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

been measured only before and after enzymic attack (Wood, 1954). Here such measurements have been attempted during elastolysis.

METHODS AND MATERIALS

Elastin. Fresh ox ligament was cut into longitudinal sections 500 μ thick and about 8 cm. long on a freezing microtome. These sections were then extracted repeatedly at 100° with aq. 1% acetic acid in a Soxhlet apparatus to remove most of the collagen. The sections were spread on polythene sheets, frozen and cut with scissors into strips measuring approximately 1 cm. by 8 cm.

Enzyme. The elastase used was purified as described by Hall & Czerkawski (1959).

Buffer. The load-extension curves were determined in borate buffer (pH 8.5, I 0.364), since preliminary experiments had shown that the Young's modulus of elastin remained constant in the pH range 6–10.

Load-extension apparatus. This consisted of a jacketed vertical tube fitted with an adapter, which was provided with a stopcock and a glass hook at its lower end. A strip of elastin was suspended in acetone, and, when nearly dry, its ends were folded over and cemented into two small glass triangles. When quite dry the ends were painted with the same cement, to make them impermeable to water, and the complete systems were finally suspended for 16 hr. in borate buffer. The lower glass triangle was attached to the upward-pointing glass hook and the upper one was connected to a fine silk thread, which passed out of the top of the apparatus over a light pulley and was secured at the end to a light pan for weights. The extension was measured by means of a travelling microscope focused on the top of the strip. The strip was completely immersed in the buffer solution, which could be easily withdrawn through the tap at the bottom for analysis (ultraviolet absorption at 275 $m\mu$) and subsequently replaced in the vessel.

The temperature was kept constant by passing water from an external thermostat through the outer jacket.

RESULTS

Extension at constant load. The strip was suspended in borate buffer at 37° under a small constant load of 2 g. The enzyme was then added. The amount of elastin passing into solution was measured (extinction at 275 $m\mu$) and also the extension. The rate of solubilization had a low but constant value (3.6%/hr.), and there was initially no change in extension. However, the latter began to increase rapidly, eventually becoming very great (about tenfold increase), resulting finally in breakage. The break occurred in the central portion of the strip and no previous irregularities of the strip could be detected visually. The time interval elapsing before break varied with the enzyme concentration.

Effect of elastase on the Young's modulus of elastin. The above results suggested that elastase produces a marked decrease in the Young's modulus (E) of elastin, and subsequently this was demonstrated directly. The strip was suspended in borate buffer in

the same apparatus as described above, but the maximum extension was limited purposely by permitting the weighted pan to travel a distance of only 0.7 cm. The measurements for the load-extension curve, which was linear up to about 30% extension, took 2–4 min. and the measurement of the extent of solubilization took about 1 min. The experiment was carried out at 37° in elastase solution containing 10 μ g. of the enzyme/ml. The load-extension curves remained linear throughout and E could be calculated from the slope and from the dimensions of the strip. Two corrections were applied to each of the values, both relating to changes in cross-sectional area, due to a small amount of plastic creep and to solubilization. The corrections were only of considerable proportions after prolonged elastolysis; for instance, when E has decreased by 80% these amounted to only 4 and 10% respectively. The results are shown in Fig. 1.

DISCUSSION

Preliminary reaction. It can be shown thermodynamically (Alfrey & Gurnee, 1956) that a decrease in the E at constant load is accompanied by an increase in entropy and consequently in the amount of disorder. This could be brought about by the severance of certain strategic lateral bonds and

Fig. 1. (a) Change in Young's modulus of elastin during the early stages of elastolysis; (b) amount of protein released into solution.

the lengthening of the loosened longitudinal peptide chains. Treolar (1958) gives a formula which relates the molecular weight of a free chain fixed at both ends to the modulus of rigidity of the elastic substance of which it is composed, namely $n = RT\rho/M$, where n is the modulus of rigidity, σ is density and M is the molecular weight of such a chain. Now n is related to E and the Poisson's ratio ρ by the well-known relation $n = E/2(1 + \sigma)$. For elastin, the necessary calculations give a value for M of 2600. The significance of this value is that it is doubled when only a very small fraction of the elastin has been dissolved under the action of elastase. Thus the early stages of elastolysis appear to be associated with the severance of lateral bonds together with an increased looseness of structure (cf. Banga & Baló, 1960).

Mechanism of elastolysis. The complete elastolytic reaction may therefore be at present pictured as follows. The enzyme is adsorbed on the available side-chain carboxyl groups of elastin (Hall & Czerkawski, 1961b). This may result in reaction or it may not. If there is no reaction the enzyme is simply attached to the substrate. On the other hand, another part of the enzyme (active centre) may come into contact with a specific site of the substrate and this may result in the fission of bonds and loosening of structure, but not necessarily in solubilization. This should be called the preliminary reaction and it would correspond to the slow phase of the elastolysis-time curve (Hall & Czerkawski, 1959). After a time a sufficient number of bonds will have been broken for particles to start passing into solution. The difference between preliminary reaction and solubilization might be only one of degree or it might involve quite different bonds. Nevertheless, an increase in the extent of the preliminary reaction must result in an increase of the rate of solubilization. The latter process results in production of α -elastin molecules, which then combine with some of the enzyme in solution.

The highly localized solubilization may also be speeded up by preferential adsorption on the carboxyl groups of the substrate, since reaction would be very slow if the process were purely random. The α -elastin is subject to a relatively slow stepwise degradation and therefore this material accumulates partly in solution and partly within the undissolved solid. This product eventually becomes inhibitory. However, the diminution in the rate of solubilization is caused mainly by a decrease in the availability of the solid.

Elastolysis at macroscopic level. Czerkawski (1960) has shown that relatively intact elastin is attacked preferentially from the ends of the fibres and that the rate of reaction depends on the exposed surface. It has long been known that the fresh tissue is attacked only very slowly by elastase. This agrees

with the fact that the rate of metabolism of elastin in the tissues is very low (Slack, 1954). However, like any other tissue it must metabolize and there must be some process by which solubilization can be brought about. Moreover, arteriosclerosis leads eventually to lesions and it is difficult to visualize such a tough substance as elastin breaking down mechanically without some previous degradation brought about by a catalytic agent.

It is uncertain what causes the sclerotic lesion in the aorta. It may be that the tissue is weakened mechanically by deposition of fat or by calcification. The perpetually fluctuating stresses may result eventually in a small mechanical damage. Such a break could act as a trigger and open the way for elastolytic attack that will primarily produce a marked lowering of the elastic modulus of the affected part, and thus result in distension and eventually in dissection of the vascular wall.

A similar process, scaled down suitably, may take place in healthy tissues. Under such conditions all three reactions (the primary loosening, solubilization and stepwise degradation of soluble elastin) would proceed slowly, subject to many controlling factors, such as auxiliary enzymes or serum inhibitors. In spite of its slowness, the whole process is economic and efficient. The stepwise requirement prevents complete degradation and appears to leave units for possible resynthesis.

SUMMARY

1. Many observations on the reaction between elastase and elastic tissue indicate that, in addition to solubilization and secondary degradation in solution, there is a third reaction that may be termed preliminary. Its presence may be demonstrated by a marked change in elasticity of elastin during early stages of elastolysis.

2. The preliminary reaction is correlated with the other two, and a possible complete mechanism of elastolysis *in vitro* and at macroscopic level is discussed.

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