A CONTRIBUTION TO THE NATURE OF THE ELASTOLYTIC ENZYME AND THE STRUCTURE OF ELASTIN.

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THE presence of a bacterial elastolytic enzyme was discovered as early as 1904 by Eijkman. He prepared culture media from the lungs of calves, from ligamentum nuchae and from arteries. With these he demonstrated the lysis of elastin as the result of bacterial action. Of the bacteria that he studied *Ps. pyocyanea* showed the highest activity. In 1949 Baló and Banga reported that extracts of fresh ox pancreas as well as that of acetone-dried pancreas powder contain a specific elastolytic enzyme which they named elastase. In further publications Baló and associates demonstrated that the enzyme does not attack collagen and that their pure elastase differs from the other proteolytic ferments of the pancreas in that neither trypsin nor chymotrypsin have any comparable dissolving action on elastic tissue (Baló and Banga, 1950; Banga, Baló and Nowotny, 1948–49; Banga, 1951). Banga (1951) suggested that the effect of elastase was not proteolysis, but rather depolymerization, and that this was effected by the splitting of H-bonds, fibrous elastin being transformed into globular elastin.

The next important advance in our knowledge of the nature of elastin was taken when Hall, Reed and Tunbridge (1952) observed that polysaccharide and sulphuric acid are intimately associated with the protein in elastic tissue. In their view elastic tissue consists of linear aggregates of pro-elastin protected on the outer side by a layer of pro-elastin molecules bound together by union with polysaccharide molecules. In support of this they found that during the action of elastase on elastin all the acid is liberated during the dissolution of only half the protein. Furthermore, they also expressed doubt as to the function of elastase and suggested that it is a mucase rather than a proteinase. According to them the function of the enzyme would be to attack the elasto-mucin chains, liberating the pro-elastin therefrom.

These observations of Hall *et al.* were further substantiated by Banga and Schuler (1953). From their chemical experiments, they concluded that elastin was a glucoprotein, the fibrous character of which is determined by the carbohydrate molecule of elastin. Following dissolution of elastin by elastase, they demonstrated a carbohydrate molecule in the deproteinized filtrate.

Neuberg and Hoffmann (1931) demonstrated the presence of a chondrosulphatase by obtaining a specific enzyme preparation from an organism related to *Ps. eisenbergii*. These authors also found chondrosulphatase in *Proteus vulgaris* and *Ps. pyocyanea*. This, taken in conjunction with the earlier work of Eijkman (1903–04) would suggest that extracts of *P. vulgaris* contain a chondro-

sulphatase as well as an elastolytic enzyme. Our own observations as regards pancreatic elastase and the bacterial chondrosulphatase and/or elastase have led us to believe that they are identical enzymes, attacking primarily the mucopolysaccharide bonds between the pro-elastin molecules.

MATERIALS AND METHODS.

Preparation of pancreatic elastase.

Pancreas powder was prepared according to the method of Willstätter and Waldschmidt-Leitz (1923). The fine powder was stored in a glass-stoppered bottle in a refrigerator at 4°. In all the experiments a 1 per cent solution of this powder in distilled water was used. The solution was put into a Coplin jar containing the deparaffinized sections to be digested and incubated at 37° for varying periods of time. The solution of the enzyme remained stable for 2–3 days. The pH of a freshly prepared aqueous solution of the enzyme as determined with a Beckman pH meter was 8·8. After incubation for 2 hr. at 37° the pH was found to be 6·6. Although the optimum pH for elastase is given as 10·3, we found that buffering of the solution was not necessary as the enzyme activity on tissue sections was not significantly affected at this pH. Control sections were incubated at 37° in distilled water.

In order to see whether the pH change during incubation would have any effect on the staining reactions used, sections of aorta and costal cartilage were incubated at 37° for 6 hr. in McIlvaine Na_2HPO_4-citric acid buffer solutions in which the pH range varied from 2·2–8·0. No interference was found in the subsequent staining of the aortic elastic tissue with Weigert's or Verhoeff's elastic tissue stains or of the metachromatic material in the aortic wall and cartilage on staining with a watery solution of toluidine blue.

Preparation of chondrosulphatase.

A 48-hr. nutrient agar growth of *P. vulgaris* and *Ps. pyocyanea* was suspended in acetone, allowed to stand for 2 hr. and then washed in an ether-acetone mixture and finally left in pure anaesthetic ether for another 2 hr. The suspension was then filtered, and the bacteria left to dry on filter-paper overnight. The powder so obtained was then ground with a pestle and mortar. A 2 per cent solution of this powder in distilled water was used in the subsequent experiments. Sections of aorta and cartilage were deparaffinized and incubated in the solution contained in Coplin jars for varying periods of time. Control sections were incubated in distilled water at 37° for the same periods. Where sections were incubated for periods exceeding 6 hr. it was found useful to cover the slides with a thin layer of celloidin to prevent the tissue lifting from the slide. In addition the hyaluronidase and trypsin extractions were done according to the methods suggested by Pearse (1953).

Substrates.

The aorta and costal cartilage used in this experiment were fixed in 10 per cent formol-saline, dehydrated in alcohol and embedded in paraffin wax. Sections were cut at 4μ, dewaxed and subjected to the action of the different enzymes.

The metachromatic substance of the aorta is composed of the acid mucopolysaccharides chondroitin sulphate B and C, whereas that of hyaline cartilage consists of chondroitin sulphate A (Meyer and Rapport, 1951). From a histological point of view elastic tissue is characterized by a specific staining reaction. The exact nature of the reaction of these different stains is not known. Chemically elastin differs from collagen in having less arginine, histidine and lysine and more leucine, valine and tyrosine. No tryptophan is present in elastin. Davies and Dubos (1947) have shown that leucine residues are lipophilic and that elastic tissue might therefore contain more lipid than collagen. In addition it has recently been shown that elastic tissue contains a carbohydrate.

Staining methods.

The staining methods used to demonstrate elastic tissue were Weigert's elastic tissue stain, prepared from basic fuchsin and resorcin, and Verhoeff's haematoxylin method. The metachromatic substance was stained by aqueous toluidine blue (Pearse, 1953). Masson's

trichrome stain and periodic-acid Schiff reactions were used to differentiate collagen and fibrous tissue.

The results are presented in the Table.

DISCUSSION.

The following two observations detailed in the Table led us to suspect that the enzyme prepared from ox pancreas is similar to the bacterial chondrosulphatase.

Firstly, both these enzymes, when allowed to react for a short period with the chondroitin sulphate present in the wall of the aorta and costal cartilage, cause disappearance of this acid mucopolysaccharide as shown by the absence of metachromatic staining with a watery solution of toluidine blue.

Secondly, when these enzymes are left in contact with the elastic tissue of the aorta for a longer period, they both cause elastolysis. The proof that elastolysis had actually taken place and that the failure of elastic lamellae to stain with the usual stains was not due to a chemical reaction of the enzyme with elastin, has been proved by the absence of such lamellae on examination of sections after extraction by means of phase-contrast microscopy.

These observations would then suggest that pancreatic elastase is in reality a chondrosulphatase and would lend support to the work of Baló and Banga, and Hall *et al.* in explaining the chemical nature of the reaction of the enzyme. Removal of the binding polysaccharide molecules in the elastin chain would then result in the setting free of pro-elastin molecules. Free amino-acids would not be set free in this reaction.

The pancreatic extract used in this experiment contains in addition to elastase other enzymes, the most important of which is trypsin. As the pH of the 1 per cent solution of pancreatic extract varied between 8·8 and 6·6 during incubation, it was necessary to do control histochemical experiments with crystalline trypsin, as this enzyme has its optimum effect at pH 8·0. It was found, however, that pure crystalline trypsin had no elastolytic effect, and furthermore that the amount of trypsin in the 1 per cent solution of pancreatic extract was relatively small, the collagen in the aortic wall not being significantly affected after incubation for 6 hours.

It was also noted that the elastolytic enzymes removed the free acid mucopolysaccharides before attacking elastic tissue itself and therefore the possibility that it might be a hyaluronidase had to be investigated. Using testicular hyaluronidase according to the method described by Pearse it was found that it removed the free mucopolysaccharides of both cartilage and aorta, but the elastic tissue was unaffected after prolonged incubation.

The fact that elastic tissue contains a chondroitin sulphate is important in so far that the age increase of mucopolysaccharide in the aorta might be due to degeneration of elastic tissue and liberation of the acid mucopolysaccharide therefrom. Many theories have been advanced as regards the origin of the mucopolysaccharides of the aorta but as yet none of these has been definitely proved. In none of these has an origin in elastic tissue been suggested. Schultz (1922), however, suggested that the metachromatic substances might furnish material for the building up of elastin and that chondroitin might be among them.

A further interesting observation has been that staining of elastin before

digestion with the enzyme inhibits the latter action. In addition it is also well known that cartilage gives a false positive staining reaction with the usual elastic tissue stains. These two observations would suggest that the specificity of the elastic tissue stains is possibly dependent on the presence of the acid-mucopolysaccharide in elastin. Schultz (1922) suggested that the staining reaction of elastic tissue by the elastic stains is due to the impregnation of elastin by mucin.

TABLE.—*Comparison of Results.*

Substrate.	Enzyme.	Time of reaction in hours.	Staining method.	Results.
A.M.P.A.	Pancreatic elastase	1	Toluidine blue	Total removal of metachromatic material.
,,	Hyalase	3	,,	Total removal of metachromatic material.
,,	Trypsin	1	,,	Normal amount of metachromatic material.
,,	Bacterial chondrosulphatase	2	,,	Total removal of metachromatic material.
A.M.P.C.	Pancreatic elastase	½	,,	Normal amount of metachromatic material present.
,,	,,	¾	,,	Small amount of metachromatic material still present.
,,	,,	1	,,	Total removal of metachromatic material.
,,	Hyalase	3	,,	Total removal of metachromatic material.
,,	Trypsin	1	,,	Normal amount of metachromatic material present.
,,	Chondrosulphatase	1	,,	Total removal of metachromatic material.
Elastin (aorta)	Pancreatic elastase	1	Weigert	No effect.
,, ,,	,,	2	,,	Fibres and lamellae begin to swell and early fragmentation can be seen.
,, ,,	,,	3	,,	Patchy disappearance of fibres. Remaining fibres have moth-eaten appearance.
,, ,,	,,	4	,,	Occasional poorly stained fibres remain.
,, ,,	,,	5–6	,,	Total removal of elastin.
Elastin	Hyalase	6	,,	Normal amount of elastin present.
,,	Trypsin	6	,,	Normal amount of elastin present.
,,	Chondrosulphatase	6	,,	Total removal of elastin.
Aorta	Pancreatic elastase	6	Masson	Collagen still present.
Elastin first stained with Weigert	,,	6	Weigert	Normal amount of elastin still present.
Elastin extracted in chloroform methanol for 6 hr.	None	6	Weigert PAS Toluidine blue	Normal staining reaction.
Elastin (aorta)	Pancreatic elastase Chondrosulphatase	6	None	No elastic lamellae seen by phase contrast microscopy.

A.M.P.A. = Acid mucopolysaccharide of aorta.
A.M.P.C. = ,, ,, of cartilage.

Further morphological, histochemical and experimental studies are being carried out to determine the relationship between the break down of elastic tissue, the increase of metachromatic ground substance and the ultimate collagenization in the pathogenesis of the degenerative arterial diseases.

SUMMARY.

Specific elastase prepared from ox pancreas and bacterial chondrosulphatase obtained from *Ps. pyocyanea* and *P. vulgaris* appear to be identical enzymes.

When these enzymes act on tissue sections for a short time, they have a chondrosulpholytic effect, whereas longer exposure to the enzymes results in elastolysis.

Staining of elastic tissue before exposure to the enzyme inhibits the elastolytic effect.

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