- Morgan, W. T. J. (1959a). *Naturwissenschaften*, **6**, 181.
- Morgan, W. T. J. (1959b). *Ciba Foundation Symp., Biochemistry of Human Genetics*, p. 201.
- Morgan, W. T. J. (1959c). *Proc. int. Symp. Biologically Active Mucoids*, Warsaw (in the Press). Warsaw: Polish Academy of Science.
- Morgan, W. T. J. (1960). *Proc. Roy. Soc. B*, **151**, 308.
- Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
- Morgan, W. T. J. & Watkins, W. M. (1948). *Brit. J. exp. Path.* **29**, 159.
- Morgan, W. T. J. & Watkins, W. M. (1953). *Brit. J. exp. Path.* **34**, 94.
- Pusztai, A. (1960). Ph.D. Thesis: University of London.
- Pusztai, A. & Morgan, W. T. J. (1958). *Nature, Lond.*, **182**, 648.
- Pusztai, A. & Morgan, W. T. J. (1960). *Biochem. J.* **74**, 31P.
- Pusztai, A. & Morgan, W. T. J. (1961). *Biochem. J.* **78**, 135.
- Schachman, H. K. (1957). In *Methods in Enzymology*, vol. 4, p. 32. New York: Academic Press, Inc.
- Schellman, J. A. (1958). *C.R. Lab. Carlsberg, Sér. chim.* **30**, 21.
- Schiff, F. & Adelsberger, L. (1924). *Z. Immunforsch.* **40**, 335.
- Watkins, W. M. & Morgan, W. T. J. (1952). *Nature, Lond.*, **169**, 825.
- Watkins, W. M. & Morgan, W. T. J. (1954). *Lancet*, i, 959.
- Watkins, W. M. & Morgan, W. T. J. (1955). *Vox Sanguinis*, **5**, 1.

Biochem. J. (1961) **80**, 121

The Reaction between Elastase and Elastic Tissue

4. SOLUBLE ELASTINS*

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Hall (1951) reported that, if the ratio of solution to solid was raised sufficiently, elastin could be made to pass into solution by prolonged treatment with boiling 40% urea solution. Adair, Davis & Partridge (1951) also reported the dissolution of elastin by dilute oxalic acid. Before this, elastin was considered to be an exceptionally stable protein, in that the normal solvents were without effect on it. Horbaczewski (1882) described a material to which he gave the name hemielastin, derived from native elastic tissue by treatment with hydrochloric acid and pepsin, and further details of a similar material (elastose) were given by Richards & Gies (1902). These authors indicated that the soluble material obtained from elastin exhibited decreased solubility at elevated temperatures. This particular property was further studied by Adair *et al.* (1951), who showed that the phenomenon was due to the separation of the solution into protein-rich and protein-poor fractions, presumably by the production of coacervates between the molecules of the soluble elastin. Bowen (1953), by ultracentrifuge and diffusion studies, revealed that soluble elastin prepared with urea consisted of polydisperse protein of relatively high molecular weight. At least two molecular species appeared to be present and the results were consistent with the existence of an equilibrium between the two. Partridge, Davis &

Adair (1955) separated the products of oxalic acid action into two main components and characterized their physical and chemical properties (Partridge & Davis, 1955). The high-molecular-weight protein (α -elastin) was polydisperse and capable of forming coacervates resulting in a two-phase system similar to that observed by Bowen. Low-molecular-weight material (β -elastin) did not form coacervates on warming and was monodisperse. It is significant that the two proteins were reported as having almost identical amino acid analyses, and the terminal amino and carboxyl group content of the two species was consistent with the view that if α -elastin were built up from β -elastin residues, then the latter must be joined through unknown cross-linkages. These same authors showed that such linkages could not be disulphide in nature.

Wood (1958) obtained soluble elastin by dissolution in a variety of solvents and showed that a material, having many of the properties of native elastin, could be reconstituted by maintaining the soluble material in a coacervated state at an elevated temperature for long periods.

The present paper reports new methods of preparing α -elastin and additional observations on its properties. The effect of detergents throws light on the phenomenon of coacervation and the results of studies on the proteolytic action of elastase on α -elastin may contribute to the general theory of proteolysis.

* The paper by Hall & Czerkawski (1959) is to be regarded as Part 3.

MATERIALS AND METHODS

Elastin and elastase. A standard elastin powder derived from ox ligamentum nuchae by the methods described by Hall (1955, 1957) was used as starting material for all the preparations of soluble elastin. The enzyme employed was the electrophoretically pure preparation of elastase described by Hall & Czerkawski (1959).

Measurement of the extent of coacervation. The nature of the soluble elastin produced by the various methods was determined by the degree of coacervation obtained on warming. In certain key experiments, a constant-temperature cell housing (constructed in these laboratories) was used in a Unicam SP. 500 spectrophotometer to control the temperature to within 0.2° over the range 20 – 40° . Extinction was measured at $400\text{ m}\mu$ and a pinhole attachment was fitted in front of the shutter opening of the photocell housing to minimize the effect of non-specific scattering. When expedient, however, especially in serial experiments, the extent of coacervation was measured in a more qualitative fashion. The protein solutions were brought to the required temperature in matched $\frac{1}{2}$ in. test tubes in a water bath. The turbidity was measured in an EEL photoelectric colorimeter calibrated directly in extinction units. With a little practice, any individual reading could be completed before the temperature dropped by more than 0.5° .

Measurement of the rate of proteolysis. Three methods were used: two measured the extent of proteolysis in terms of amino group release and the third utilized trichloroacetic acid precipitation, the extent of which was taken as a measure of the molecular size of the protein.

In the first method (Spies, 1952) a copper complex which absorbed strongly at $230\text{ m}\mu$ was formed at pH 9.3. The magnitude of absorption depends on the number of amino acid residues in the peptide or protein when these are of relatively low molecular weight, but with larger protein molecules the extinction is proportional to the number of free amino groups. The second method used the reaction with ninhydrin (Moore & Stein, 1948), alanine being employed as standard.

In the third method, 2 ml. of 30% (w/v) trichloroacetic acid was added to 2 ml. of protein solution in a centrifuge tube, and the mixture was kept at room temperature for 10 min. After centrifuging, 2 ml. of the supernatant was withdrawn and the non-precipitable protein determined by means of the biuret reaction. With dilute protein solutions, the supernatant was poured off, the tubes were inverted to drain, 2 ml. of borate buffer (pH 8.5) was added to dissolve the precipitate, and the biuret reagent added directly to this.

Preparation of soluble elastin

Several methods are now available for the preparation of α -elastin, in addition to the methods with oxalic acid and urea.

Alkaline hydrolysis. Although treatment with 0.1N-alkali at 100° has been used by several workers as a method for the purification of elastin, Wood (1952) has shown that a sufficiently prolonged treatment with alkali even at lower temperatures results in the dissolution of the elastin. The solution so obtained consists in part of α -elastin.

Standard elastin powder (0.5 g.) was suspended in 50 ml. of 4N-NaOH and incubated at 37° . Dissolution was com-

plete in 20 hr., and the curve relating the amount of protein dissolved to the period of incubation was sigmoid and resembled similar curves relating to the solubilization of elastin by elastase. The product was a yellow solution which, when neutralized, dialysed and then brought to pH 4, had the power of coacervation. No attempts were made to fractionate this material since the presence of an appreciable amount of free amino groups indicated that the partial hydrolysis resulting from the action of alkali had degraded a relatively high proportion of the α -elastin.

Treatment with strong mineral acid. The action of strong acids for short periods was as effective as that of weak alkalis for longer periods.

Elastin (10 g.) was added with rapid stirring to 254 ml. of 80% (w/v) H_2SO_4 at 95° and stirred until most of the solid dissolved (3–4 min.). The solution was poured into 2750 ml. of cold water and neutralized with conc. aq. NH_3 solution. The pH was adjusted to 4 with dilute H_2SO_4 and the solution warmed to a temperature of 50° . The coacervated protein separated in the form of a brownish yellow, viscous mass, which readily adhered to a glass rod. The protein was then dissolved in 50 ml. of water; the solution was filtered to remove small amounts of unattacked elastin, dialysed against frequent changes of distilled water for two days, and freeze-dried to give 5.5 g. of a very soluble powder having all the properties of α -elastin.

After neutralization, the $(\text{NH}_4)_2\text{SO}_4$ concentration was approximately equivalent to the addition of 0.2 vol. of a saturated solution of the salt. The protein was used extensively in subsequent experiments because of the ease of preparation and because it exhibited a high degree of chemical homogeneity. Fractional precipitation with trichloroacetic acid showed that material precipitated by the addition of an equal volume of 5–40% (w/v) trichloroacetic acid had a constant amino group content of 32 moles/105 g. of protein. These fractions comprised more than 90% of the total protein.

Hydrolysis by ethanol. Partridge *et al.* (1955) observed that solubilization of elastin with oxalic acid is accompanied by the liberation of amino and carboxyl groups in about equal numbers and therefore deduced that the process consisted essentially of a hydrolytic reaction involving peptide bonds. As the protein can be dissolved under relatively mild conditions in the presence of alkali, it might be expected that elastin is stabilized in the solid state by unknown but highly significant bonds. Ester linkages have many of the properties which are necessary to produce the observed stability and the truth of such a hypothesis was tested by recourse to hydrolysis with hot acidified ethanol.

Gaseous HCl was passed into 100 ml. of absolute ethanol to render it 0.5N with respect to the acid. Elastin powder (2.5 g.) was suspended in this solution and the mixture boiled under reflux for 3 hr., after which nearly all the elastin had passed into solution. The residue was removed by filtration while hot, and the filtrate allowed to cool to 10° . After 1 hr. at this temperature a precipitate formed which was removed by filtration, dissolved in water and freeze-dried to give 0.8 g. of protein (designated AP). The cool ethanolic supernatant was treated with 1.5 vol. of ether with the resulting formation of a bulky precipitate, which was dissolved in water, dialysed and freeze-dried to give a second product (0.6 g.; designated EP). Both proteins were strongly esterified (AP 48 moles of ethoxy groups/10⁵ g. of protein, and EP 56 moles) and showed maximum coacerva-

tion at pH 9. Mild alkali treatment at room temperature resulted in a shift of this maximum to pH 4.

Action of pepsin. Horbaczewski (1882) prepared soluble elastin by the action of pepsin on elastin. During the present investigation pepsin dissolved solid elastin at pH 2, but the activity-time curve was unlike that with elastase at pH 8.5. The rate of dissolution at pH 2 is about one-fifteenth of that with an identical amount of elastase at pH 8.5. Nevertheless, the product can be made to coacervate at pH 5.

Elastin powder (1.0 g.) was incubated at 3° in 200 ml. of 0.1 M-acetate-HCl buffer (pH 2.0) containing 50 µg. of crystalline pepsin/ml. After 1 hr. the pH was raised to 7.0 and the mixture heated at 100° for 4 min. The unattacked solid was rather gelatinous and was removed by filtration in the presence of cellulose powder. The volume was made up to 330 ml. with water and 0.2 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added. The precipitate was then dialysed and freeze-dried to give 0.5 g. of powder.

Controlled elastolysis. The reaction between elastin and elastase takes place in two consecutive stages (Hall & Czerkawski, 1961). During the first of these, α -elastin is produced and thereafter this intermediate is converted into lower-molecular-weight material. The second half of the overall reaction can be inhibited by sodium dodecyl sulphate, in which case the α -elastin content of the system builds up.

Elastin (5 g.) was suspended in 380 ml. of borate buffer, pH 8.5; to this 0.5 g. of sodium dodecyl sulphate was added and the mixture kept at 37° for 1 hr. It was then chilled to 4° and mixed with 20 ml. of an elastase solution containing 0.2 mg. of pure elastase/ml. (Hall & Czerkawski, 1959) in borate buffer at the same pH. After 30 min. at 4° the mixture was incubated at 37° until dissolution was nearly complete (4–5 hr.) and heated at 100° for 4 min. to inactivate the enzyme.

The solution was dialysed for 24 hr. against running tap water to remove the greater part of the detergent, chilled to 4° and treated with 0.2 vol. of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. After 2 hr. the precipitate was separated by centrifuging and redissolved in 100 ml. of water. More detergent crystallized out on standing at -5°. The protein solution was then dialysed further, first against 20% NH_4HCO_3 , water and aqueous 30% acetone and then against distilled water at 37°. The freeze-dried protein was a yellowish white powder (yield 4.2 g.).

RESULTS

Conditions most suitable for optimum coacervation

Coacervation is a function of pH, temperature, ionic strength and the concentration of the protein. Partridge *et al.* (1955) investigated the variation of this phenomenon with pH and ionic strength by noting the temperature at which the coacervate was either just noticeable or had just disappeared. Here the problem has been approached in a different fashion. Into each of a series of matched tubes were placed 2 ml. of a solution of soluble elastin, 2 ml. of buffer solution of a suitable pH and 4 ml. of water. The final protein concentration was 0.2 mg./ml. These mixtures were then heated at

various temperatures and the turbidity was determined. The results of a typical experiment are shown in Fig. 1a. If an arbitrary line is drawn horizontally at any given value for extinction it cuts the family of curves in a number of points. This enables one to relate the temperature at which the degree of coacervation equals a given value against pH (Fig. 1b). Such results can then be compared directly with those of Partridge *et al.* (1955), and it can be concluded that there is a minimum temperature at finite pH, at all ionic strengths, but that the curves become more asymmetrical at higher ionic strengths until one side of the curve is almost lost. The optimum pH increases with temperature and decreases with ionic strength. If the pH, ionic strength and temperature are kept constant, the amount of coacervate increases steadily but not linearly with initial protein concentration.

Effect of detergents on coacervation

Since the phenomenon of coacervation consists in the interaction between molecules and their association through groupings which must be located on their surfaces, the effect of various surface-active agents on the progress of the phenomenon was determined. Neutral detergents such as the Tweens did not affect either the rate of coacervation or the amount of product formed. This was not so, however, with cationic and anionic surface-active

Fig. 1. (a) Variation of the extent of coacervation (measured as extinction) with the pH of solution at I 0.075. A, 28°; B, 35°; C, 41°; D, 60°; E, 80°. (b) A derived curve showing the variation of the temperature required for equal coacervation (arbitrarily chosen as 6 in Fig. 1a) with pH (cf. Partridge, Davis & Adair, 1955).

agents. The effect of sodium dodecyl sulphate and Cetavlon (cetyltrimethylammonium bromide) was determined at pH values both below and above the isoelectric point of the protein (Figs. 2*a, b* and 3*a, b*). At pH 3.4 the anionic sodium dodecyl sulphate

causes an initial increase in coacervation followed by a pronounced drop as the concentration increases. At pH 5.4, however, the effect of this detergent is to inhibit coacervation steadily at all the concentrations tested (Figs. 2*a, b*). Cetavlon, having a positive charge, displays the opposite effect, inhibiting at pH 3.4 and activating at pH 8.4 (Figs. 3*a, b*). Similar results were obtained with other cationic and anionic detergents such as sodium dodecylbenzenesulphonate and cetylpyridinium bromide.

Effect of elastase on coacervation

Soluble elastin was dissolved in borate buffer, pH 8.45, mixed with elastase dissolved in the same buffer and incubated at 37°. Samples were withdrawn after various intervals and the enzyme was inactivated by heating for 4 min. at 100°. Part of each sample was adjusted to pH 4, diluted and heated to 40°. There was a steady release of amino groups, whereas the power to coacervate was lost much more rapidly. The addition of previously inactivated enzyme was without effect.

Chemical and physical characterization of soluble elastin

Amino acid distribution. No attempt was made to perform a quantitative analysis. The solid elastin and the preparations of α -elastin were hydrolysed with 5*N*-hydrochloric acid under the same conditions, and the resulting amino acid mixtures were subjected to two-dimensional paper chromatography. The qualitative amino acid spectrum of both types of protein is the same.

Electrophoresis and isoelectric point. When a crude product of elastolysis is subjected to paper electrophoresis at pH 8.6, the separation is poor. At pH 5.2 three fractions can be distinguished; the main component (α -elastin) migrates with the same speed as glucose. The best enzymic preparation of α -elastin moves as a well-defined band and its mobility is consistent with an isoelectric point of 5.0. This agrees with the isoelectric point obtained from the titration curve in 1% sodium chloride solution (4.8).

Ultraviolet spectrum. The solution of α -elastin in borate buffer gives a smooth absorption curve with a rather flat but definite maximum at 275 $m\mu$. The solution obeys Beer's law and the absorption coefficient at 275 $m\mu$ is 6.5. This low value is not unexpected in view of the small amount of tyrosine and the absence of tryptophan in both solid and soluble elastin.

Viscosity and sedimentation. An aqueous solution of α -elastin is not very viscous (intrinsic viscosity at 37° in borate buffer, pH 8.6, is 7.1 ml./g.). There is no increase in viscosity if the elastin solution is heated at pH 4.5 in the presence of sufficient sodium

Fig. 2. (a) Effect of the anionic detergent sodium dodecyl sulphate on coacervation of α -elastin solution at pH 3.4 and *I* 0.15. A, 18°; B, 25°; C, 33°; D, 52°. (b) Effect of sodium dodecyl sulphate on coacervation at pH 5.4 and *I* 0.15. A, 41°; B, 57°; C, 69°; D, 85°.

Fig. 3. (a) Effect of the cationic detergent Cetavlon on coacervation of α -elastin solution at pH 3.4 and *I* 0.15. A, 28°; B, 40°; C, 53°; D, 67°. (b) Effect of Cetavlon at pH 8.4 and *I* 0.15. A, 14°; B, 24°; C, 38°; D, 68°.

Table 1. *Properties of preparations of α -elastin*

Values in column 4 are the percentages of sample precipitated from a 0.5% solution by 30% trichloroacetic acid.

Sample or treatment	pH optimum for coacervation	Free amino group content (moles/10 ⁶ g.)	Ppt. (%)
Treatment with H ₂ SO ₄	4.2	32	94
Treatment with NaOH	About 4.5	—	—
AP	9.2	37	87
EP	9.2	42	83
AP treated with NaOH	4.5	—	—
Elastolysis (crude)	4.5	65	65
Treatment with pepsin	5.5	29	98
Elastolysis (controlled)	5.2	30	98
Deaminated	2.0	2	93

Fig. 4. A curve (A) relating the activity (expressed as moles of free amino groups/ml.) to time (●). The linear plot (B) of the ratio time/activity against time (○) shows that activity is a hyperbolic function of time. The substrate was elastin after H₂SO₄ treatment, 40 mg./ml., enzyme E₂I (Hall & Czerkawski, 1959), 25 μg./ml., in borate buffer pH 8.5 and *I* 0.364 at 37°.

dodecyl sulphate to prevent coacervation. The implications of this observation are discussed below. Two samples of soluble elastin were subjected to sedimentation in a Spinco model E ultracentrifuge (1% solution in acetate buffer, pH 5.2). The α -elastin prepared with sulphuric acid gave a diffuse peak, which rapidly flattened and eventually disappeared. This material was obviously polydisperse. The soluble elastin prepared by the controlled action of elastase, however, gave a sharp symmetrical peak, indicating a high degree of homogeneity and confirming the electrophoresis results. The value of $S_{22,w}$ for these two proteins was 1.67 and 1.68s respectively. A summary of the properties of various preparations is given in Table 1.

Kinetics of the reaction between elastase and soluble elastin

The initial rate of the reaction between elastase and soluble elastin is very fast (curve A, Fig. 4) and

Fig. 5. A curve (A) relating the activity (expressed as the moles of free amino groups released in 1 hr.) to the initial concentration of elastase. The linear plot (B) of the ratio enzyme/activity against enzyme shows that this activity is a hyperbolic function of the enzyme concentration. The conditions are the same as in Fig. 4.

then falls off gradually, but does not proceed to completion within reasonable limits. Thus it is difficult to measure the initial reaction velocity and it is more convenient instead to measure the activity over a finite period. This is defined as the number of amino groups released in 1 hr. at 37° at the optimum pH. This optimum was 8.45 and was identical both with that for the elastolysis of the solid protein and with the isoelectric point of elastase. The activity is directly proportional to the starting concentration of substrate up to 30 mg. of α -elastin/ml. and varies hyperbolically with the enzyme concentration at the commencement of the reaction (curve A, Fig. 5). These relationships between elastin and elastase are equally applicable if the activity is measured in

terms of the material precipitable by trichloroacetic acid; this indicates that the proteolytic fission of the α -elastin molecule is stepwise rather than random, since precipitability by trichloroacetic acid is an approximate measure of particle size and, in random splitting, the decrease in molecular weight per unit of released amino group would increase rapidly.

A typical example is shown in Fig. 6, which relates the release of amino groups to the amount of non-precipitable material produced. Although there is good linearity, the straight line does not pass through the origin. The intercept on the ordinate is even greater with substrate prepared by pepsin digestion of solid elastin and is almost negligible for the material produced by attack with elastase in the presence of sodium dodecyl sulphate. The linear portions of the various curves are, however, approximately parallel.

Since the non-precipitable material produced is expressed in g./ml. and the amino group release is given in moles/ml., the slope of the line gives

Fig. 6. Variation of production of peptides not precipitated with an equal volume of 30% trichloroacetic acid, with the number of peptide bonds broken during proteolysis with elastase of α -elastin from treatment with H_2SO_4 . ●, Estimated by ninhydrin; ○, estimated by copper complex. The numbers on the curve refer to the time of elastase action.

Table 2. *Relative rates of digestion of various soluble elastin preparations*

Values are expressed in terms of the release of amino groups/ 10^5 g. of elastin protein/min.

Substrate	Rate of release of amino groups (moles of $NH_2/10^5$ g. of elastin/min.)
α -Elastin from H_2SO_4 treatment	1.0
AP elastin	0.4
α -Elastin from elastolysis	0.6
Later stages of elastolysis	0.3

directly the approximate molecular weight of the unit which is split off in a stepwise fashion. The value is approximately 600, and is independent of the method of preparation within the limits of experimental error.

The various detergents show marked differences in their ability to affect the rate of attack by elastase on α -elastin compared with their effect on elastolysis. The action of elastase on soluble elastin is only slightly affected by even high concentrations of Cetavlon, whereas the anionic detergents have almost no effect.

In Table 2 are shown the relative rates of digestion of various preparations. The slightly lower rate for the esterified fraction AP is probably due to the fact that at pH 8.45 and at a temperature of 37° most of this fraction is in the form of a coacervate and hence, in contradistinction to the other reactions, this particular one is heterogeneous and not homogeneous.

DISCUSSION

Coacervation

The pH optimum for coacervation is close to the isoelectric point of α -elastin and the extent of coacervation increases with temperature. Thus there are apparently two main requirements for coacervation, namely net neutrality of charge on the protein and a high degree of ionization. Other factors may, and in all probability do, contribute to the general mechanism, but for simplicity it may be assumed that coacervation is a reaction between positive and negative charges on adjacent molecules with the formation of salt linkages. The rapid disappearance of coacervation under the action of elastase may indicate that the phenomenon cannot take place when the particle size is below a critical value. On the other hand, the action of elastase may be to remove certain important polar groupings from the molecules. Below its isoelectric point the protein carries a net positive charge which will attract and combine with anionic detergents via their polar groupings and partially via hydrophobic acids. This combination will tend to make the protein molecule more neutral and will result in increased coacervation. The elastin molecule is, however, mainly non-polar and more of the detergent will be adsorbed through its non-polar hydrophobic regions by van der Waals forces than by salt linkages through its limited supply of polar groups. Consequently a net negative charge will be imparted to the molecule as a whole and there will be a progressive decrease in coacervation as the concentration of detergent increases.

At pH values below the isoelectric point of the protein, cationic detergents will combine solely by non-specific adsorption, thus continuously increas-

ing the net positive charge and steadily inhibiting coacervation. Above the isoelectric point, however, the protein carries a negative charge. It follows that the effects of the anionic and cationic reagents will be reversed: the former will show inhibition and the latter activation followed by inhibition as the concentration increases.

The phenomenon of coacervation is thus markedly dependent on the electric charge of the particles. Although the purely physical uncoiling and inter-twining of particles proposed by Partridge *et al.* (1955) might be a contributing factor, the process is probably mainly due to a salt-like combination of charges on the protein.

The fact that there is no increase in viscosity under the conditions which would normally lead to coacervation indicates that it is not a purely physical process. However, the main argument against such a mechanism is the rapid reversibility of the process of coacervation (only a little shaking would intertwine a number of short helical springs and no amount of random movement would separate them again, unless influenced by a directive force).

Wood (1958) has prepared the so-called regenerated elastin by prolonged heating of the coacervate. The regenerated elastin is not soluble in water or dilute buffer, but it dissolves rapidly when acted on by elastase. This material may be the product of a purely physical and irreversible inter-twining of α -elastin molecules.

Kinetics of homogeneous reaction

Preliminary results of the study of the homogeneous reaction between elastase and α -elastin have been reported by Czerkawski (1958) and the present observations agree with those advanced earlier. The reaction can be represented accurately by an empirical relationship which is of the same form as equation (3) [see the straight lines (B), in Figs. 4 and 5].

The elastin prepared with sulphuric acid is probably homogeneous chemically, but there is no doubt that it is polydisperse with respect to the molecular size. At first sight it might be considered a poor choice of substrate for kinetic studies, but the fact that it is produced by a predominantly random fission of solid elastin turns out to be advantageous. It means that the resulting material is chemically very similar to the solid elastin and that, at least in theory, any kinetic results obtained from its reaction with elastase may be extrapolated to the more complex solubilization of elastin. In this respect the soluble protein produced by the action of elastase is inferior, although it is quite monodisperse.

The kinetic studies of the homogeneous reaction are helpful in the theoretical derivation of an equa-

tion which is very similar to the empirical one. The fact that the reaction is first-order with respect to the substrate indicates the fast formation of an enzyme-substrate complex, and the fact that it remains of this order throughout implies a stepwise degradation of the substrate.

The reaction appears to consist of two processes, one fast, one slow. The former may be identified with the fission of specific bonds and may lead to the solubilization of solid elastin (bonds which have survived the indiscriminate acid hydrolysis), and the latter appears to be the stepwise degradation of the soluble material.

If it is assumed that the enzyme is adsorbed instantaneously on elastin according to the Langmuir isotherm, then the fraction of sites occupied by the enzyme, θ , is $E_0/(K + E_0)$, where E_0 is the initial concentration of the enzyme. If it is also assumed that the substrate contains n_1 bonds which are attacked rapidly and n_2 that are attacked slowly, and that the latter predominate, then if x is the number of bonds split in time t , the reaction velocity is given by

$$dx/dt = k_1(n_1\theta - x) + k_2n_2\theta, \quad (1)$$

where k_1 and k_2 are the velocity constants for the fast and slow reactions respectively.

The above equation can be integrated and if the time interval used is not too large it can be simplified, giving finally

$$x = \left(R + \frac{k_1}{k_2}\right) n_2 \frac{E_0}{K + E_0} \frac{t}{1/k_2 + t} \quad (2)$$

where $R = n_1/n_2$.

All the constants in the above equation can be obtained experimentally. If t/x is plotted against t at constant enzyme concentration the intercept on the t axis gives $1/k_1$, and is expressed in reciprocal time units. Similarly a plot of E_0/x against E_0 gives K which is measured in the same units as the enzyme concentration. If the smallest degraded elastin unit has a molecular weight of 600, the solution containing a mg. of soluble elastin/ml. must contain $1.67a$ μ moles of such units/ml., this may be identified with n_2 . The value of n_1 may be estimated from the steep portion of the activity-time curve, and so one may determine R . Thus all the quantities except k_2 are known and therefore this may also be calculated. However, if one puts $n_1 = 0$ (for the substrate prepared by controlled elastolysis), one obtains:

$$x = n_2 \frac{E_0}{K + E_0} \frac{t}{1/k_2 + t} \quad (3)$$

and thus k_2 may also be obtained directly.

The derived equation contains a number of constants which have a definite physical meaning and to which one can assign the appropriate dimensions.

The effect of detergents on the homogeneous reaction is interesting practically. The elimination of the secondary reaction by sodium dodecyl sulphate can be made use of in the preparation of an α -elastin which is far more uniform than that obtained by any of the more drastic chemical methods.

SUMMARY

1. Several methods of preparation of soluble elastin are described and it is shown that the best material is obtained by controlled elastolysis.

2. Coacervation is taken as best characterization of α -elastin and the most suitable conditions for the measurement of this phenomenon are investigated. The effect of detergents and elastase on this phenomenon are also studied.

3. It is concluded that the process of coacervation of α -elastin is complex and is mainly brought about by salt-like interaction between charged centres of the protein molecules.

4. The kinetic studies of the reaction between α -elastin and elastase give rise to an empirical equation. It is shown that only a few reasonable assumptions and the observed facts lead to a theoretical equation which is of the same form as the empirical one. The constants of the equation have a sound

physical meaning and can be obtained experimentally.

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REFERENCES

- Adair, G. S., Davis, H. F. & Partridge, S. M. (1951). *Nature, Lond.*, **167**, 605.
 Bowen, T. J. (1953). *Biochem. J.* **55**, 766.
 Czerkawski, J. W. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, nos. 4-45, p. 43.
 Hall, D. A. (1951). *Nature, Lond.*, **168**, 513.
 Hall, D. A. (1955). *Biochem. J.* **59**, 459.
 Hall, D. A. (1957). *Arch. Biochem. Biophys.* **67**, 336.
 Hall, D. A. & Czerkawski, J. W. (1959). *Biochem. J.* **73**, 356.
 Hall, D. A. & Czerkawski, J. W. (1961). *Biochem. J.* **80**, 128.
 Horbaczewski, J. (1882). *Hoppe-Seyl. Z.* **6**, 330.
 Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
 Partridge, S. M. & Davis, H. F. (1955). *Biochem. J.* **61**, 21.
 Partridge, S. M., Davis, H. F. & Adair, G. S. (1955). *Biochem. J.* **61**, 11.
 Richards, A. N. & Gies, W. J. (1902). *Amer. J. Physiol.* **7**, 93.
 Spies, J. R. (1952). *J. biol. Chem.* **195**, 65.
 Wood, G. C. (1952). Quoted by Hall, D. A., Reed, R. & Tunbridge, R. E. *Nature, Lond.*, **170**, 264.
 Wood, G. C. (1958). *Biochem. J.* **69**, 538.

Biochem. J. (1961) **80**, 128

The Reaction between Elastase and Elastic Tissue

5. GROUPINGS ESSENTIAL FOR ELASTOLYSIS

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The amino acid distribution of elastin has been determined by numerous workers (for review see Hall, 1959), and the values obtained show that the molecule is built up from predominantly non-polar amino acids. Thus 10⁵ g. of the protein contain only 28 moles of dibasic acids, 12 moles of diamino acids, 33 moles of aliphatic hydroxy acids and 7 moles of tyrosine (calculated from the figures reported by Partridge & Davis, 1955). The remainder (1120 moles) consists of various non-polar amino acids. Of the former group the amino acids containing the weakly polar aliphatic hydroxyl groups predominate. This rather unusual distribution of amino acids confers a considerable degree of chemical stability on the protein, thus making it admirably

suited to chemical studies. Elastin is dissolved by the proteolytic component of elastase (Hall & Czerkawski, 1959) and to a far smaller extent by certain other proteolytic enzymes of plant origin (Thomas & Partridge, 1960). This process, commonly known as elastolysis and which we wish to distinguish from the subsequent degradation in solution (Hall & Czerkawski, 1961), is a striking example of a reaction in which a solid substrate is made to pass into solution.

Banga & Schuler (1953) were unable to identify any non-protein amino groups in the products of the reaction between elastase and elastin. They interpreted this as indicating that the solubilization of elastin was accomplished without the fission of