OBSERVATIONS ON COACERVATION OF SOLUBLE ELASTINS

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Coacervation of soluble elastins has been studied using a derived protein, α-elastin, and the precursor of mature elastin, tropoelastin. The occurrence and the degree of coacervation are related to pH, ionic strength, and the composition of buffer. In comparison with other common ions, acetate ions have proved to affect favourably the degree of coacervation. The results enable some suggestions to be made concerning the procedure for the isolation of soluble elastin.

Soluble elastins undergo coacervation at elevated temperatures[1]. As other components of connective tissue do not exhibit this behaviour, coacervation has become an important step in the preparation of native soluble elastin from tissue[2-4]. The aim of the present work is to contribute to the knowledge of the conditions of elastin coacervation, and so to improve the effectiveness of the procedure for the preparation of soluble elastin.

For the study of some general features of coacervation, α-elastin has been used as a model protein. The conclusions reached have been applied to tropoelastin considered to be the true native precursor of mature elastin[3].

EXPERIMENTAL

Materials and Methods

Bovine ligamentum nuchae was taken from adult animals immediately after slaughter, freed of adherring fat and muscle tissues, and kept at −40°C until required. Tropoelastin was prepared from aorta of Cu-deficient pig.

Insoluble elastin was prepared from minced ligaments by hot alkali procedure substantially after Lansing and coworkers[5]. The material was dispersed in 0·1M-NaOH and heated in a boiling water bath for 45 min. The suspension was filtered and the residue (insoluble elastin) was washed several times with hot water to neutral reaction, then several times with absolute ethanol, twice with acetone, twice with ether, and was finally dried *in vacuo* at room temperature. α-Elastin was prepared from the insoluble elastin by a slightly modified method of Partridge and coworkers[1]. Insoluble elastin was hydrolyzed with 0·25M oxalic acid in a boiling water bath until all the material was dissolved. The solution was neutralized with 1M-NaOH, dialyzed and freeze-dried. The dried hydrolysate was dissolved in water (1 : 20 weight ratio) and heated to 80°C

in a slowly revolving flask in a water bath. Superhyphlocell was added (approx. 1 g per 20 ml) preheated to the same temperature. Coacervated α-elastin was adsorbed to Superhyphlocell within a few minutes and the mixture became clear. The suspension was filtered through Whatman No 1 filter paper preheated to 80°C together with the funnel. The filtrate containing low molecular weight components (β-elastin) was discarded. α-Elastin was extracted from the residue with cold water. The procedure of coacervation and extraction was repeated three times. The resulting extract was dialyzed and freeze-dried yielding pure α-elastin. Acetoacetylated α-elastin was prepared by the diketene treatment of α-elastin after Marzotto and coworkers[6]. Esterified α-elastin was prepared by methylation of α-elastin with 0·1M-HCl in absolute methanol as described by Chibnall and coworkers[7]. Guanidylated α-elastin was prepared by the method of Baret and coworkers[8], using treatment of α-elastin with S-methylthioisourea.

The degree of coacervation was estimated from the optical density at 440 nm of the protein solutions exposed to temperatures of 20 to 80°C. Spectrophotometer Optica was used throughout the study. Concentration of α-elastin and modified α-elastins was 1 mg per 5 ml, that of tropoelastin 0·25 mg per 3 ml. The optical path was 1 cm in all cases.

RESULTS

The Influence of Various Cations and Anions on α-Elastin Coacervation

In preliminary experiments (not described) pH 5·0 was chosen for this part of the work. The set of cations studied comprised Na^+, K^+, Ca^{+2}, and Mg^{2+} (in the form of chlorides), that of anions F^-, Cl^-, SO_4^{2-}, PO_4^{3-}, acetate, and citrate (in the form of sodium salts). pH was adjusted with NaOH or the respective acid. Coacervation of α-elastin was followed in solutions which were either 0·025M with respect to the ion tested or had ionic strength 0·025. The results expressed as a plot of optical density *vs* temperature at the pH values of the occurrence of coacervation are given in Figs 1–3.

As obvious, the differences between the cations used are relatively small (Fig. 1). On the other hand, the degree of coacervation differed considerably in the dependence

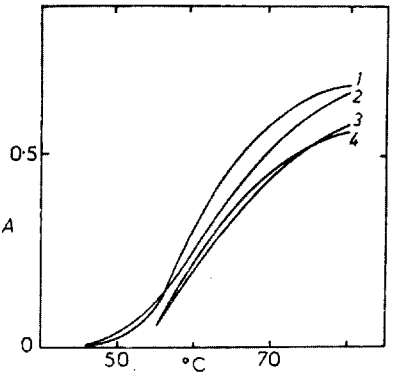

FIG. 1

The Influence of Cations on the Degree of Coacervation of α-Elastin

1 mg of α-elastin per 5 ml of 0·025M salt solution, pH 5·0. *A* Absorbancy at 440 nm. Curve: *1* $CaCl_2$; *2* $MgCl_2$; *3* KCl; *4* NaCl.

on the anion quality, mainly in the temperature range of 50–70°C (Fig. 2). At identical ionic strength, the extent of coacervation was much higher in sodium acetate solution and, to a smaller extent, in sodium fluoride solution than in any other salt solution tested (Fig. 3).

The Influence of pH *and Ionic Strength on α-Elastin Coacervation*

On the ground of the results given above sodium acetate buffers were used in this part of the work. The concentration of sodium acetate was 0·02M. Ionic strength was adjusted with sodium chloride to 0·02, 0·05, 0·10, 0·50, and 1·00, pH was adjusted with HCl, acetic acid or NaOH to 3·0–8·0 at one unit intervals. The results are shown in Fig. 4.

It can be seen that increasing ionic strength results in a decrease in the temperature and pH value of the first occurrence of coacervation. Thus, at ionic strength 0·02 coacervation appeared first at 45°C and pH 5·0, at ionic strength 1·0 coacervation was observed below 20°C at pH 3·0. Further, the higher ionic strength the broader the pH range in which coacervation occurs. At ionic strength 0·02 the occurrence of coacervation was limited to pH values 4·0 and 5·0 only, at ionic strength 1·0 coacervation took place at all pH values used (3·0–8·0).

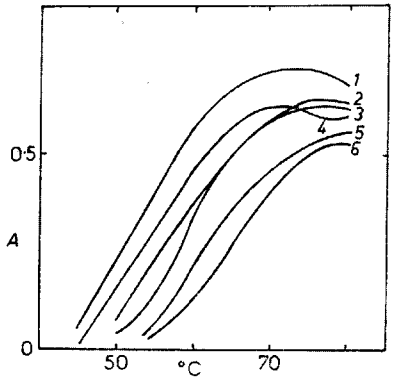

FIG. 2

The Influence of Anions on the Degree of Coacervation of α-Elastin

1 mg of α-elastin per 5 ml of 0·025M salt solution, pH 5·0. *A* absorbancy at 440 nm. Curve: *1* Na-acetate; *2* Na-phosphate; *3* Na_2SO_4; *4* NaF; *5* NaCl; *6* Na-citrate.

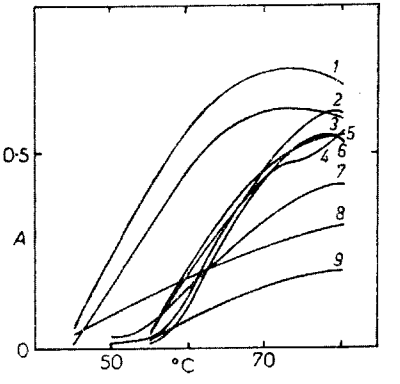

FIG. 3

The Influence of Salts on the Degree of Coacervation of α-Elastin

1 mg of α-elastin per 5 ml of the salt solution, $\Gamma/2 = 0{\cdot}025$, pH 5·0. *A* absorbancy at 440 nm. Curve: *1* Na-acetate; *2* $CaCl_2$; *3* NaF; *4* KCl; *5* NaCl; *6* $MgCl_2$; *7* Na_2SO_4; *8* Na-phosphate; *9* Na-citrate.

Coacervation of Modified α-Elastins

Solutions in acetate buffer of ionic strength 0·1 were used. The measurements were carried out at pH values 3·0 to 8·0 at intervals of 0·5 units. The results are shown in Fig. 5. As evident, after the blockage of ε-NH_2 groups by acetoacetylation coacervation took place at pH values 3·0 to 4·0 only with maximum at pH 3·5 and 50°C.

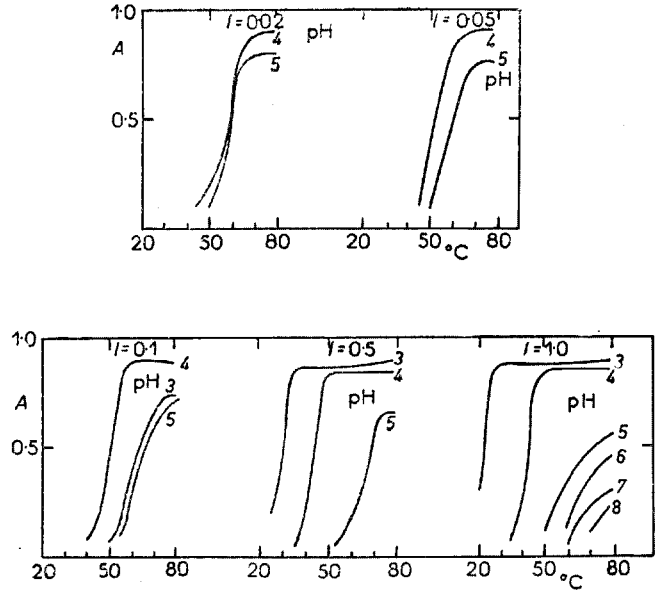

FIG. 4

The Influence of pH and Ionic Strength on Coacervation of α-Elastin
1 mg of α-elastin per 5 ml. *A* absorbancy at 440 nm.

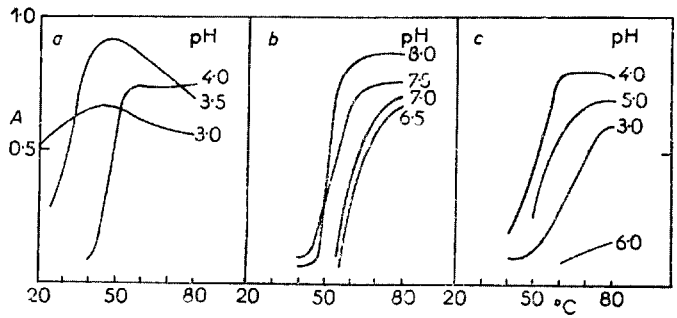

FIG. 5

Coacervation of Modified α-Elastins
1 mg of the modified protein per 5 ml. *A* absorbancy at 440 nm.
a Acetoacetylated α-elastin, *b* esterified α-elastin, *c* guanidylated α-elastin.

Esterification, on the other hand, shifted the occurrence of coacervation to pH values of 6·5 to 8·0 with the first appearance at pH 7·5 and 40°C and with maximum at pH 8·0 and 70°C. Guanidylation did not modify the pattern of coacervation obtained with unmodified α-elastin apart from some minor differences.

Coacervation of Tropoelastin

Due to a small amount of the protein available, coacervation was followed only at ionic strength 0·5 and pH 3·0 to 8·0 at one unit intervals, and at ionic strength 1·0 and pH 3·0, 4·5, 6·5, and 8·0. For comparison, coacervation was observed also in 0·5M potassium phosphate buffer (1·0M with respect to potassium ions), pH 7·0, used by Sandberg and coworkers in the coacervation step in tropoelastin preparation[3]. The results are given in Fig. 6.

Compared to α-elastin, tropoelastin coacervated in a broader pH range which covered all the pH values used (3·0–8·0) even at the lower ionic strength (0·5).The dependence of the degree of coacervation on pH was much less pronounced than with α-elestin, mainly at ionic strength 1·0 at which there were only slight differences in the optical density at different pH values. The dependence of coacervation on ionic strength revealed a trend similar to that of α-elastin coacervation: an increase in ionic strength shifted the first appearance of coacervation to a lower temperature and a lower pH value. The extent of tropoelastin coacervation in phosphate buffer was comparable to that in acetate buffer at pH 3·0 or 4·0 and ionic strength 0·5. On the other hand, it did not reach the extent of coacervation obtained in acetate buffer of ionic strength 1·0, especially at lower temperatures.

DISCUSSION

The primary structure of elastin exhibits an unusually high proportion of apolar amino acids[9]. Due to this, extensive hydrophobic associations can take place in

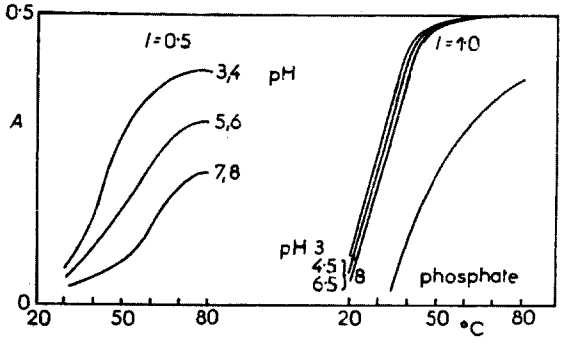

FIG. 6
Coacervation of Tropoelastin
1 mg of tropoelastin per 3 ml. *A* absorbancy at 440 nm.

soluble elastins solutions at elevated temperatures resulting in coacervation[1] accompanied by fibrillation[10] or, on prolonged heating, in insoluble product formation[11].

The general conditions of coacervation of soluble elastins have been studied in this work using α-elastin as a model of soluble elastin. As expected, maximum coacervation has been found to occur in or close to the isoelectric point of the protein. Partridge and coworkers[1] have determined the isoelectric point of α-elastin to be 4·7 at ionic strength 0·02. Podrazký has resolved α-elastin into five fractions[12] each of which could be further separated by isoelectric focusing into several fractions having isoelectric points near to 5 (ref.[20]). In accordance with this, coacervation of α-elastin has been observed at pH values 4·0 to 5·0 at low ionic strength.

The dependence of coacervation on isoelectric point can be derived also from the experiments with modified α-elastins (Fig. 5). The blockage of free aminogroups (resulting in a decrease in the isoelectric point) has limited the occurrence of coacervation to low pH values only while esterification has had an adverse effect. Guanidylation, which does not alter the overall distribution of charged groups, has not given rise to any marked changes in the pH- and temperature dependence and the degree of coacervation.

The shift of the occurrence of coacervation to lower pH values at increasing ionic strength can be explained by a decrease of isoelectric point. Partridge and coworkers[1] have shown that isoelectric point of α-elastin decreased from 4·7 at ionic strength 0·02 to 4·0 at ionic strength 0·2. A similar observation has been made by Jackson and Neuberger[13] on the other connective tissue protein, collagen.

With tropoelastin, the conditions for maximum coacervation are much less dependent on ionic conditions. The pH range of the occurrence of coacervation is markedly broader than that of α-elastin coacervation at identical ionic strength. The possible explanation for this difference between α-elastin and tropoelastin behaviour must be looked for in the molecular organization of both proteins as the differences in the amino acid composition are very small except for the presence of crosslinks in α-elastin, and as the molecular weights of both substances are very similar, namely near 70000 (ref.[1,13]). α-Elastin is composed of relatively short polypeptide chains covalently bound together by cross-links[14]. Apart from a considerable restriction of the free movement of the polypeptide chains due to crosslinking, the spatial arrangement of the molecule causes inaccessibility of a number of groups capable of interaction. Polypeptide chains of tropoelastin, on the other hand, are not restricted in their free movements by cross-linkages and a high number of reactive groups on them are available for the interaction.

An important observation concerns the influence of various ions on the degree of coacervation. It can be traced from Figs 1 – 3 that the effect on coacervation may be attributed mainly to anions.

The influence of the quality of anion on the isoelectric pH value has been shown for example by Longsworth and Jacobsen[15] on β-lactoglobulin and bovine serum

albumin. Similarly, Cann and Phelps[16] and Phelps and Cann[17] have given evidence on different effectiveness of anions in bringing about conformational changes, changes in isoelectric point, the extent of aggregation or sedimentation behaviour. The comparison of the effectiveness of various anions on α-elastin coacervation leads to the conclusion that the binding of acetate ions results in such changes in the protein conformation that favour a high degree of coacervation.

This findings seems very important in terms of the isolation of native soluble elastin. Tropoelastin, although detectable by sensitive methods[18] is present in normal tissue in extremely low amounts. The only possible way to achieve its accumulation is the prevention of the cross-link formation, for example by Cu-deficiency[2] or lathyrism[19]. Coacervation has been used in most of the works attempting at tropoelastin isolation as an important step for obtaining a crude product rich in tropoelastin[2-4]. The use of optimum conditions for coacervation will ensure higher yields of the protein. The comparison of the effectiveness of the phosphate buffer used in some of the isolation procedures[3] with that of the acetate buffer (Fig. 3) has shown clearly the advantage of the latter. Accordingly, the findings given here give suggestions for the choice of the proper conditions for the step of coacervation in the procedure of tropoelastin isolation.

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