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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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

peptide linkages. They suggested that a fibrous form of elastin was converted into a globular form merely by the fission of hydrogen bonds. Partridge & Davis (1955) showed that the complete solubilization of elastin was accompanied by the release of a polydisperse material that had a very high amino group content [(121 moles/ 10^5 g. of protein as compared with 17 moles deduced from titration curves for native elastin by Bendall (1955)]. The production of this material was, however, only accomplished by incubation with the enzyme for a period which was three times as long as was necessary for the dissolution of the solid. Thus it is not strictly true to say that these terminal amino groups were liberated during elastolysis, since many of them may have been formed by the fission of peptide linkages after the elastin had passed into solution. In view of the preliminary results obtained for the kinetic analysis of the reaction between elastase and the soluble elastin (Czerkawski, 1958) and the differences that could be observed between the rates of amino acid release during the two stages of the reaction, it was decided to investigate the chemistry of these two component stages of the overall reaction.

MATERIALS AND METHODS

Standard enzyme and substrate. The enzyme employed throughout these experiments was the very active preparation E₂I, obtained by the method described by Hall & Czerkawski (1959). The preparation employed had an activity of 370 units/mg. (cf. definition of activity in the paper quoted above) and was used at a concentration of 5–10 μ g./ml. in a system containing 50 mg. of substrate suspended in 10 ml. of borate buffer (I 0.364, pH 8.45).

The standard substrate preparation was from ox ligamentum nuchae, the powder (40–60 meshes/in.) being freed of collagen by the method described by Hall (1955).

Estimation of elastolytic activity. Elastolysis was carried out under the conditions of Hall & Czerkawski (1959), and the amount of protein passing into solution measured by means of the biuret reaction. If pretreatment of the substrate resulted in the formation of a coloured product which gave a coloured solution after elastolysis, the degree of solubilization was determined by the interferometric method of Hall & Czerkawski (1959).

Proteolytic activity measurements. Proteolysis as opposed to solubilization was measured by the methods described by Hall & Czerkawski (1961), as also were assessments of molecular size based on the ability to form coacervates and precipitation with trichloroacetic acid.

Determination of methoxy and acetyl groups. The extent of esterification of certain modified substrates was measured by means of the Zeisel method adapted by Grant (1945). Acetylation of the substrate was determined by the method of Herriot & Northrop (1934), in which acetic acid is displaced by citric acid and distilled at a pressure of 20 mm. Hg. There are no complications due to atmospheric CO₂.

RESULTS

Proteolytic activity during elastolysis

The amount of protein released and the number of terminal amino groups liberated during the progress of the elastolytic reaction are shown in Fig. 1*a*. The number of amino groups decreases, passes through a minimum and then increases again towards the end of the reaction when all the solid has been taken into solution. The lowest value reached is about 70 moles/ 10^5 g. of protein as compared with α -elastin which has about 30. The percentage of trichloroacetic acid-precipitable material (Fig. 1*b*) and the ability to form coacervates (Fig. 2) pass through maxima which are coincident with the minimum in Fig. 1*a*.

Elastolysis in the presence of sodium dodecyl sulphate

Sodium dodecyl sulphate was added to a standard system containing 50 mg. of elastin in 8 ml. of borate buffer, pH 8.45, so that its final concentration in 10 ml. was 2 mM, and the mixture incubated

Fig. 1. (a) The amino group content (measured by the ninhydrin reaction) during various stages of elastolysis under normal conditions. (b) The extent of precipitation by trichloroacetic acid (equal volume of 30% trichloroacetic acid) of the protein released during elastolysis.

at 37° for 30 min. Before the addition of the enzyme (in a further 2 ml. of buffer) the system was chilled to 4° and this temperature was maintained for 10 min. after the enzyme had been added. This enabled the enzyme to react with the substrate without suffering denaturation by the detergent. Subsequent incubation was carried out at 37° for various periods. In controls the detergent was replaced by 2 mM-NaCl. Fig. 3a shows that under these conditions the average α -amino group content of the liberated protein remains approximately constant at the low value of 30 moles/10⁵ g., and similarly the material passing into solution is almost completely precipitated by trichloroacetic acid at any stage of the reaction (Fig. 3b). It should be noted that sodium dodecyl sulphate is a powerful activator of elastolysis (Table 1).

Ability to coacervate during the course of elastolysis

The pH of the filtrates from elastolysates obtained after various periods of incubation was adjusted to 4.0; the solutions were diluted suitably and warmed to 40°. The degree of coacervation per unit mass of protein in solution was maximum during the middle third of the reaction. It was difficult to measure the extent of coacervation in the presence of sodium dodecyl sulphate, for this reagent suppresses this process at pH 4 and increases it at pH 3. After exhaustive extraction of the detergent, how-

Fig. 2. The solubilization-time curve (O) for the system containing 50 mg. of elastin in 10 ml. of borate buffer, pH 8.5, I 0.364, and 8 μ g. of elastase/ml. The power to form a coacervate is expressed as the ratio: turbidity/protein concn. (●).

ever, the material obtained by elastolysis in its presence demonstrated coacervation in the normal way at pH 4.0.

Function of carboxyl groups in elastolysis

Preliminary observations showed that the cationic detergent Cetavlon acts as a powerful inhibitor of elastase (0.1 mM-Cetavlon produces 50% inhibition). This proved to be a general property of cationic detergents and appeared to be due to the stable binding of the detergent molecule to the substrate. There were two possibilities: either the detergent was bound by its long non-polar tail to the non-polar regions of elastin, or it was bound through its positively charged head to the relatively few negatively charged centres. The results obtained with neutral and acidic detergents indicated that probably the latter was correct; thus the effect of other methods of blocking the negatively charged

Fig. 3. Amino group content (a) and extent of trichloroacetic acid precipitation (b) of material released during various stages of elastolysis in the presence of sodium dodecyl sulphate (●) and controls in the presence of NaCl of the same ionic strength (O).

Table 1. *Effect of detergents on the action of elastase on the solid and soluble elastins*

Detergent (0.5 mM)	Change in activity	
	Homogeneous	Heterogeneous
Sodium dodecyl sulphate	4% activation	105% activation
Cetavlon	9% inhibition	81% inhibition

groups in elastin on the progress of elastolysis was studied.

Esterification. Elastin powder (2.5 g.) was suspended in 100 ml. of methanol containing 0.1N-HCl and the mixture kept at room temperature with

intermittent stirring for 3 days. It was then filtered and washed alternately with many changes of water and acetone. The solid was finally washed with acetone and dried at 37°. Elastin thus esterified was quite resistant to elastase, being only one-fifth as susceptible as the standard substrate (Table 2). The ester could be saponified with 0.01N-NaOH at 37°, and yet quantitative estimations of methoxy groups showed that esterification of 14 of the possible 28 sites had occurred (Fig. 4a). However, original activity could be regenerated in only a few minutes, and further mild hydrolysis gives a vastly increased susceptibility to elastase (Fig. 4b).

To decide whether the carboxyl groups were terminal or those of amino acid side chains, a thiohydantoin derivative was prepared. The conditions under which this was attempted (30 min. at 100°) were considerably more drastic than those employed for the introduction of methoxy groups. Elastin (1.0 g.) was suspended in 40 ml. of a mixture of acetic acid and acetic anhydride (8:1) containing 500 mg. of NH_4CNS , and heated for 30 min. at 100°. After chilling and washing, the protein was dried with acetone. The product was 20% less susceptible to elastase than was the starting material (Table 1). The whole of this loss of susceptibility could, however, be attributed to acetylation, since a similar experiment under identical conditions but in the absence of the NH_4CNS afforded a modified substrate that also had 20% less susceptibility to elastase than had native elastin.

Function of amino and hydroxyl groups

Acetylation under the conditions of the control experiment for the thiohydantoin reaction, although indicating that acetylation affects certain groupings that are essential for the elastase-elastin reaction, was too drastic a method since although saponification removed the acetyl groups, the susceptibility of the regenerated substrate was considerably greater than that of the starting protein. From this it would appear that during the acetylation process degradation may occur.

Milder conditions of acetylation can be used to acetylate particular reactive groups and two such methods have been employed. Elastin (2.0 g.) was suspended in 15 ml. of citrate buffer, pH 4.0, and 5 ml. of acetic anhydride added in small portions at 5° throughout a period of 5 hr. The mixture was kept at 5° for 18 hr., filtered, washed and dried as before. The other method, which is claimed to acetylate only hydroxyl groups (Bello & Vinograd, 1956), also utilizes acetic anhydride at low temperatures, but the reaction is allowed to proceed in the presence of small amounts of HClO_4 .

Quantitative estimation showed that 56 acetyl groups had been introduced/10⁵ g. of fully acetyl-

Table 2. *Effect of various treatments of elastin on its susceptibility to elastase*

Pretreatment of substrate	Change in susceptibility (%)		% of enzyme adsorbed*
	Increase	Decrease	
Standard elastin	0	0	25
Esterification	0	80	0
O-Acetylation	0	50	59
Thiohydantoin	0	20	—
Full acetylation	0	20	—
Dinitrophenylation	0	30	—
N-Acetylation	50	0	37
Deamination	50	0	—
Glyoxylation	190	0	80
Iodination	0	0	—

* Dashes indicate that adsorption experiments were not performed on these substrates.

Fig. 4. Saponification of esterified elastin. (a) Quantitative release of methoxy groups during hydrolysis with 0.01N-NaOH at 37°. (b) Susceptibility of esterified elastin to elastase. (Both the test samples and standard elastin powder were treated with 0.01N-NaOH for the indicated time interval.)

ated elastin. Saponification of these groups indicates that they are not all identical. About 40 can be saponified easily, whereas the remaining 16 are only removed with difficulty (Fig. 5). *O*-Acetylation reduces the activity by about 50% (Table 1) and it is significant that a 2 hr. treatment with 0.1N-NaOH at 37° results in complete regeneration of the elastase susceptibility of this substrate. Acetylation under milder conditions blocks all the amino groups and about 13 hydroxyl groups, resulting in a 50% increase in elastolysis.

Deamination. Elastin (1.0 g.) was suspended in 15 ml. of citrate buffer, pH 5.0, and chilled to 5°. The suspension was mixed with 50 ml. of water containing 1.0 g. of NaNO₂ and kept at a temperature of between 2° and 5° for 18 hr. When filtered off, washed and dried, the product was a yellow powder. Treatment of this substrate with elastase resulted in increased elastolysis (Table 1), an observation which was in agreement with the results of *N*-acetylation. Although the amount of elastin protein passing into solution from the deaminated substrate was increased, the number of amino groups released during the initial stages of the same reaction decreased.

Iodination. The introduction of I₂ into elastin, under conditions favouring substitution in the

tyrosine residue (pH 9.0, 0.01M-I₂ in 0.5M-KI at room temperature for 40 min.), has no effect on the rate of elastolysis, although the amount of I₂ consumed (15 moles/10⁵ g. of protein) is consistent with disubstitution of the seven tyrosine molecules of elastin.

Dinitrophenylation. Elastin was treated with 1-fluoro-2:4-dinitrobenzene according to Sanger (1945). The susceptibility of this yellow material to elastase was decreased by 30%. The amount of dissolved protein was measured in the interferometer, because the coloured protein interfered with the biuret reaction.

Introduction of new carboxyl groups. Elastin was treated with glyoxylate at pH 9.3 and with monobromoacetate at pH 10 for 4 hr. as recommended for collagen by Sykes (1952). Both these procedures, which introduce new carboxyl groups as well as blocking amino groups, increase the susceptibility of elastin to elastase (Table 1).

Adsorption of elastase on elastin

Hall & Czerkawski (1959) showed that elastase is rapidly adsorbed on elastin at 4° at the optimum pH of the system without any appreciable elastolysis occurring. This phenomenon has been used to assess how modification of the substrate affects its ability to pick up elastase.

Elastin (50 mg.), esterified elastin and *O*-acetylated elastin were suspended in 2 ml. of borate buffer, pH 8.45, and chilled to 4°. The enzyme solution was then added to bring the volume to 5 ml. and the mixture kept at 4° for 5 min. The solid was removed by centrifuging at the same temperature. A sample of the supernatant was added to standard elastin suspended in fresh buffer, and incubated at 37° for the standard period of time. The difference between the degree of elastolysis of a control and the test sample was a measure of the amount of enzyme adsorbed on the substrate. Typical results are presented in Table 1 (third column).

DISCUSSION

The empirical equation which was derived to explain the reaction between elastase and elastin (Hall & Czerkawski, 1961) consists of three terms: a linear function of substrate concentration, a hyperbolic function of enzyme concentration and a function of time. Differentiation of this equation with respect to time gives an equation representative of the rate of the reaction and directly proportional to the amount of substrate available, to the same hyperbolic function of enzyme concentration, and again to a function of time. The amount of solubilized elastin increases rapidly owing to the sigmoid shape of the elastolysis-time curve. Part of

Fig. 5. Release of acetyl groups from acetylated elastin during mild hydrolysis with 0.05N-NaOH at 37°. (a) The sum of acetyl groups held and released during hydrolysis of fully acetylated (●) and *O*-acetylated (○) elastin. (b) The amount of acetyl group released during saponification of fully acetylated (●), *O*-acetylated (○) and mildly *N*-acetylated (◐) elastin.

the enzyme may be assumed to become adsorbed on the surface of the solid elastin, part will diffuse more slowly into the solid and part will remain in solution. Thus the solution becomes progressively poorer in enzyme and richer in soluble elastin. This latter material will also be able to pick up elastase from solution and the direct outcome will be a relatively small increase in the rate of the secondary reaction between elastase and the soluble product of the first reaction. Consequently the solubilized protein becomes progressively richer in the un-attacked soluble material in agreement with the experimental results given in Fig. 1.

In the later stages of elastolysis the substrate for the secondary reaction begins to predominate, and the process of diffusion, which tends to diminish the amount of enzyme available in the solution, not only stops but is reversed as the solid within which it is trapped passes into solution. At the same time as the amount of substrate for the first reaction decreases the rate of solubilization decreases until the rate of the second reaction eventually surpasses it. When all the elastin is dissolved the second reaction slows down but does not stop. The number of terminal amino groups approaches the value of 120 moles/10⁶ g. of protein, in agreement with the results of Partridge & Davis (1955). The terminal amino acid content of solid elastin is negligible (0.3 mole/10⁶ g. according to Partridge) and when the secondary reaction is inhibited by detergent the product of elastolysis contains about 30 such groups. It is obvious that if both reactions are brought about by the same enzyme, as would appear to be the case in view of the purity of the elastase preparation (Hall & Czerkawski, 1959), the respective reactions must be different.

The reaction with the solid substrate appears to require the initial adsorption of the enzyme on the substrate followed by their interaction. The results of the adsorption experiments indicate that the presence of a free carboxyl group is essential for this first half of the reaction. It is therefore not surprising that esterification has such a marked effect on elastolysis, since if the enzyme and its substrate are not able to combine no reaction can take place in such a heterogeneous and consequently highly localized system. The nature of the carboxyl groups involved in this adsorption reaction can be deduced from the failure of thiohydantoin reaction. Such cyclizations take place only with α -carboxyl groups and hence the ones which are available in elastin must be the side-chain carboxyl groups of glutamic acid and aspartic acid. Although the carboxyl group appears to be all-important in attracting the enzyme to the substrate, the presence of an unsubstituted hydroxyl group also seems to be necessary for the actual reaction to proceed. It need not necessarily be inferred that the carboxyl and hydroxyl

groups are present on adjacent amino acid residues nor even that they are on the same peptide chain. They may merely be brought into close proximity by the spatial convolutions of the elastin molecule. The net result must be, however, the production of a hydroxyl-carboxyl complex capable of reacting with a single centre on the enzyme and not two separate groupings reacting with widely separate centres, since the incorporation of glyoxylate into the molecule, which introduces fresh hydroxyl and carboxyl groups adjacent to one another, results in a marked increase in activity. But monobromoacetic acid introduces no hydroxyl group and yet gives an increase.

Both the hydroxyl and the carboxyl groups must therefore react with a single relatively small centre in the enzyme. Hall (1954) suggested that the enzyme and the substrate might be united through a calcium atom and that the calcium atom was held by chelate bonds to both proteins. The essential nature of carboxyl and hydroxyl groups which could afford such a chelating centre on the substrate is very favourable to this hypothesis.

Elastolysis results not only in the dissolution of elastin but also in its degradation whilst in solution. Table 1 indicates that although the enzyme appears to be unitary in nature it has quite a different mode of action on solid and soluble elastin. Carboxyl and hydroxyl groups are essential for the reaction with native elastin. The presence of free amino groups appears to have an adverse effect on elastolysis, but does not affect the secondary reaction. Involvement of amino groups may explain the anomalous results of the adsorption experiments with *O*-acetylated substrate. Partial acetylation of the amino groups could result in a charge redistribution and an enhanced adsorption on the carboxyl group.

The reaction between elastase and elastin is therefore a complex system with at least two parts, which are to a certain extent in competition. The first reaction consists in adsorption of elastase on solid elastin through the side-chain carboxyl groups of glutamic acid and aspartic acids and the possible formation of a chelated link through calcium ions and the hydroxyl groups of tyrosine and possibly other residues bearing such groups. As the reaction proceeds and soluble elastin appears in solution, an increasing proportion of elastase becomes bound to this second substrate. This partition of the enzyme between the two substrates can account for some of the changes occurring during the course of the overall reaction. The adsorption of elastase is increased by sodium dodecyl sulphate, which imparts a net negative charge to the protein. This results in an increased elastolysis (appearance of protein in solution) at the expense of proteolysis (appearance of amino groups). This differentiation between elastolysis and proteolysis is more apparent than real

since the appearance of 30 amino groups in elastin indicates a limited proteolytic reaction in the first stage.

When esterified elastin is subjected to very mild alkaline hydrolysis its susceptibility to elastase reverts to normal long before all the carboxyl groups are unmasked. Moreover, when all the methoxy groups are removed, the activity is much greater than that of the standard powder treated with alkali under identical conditions. It is possible that elastin contains some linkages (probably ester) which are particularly susceptible to hydrolysis by ethanol. Such linkages may be purely internal or might be utilized in binding traces of other substance, such as lipid or polysaccharide. The enzymic cleavage of such strategic bonds might constitute an additional third reaction.

SUMMARY

1. The proteolytic activity of elastase during solubilization of elastin is investigated by measuring the release of free amino groups and the magnitude of the average particle size. It is shown that the secondary process may be largely eliminated by elastolysis in the presence of an anionic detergent.

2. Chemical modification of certain side-chain groups of elastin shows that the side-chain carboxyl and the tyrosine hydroxyl groups are necessary for elastolysis. The former are directly concerned in

adsorption of elastase on elastin. The amino groups are not directly concerned in elastolysis, but their blockage or removal gives an increase in activity probably due to the increased electronegativity of elastin.

3. The results of chemical studies are discussed in the light of the postulated two reactions.

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The Reaction between Elastase and Elastic Tissue

6. THE MECHANISM OF ELASTOLYSIS

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Some hypotheses on the mechanism of elastolysis have been discussed by Hall & Czerkawski (1959, 1961*a, b*). New information has been given about the two postulated reactions, namely solubilization and secondary degradation in solution. The present paper introduces data in support of a postulated preliminary reaction and correlates it with the other two.

The reaction between solid elastin and elastase is preceded by the adsorption of the enzyme on the substrate through the side-chain carboxyl groups. Blockage of such groups prevents adsorption and,

therefore, reaction, whereas an increase in their number increases both these considerably. However, such adsorption is normally confined to the surface or the layers close to the surface of elastin particles (Czerkawski, 1960), and solubilization would be very inefficient unless there were a loosening, permitting the ensuing permeation of elastin by elastase. Such a process, although important, would be difficult to detect chemically; but, it would result in structural changes, which are bound to produce large changes in the elastic properties of elastin. The elasticity of this material has previously