide fraction is unlikely to be the same as that of the parent protein because of the marked variations in the rates of hydrolysis for different peptide bonds. All the results for proline determined during the present work are lower than those given by Newman, but closer to the value (15.6%) given by Graham *et al.*, and it is thought that the

microbiological determination with *Leuconostoc mesenteroides* used by Newman may be in error in giving high results.

A careful analysis of the sulphur distribution in elastin was made by Stein & Miller (1938). These authors found the total S content of a carefully purified elastin powder to be 0.16%; methionine S was 0.08% (volatile iodide method), cystine S, 0.06% (photometric Folin method) and sulphates, 0.02%. These figures correspond to methionine 0.38% and cystine 0.23%. In the present work, the methionine after hydrolysis is shown to be less than 0.05% (in agreement with Newman, 1949) while the cystine is higher than the earlier indications at 0.35–0.40%.

obtained by partial hydrolysis of elastin powder with oxalic acid. The material thus contained both α - and β -proteins.

Solutions of the protein (approx. 0.5%, w/v) were made up in 0.1N-HCl and 0.1N-NaOH and the protein content was determined by estimation of N (Kjeldahl). For measurement of u.v. absorption, the solutions were diluted 1:10 with 0.1N-HCl or 0.1N-NaOH. Measurements were made using the Hilger Uvispek spectrophotometer. The spectral absorption curves, after correction to protein concentration 0.5 mg./ml., are shown in Fig. 1.

It is usually assumed that absorption shown by proteins between wavelengths 2700 and 3150 Å is due to tyrosine

Table 1. *Amino acid analysis of elastin and the derived α - and β -proteins*

	g. amino acid/100 g. protein dry wt.				
	Present work				Newman (1949) Elastin
	Elastin, prep. 1	Elastin, prep. 2	α -Protein	β -Protein	
Glycine	26.7	27.0	23.2	29.0	29.9
Alanine	21.3	23.1	24.5	20.8	18.9
Leucine	9.0	8.9	8.3	8.1	8.7
Isoleucine	3.8	3.7	3.0	3.8	4.0
Valine	17.7	17.0	15.5	20.6	17.4
Serine	0.85	1.0	0.89	0.85	0.82
Threonine	1.12	1.16	1.13	0.98	0.96
Proline	13.5	12.8	12.3	13.8	18.0*
Phenylalanine	6.2	6.2	—	4.6	5.0
Tyrosine	1.5	1.2	1.6	1.1	1.6
Methionine	Trace	Trace	Trace	Trace	0.06
Histidine	—	0.09	0.03	0.05	0.07
Arginine	1.3	1.2	1.2	0.93	0.89
Lysine	0.50	0.54	0.56	0.48	0.39
Aspartic acid	1.1	1.1	0.66	1.0	0.63
Glutamic acid	2.4	2.6	2.6	2.1	2.1
Hydroxyproline	1.6	1.6	1.5	—	1.84*
Cystine	0.35	—	0.40	—	0.25
Glucosamine	<0.05	—	—	—	—
Galactosamine	<0.05	—	—	—	—

* Newman & Logan (1950).

Taking into account the loss of amide nitrogen, estimated at 0.04 g. N/100 g. protein by Stein & Miller (1938), the nitrogen recoveries during the analysis were 91–95%. The values quoted in Table 1 are not corrected for the known losses of serine and threonine during hydrolysis (Rees, 1946) but it still remains possible that elastin contains some 5% of a nitrogenous substance which has not been detected during the analysis. This is important since, as will be indicated later, there is reason to suppose that peptide chains in elastin are bound together by some form of cross-linkage involving covalent bonds, and the presence of such a N-containing compound might provide a clue to the nature of the linkages involved.

Ultraviolet absorption spectra

The preparation of a soluble form of elastin renders possible the examination of the u.v. absorption spectrum of the protein by the methods normally in use for soluble proteins. The measurements were made using a freeze-dried preparation of the primary extraction product

and tryptophan only. The absorption of tryptophan at 2800 Å is not much affected by change in pH but the peak absorption of tyrosine in acid solution at 2750 Å is shifted to 2940 Å when the solvent is changed to 0.1N-NaOH, the effect being due to ionization of the phenolic hydroxyl. As shown by Holiday (1936), the acid and alkaline curves may be used as a basis for the estimation of the tyrosine and tryptophan content of proteins, but in the case of elastin and its degradation products the analysis is complicated by the presence of traces of a yellow pigment. This appears to be firmly bound to the protein since it is not removed by the action of solvents or the various purification procedures applied to the soluble products.

Correction for the yellow pigment was applied by extrapolating back the straight-line section of the curve above 3300 Å, on the assumption that absorption in this range was due to the pigment. From the corrected measurements at 2944 and 2800 Å, using the equations of Goodwin & Morton (1946), the calculated tyrosine content was 1.1% and the value for 'tryptophan' 3.2%. The tyrosine figure is in fair agreement with the values given in Table 1, but

chemical analyses of elastin (Stein & Miller, 1938) and microbiological estimation (Newman, 1949) have shown tryptophan to be absent. It thus appears that the absorption peak at *ca.* 2750 Å, which is unchanged on making the solution alkaline, is due to a chromophore other than tryptophan, and this has not been detected during the analysis reported in Table 1. Since the yellow colour of native elastin has been associated with porphyrin pigments (With, 1947), it is tempting to suggest that the absorption at 2750 Å may be due to the presence of a structure containing the pyrrole nucleus. However, it is interesting that Anslow (1945) attributed absorption at *ca.* 2800 Å to the peptide fabric in certain proteins and suggested that the absorption may arise as a result of structures of the 'cyclol' type proposed by Wrinch (1937).

Fig. 1. Ultraviolet absorption of primary extraction product from elastin corrected to protein concentration 0.5 mg./ml.: I, spectrum in 0.1 N-NaOH; II, spectrum in 0.1 N-HCl.

Since the tyrosine content calculated from the shift in the spectral curve in alkaline solution is in agreement with tyrosine determined chemically, it follows that the phenolic groups of tyrosine are available for ionization, and thus are not involved in inter-chain cross-links (cf. Crammer & Neuberger, 1943). As reported later, this result has been confirmed by the isolation of *O*-DNP-tyrosine from the products of reaction of elastin with FDNB.

Analysis of N-terminal groups

In Table 2 the results are expressed for each protein as the number of *N*-terminal residues in the protein molecule assuming its molecular weight to be 100,000 in each case. The results given are the mean of two or three determinations for each protein. The small number of *N*-terminal residues in the elastin preparation is probably not significant since they may be due to traces of collagen breakdown

products remaining in the elastin preparation after removal of collagen by autoclaving. The result can be taken to mean either that the peptide chains in elastin are very long, or alternatively that the absence of *N*-terminal residues is due to cyclization. Both the α - and β -protein contain much larger amounts of *N*-terminal residues than the native elastin, showing hydrolytic cleavage of the peptide chains during the process of extraction from elastin powder.

Table 2. Number of moles of *N*-terminal residues in 100,000 g. protein

	Elastin	α -Protein	β -Protein
Leucine	Trace	2.6	2.9
Isoleucine			
Valine	Trace	4.0	6.6
Alanine	0.08	8.6	11.0
Glycine	0.12	8.8	12.0
Serine	0.05	Nil	Nil
Aspartic acid	0.04	0.4	Trace
Total	0.29	24.4	32.5

The *N*-terminal amino acids found in greatest amount in the β -protein are the same as those in the α -protein and the relative quantities are similar. With the exception of proline, they correspond to the amino acids which are present in greatest amount in the native protein (Table 1). Extensive destruction of DNP-proline is invariably observed during the hydrolysis of DNP-proteins. However, Porter & Sanger (1948) showed that complete destruction could be avoided by employing 12N-HCl at 105°, and Bowes & Moss (1953) recovered 20% of DNP-proline using 12N-HCl for 16 hr. in the presence of gelatin. In the present work no DNP-proline was detected after hydrolysing the DNP derivatives of the α - and β -proteins under these conditions for periods as short as 2 hr. and it is concluded that proline is absent as an *N*-terminal residue.

Recovery of ϵ -DNP-lysine. Assuming the lysine content of the three proteins to be 0.5 g./100 g., the recoveries of lysine as the ϵ -DNP derivative represented 65% of that originally present in the native protein. For the α - and β -proteins the recoveries were 60 and 65% respectively. Experiments in which untreated elastin powder was hydrolysed under the same conditions in the presence of added ϵ -DNP-lysine gave recoveries as low as 60–70% in different experiments. It therefore seems probable that in all three proteins the whole, or almost the whole, of the ϵ -amino residues of lysine are available for reaction with FDNB.

Recovery of *O*-DNP tyrosine. In view of the possibility of the presence of cross-links involving the phenolic hydroxyl groups of tyrosine it was of interest to ascertain if these groups are available for reaction with FDNB. It has been shown (Crammer & Neuberger, 1943) that with some proteins the phenolic hydroxyl groups of tyrosine are involved in hydrogen-bond formation; these authors compared the u.v. spectra at different time intervals and showed that several hours treatment with 0.1N-NaOH at room temperature is required to complete the dissociation of hydroxyl hydrogen. Accordingly in the present work the elastin powder preparation was treated with dilute NaOH before carrying out the reaction with FDNB.

Elastin powder (1.93 g., ash and moisture free) was allowed to stand for 2 hr. with 0.1N-NaOH (25 ml.). The

suspension was then centrifuged and washed with distilled water. Treatment with FDNB and hydrolysis with 11·4N-HCl followed the procedure given above. The hydrolysate was extracted thoroughly with ether and the ether-soluble components were set aside; the aqueous solution was evaporated to dryness *in vacuo* and extracted with a water-saturated mixture of methyl ethyl ketone (2 vol.) and ether (1 vol.) (180 ml.). A sample (10 ml.) of the methyl ethyl ketone extract was then evaporated to dryness and made up to a known volume with N-HCl. Measurement of the *O*-DNP-tyrosine content of this solution by the u.v. spectrographic method of Sanger (1949b) showed that 1·32 g. tyrosine/100 g. dry proteins had been recovered as the *O*-DNP derivative. Since the tyrosine content of the elastin powder preparation was 1·35% as determined by the method of Moore & Stein (1951) the result indicates that substantially the whole of the tyrosine is available for reaction with FDNB. However, the spectrographic procedure is open to the objection that unknown substances with absorption in the ultraviolet may be present in the mixture, and for this reason the analysis was repeated using a paper-chromatographic method for the separation of *O*-DNP-tyrosine from other components of the methyl ethyl ketone extract.

A sample of the methyl ethyl ketone extract (20 ml.) was percolated through a column of Celite 545 (5 × 2 cm.) buffered with phosphate at pH 4 (Perrone, 1951) and the chromatogram developed with a further quantity of the same solvent. The bands due to *O*-DNP-tyrosine and ϵ -DNP-lysine separated only partially and were collected together. The combined solutions were evaporated to dryness and the residue was taken up in 1 ml. of methanol-HCl (25 ml. of methanol + 5 ml. of N-HCl). Samples (7·8 μ l.) were placed in a series of spots on the base line of a sheet of Whatman no. 1 filter paper and the chromatogram developed with *n*-butanol-acetic acid-water (4:1:5 by vol.). The band due to *O*-DNP-tyrosine was located by spraying narrow strips cut from the sides of the paper with ninhydrin. Separation from ϵ -DNP-lysine was substantially complete, and a transverse strip carrying the spots due to *O*-DNP-tyrosine was cut from the paper and eluted with N-HCl. The eluate was made up to known volume with N-HCl and the *O*-DNP-tyrosine was estimated at 300 $m\mu$, using the Hilger Uvispek spectrophotometer.

The content of *O*-DNP-tyrosine found was equivalent to 1·16 g. of tyrosine/100 g. of dry protein. Since losses during the isolation procedure would be expected to be high, this figure confirms the result given above (1·32 g./100 g.) obtained by direct measurement of *O*-DNP-tyrosine in the presence of ϵ -DNP-lysine and suggests that substantially the whole of the tyrosine hydroxyl residues in the protein are unsubstituted and available for reaction with FDNB at pH 8.

Enzymic dissolution of elastin

Balo & Banga (1950) showed that pancreatic extract contains an enzyme which dissolves the elastic fibres of aorta wall and leaves native collagen unchanged. The enzyme, which they call 'elastase', was shown to differ from trypsin, pure specimens of which have little effect on elastin. Since these authors could not detect the liberation of free amino acids during the dissolution of elastin by the enzyme, they concluded that its action was not proteolytic. Banga (1951) showed that during the action of the enzyme there is no increase in amino nitrogen as measured in the

Van Slyke apparatus, and offered this as confirmatory evidence that solution is not due to hydrolytic cleavage of peptide links. In the view of Banga, elastin fibrils are built up from chains of globular protein molecules, and the effect of elastase is to break down the forces holding the globular molecules together, thus producing a soluble protein.

Since the measurement of amino N by the method of Van Slyke could not be expected to show the rupture of small numbers of peptide bonds, it was considered desirable to study the products of the enzymic reaction using the FDNB technique of Sanger.

Preparation of the enzyme. Two preparations of the enzyme were used in these experiments. Preparation A, which was kindly supplied by Professor I. Banga, was the highly purified fraction FP prepared from ox pancreas (Banga, 1952). Preparation B was a fraction of lower purity prepared in this laboratory following Banga's procedure. The enzymic activity of preparation B was 12 'elastase units' (EU)/mg. as measured by the procedure of Banga (1952). The activity of preparation A was given as 22 EU/mg.

Proteolytic activity of the elastase preparations. The proteolytic activity of the two preparations of elastase was compared with that of crystalline trypsin (Armour and Co.) using a modification (Snoke & Neurath, 1950) of the method of Anson (1938). Expressed in terms of the amount of trichloroacetic acid-soluble tyrosine liberated from a standard preparation of urea-denatured haemoglobin in 30 min. at pH 8·67, the activity of the two elastase preparations was identical, and was higher than that of the preparation of crystalline trypsin. When relatively large amounts of the three enzymes were incubated with haemoglobin (10 mg. enzyme to 100 mg. haemoglobin) the activity of the two preparations of elastase was 1·8 times that of the crystalline trypsin.

Action of the enzymes on elastin from ligamentum nuchae. Routine estimations of the elastase activity of various enzyme preparations were carried out with elastin powder prepared from aorta of cattle as described by Banga (1952). Since this material may not be identical in properties with the elastin powder from *ligamentum nuchae* studied in the present work, the action of elastase on the latter was investigated.

Elastin powder (40 mg.; standard preparation from *ligamentum nuchae*) was mixed with 2 ml. of 0·1M sodium carbonate-HCl buffer (pH 8·9) and 3 ml. of a solution (0·1%, w/v) of elastase were added. The mixture was incubated at 37° with gentle shaking. Solution was complete in 2 hr. with elastase preparation A and 2·5–3 hr. with preparation B. During the reaction the pH of the solution fell to 8·0.

It was found that in quantitative estimations of the enzyme activity of preparations of elastase following the method of Banga, elastin powder from aorta could be replaced by powder of similar particle size from *ligamentum nuchae* without affecting the result.

The action of trypsin was investigated as follows: elastin powder from *ligamentum nuchae* (0·5 g.) was mixed with 50 ml. of an aqueous solution containing 0·05 g. of crystalline trypsin (Armour and Co.) and 50 ml. of 0·1M sodium carbonate-HCl buffer (pH 8·4). The mixture was incubated at 37° for 18 hr. under toluene. The elastin powder did not dissolve and was recovered quantitatively by centrifuging, dehydration with ethanol and drying. In

order to investigate if there had been any alteration in the stability of the elastin fibres, a portion of the trypsin-treated elastin was refluxed for 6 hr. in 1% (v/v) acetic acid solution. The fibres appeared unchanged and the elastin was recovered quantitatively after the experiment. A second sample was used to determine the course of hydrolysis in 0.25M oxalic acid. The curve relating the quantity of elastin dissolved to the time of hydrolysis was the same as for the untreated elastin preparation (Adair, Davis & Partridge, 1951).

Action of elastase and trypsin on the α -protein. The action of elastase (preparation A, 22 EU/mg.) and trypsin (Armour and Co., crystallized trypsin, containing 50% w/w, MgSO_4) on the α -protein was compared under identical conditions.

The enzyme solution (10 mg. enzyme dissolved in 1 ml. 0.04M- NaHCO_3 - Na_2CO_3 buffer of pH 8.7) was diluted serially by taking 0.5 ml. of each successive solution and diluting with 0.5 ml. of buffer. The final dilution was 1:64. Portions (0.1 ml.) of each of these dilutions were then added to a series of tubes, each containing 0.9 ml. of the carbonate buffer and 1 ml. of an aqueous solution (1.26%, w/v) of the α -protein. The tubes were then incubated at 37° for 1 hr. After cooling they were titrated to pH 4.5 by addition of three drops 0.5N acetic acid to each tube.

The 'coacervation temperature' (Partridge *et al.* 1955) of the α -protein in each tube was then estimated by the procedure already given. None of the mixtures containing elastase gave any precipitate of α -protein on raising the temperature, and thus it was concluded that the α -protein had been degraded by the action of elastase. In the case of the experiments with trypsin, coacervate separated in all the tubes on raising the temperature to 25–30°, but in tubes 1 and 2 (containing 0.05 and 0.025% trypsin respectively) the precipitates were lighter than the controls. From these results it was concluded that the sample of trypsin was substantially without effect on the α -protein, although a few bonds may be split. This was perhaps to be expected since the action of pure trypsin is said to be specific for those bonds in which the carboxyl groups of arginine and lysine are involved (Bergmann & Fruton, 1941; Sanger & Tuppy, 1951) and the content of these amino acids in the protein is low.

Liberation of N-terminal residue by the action of elastase. Elastin powder from *ligamentum nuchae* (0.675 g., dry wt. of ash-free protein) was suspended in 36 ml. 0.2M- Na_2CO_3 -acetic acid buffer (pH 8.6) and 72 ml. distilled water. To this, 9.4 mg. elastase (12 EU/mg.) in 2 ml. buffer was added. The suspension was gently shaken in the presence of toluene at 37° for 42 hr., by which time the whole of the elastin had dissolved leaving a clear pale-yellow solution. The solution was evaporated to 15 ml., ethanol (30 ml.) and NaHCO_3 (0.5 g.) were added and the mixture was treated with FDNB (2 ml.) by shaking for 2 hr. in the dark. The mixture was then extracted with ether to remove excess FDNB and the residue acidified and evaporated to dryness. The dry residue was hydrolysed with HCl and liberated DNP-amino acids were identified and estimated by the procedures already given. The results are shown in Table 3.

As in the case of the α - and β -proteins, no DNP-proline was found. The distribution of N-terminal residues was rather similar in both cases (cf. Table 2), but there was relatively less valine and more aspartic and glutamic acid end-groups in the product of enzyme hydrolysis. The most striking feature of the result was that the elastase-treated

product contained roughly four times the number of N-terminal residues found in the same weight of α - or β -protein. This shows that rupture of the peptide chains proceeds further in the enzyme reaction than with controlled hydrolysis by 0.25N oxalic acid. The estimate of N-terminal residues liberated by the enzyme is subject to an error due to the possibility of autohydrolysis of the enzyme itself, but since only 9.4 mg. of enzyme was used for hydrolysis of 675 mg. of elastin, the error could not account for more than 10–15 N-terminal residues in 100000 g. of protein.

Table 3. Release of N-terminal residues from elastin by elastase

	Moles N-terminal residue in 100000 g. protein
Glycine	55.9
Alanine	42.5
Valine	4.1
Leucine + isoleucine	13.4
Aspartic acid	2.9
Glutamic acid	3.1
Total	121.9

DISCUSSION

In the preceding paper (Partridge *et al.* 1955) three possible views of the macromolecular structure of the protein comprising the elementary elastin fibre were outlined.

(1) The protein is composed of two components of differing chemical composition.

(2) The protein is composed of two components which consist basically of the same kind of polypeptide chain, but differ as a result of differences in structural configuration.

(3) The protein is homogeneous, and the production of two degradation products of different molecular weight is due to the loosening of a network by removal of parts of it as low-molecular material.

A clear decision on hypothesis (1) has been obtained by determining the amino acid composition of the original elastin powder, the α -protein and the β -protein. The results given in Table 1 show that the amino acid composition of the three proteins is substantially the same, and thus it appears that elastin powder as prepared in this work may be regarded as essentially homogeneous for the purpose of purely chemical investigations.

As regards the molecular structure of the two soluble degradation products, previous work has shown that a considerable degree of hydrolysis of peptide links occurs during the extraction with 0.25M oxalic acid. The hydrolytic procedure releases aspartic and possibly glutamic acid in a free state, and at the same time cleaves the protein chains at other positions where labile peptide linkages are to be found (Adair *et al.* 1951).

Titration curves carried out on elastin and the α - and β -proteins by Bendall (1955, fig. 1) show the presence of many α -carboxyl and α -amino groups in both the soluble proteins. From the curves the α - and β -proteins contain 26 α -amino equivalents and 31 α -amino equivalents in 100 000 g. of protein respectively.

This result accords rather well with the information obtained by application of the FDNB technique of Sanger (1945) which is reported in Table 2. The number of *N*-terminal amino acid residues found in 100 000 g. of protein for elastin, α -protein and β -protein was 0.29, 24.4 and 32.5 respectively.

It is significant that the α -protein (mol.wt. 67 000) and the β -protein (mol.wt. 5500) contain not very different proportions of *N*-terminal residues, in spite of the very great difference in their molecular weights as determined osmotically (Partridge *et al.* 1955). This probably means that they both consist of polypeptide chains of roughly equal length, and the larger protein molecules must be built up by the binding together of a number of such unit polypeptide chains.

The relationship between chain length and molecular weight is brought out in Table 4. The data in this table shows that the β -protein consists of molecules composed, on the average, of 2 chains each containing about 27 residues, the *N*-terminal

Table 4. *Moles of N-terminal residues in amounts of elastin derivatives corresponding to the mean molecular weight determined osmotically*

	α -Protein (67 000 g.)	β -Protein (5500 g.)
From titration curve*	17.4	1.72
From FDNB determination	16.2	1.84

* Bendall (1955).

groups of which are variable. The α -protein has, as a mean value, 17 such chains containing, on an average, 35 residues each, the *N*-terminal residues being glycine, alanine, valine and leucine with a small proportion of aspartic acid.

As yet there is little indication as to the nature of the cross-links which hold the chains together. Since the α -protein is derived from native elastin by a process involving rupture of the peptide chains, it seems probable that the cross-links in the soluble protein are the same as those existing in the natural fibrous protein. In this case the cross-links must be rather stable to acid hydrolysis, and the α -protein may then represent a 'resistant core' derived from the more highly cross-linked sections of the elastin fibril.

The rubber-like elasticity of the hydrated elastin fibril suggests that it is a disordered structure

composed of bundles of randomly contorted peptide chains lying generally parallel to the fibre axis. For the greater part of their length the peptide chains must be free to take up independent thermal motion; but to account for the insolubility and the swelling properties of the fibre, the presence of cross-links at rather wide intervals must be assumed.

It is possible that such cross-links could be established by the presence of crystalline regions containing polar groups in close apposition, but there appears to be little evidence for such a structure from published X-ray diffraction studies. However, since elastin, unlike collagen, is resistant to the action of prolonged autoclaving, and shows no tendency to dissolve either in hydrogen bond-breaking solvents such as neutral urea solution or in organic solvents such as phenol or cresol, it seems more probable that primary covalency is involved in the relatively small number of cross-links present.

The only type of covalent cross-link that has so far been proved to exist in proteins is the —S—S— bridge present in cystine residues. In elastin, however, cystine represents not more than 0.4 % of the protein dry weight, and this content would not provide sufficient cross-links to account for the stability of the structure. In order to ascertain if cystine bridges are an important factor in stabilizing the protein, elastin powder was treated with performic acid under conditions which ensured the conversion of the whole of the cystine half-residues into cysteic acid, thus breaking all —S—S— bridges. The oxidized protein remained insoluble after prolonged autoclaving, and was also insoluble in neutral 40 % (w/v) aqueous urea and 90 % (w/v) aqueous phenol both at room temperature and 100°. It thus appears that —S—S— bridges have little effect on the solubility properties of the protein, and other stable cross-links must be present.

Cross-linking at the ϵ -amino groups of lysine or the phenolic hydroxyl groups of tyrosine appears to be ruled out since it is shown that these groups are available for reaction with FDNB. The amino acid analysis of elastin and the α - and β -proteins shows them to have a remarkably low content of polar side chains. Aspartic and glutamic acids together account for 3.5 % of the dry protein; serine and threonine account for 2 % and hydroxyproline 1.6 %. It is possible that ester links formed between the hydroxyamino acids and such of the dicarboxylic amino acid residues as are not combined with ammonia may account for the stability of the structure, but no evidence for the presence of such bonds has as yet been obtained. However, it should not be forgotten that since only approximately 95 % of the protein dry weight has been

accounted for by amino acid analysis, an unidentified substance may be involved in cross-bond formation.

A further possibility remains that the peptide fabric itself is involved either in some form of covalent cross-linking or, more probably, in aggregation at the molecular level by the intertwining or interpenetration of helical chains at certain points. Most workers now agree that the basic chain configuration in many synthetic polypeptides and the α -forms of fibrous proteins is a helical structure, and various model systems involving molecular aggregation with the production of 'super-helices' have been discussed by Crick (1952) and Pauling & Corey (1953). In the case of elastin, a highly ordered structure appears to be unlikely; but the production of a network, with some degree of order, by the interpenetration of helical structures at specific points in the peptide chains could be envisaged. Such a model would conceivably explain both the elastic and swelling properties of the natural fibre and also the production from it of two types of soluble protein by hydrolytic cleavage of the chains; the high-molecular α -protein would then be regarded as composed of fragments of the three-dimensional network, and the β -protein as loose strands of the polypeptide fabric.

Recently Bowen (1953) has published a study of a soluble protein derived from elastin by prolonged boiling with 40% (w/v) urea (Hall, 1951). This protein shows reversible phase separation on warming, and in some of its other properties resembles the α -protein described here. From the results of sedimentation and diffusion experiments carried out with the protein derived from urea treatment, Bowen suggested that the material may exist in solution at pH 4 as a dynamic equilibrium between more than one molecular species, dilution favouring breakdown into smaller molecules. By extrapolation to zero concentration a value for the mean molecular weight at infinite dilution of 6870 was obtained, and Bowen suggests that this may mean identity with the β -protein produced by oxalic acid treatment (mol.wt. 5500). A reliable value for the maximum molecular weight approached at high concentration could not be given, but in Bowen's view a value of 84000, corresponding with the α -protein, would be in reasonable accord with his data.

In our work with the α - and β -proteins we have not observed any tendency towards interconversion of the two proteins due to alterations in the protein concentration; this is well brought out in the data showing the effect of protein concentration on osmotic pressure (Partridge *et al.* 1955). Strong solutions of the β -protein can be stored for prolonged periods without production of any

material showing reversible phase separation on warming, and similarly, solutions of the α -protein will still show a milky appearance on raising the temperature at dilutions of 0.05% (w/v) or less. We have had no opportunity of repeating the experiments of Bowen using the product derived from oxalic acid treatment, but the present evidence suggests that the proteins are not identical. It should perhaps be pointed out that during the treatment with boiling urea, ammonia is produced and the solution becomes alkaline. Solubility in urea may be brought about by partial alkaline hydrolysis, and the alkaline conditions may cause the rupture of interchain linkages which are more stable under acid conditions. The difference in properties of the two types of soluble degradation product may thus be due mainly to the absence in the urea product of stable cross-bonding. Work is continuing in an attempt to obtain further information on this aspect of the problem.

Action of elastase on elastic tissue and purified elastin

The state of combination of the polysaccharide in yellow connective tissue and its possible effects in stabilizing the protein structure has been discussed by several authors, but some confusion has arisen largely as a result of inadequate definition of the preparations of elastin used for the experiments. Hall, Reed & Tunbridge (1952) found that polysaccharide and sulphuric acid are intimately associated with protein in elastic tissue and noticed the liberation of both during dissolution of elastin preparations by elastase or other agents. As a result they suggested that pancreatic elastase is not a proteolytic enzyme, but rather a mucase. Banga & Balo (1953) observed the liberation of reducing substances during the action of elastase and at first considered that purified elastin contained some 10–12% of a carbohydrate resembling glucosamine, but this view was later withdrawn (Banga, 1953). Recently Wood (1953) showed that elastic tissue (from ox *ligamentum nuchae*) from which collagen had been removed by treatment with boiling dilute acetic acid still contains a polysaccharide which is bound interfibrillarly. The polysaccharide can be removed in large part by extraction with alkaline 10% (w/v) calcium chloride and appears to be similar to chondroitin sulphate. Prolonged treatment of strips of ox *ligamentum nuchae* with alkaline calcium chloride solution at 37° had no observable effect on the elastic properties of the fibres, and from this and other evidence it was concluded that there is doubt as to whether the polysaccharide associated with elastin plays an important part in structural stability. About the same time Hall (1953) showed that the elastase preparations commonly used are

probably complex and that at least one component has mucolytic activity.

The results of the present work show that the elementary fibres comprising the bulk of the native ligament consist of a protein which may be regarded as chemically homogeneous and which contains not more than about 0.3% of carbohydrate and only small amounts of ester-bound sulphate. Estimations of free α -amino groups liberated during the action of elastase on this protein shows that enzymic degradation involves the rupture of peptide bonds, and thus at least one of the components of 'elastase' must be regarded as a proteolytic enzyme.

SUMMARY

1. Amino acid analyses are given for purified elastin from *ligamentum nuchae* of cattle and for the α - and β -proteins derived from it by mild acid hydrolysis (Partridge *et al.* 1955). The amino acid composition of the three proteins is closely similar, and thus the intact fibrous protein must be regarded as substantially homogeneous.

2. The glucosamine and galactosamine content of purified elastin has been estimated by a chromatographic method; the two amino sugars together account for not more than 0.1% of the dry protein.

3. The ultraviolet absorption spectra of the mixture of soluble proteins derived from elastin show the presence of an unidentified substance with an absorption peak in the region of 2750 Å.

4. *N*-Terminal groups in elastin and the two derived proteins were estimated by the fluorodinitrobenzene technique. Elastin contains 0.29 mole of *N*-terminal residues in 100 000 g., the α - and β -proteins contain 24.4 and 32.5 moles of *N*-terminal residues respectively in the same weight of protein. It is deduced that the β -protein consists of molecules composed on the average of two chains each containing 27 residues; the α -protein has, as a mean value, 17 such chains containing 35 residues each.

5. The nature of the cross-links binding adjacent peptide chains in elastin and the two soluble proteins is discussed.

6. The action of pancreatic elastase on purified elastin has been studied. It is concluded that dissolution of the fibrous protein is brought about by the rupture of peptide links and the active enzyme must be regarded as proteolytic.

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