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Physical Studies on a Soluble Protein Obtained by the Degradation of Elastin with Urea

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By the treatment of elastin with oxalic acid at 100°, Adair, Davis & Partridge (1951) obtained a soluble protein which, on gentle warming in pH 4–6 buffer solution, turned milky in appearance; cooling reversed the phenomenon. The effect was due to the separation of a second phase consisting of minute drops of protein solution at a different concentration. Electrophoresis failed to show the existence of more than one component except at an ionic strength of 0.02 and pH 3–5 when there was partial separation of two components. Porous dialysis separated the protein into two components of molecular weight (by osmometry) 6000 and 84000. Only the component of higher molecular weight showed the reversible separation of another phase on warming. Both fractionated and unfractionated material showed only one boundary on sedimentation in the ultracentrifuge, the sedimentation constant being the same in each case.

Hall (1951) showed that elastin could also be brought into solution in boiling 40% (w/v) urea. The protein obtained after removing the urea by dialysis showed great similarity to that obtained by oxalic acid degradation. This protein has been studied in the work described here. Evidence is presented which can be interpreted in terms of a reversible dissociation of the protein. The dissociation is favoured by dilution.

METHODS AND RESULTS

Preparation of the protein

Bovine ligamentum nuchae was stripped of extraneous material as thoroughly as possible by mechanical means, then finely minced. Approx. 500 g. of the minced material

was mixed with 1.5 l. of 1% aqueous acetic acid and boiled under reflux for 2–3 hr. to convert collagen into gelatin. The mixture was filtered through cloth, squeezed as dry as possible and then weighed approx. 300 g. In order to prepare the urea breakdown product, about 100 g. of this elastin was refluxed with 5½ l. of 40% (w/v) aqueous urea until a clear solution was obtained. This usually took about 40 hr. The mixture was filtered through cloth and the filtrate dialysed in cellophane for 4 days against running tap water to remove urea, amino acids, etc. The solution was next evaporated under reduced pressure to 2 l. The concentrated solution was brought to pH 10 with NaOH and any residue spun off. HCl was then added to bring the solution to pH 2, any residue being spun off and rejected. The calculated quantity of solid NaCl was added to make the solution 2.5 M, when the protein separated out as a dense opalescent suspension which was centrifuged. The protein was redissolved most easily, giving a pale-yellow solution, in water brought to pH 10 with NaOH. The material obtained resembled that obtained by treating elastin with oxalic acid (Adair *et al.* 1951) in showing the reversible separation of another phase on heating. The temperature at which the milky appearance due to the separation of fine droplets of the second phase appears depends chiefly on the pH. The precise nature of the buffer present is of much less importance than the pH and the concentration of the material. At pH 2–3 an approx. 1% (w/v) solution shows the phenomenon when merely warmed in the hand, but the higher the pH of the solution the higher is the temperature required. The material gave a negative result when tested for carbohydrate by Molisch's test. It gave an intense biuret reaction.

Physical properties

Sedimentation in the ultracentrifuge. The experiments were carried out with the Spinco electrically driven ultracentrifuge using the analytical cell with 12 mm. channel. Observations were made on solutions containing 0.259, 0.106, and 0.042% N (w/v). Estimation of concentration was by

micro-Kjeldahl determinations, but biuret colours were used as a secondary method for checking the dilutions of stock protein solutions. All runs were carried out in the buffer of Miller & Golder (1950) at pH 4 and ionic strength 0.2 against which the samples of material had been dialysed for 2 days. The micro-Kjeldahl analyses were carried out on the dialysed material. Rotor speeds giving gravitational fields of approx. 250 000 *g* were used (but revolution-counter readings were used for the actual calculation of sedimentation constants). Fig. 1 shows the variation of the sedimentation constant with concentration after correcting values to pure water as solvent at 20°. No separation into more than one component was observed on runs lasting 1–1.5 hr. after reaching top speed. A typical sedimentation diagram is shown in Fig. 2.

Fig. 1. The variation of the sedimentation constant (○—○) and diffusion coefficient (●—●) with concentration for the elastin degradation product dissolved in buffer at pH 4 and ionic strength 0.2.

Fig. 2. Sedimentation diagram for protein solution in buffer (pH 4, ionic strength 0.2) after 1.5 hr. at approx. 250 000 *g*. Solution contains 0.042% (w/v) N. Direction of sedimentation is from right to left.

Diffusion. A diffusion cell was used similar to that described by Claesson (1946) and made out of stainless steel. The diagonal schlieren optical system was employed and care taken that the light passing through the cell was parallel. The cell was supported in a rigid water bath standing on a concrete floor. Temperature was controlled at $20^\circ \pm 0.01$, the circulating pump being remotely situated from the bath to prevent vibration. Diffusion coefficients were calculated by the area-maximum height method. All runs were in the buffer used for the sedimentation work, and were of 2 days duration. Values of the diffusion coefficient (corrected to water as solvent at 20°) are plotted against concentration in Fig. 1.

Partial specific volume. This was found by weighing successive dilutions of protein of known weight fraction in a pycnometer. Temperature was controlled at 20° when filling the pycnometer. The results were calculated according to Kraemer (1940) and the value 0.778 obtained. If a partial specific volume is calculated from the amino acid analysis given for undegraded elastin by Neumann (1949) it is found to be 0.766.

Ultraviolet spectrum. This was determined for the material dissolved in 0.01 *N*-HCl using a Unicam SP 500 spectrophotometer and is shown in Fig. 3.

Fig. 3. The ultraviolet-absorption curve in 0.01 *N*-HCl for the protein obtained by degradation of elastin with urea.

Specific refraction increment. This was calculated from micro-Kjeldahl determinations of concentration and refractive index measurements using an Abbé type refractometer. The value obtained was $\Delta n = 0.0118$ for 1% (w/v) of N in the solution.

DISCUSSION

The evidence given above is consistent with the material described being largely polypeptide in nature. The positive variation of the sedimentation constant with concentration, and the converse variation of the diffusion coefficient suggest 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. Reversible protein dissociations are well known, e.g. arachin (Johnson, 1946) and insulin (Harfenist & Craig, 1952; Creeth, 1953; Fredericq, 1953). If the rate at which

equilibrium is established between dissociated and undissociated molecules is rapid compared with the rate of separation during sedimentation no resolution of the components is observed and the sedimentation constant assumes an intermediate value.

Adair *et al.* (1951), in their studies on the oxalic acid degradation product of elastin, failed to detect the component of molecular weight 6000 in the ultracentrifuge and obtained identical sedimentation constants for material fractionated by porous dialysis and also for the unfractionated material. This would be explained by assuming an equilibrium between the components.

On extrapolation to zero concentration the sedimentation constant is found to be 0.71 Svedberg unit and the diffusion coefficient $11.33 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. These values taken with the value for the partial specific volume lead to a molecular weight at infinite dilution of 6870. In view of the errors involved in the estimation of such a low molecular weight by sedimentation velocity and the nature of the extrapolations this may mean identity with the 6000 molecular-weight component described by Adair *et al.* (1951). A reliable value of

the maximum molecular weight approached at high concentrations cannot be given since sedimentation and diffusion coefficients depend on concentration in cases where there is no possibility of dissociation occurring. By analogy with the material prepared by the oxalic acid route a value of 84 000 might be expected for the maximum molecular weight.

SUMMARY

1. The preparation of a soluble protein by treating elastin with hot concentrated aqueous urea is described.

2. Sedimentation, diffusion and other physical measurements have been made on this material.

3. The results have been explained in terms of a reversible dissociation favoured by dilution and a value given for the minimum molecular weight.

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Glycerylphosphorylethanolamine in Rat Brain

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The presence of glycerylphosphorylethanolamine (GPE) in aqueous extracts of pig and rabbit liver has been reported by Campbell & Work (1952). More recently, Walker (1952) in carrying out an investigation of the amino compounds in various animal tissues has shown a small amount of glycerylphosphorylethanolamine to be present in ox brain but was unable to detect it in rat brain. Interest in this compound lies chiefly in its possible role as an intermediate in the synthesis of phosphatidylethanolamine, one of the constituents of the kephalin fraction of the phospholipids.

Hahn & Hevesy (1937) demonstrated by means of experiments with radioactive phosphorus (^{32}P) that there is an uptake of phosphorus by the phospholipids of brain tissue, and it has been further shown by Dawson & Richter (1950) that when due allowance is made for the slow rate of the entry of ^{32}P into the brain this uptake is comparable with that of other tissues. Very little, however, is known of the route by which these compounds are synthesized in the tissues. Popják & Muir (1950) investigated possible precursors of the phospholipids using ^{32}P and calculated that α -glycero-