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The Chemistry of Connective Tissues

2. SOLUBLE PROTEINS DERIVED FROM PARTIAL HYDROLYSIS OF ELASTIN*

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(Received 17 December 1954)

By contrast with other components of connective tissue, relatively few chemical studies on the structure of elastin have been reported. This may be due to the inherent difficulty of studying a protein which is characteristically insoluble and resistant to the attack of mild reagents. As in the case of other fibrous proteins, the only feasible approach to a chemical study of structure appears to be by a process of stepwise degradation, and the aim of this work was to prepare the ground for such a study by an examination of the products of mild acid hydrolysis.

During an investigation of the early stages of hydrolysis of various mucoids and connective-tissue proteins, it was observed that, on treatment with dilute acetic acid or oxalic acid at 100°, aspartic acid was in each case the first free amino acid to appear, followed at a later stage by glutamic acid (Partridge & Davis, 1950). Since elastin has an unusually low content of the dicarboxylic amino acids (Stein & Miller, 1938; Neuman, 1949) and has a low content of serine and threonine, which also give rise to easily hydrolysable peptide bonds (Desnuelle & Bonjour, 1952), it was thought that the first products of hydrolytic cleavage might consist of a mixture of soluble degradation products of high molecular weight. It was later observed (Adair, Davis & Partridge, 1951) that six successive extractions, each with 0.25M oxalic acid at 100° for 1 hr., were sufficient to bring dried and powdered elastin from *ligamentum nuchae* of cattle completely into solution.

If a study of the degradation products is to give information which is relevant to the elastin fibre as it exists in elastic tissue, it is important to know if the fibre is itself homogeneous. Morphologically,

the elastin fibre has for many years been recognized as an entity, and autoclaved preparations of elastin, particularly those from *ligamentum nuchae*, are usually considered to be homogeneous chemically. The work reported in this series of papers supports the view that purified preparations of elastin are chemically homogeneous, although there seems reason to suppose that there may be structural heterogeneity at macromolecular level.

The protein fibre as it exists in elastic tissue is closely associated with collagen. Thus Astbury (1940) observed a typical collagen X-ray diffraction pattern in stretched *ligamentum nuchae*, but this changed to amorphous rings when collagen was removed by autoclaving.

Wolpers (1944) treated *ligamentum nuchae* with pepsin for 24 hr. in order to remove collagen and obtained fibres of a size suitable for study with the electron microscope. The preparation was revealed as large branching amorphous fibres ranging in width from 2500 Å to 200 Å. The fibres appeared to have no axial periodicity.

McCartney (1914) showed that after removing collagen from *ligamentum nuchae* by peptic digestion the fibres contract linearly with increase in temperature and return to their original length on cooling. Jordan Lloyd & Garrod (1948) compared the thermoelastic behaviour of elastin to that of rubber and deduced that in the unstretched fibre the peptide chains are highly contorted and are cross-linked at a few points only. They found that if the fibre is dried in the stretched condition it remains in the extended state after release of the tension. This was explained as being due to the polar properties of the peptide bonds, which introduce further cross-links when water is removed. Imbibition of water is necessary to keep the chains apart, and thus to permit thermal

* Part 1: *Biochem. J.* **43**, 387 (1948).

contraction after stretching. In this, elastin fibres differ from rubber, which being non-polar will not 'set' in the extended state at room temperature.

Recently Balo & Banga (1950) showed that pancreatic extracts contain an enzyme 'elastase' which dissolves elastin but not native collagen fibres. Pure trypsin and chymotrypsin have no action on the elastin of aorta wall and the dissolution of elastin by elastase was thought to be associated with a change in molecular state from rod-shaped to globular particles rather than to proteolysis. Hall, Reed & Tunbridge (1952) observed that small amounts of polysaccharide and bound sulphuric acid are intimately associated with protein in elastic tissue, and that on treatment with elastase these substances are released.

Banga & Schuler (1953) showed that, in the course of digestion with elastase, reducing substances thought to be carbohydrate in character are split off from elastin. This result led them to the suggestion that elastin is a glycoprotein, the fibrous character of which is determined by a carbohydrate group built into the molecule. Later (Banga, 1953) it was shown that the reducing substances split off during elastolysis could not be ascribed to carbohydrate.

The results of the work described in this and the following paper (Partridge & Davis, 1955) have led to the view that the substance of the elementary elastin fibre is a chemically homogeneous protein, although it may exhibit heterogeneity at macro-molecular level; the physical properties of elastic tissue may be a result of the macromolecular structure of the elastin comprising the elementary fibres and of the way in which these fibres are cemented by collagenous structures and mucoproteins.

A preliminary report on the work described in this and the following paper was read at the Second International Congress of Biochemistry (Partridge, 1952).

METHODS

A modification (Hiller, Plazin & Van Slyke, 1948) of the micro-Kjeldahl method was used for the determination of N. Total S was estimated by a modification (Lugg, 1938) of the peroxide fusion method. Ester-bound S was determined as $\text{SO}_4\text{-S}$ after hydrolysis with 5N-HCl for 20 hr. at 100° , using the gravimetric procedure described by Lugg (1938). Moisture was determined by drying to constant weight over P_2O_5 at 80° under reduced pressure; in all cases ash was weighed as sulphate. Protein concentrations were determined refractometrically (Adair & Robinson, 1930a) using $\Delta n = 1.84 \times 10^{-3}$ as the refractive increment of the protein (1%, w/v solution at 25°).

The analyses quoted in the text are corrected for ash and water.

Electrophoresis experiments. The experiments were carried out with the apparatus of Tiselius (1937) using the Philpot-Svensson optical arrangement (Svensson, 1939, 1946). The bath temperature was 3.2° . Two series of buffers were used. The series at ionic strength 0.02 were sodium acetate, glycine-HCl and sodium phosphate buffers chosen to cover the range pH 2-9. The solutions at ionic strength 0.20 were prepared by addition of sufficient NaCl to the same buffers to raise the ionic strength to the required value. The protein solutions were dialysed against the appropriate buffer for 3-4 days until equilibrium was reached, the concentration of protein was then measured refractometrically and the solutions were adjusted to 0.5-1.0% (w/v) by dilution with the buffer.

The conductivities of the buffer and protein solutions were measured at 0° and the mean of the two values was used in conjunction with the current readings in order to determine a mean value for the potential gradient at the boundaries. Since conductivity was measured at 0° the values for mobility refer to 0° rather than to the temperature of the bath. The pH of the buffer solutions was measured by means of a glass electrode, checked by hydrogen electrode at 0° .

Membrane potentials and osmotic pressure measurements. The apparatus and the experimental methods for the measurement of membrane potentials were as described by Adair & Adair (1934). The measurements were carried out at $0 \pm 0.2^\circ$ and equilibration was continued for several days with repeated changes of buffer mixtures until constant readings were obtained, or until the solution gave constant osmotic pressures.

Osmotic pressures were measured at temperatures ranging from 0.5 to 1.5° using the toluene osmometers of Adair (1949). Collodion membranes (Adair, 1925) were employed in all experiments. Equilibrium was reached quickly (sometimes overnight) but in all cases readings were taken over extended periods to ensure that solutions were truly equilibrated. Pressures were reduced to mm. Hg at 0° by applying the gas laws.

At the end of each experiment, samples of the protein solution and the external solution were warmed to room temp. (about 18°) and their refractive indices were measured by means of an immersion refractometer.

The protein concentration, c , expressed as g. protein/100 ml. solution, was calculated by the formula:

$$c = (n - n')/\alpha',$$

where n = refractive index of protein solution, n' = refractive index of external solution, α' = apparent refractive increment.

A preliminary determination of α' by a gravimetric method is recorded in Table 1. c was determined by drying the solution over P_2O_5 *in vacuo*, and deducting the weight of sodium and phosphate ions.

Table 1 gives additional data for densities, density increments and apparent specific volumes at 0.9° and at 19° . For definition and measurements of density increments see Adair & Adair (1947).

Table 1 also includes a calculation of a factor k_w , the weight of water displaced by 1 g. of protein. This factor k_w may be used to calculate the protein content, in g./100 g. of water, from measurements of the concentration c .

(a) Typical appearance of the fibres forming the purified elastin powder (unstained, $\times 100$).

(b) Purified elastin powder: a field in which several bundles of fibres have escaped separation during the process of purification (unstained, $\times 100$).

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Table 1. Gravimetric determination of refraction and density increments for the primary extraction product using a solution in sodium phosphate buffer

Symbol	Definition	Value found
n'	Refractive index of buffer (19°)	1.33414
n	Refractive index of protein solution (19°)	1.34559
c	g. protein/100 ml. protein solution (19°)	6.24000
α'	Refractive increment*	0.00184
ρ	Density of protein solution (19°) (g./ml.)	1.01920
ρ'	Density of buffer (19°) (g./ml.)	1.00340
k'_d	Density increment (19°)†	0.25430
ρ	Density of protein solution (0.9°) (g./ml.)	1.02180
ρ'	Density of buffer (0.9°) (g./ml.)	1.00510
c	g. protein/100 ml. protein solution (0.9°)	6.25600
k_d	Density increment at 0.9°†	0.26700
v'	Apparent volume, 1 g. protein (19°) (ml.)‡	0.74300
v	Apparent volume, 1 g. protein (0.9°) (ml.)‡	0.72900
c_w	g. water/ml. protein solution (19°)	0.95190
c_w'	g. water/ml. buffer (19°)	0.99770
k_w	g. water displaced/g. protein§	0.73400
S	g. (Na ⁺ + HPO ₄ ²⁻ + H ₂ PO ₄ ⁻)/100 ml. protein soln.	0.49000
S'	g. (Na ⁺ + HPO ₄ ²⁻ + H ₂ PO ₄ ⁻)/100 ml. buffer	0.54600

* $\alpha' = (n - n')/c$.

† $(\rho - \rho')/(0.01c)$.

‡ $v' = (1 - k'_d)/\rho'$.

§ $k_w = (c_w' - c_w)/(0.01c)$.

MATERIAL

Ligamentum nuchae from ox, obtained fresh from the abattoir, was carefully freed from adhering fatty tissue and membrane. It was then cut into thin strips and comminuted finely in a power mincer. The product was then shaken gently with about 20 vol. of 1% (w/v) NaCl and the treatment continued with fresh salt solution until the extract gave no test for protein with trichloroacetic acid solution. The mince was washed free from NaCl with distilled water and was then extracted with a further quantity of distilled water in an autoclave at 1 atmosphere pressure for 45 min. Autoclaving was continued with fresh distilled water for further periods of 45 min. until the extract gave a negative biuret test. Usually three or four extractions were necessary.

After washing with warm distilled water, the material was dehydrated with ethanol and thoroughly extracted with 50% (v/v) ethanol-ether to remove lipids. It was extracted finally with ether and dried *in vacuo* at 30–40°. Final drying was carried out *in vacuo* over H₂SO₄ and the dry mince was then milled to a fine free-flowing powder by passage through a stainless-steel hammer mill.

The fine powder was again treated in the autoclave at one atmosphere pressure for two further periods of 45 min. to remove remaining traces of collagen and mucopolysaccharide. It was then washed with warm distilled water, dehydrated with ethanol and again extracted with ethanol-ether. After drying to a low water content it was again passed through the hammer mill using a fine-mesh sieve.

EXPERIMENTAL AND RESULTS

The purified preparation of elastin from *ligamentum nuchae* was a free-flowing cream-coloured powder, which under the microscope appeared as short, smooth, rod-like fibres of almost circular cross-section. The fibres appeared to be remarkably uniform in thickness; measurement of 50 fibres gave a mean value of 65 μ . for the diameter with a

variation from 36 to 99 μ ., whilst the lengths were of the order of 0.15–0.50 mm.

The fibres forming the dried powder from *ligamentum nuchae* were much thicker than the elastin fibres to be seen in loose connective tissue. These latter are observed as long thin threads, branching in many directions and staining strongly with orcein- or resorcin-fuchsin. Histological preparations of the fibres from *ligamentum nuchae* were made by embedding some of the powder in gelatin and fixing in neutral formalin solution; alongside this a number of preparations were made from abdominal areolar tissue from freshly killed rats and these were fixed with the same solution. Sections were then cut and stained both by Verhoeff's and Weigert-French's procedures for elastin. The sections were finally treated with the counterstains of Van Gieson and Mallory.

The fibres from the powder preparation showed the same staining properties as the elastin fibres in the areolar tissue sections; the collagen and ground substance in the latter preparations did not retain the orcein-fuchsin stain but were well demonstrated by the counterstains. Plate 1a shows the typical appearance of the fibres forming the purified elastin powder. Plate 1b shows a field in which there are several bundles of fibres which have escaped separation during the process of purification.

Composition of the elastin powder

For elementary analysis the powder was dried over P₂O₅ at 80° to constant wt. (56 hr.). The ash content (as sulphate) was less than 0.02%. The ash-free protein had N, 17.3 (Dumas); C, 51.8; H, 7.4% (combustions by Weiler and Strauss, Oxford). The S content varied in different preparations from 0.17 to 0.28%. Determinations of inorganic and ester-bound S (weighed as BaSO₄ after hydrolysis with 5N-HCl for 20 hr. at 100°) also gave variable results, different preparations yielding values in the range 0.02–0.13%; it thus seems possible that the variation in both sets of figures is due to the presence of small and variable amounts of ester-bound and inorganic sulphur, P,

determined by the method of Fiske & Subbarow (1925) was less than 0.02%.

The preparation gave negative Molisch and Seliwanoff tests but with the anthrone reagent (Morris, 1948) gave a colour corresponding to 0.17% carbohydrate (as glucose) and with the orcinol reagent (Sørensen & Haugaard, 1933) 0.30% (as glucose). Since both these reagents give high values in the presence of a large excess of certain proteins, due to the production of coloured degradation products, it is probable that the actual carbohydrate content is considerably lower. Analysis for hexosamine by the method of Elson & Morgan (1933) gave a colour equivalent to 0.25% (as glucosamine). Here again the colorimetric procedure is known to be unreliable in the presence of a large amount of protein (see, for example, Immers & Vasseur, 1952). Since the presence or absence of this content of hexosamine may be important, amino sugars were also determined chromatographically. The procedure used was a modification of the method of Moore & Stein (1951; see Partridge & Davis, 1955). Small elution peaks due to both glucosamine and galactosamine were observed, but the amount in each case corresponded to less than 0.05% of the protein. Small peaks corresponding to other substances giving a red colour with the Elson & Morgan reagents were also found. A full amino acid analysis, carried out on the hydrolysed protein, is given by Partridge & Davis (1955).

Partial hydrolysis with oxalic acid

The elastin powder (20 g.), prepared as above, was mixed with 0.25M oxalic acid (150 ml.) in a round-bottomed flask provided with an air condenser. The mixture was heated on a steam bath for 1 hr. and then rapidly cooled and centrifuged. The precipitate was washed on the centrifuge with 0.25M oxalic acid, and the washings were added to the supernatant. The residue was again treated with 0.25M oxalic acid for 1 hr. at 100°, and the extract and washings were collected as before. This was repeated five times in all. The whole of the powder dissolved to give a clear yellow solution which required no filtration. The solution was then dialysed in cellophan tubes against changes of distilled water until free from oxalate. The solution then contained about 1.25% (w/v) protein, as determined refractometrically, and was stored in the frozen state at -10°.

The diffusible material, which passed through the cellophan tubes, was investigated by evaporating the solution to small bulk and determining total N (Kjeldahl). The material represented 4.9% of the N originally taken. Oxalic acid was removed as the Ca salt and the solution evaporated to about 10 ml. Paper chromatograms prepared with this concentrated solution showed the presence of only one free amino acid, aspartic acid. The remainder of the material contained peptides, which on hydrolysis gave two-dimensional chromatograms similar to those of the hydrolysis products of the elastin powder.

Properties of the soluble protein

For the preparation of a dry product, the protein solution was evaporated from the frozen state *in vacuo*. This yielded a light foliated powder: ash, 0.03; C, 51.6; H, 7.4; N, 16.3%. Orcinol reaction for carbohydrate, 0.20% (as glucose).

The dry powder, which is called here 'the primary extraction product', was readily soluble in cold water, yielding a pale-yellow mobile solution, from which protein

was precipitated by the addition of trichloroacetic acid to 5% (w/v). In this respect the behaviour of the protein differs from that of the soluble protein produced by the action of elastase on elastin, which gives no precipitate with trichloroacetic acid at 5% (w/v) but gives an oily precipitate at 10–18% (w/v) (Banga, 1951). On raising the temperature of the solution of the primary extraction product in dilute buffer at pH 4–6, a precipitate, or 'coacervate', consisting of liquid droplets separated. The droplets showed no birefringence under crossed nicols and immediately dissolved again on lowering the temperature.

Effect of pH and ionic strength on the temperature of coacervate formation

For these experiments a solution of the primary extraction product containing 1.43% (w/v) was used. Equal volumes of protein solution and buffers of various pH and ionic strength were well mixed in small beakers and the pH was determined by glass electrode in each case. The final protein concentration was 0.715% (w/v). A number of lengths of thin-walled tube about 2 mm. diameter, were sealed off at one end, and a small quantity (0.3 ml.) of the various protein solutions was introduced into each. The tubes were attached to the bulb of a standardized thermometer by means of rubber bands, and the temperature was raised slowly by immersion in a water bath provided with a mechanical stirrer. The temperature at which opacity first appeared was noted in each case. The bath was then allowed to cool and the temperature at which the solution cleared was noted. These two temperatures were the same within 0.1°.

The results of the determinations are shown in Fig. 1. For the determinations at ionic strength 0.01, sodium acetate buffers were used over the range pH 3.6–5.8, glycine-HCl buffers over the range pH 2.5–3.6 and sodium phosphate buffers for the remainder of the experiments. The experiments at ionic strength 0.10 were carried out with

Fig. 1. Temperature of first appearance of coacervate. Primary extraction product, protein concentration 0.715% (w/v). Δ , acetate or phosphate buffer, ionic strength 0.01; $\circ$, buffer of ionic strength 0.01 with addition of NaCl to total ionic strength 0.10. The coacervate appeared on raising and disappeared on lowering the temperature.

the same range of buffers, but NaCl was added to give a concentration of 0.18 M. The final ionic strength was therefore 0.09 with respect to NaCl and 0.01 with respect to buffer.

To test the effect of protein concentration a parallel series of experiments was carried out at higher dilution (final protein concentration, 0.358% w/v). Within the accuracy of the determinations the points lay on the same curve; thus the concentration of protein appears to have no effect on the precipitation temperature in the range of concentrations studied.

Fig. 1 shows that at low ionic strength there is a marked minimum in the precipitation temperature at pH 5.3, the curve being nearly symmetrical about this point. As is shown later, the minimum lies close to the isoelectric point of the protein in the buffer used. At higher ionic strength the curve is much flattened and the minimum disappears.

Composition of the protein-rich phase. A solution of the primary extraction product (10 ml.) was mixed with 0.1 ml. of M sodium acetate buffer (pH 4.7). The protein concentration of the buffered solution was 1.408% (w/v) as determined refractometrically.

The solution was warmed to 37° and then centrifuged at the same temperature. The droplets of coacervate collected on the bottom of the centrifuge tube as a viscous syrup. The clear upper layer was carefully poured off and the refractive index of both the supernatant and the lower layer was determined. If the salt content of both layers is assumed to be the same, the protein concentration of the supernatant was 0.87% (w/v) and that of the lower layer $43.1 \pm 0.25\%$ (w/v). Assuming the specific volume of the protein to be 0.74 ml. (Table 1), calculation of the hydration value of the protein-rich phase gives 1.57 g. water/g. dry protein.

width and spreading of the boundaries in sodium acetate-NaCl buffer at pH 4.64.

In Fig. 3 the mobility readings are plotted against the buffer pH. At ionic strength 0.2 the value for the isoelectric point of the protein was 3.9, but the determinations carried out in the more dilute buffer showed a considerable change in isoelectric point, the value found being 4.8. Since a change in isoelectric point of this order is a rather unusual property, the isoelectric points of the protein were also determined in the same buffers by means of membrane potential measurements.

Fig. 3. Electrophoretic mobility of the primary extraction product: Δ , buffer of ionic strength 0.02; $\circ$, buffer of ionic strength 0.20 with the addition of NaCl to total ionic strength 0.20.

Fig. 2. Electrophoresis pattern of the primary extraction product in sodium acetate-NaCl buffer at pH 4.64 (I, 0.20). Left, negative, descending; right, positive, ascending. Top, pattern after 190 min.; bottom, pattern after 355 min. Potential gradient, 3.1 v/cm.^{-1} . Distance from A to B, 8.6 cm.

Electrophoresis experiments

When dialysed against buffer mixtures of ionic strength 0.20 the protein (primary extraction product) showed a single symmetrical peak in the electrophoresis apparatus at all pH values in the range pH 2-9. When the ionic strength was decreased to 0.02 the protein showed a single peak in the range pH 4.7-7.5, but below pH 4.7 a boundary anomaly was encountered which resulted in unsymmetrical peaks, particularly in the ascending limb. Fig. 2 shows the

Fig. 4. Molecular charge (assuming mol.wt. 67 000) from membrane potential measurements with the primary extraction product: I, $\circ$, charge at ionic strength 0.02 from membrane potentials; Δ , plot of electrophoretic mobility ($\text{cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$) multiplied by an arbitrary factor (3.11×10^6); II, $\circ$, charge at ionic strength 0.2 from membrane potentials.

Isoelectric point from membrane potential measurements

Fig. 4 (curve I) shows the valency of the protein (primary extraction product) when in equilibrium with acetate and phosphate buffer mixtures at various pH values in the pH range 3.0-7.5. The charge on the protein was calculated from measurements of membrane potential at 0° assuming an average particle weight of 67 000 derived from osmotic-pressure determinations reported later. The ionic strength

of the buffer solutions was 0.02; the readings were corrected for variations in protein concentration as described by Adair & Adair (1934). By interpolation (curve I) the isoelectric point of the protein at ionic strength 0.02 was 4.7. When the ionic strength of the buffer solution was raised to 0.20 by the addition of NaCl, similar determinations (curve II) showed that the protein was isoelectric at pH 4.0.

The broken line in Fig. 4 is a plot of values for the electrophoretic mobility of the protein measured in the same buffer mixtures as were used for the determination of membrane potentials. The values for mobility have been multiplied by an arbitrary factor in order to bring the ordinates to the same scale.

Fractionation of the primary extraction product

Several methods of fractionating the primary extraction product were attempted. These included fractional precipitation with ethanol at 20°, precipitation with ammonium sulphate at 0°, fractionation by ultrafiltration through highly porous collodion membranes, and precipitation as the coacervate at pH 4 (ionic strength 0.01) by raising the temperature.

The experiments resulted in the separation of two components with differing physical properties. The major component, designated the 'α-fraction', showed the characteristic property of phase separation on raising the temperature. Osmotic-pressure determinations for this fraction (which represented 60–75% of the whole) gave a mean molecular weight of 60 000 to 84 000 in different preparations. The minor component (designated the 'β-fraction') gave no precipitate on raising the temperature of an isoelectric solution and had a mean molecular weight of 6000 from osmotic-pressure measurements.

Ultrafiltration with a highly permeable collodion membrane. A solution (11 ml., containing 3.16% (w/v) protein) of the primary extraction product (mean mol.wt. 60000) in sodium phosphate buffer (pH 7.4; ionic strength 0.1) was filtered under pressure through a highly permeable collodion membrane. A mercury manometer was adjusted to provide an internal pressure in the sac sufficient to complete the filtration in about 8 hr. The residue in the sac (2–3 ml.) contained 12.1% protein. Osmotic-pressure determinations in the same buffer solutions showed that the diffusible fraction (which represented approximately 20% of the whole) had mean mol.wt. 9000.

The fraction of low molecular weight, after dialysis against 0.01M sodium acetate buffer (pH 4.7) in a cellophan membrane, gave no visible precipitate on raising the temperature. These results are interpreted as giving a clear indication that the primary extraction product contains a minor component of very low molecular weight and markedly different physical properties from those of the remainder of the material.

Fractionation with ammonium sulphate. A solution of the primary extraction product (10 ml.) containing 3.8% (w/v) protein was cooled to 0° and $(\text{NH}_4)_2\text{SO}_4$ solution, saturated at 0°, was added in successive amounts of 1 ml. The solution remained clear until 0.4 vol. of sat. $(\text{NH}_4)_2\text{SO}_4$ had been added, when a heavy precipitate of liquid droplets separated. This was removed by centrifuging at 0°. No further precipitate appeared until 1.0 vol. of $(\text{NH}_4)_2\text{SO}_4$ solution had been added when precipitation of liquid droplets was again observed. A further addition of 0.1 vol. of $(\text{NH}_4)_2\text{SO}_4$ resulted in a further light precipitate and a little more

material separated on saturating the centrifuged solution with $(\text{NH}_4)_2\text{SO}_4$ crystals. The precipitate which appeared after addition of 0.4 vol. of sat. $(\text{NH}_4)_2\text{SO}_4$ represented about 60% of the protein originally present, and after dialysis against water followed by 0.01M sodium acetate buffer (pH 4.7) it showed the property of reversible coacervate formation characteristic of the α-fraction. The precipitates which appeared after the addition of 1 vol., 1.1 vol. and at saturation with $(\text{NH}_4)_2\text{SO}_4$ were combined and dialysed against water, followed by the acetate buffer. On raising the temperature of the buffered solution no precipitate formed. It was thus concluded that this substance was the minor protein component (β-fraction) observed during the ultrafiltration experiments. It is significant that no precipitate was formed between the addition of 0.4 and 1.0 vol. of sat. $(\text{NH}_4)_2\text{SO}_4$, and this was taken to indicate that the molecular-weight distribution in the primary extraction product takes the form of two discrete peaks, corresponding to the α- and β-protein fractions.

A similar fractionation carried out with ethanol at 20° gave no clear separation between the α- and β-proteins.

Fractionation by precipitation of the coacervate at higher temperatures. A solution of the primary extraction product (340 ml., 1.25% (w/v) protein) was adjusted to pH 4.7, ionic strength 0.01 with sodium acetate buffer. A large round-bottomed flask was arranged on the shaft of a geared motor so that it could be rotated slowly in a bath of water with its neck at an angle of about 45°. About 100 g. of glass spheres (approx. 4 mm. diam.) were introduced into the flask together with the protein solution. The water bath was raised to 50° and maintained at this temperature for 10 min. while the flask was rotating. The droplets of coacervate which formed in the solution were transferred to the surface of the beads as a thick viscous layer and the supernatant solution finally became clear. A glass filter funnel, containing a fluted paper, was arranged in a constant-temperature oven at 60°, and the clear supernatant in the flask (still at 50°) was poured off the beads directly into the funnel. After draining, the viscous coacervate was dissolved in ice-cold water by slowly rotating the flask in a bath containing ice and water.

The coacervate solution was then adjusted to the original pH and ionic strength by the addition of sodium acetate buffer and the process repeated twice more. The supernatant solutions from the second and third precipitation contained less than 0.06% (w/v) of protein and were rejected. The supernatant from the first precipitation was heated to 80°; a further light precipitate of coacervate formed, and this was removed by shaking with a little Hyflo-Supercel (Johns-Manville Co., London) and filtering at 80°. The solution was evaporated to 34 ml. by use of a rotary evaporator (Partridge, 1951). It was then dialysed against 0.01N sodium acetate buffer (pH 4.7) to equilibrium and again raised to 80°. The slight opalescence which formed was removed by shaking at 80° with Hyflo-Supercel and filtering at the same temperature.

The protein from the supernatant was recovered by dialysing against water and drying from the frozen state (yield 0.73 g.). Its solution in 0.01N acetate buffer gave no opalescence on heating to 90° and the product was therefore identified as the β-protein. Osmotic pressure determinations on this product gave a value of 6000 for the mean mol.wt.

The thrice-precipitated coacervate was recovered by dialysing the solution against distilled water, filtering to remove traces of insoluble matter and finally drying from the frozen state. It showed the typical property of coacervate formation, and was therefore regarded as consisting substantially of the α -fraction. Molecular weight determinations by osmotic-pressure measurements gave a value of 70 000. As a test for degree of homogeneity, its behaviour on fractional precipitation with ethanol and with ammonium sulphate was determined. The whole of the material in an aqueous solution containing 0.7% of protein was precipitated sharply on addition of 0.2 vol. of ethanol. After standing for several hours, the precipitate was removed by centrifuging, and no protein could be detected in the clear supernatant. A similar result was given with ammonium sulphate fractionation at 0° according to the procedure already given; the whole of the protein was precipitated sharply on addition of 0.4 vol. of saturated ammonium sulphate, and no fraction corresponding to the β -protein was found.

The results of these and other fractionation procedures attempted are generally in accordance with the view that the primary extraction product is a mixture of two gross components, one of very high mean molecular weight and one of low molecular weight. The absence of centre fractions suggests that components of intermediate molecular weight are absent.

In order to obtain some further indication of the degree to which the α -protein was itself polydisperse, the product from this fractionation was again fractionated by coacervate formation at a lower temperature. A solution of the α -protein (78 ml.) containing 0.7% protein (0.585 g.) was adjusted to pH 4.7, ionic strength 0.01, with acetate buffer and centrifuged at 37° in a constant-temperature room. The coacervate which formed collected as a viscous layer in the bottom of the centrifuge cups. The clear supernatant was poured off and contained 0.22% protein (0.17 g.). On evaporating this solution to small bulk and dialysing it again against 0.01 N acetate buffer (pH 4.7), the temperature at which droplets of coacervate appeared in the solution (protein concentration 0.9%) was 35.2°. This material was therefore regarded as a fraction of the α -protein having a mean molecular weight lower than that of the starting material. Owing to an accident, osmotic pressure determinations could not be carried out on this fraction.

The coacervate which had been precipitated at 37° was taken up in the ice-cold acetate buffer and was again precipitated at 37°. The process was then repeated for a third time. The second and third supernatants contained less than 0.06% (w/v) protein and were rejected. The thrice-precipitated coacervate was taken up in 37 ml. of ice-cold buffer (protein concentration 1.1% (w/v), wt. of protein 0.41 g.) and a sample taken for a precipitation-point determination at pH 4.7. The temperature at which opalescence first appeared was found to be 22.2°. Osmotic-pressure determinations on this fraction gave a value of 130 000 for the mean molecular weight. This result indicates that the α -protein is itself polydisperse over a considerable range.

Osmotic pressure experiments with solutions of the β -fraction

The simplest results were obtained with solutions of the β -fraction, specified in Table 2, with concentrations of

electrolyte controlled by dialysis with a sodium phosphate buffer mixture containing traces of sodium bicarbonate.

In the solutions referred to in Table 2, the observed osmotic pressures were directly proportional to the protein concentration. The third column gives values for M/ϕ , where M is the number-average molecular weight of the protein, and the symbol ϕ represents the effects on the membrane equilibrium of diffusible ions and other factors which may cause deviations from van't Hoff's law (Adair & Robinson, 1930*b*).

Table 2. *Osmotic pressure of the β -protein*

Buffer mixture: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 10.747 g./l. (0.03 M); $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.561 g./l. (0.01 M). The initial ionic strength was 0.10 and the initial pH value 7.42 at 1.0°. II, osmotic pressure in mm. Hg reduced to 0°; c , percentage concentration in g. anhydrous protein/100 ml. protein solution; M/ϕ , 'apparent molecular weight' (see text). Calculated on basis that $M/\phi = 17034$ (10 c/II). Final pH values of dialysates containing traces of CO_2 ranged from 7.37 to 7.40.

c	II	M/ϕ
0.187	5.82	5500
0.578	17.72	5600
0.932	28.35	5600
1.094	34.35	5400

The observed variations in M/ϕ are unusually small; it may be inferred that the factor ϕ is near to unity and that the mean value for M/ϕ , 5500 ± 100 , is nearly equal to the molecular weight.

Osmotic pressures of solutions of the primary extraction product

Table 3 records results for a preparation of the primary extraction product. It will be seen that the apparent molecular weights for this unfractionated protein are much more variable than the figures for the β -fraction.

The measurements in Table 3 show two types of variations. (1) The values for the apparent molecular weight

Table 3. *Osmotic pressure of solutions of the primary extraction product*

pH values of the dialysates ranged from 7.37 to 7.40; ionic strength 0.10. Conditions as in Table 2. II, osmotic pressure in mm. Hg reduced to 0°; c , g. anhydrous protein/100 ml. protein solution; M/ϕ , 'apparent molecular weight'. (For basis of calculation see text.) $\Pi_{(\text{calc.})} = 2.839 c/(1 - 0.084 c)$ (see text).

c	$\Pi_{(\text{obs.})}$	$\Pi_{(\text{calc.})}$	$M/\phi_{(\text{obs.})}$
0.47	1.51	1.40	53 000
0.53	1.47	1.59	62 000
0.87	2.43	2.65	61 000
1.26	4.40	4.00	49 000
1.53	4.87	4.98	34 000
1.97	7.24	6.71	47 000
2.03	6.71	6.94	51 700
3.08	12.10	11.77	43 500
5.43	29.20	28.40	31 800
5.54	29.10	29.41	32 500

M/ϕ tend to diminish as the protein concentration is increased as shown in the last column in Table 3. (2) There are erratic variations, shown by comparing observed osmotic pressures, listed in column 2, with calculated pressures recorded in column 3. These pressures were computed by making use of an empirical formula

$$\Pi_{\text{calc.}} = 2.839c/(1 - 0.084c).$$

The mean difference between observed and calculated pressures is 0.3 mm. Hg, or 5.5 % of the calculated pressure.

These variations are much larger than the experimental errors observed in earlier series of measurements made with haemoglobin, where the mean difference between observed and calculated pressures was 0.015 mm. Hg (Adair, 1949).

The larger variations observed may be due to the heterogeneity of the protein. In the process of measuring the osmotic pressure of a protein, a part of the protein may be adsorbed by the membrane. If the protein be heterogeneous, the process of adsorption may lead to alterations in the mean molecular weight. The quantities of protein adsorbed are influenced by the properties of the membranes, and it follows that the mean molecular weight observed in a series of measurements made with different membranes may show measurable variations.

Experiments with the ultracentrifuge

These experiments were carried out by Dr R. A. Kekwick at the Lister Institute of Preventive Medicine, London. Since the temperature rises during ultracentrifuge runs (mean temperature about 25°), it was necessary to choose conditions of pH and ionic strength such that the protein would remain soluble during the course of the experiment. The conditions chosen were: protein concentration, 1.79 % (w/v); sodium phosphate buffer (pH 7.4); ionic strength 0.1. The protein solution was dialysed against the buffer to equilibrium at +2° and the protein concentration adjusted by the addition of more buffer. The solutions were examined in a 12 mm. cell at 270 000 g; interval between exposures, 10 min.

Three solutions were examined: (1) a preparation of the primary extraction product (mean molecular weight by osmotic pressure, 65 000); (2) an α -protein preparation derived from (1) by dialysis for several days with a collodion membrane of high permeability (mean molecular weight 81 000); (3) the diffusible fraction which passed through the membrane during the preparation of (2). This had mean molecular weight 26 000 and gave a light precipitate of coacervate on heating; it could thus be regarded as the β -fraction admixed with material usually considered as part of the α -fraction. All three solutions gave single peaks with closely similar sedimentation rates.

The spread of the peaks suggested that the sedimenting protein was to some extent polydisperse. The single peak shown by the primary extraction product and the diffusible fraction was presumably that due to the α -protein which constituted about 75 % of the original preparation, the low-molecular β -protein (mol.wt. 6000) being unobserved.

Rate of release of the two protein fractions during mild hydrolysis of elastin powder

Since two protein fractions with dissimilar physical properties are released during the extraction of purified elastin powder with hot dilute organic acids, the question arises as to whether this may be the result of structural or

perhaps chemical heterogeneity of the elementary elastin fibres comprising the tissue. If this were the case, it might be expected that the two components would be released at different rates during the process of extraction. Accordingly, the contents of α - and β -protein were estimated in each of the successive extracts produced on treatment of elastin powder with 0.25M oxalic acid at 100°.

The extractions were carried out with 5 g. of elastin powder using 50 ml. of 0.25M oxalic acid for each extract. The mixtures were in each case heated for 1 hr. and, after cooling, the residue of elastin was collected by centrifuging. The residue, without washing, was then mixed with a further 50 ml. of oxalic acid in readiness for the next extraction. The protein content of each supernatant was measured refractometrically and the results are recorded in Fig. 5.

Fig. 5. Extraction of 5 g. of elastin powder by heating with successive 50-ml. quantities of 0.25M oxalic acid at 100° for 1 hr.; Δ , g. total protein dissolved in each extract; $\times$, g. α -protein released; $\circ$, g. β -protein released.

In order to separate the β -protein, the extracts were first dialysed against repeated changes of distilled water followed by sodium acetate buffer (pH 4.7, ionic strength 0.01). The buffered solutions were then raised to 37° and centrifuged at the same temperature. The supernatant solutions were heated to 90° with the addition of a little Hyflo-Supercel and filtered hot. After cooling, the content of β -protein in each of the filtrates was measured refractometrically; the corresponding contents of α -protein were calculated by difference. The results are given in Fig. 5.

The figure shows that the total soluble protein in the extracts increases exponentially as the extraction proceeds, but the two component proteins are liberated at different rates. The first extract is almost wholly β -protein and this component also constitutes the greater part of the

second extract. In the third extract the proportions are about equal, but in subsequent extracts the α -protein is overwhelmingly the major component.

DISCUSSION

The work described in this paper has been carried out with a purified preparation of elastin powder obtained from *ligamentum nuchae* of cattle. This substance is regarded as a chemically defined fibrous protein; it is believed to be in a state of substantial purity and to represent, as such, an integral component of the fibres of elastin tissue.

Yellow elastic tissue is known to contain, in addition to elastin, considerable amounts of collagen and smaller amounts of other proteins, mucoids and mucopolysaccharides, including chondroitin sulphate. These are associated more or less intimately with the fibrous elements of pure elastin, which are themselves largely responsible for the characteristic physical properties of the tissue.

A procedure for the isolation of the protein 'elastin' must ensure complete removal of the other tissue components, but at the same time it must not result in chemical damage to the elastin elements. It has been shown (Partridge & Davis, 1950) that, with many proteins, treatment with 2% acetic acid solution at 100° for several hours results in the hydrolytic cleavage of peptide bonds at specific points in the chain. Similarly, the work of Courts (1954) shows that on heating a sample of gelatin at 75° for 24 hr. at pH 3.0, free α -amino groups were liberated and the mean molecular weight of the sample fell from 58 000 to 7000. Boiling with dilute acetic acid as a step in the purification of elastin (cf. Gross, 1949; Hall, 1951) was avoided therefore; instead, a procedure based on repeated extraction with water in an autoclave at 120° was adopted. In order to ensure the complete removal of collagen and mucopolysaccharide, the dried powder produced after the first course of extractions was finely ground in a hammer mill before treating for a second time in the autoclave. Microscopic examination showed that this intermediate fine grinding separated the fibre bundles and thus exposed fresh surfaces to the action of boiling water. The final product, after again drying and grinding, consisted essentially of morphologically intact elastin fibres, which had the same staining properties as those to be seen in tissue preparations. Comparison of the analytical data and physical properties shows that this product is closely similar to the elastin preparation described by Stein & Miller (1938).

The elastin preparation is not soluble in boiling 40% urea solution, even after 24 hr. treatment, but it should be noted that under these conditions urea

solutions develop a strongly alkaline reaction, and very prolonged treatment with boiling urea results in partial or complete dissolution (cf. Hall, 1951) which may be caused by alkaline hydrolysis of peptide bonds.

Treatment with boiling dilute oxalic acid solution results in complete dissolution of the fibrous protein and yields the mixture of two soluble proteins which is studied in this paper. The formation of a mixture of proteins with markedly dissimilar physical properties might suggest that the elementary elastin fibre is a complex structure composed of two macromolecular elements, each giving rise to a different soluble protein. Suggestions such as this have already been made in respect of other fibrous proteins, notably keratin (Alexander & Gough, 1951; Pauling & Corey, 1953). The possible existence of a complex structure of this sort is rendered more plausible when account is taken of the results of examination of successive supernatant solutions produced during extraction of elastin powder with hot oxalic acid. In these experiments it is shown that the protein fraction of low molecular weight (the β -protein) is liberated at a greater rate than the high-molecular α -protein in the early stages of the extraction, but in the later stages the position is reversed and the α -protein is liberated more rapidly. This is the opposite of what would be expected if the β -protein were simply a degradation product of the protein of higher molecular weight, and the result seems to indicate that the elastin fibre may be structurally heterogeneous. Parallel lines of evidence are not available, owing to its amorphous appearance under the electron microscope, its lack of birefringence in the unstretched condition in plane-polarized light and the amorphous fibre diagrams given in X-ray studies. However, it should be noted that, if the fibre were in fact a homogeneous structure, the results would still be consistent were it assumed that large aggregates of polypeptide chains constituting the α -protein are released only after the fibre has been weakened at certain points by the removal of exposed peptide chains as smaller fragments.

An alternative view is that some of the peptide chains composing the fibre may be different in chemical constitution from the remainder; in this case the α - and β -proteins should themselves show chemical differences which would be detectable by amino acid analysis. The paper which follows (Partridge & Davis, 1955) presents the chemical evidence bearing on these alternatives.

The reversible precipitation of the α -protein which occurs on warming its solutions is of interest as an unusual and characteristic physical property. The formation of partially miscible liquid systems is rare with solutions of native corpuscular proteins

but is of frequent occurrence with gelatin sols. These are now generally regarded as true solutions in which the linear macromolecules take up the statistically probable form: as loose skeins of random kinks or coils which occlude a large amount of solvent. Addition of a non-solvent such as an aliphatic alcohol favours both intermolecular and intramolecular association, and if sufficient is added a point is reached when intermolecular association results in the separation of a protein-rich phase which remains liquid because of the high content of occluded solvent it still contains (Bungenberg de Jong, 1949).

The behaviour of solutions of the α -protein differs from the gelatin system in that phase separation is induced by a rise in temperature, although it may also be induced by the addition of fairly high concentrations of salts or ethanol. A negative solubility increment is quite common with aqueous solutions of partially miscible organic liquids, such as *s*-collidine, and has also been observed with protein solutions, the outstanding example being Bence-Jones protein (see Putnam, 1953). In the present work no detailed study of the process of phase separation has been undertaken and its mechanism remains obscure. However, it is significant that the α -protein contains an unusually high content of non-polar amino acid residues, which would be expected to decrease its solubility. The high solubility in cold water which is in fact observed may be due to a structure in which the non-polar parts of the molecule are free to form intramolecular associations, thus reducing their effective surface. A rise in temperature would tend to reduce this type of association, and by causing the molecule to unfold would favour the formation of the intermolecular bonds which ultimately lead to the separation of a second phase. The data in Fig. 1 show that the temperature of phase separation is lowest at the isoelectric point when the repulsive charge on the molecule is least; as would be expected the pH dependence is reduced in the presence of low concentrations of salts owing to the effect of inorganic ions in reducing the coulombic repulsion.

SUMMARY

1. Elastin from *ligamentum nuchae* of cattle is transformed into a soluble protein by repeated extraction with 0.25M oxalic acid at 100° with the loss of about 5% of the nitrogen as small peptides.

2. The protein is soluble in distilled water or buffer solutions at temperatures below 25°, but on raising the temperature of the solution in dilute buffer at pH 4–6 a precipitate consisting of liquid droplets separates.

3. The protein shows a single boundary peak in the Tiselius electrophoresis apparatus in buffers of

ionic strength 0.20 at all pH values in the range pH 2–9.

4. At ionic strength 0.2 the pH values for the isoelectric point from electrophoretic mobility measurements and from membrane potential determinations are 3.9 and 4.0 respectively; at ionic strength 0.02 the values are 4.8 from electrophoresis measurements and 4.7 from membrane potentials.

5. Fractionation of the soluble protein shows it to consist of two components with markedly different physical properties; the major component (α -fraction) shows the characteristic property of reversible coacervate formation on heating and has a mean molecular weight of 60 000–84 000 in different preparations, while the minor component (β -fraction) is soluble in water at all temperatures and has a mean molecular weight of 5500.

6. Further fractionation and investigation with the ultracentrifuge suggests that the α -protein is itself polydisperse.

7. The α - and β -proteins are liberated at different rates during the extraction of elastin powder.

8. The bearing of these results on the structure of the native fibrous protein is discussed.

The authors wish to thank Dr R. A. Kekwick for his examination of the soluble proteins with the ultracentrifuge. Mr H. P. Hale carried out the histological examination of the purified elastin powder.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Chemistry of Connective Tissues

3. COMPOSITION OF THE SOLUBLE PROTEINS DERIVED FROM ELASTIN

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(Received 17 December 1954)

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