The Elastolytic Activity of Pancreatic Extracts

By J. Baló and I. Banga
Department of Pathological Anatomy and Experimental Cancer Research, University of Budapest

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According to Lowry, Rourke & Katersky (1941) the insoluble proteins which build up the wall of arteries consist of two scleroproteins, the collagen and the elastin. Of these the elastin cannot be dissolved by any inorganic or organic solvent; the collagen can be dissolved in 2 % acetic acid and when boiled with water it is converted into gelatin. In a previous paper Baló & Banga (1949) showed that histological sections prepared from elastic arteries are dissolved by pancreatic extracts, and examination of the sections stained with resorcinol-fuchsin showed that the elastic fibres building up the wall of the arteries first swell, then break up into particles and finally are entirely dissolved. The dissolving effect of the pancreatic extracts may be explained by the presence of an enzyme, which has been called elastolytic enzyme or elastase.

Work with histological sections requires time, and is not quantitative; it does not involve the isolation of the elastolytic enzyme. Since our investigations showed that dried aorta powder is dissolved by the elastase, as are histological sections, quantitative measurement of the enzyme became possible. In this paper we describe properties of the elastase and methods for the quantitative measurement of its activity.

In connexion with the dissolution of elastin by pancreatic extract the question arises whether this effect may be attributed to the trypsin or chymotrypsin contained in the extract or whether it is the result of the action of a specific enzyme. The literature contains contradictory statements as to whether trypsin dissolves elastin and collagen. There are no adequate data in the literature concerning the enzymic decomposition of the elastic fibres of human and animal arteries. Grassmann (1936) stated that collagen is not digested by trypsin. Ewald (1890) reported that trypsin digests the elastic fibres of the ligamentum nuchae of cattle; in these experiments a pancreatic extract, which might have contained trypsin as well as elastase, was used. Yet Schneider (1937) denied that proteolytic enzymes attack elastin. Thus little definite information exists on the enzymic decomposition of the wall of the arteries.

EXPERIMENTAL

Measurement of elastase activity. For measuring the activity of pancreatic extracts a gravimetric method was used, similar to that employed by Willstätter, Grassmann & Ambros (1926) for measuring the activity of papain on fibrin. These authors weighed the fibrin which remained undissolved after the action of papain. Our method is based on the observation that ethanol-dried aorta powder cannot be dissolved at neutral or slightly alkaline reaction. On treatment with elastase the elastin of aorta powder is dissolved, and the amount dissolved can be determined by weighing the dried matter before and after the action of elastase.

This estimation was done as follows: equal quantities (20 mg.) of aorta powder were put into weighed centrifuge tubes of 6 ml. capacity and into each tube 1 ml. of carbonate-HCl buffer of pH 10·32 was added. To the experimental tubes different quantities of elastase or pancreatic extract were added; no enzyme was added to the control tubes. The volume in each tube was made up with water to 2·5 ml. The tubes were incubated at 38° for 30 min. with constant shaking; they were then centrifuged and the supernatant discarded. The loss of weight from the control tubes is caused by the dissolution of the soluble proteins of the aorta wall, whilst the loss of weight from the experimental tubes is due in addition to the elastin which is dissolved by the action of the elastase. After centrifugation the sediment was washed with 5 ml. of 96 % ethanol and after drying at 110° the tube and its contents were weighed. The difference between the weights of dry substance in the control and in the experimental tubes gives the weight of the elastin dissolved.

Formaldehyde titration. Formaldehyde titration according to Sørensen was used for the quantitative estimation of trypsin activity with casein as substrate. We also examined the activity of our pancreatic extracts containing the elastolytic enzyme upon casein and upon aorta using formaldehyde titration.

Estimation of proteins. In estimating the activity of elastolytic enzyme it is necessary to know the protein contents of the pancreatic fractions. This was done as follows: 5 ml. of acidified enzyme solution containing 1 % (w/v) NaCl were added to weighed centrifuge tubes. The solutions were then heated for 30 min. at 100°. The coagulated protein was centrifuged off and washed twice with 5 ml. of 96 % ethanol. The tubes were dried at 110° and weighed.

Calculation of elastolytic activity. We took as basis the elastolytic units (E.U.) defined in a previous paper (Baló & Banga, 1949): 1 ml. of 1 : 1000 diluted saline extract of

powdered pancreas contains 1 E.U. This concentration can dissolve, in 12 hr. at 37°, histological sections of a thickness of 20 μ. prepared from elastic arteries. This unit is equal to the quantity of enzyme which dissolves, under the conditions used in our present experiments, 5% of the elastin. We determined the E.U. values and the quantity of protein in each fraction used. The activity is equal to E.U./mg. of protein.

Preparation of pancreatic extract. Defatted and powdered pancreas (1 g.) was extracted with 20 ml. of 0·1 N-phosphate buffer (pH 6·0) by shaking it for 30 min. The solution was centrifuged and the precipitate suspended again in 20 ml. of 0·1 N-phosphate buffer (pH 6·0), again shaken for 30 min. and centrifuged. The two solutions were combined and the activity determined. The mixture (1 ml.) contained 25 E.U. and 6·75 mg. of protein. The activity therefore was 25/6·75 = 3·7.

Purified elastase. To 30 ml. of the pancreatic extract described above $(NH_4)_2SO_4$ was added to 0·2 saturation and kept at 2° for 12 hr. The precipitate was centrifuged and discarded. To the solution $(NH_4)_2SO_4$ was added again to 0·4 saturation, and the precipitate, which contained the active elastase, was suspended in 15 ml. of water. The solution was dialysed until free of sulphate and centrifuged. The solution, to which NaCl was added to a final concentration of 1%, was kept at 0°. This solution contained 50 E.U./ml. and 1·55 mg. of protein/ml. (activity = 50/1·55 = 32·1). The elastase prepared in this way was 8·5 times as active as the original pancreatic extract.

Trypsin. Trypsin and chymotrypsin were prepared from fresh pig pancreas by the method of Kunitz & Northrop (1935–6). The fraction, which precipitated with $(NH_4)_2SO_4$ between 0·4 and 0·7 saturation, was used as trypsin, although it contained chymotrypsin as well. The trypsin solution so obtained contained 5 E.U./ml. and 5·4 mg. of protein/ml. (activity, 0·9); it showed very high proteolytic effect on casein.

Preparation of collagen. Human tendo Achillis was cut very finely and ground during 20 min. with 20 vol. of 2% (v/v) acetic acid. It was centrifuged, the fluid decanted and the extraction procedure repeated on the insoluble residue. The supernatant fluids which contained the collagen were mixed and the protein content determined. On adjusting the pH of the acetic acid-collagen solution to pH 8·0, the collagen precipitated in a loose form. The collagen was washed first with water and then with ethanol, and dried in a desiccator.

Preparation of elastin from the aorta wall. The purified aorta wall was ground very finely for 20 min. with 5 vol. of 2% (v/v) acetic acid and then centrifuged; the insoluble material was similarly ground with acetic acid and centrifuged again. The insoluble material was extracted twice with water, washed with 96% ethanol and dried in a desiccator.

RESULTS

The effect of concentration of pancreatic extract on the dissolution of aorta powder

Fig. 1 shows how the dissolution of aorta powder proceeded in the presence of different quantities of pancreatic extract. With increasing quantities of enzyme the dissolution of aorta powder also increased.

Effect of time on the action of elastase

The velocity with which the pancreatic extract dissolves the elastin of aorta was examined. As Fig. 2 shows, the rate of dissolution is greatest at the beginning. During the first 30 min. of incubation 50% of the aorta protein was dissolved and the maximum value was reached after 180 min. At this time 75–80% of the aorta protein was decomposed.

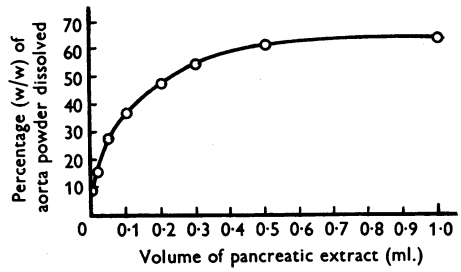

Fig. 1. Dissolution of aorta powder in the presence of increasing quantities of pancreatic extract after incubation for 30 min. at 37°. Total volume 2·5 ml.; 20 mg. of aorta powder; 0·5 ml. of carbonate-HCl buffer, pH 10·32.

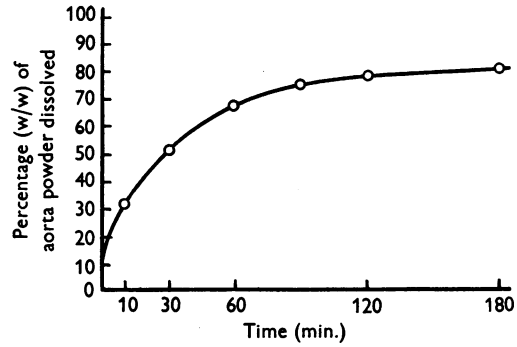

Fig. 2. Effect of time on the dissolution of aorta powder by pancreatic extract (expressed as percentage); 5 E.U. to 20 mg. of aorta powder in 2·5 ml. total volume, pH 10·32; temp., 38°.

There was no further dissolution during longer incubation. This proves that there is a protein fraction amounting to 20–25% which remains unaffected by the pancreatic extract, which contained trypsin as well as elastase. As our further experiments show, this protein is probably collagen.

pH-*activity curve of elastase*

The pH optimum of elastolytic activity was determined by dissolving aorta powder in presence of elastase at different pH's. In order to measure the initial velocity of the reactions a 10 min. incubation was chosen. Aorta powder (10 mg.), buffer (0·5 ml.) and 6 E.U. were used in a total volume of 2·5 ml.

Between pH 3·0 and 6·0 acetic acid-sodium acetate, between pH 5·0 and 8·0 phosphate, between pH 7·0 and 9·0 borate and between pH 9·0 and 11·0 sodium carbonate-hydrochloric acid buffers were used. There

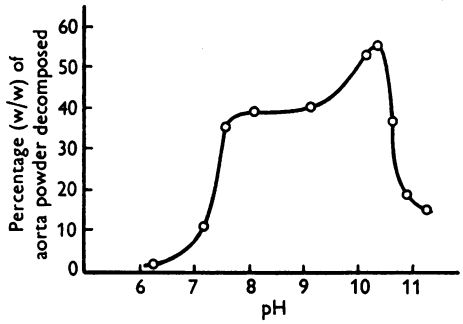

Fig. 3. Decomposition of aorta powder at different pH in the presence of elastase. In 2·5 ml. total volume, 10 mg. of aorta powder, 0·5 ml. of buffers of different pH and 6 E.U. Time of incubation, 10 min.

is practically no enzymic action below pH 6·0. Fig. 3 shows that above pH 6 the activity of elastase increases with alkalinity and that the optimum is at pH 10·3.

The hydrolytic effect of trypsin on aorta and casein

In this experiment the hydrolytic effect of trypsin on the aorta wall was compared with the effect of casein. For following the rate of hydrolysis the Sørensen formaldehyde titration was used. Fig. 4

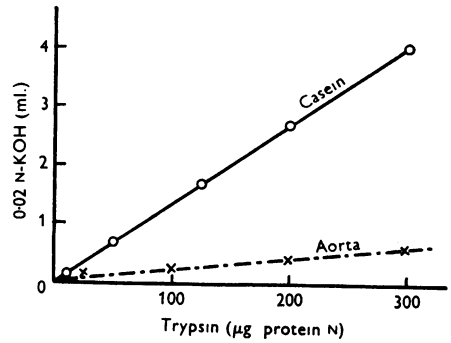

Fig 4. Proteolytic effect of trypsin on casein and aorta. In 2·5 ml. total volume, 20 mg. of casein and aorta respectively, 0·5 ml. of phosphate buffer (pH 8) and different quantities of trypsin. After 20 min. incubation the proteolytic activity of trypsin was estimated by formaldehyde titration.

shows the action of different quantities of trypsin upon casein and aorta. Trypsin liberates very small quantities of amino-acids from the aorta wall in conditions in which the hydrolysis of casein was very marked. This experiment proves that the aorta wall is not appreciably hydrolysed by trypsin. Neither can the liberation of amino-acids be demonstrated following the action of elastase. The purified elastase contains traces of trypsin so the above experiment proves that during the enzymic dissolution of the aorta no amino-acids are liberated. The effect of elastase is only to transform the insoluble protein of aorta into a soluble one.

The effect of elastase on elastin and collagen

It was interesting to know whether in the aortic wall collagen, elastin or both are attacked by elastase. We investigated this question as follows: 10 mg. of elastin and the same quantity of collagen were placed in weighed centrifuge tubes. The test tubes contained 5 E.U. while the control tubes contained no elastase. After 30 min. incubation, 6 ml. of 96 % ethanol were added to each tube and the tubes were centrifuged. The precipitates were washed once with ethanol, and dried at 110° to constant weight. In these conditions 67 % of the elastin was dissolved by the elastase, but of the collagen only a negligible proportion (about 1 %) was dissolved. This experiment proves that in the aorta wall the elastin is the protein which is decomposed by the elastolytic enzyme.

DISCUSSION

The enzymic dissolution of the aorta wall is an important phenomenon from the point of view of arteriosclerosis. The pancreatic extract contains several proteolytic enzymes. Among these trypsin and chymotrypsin are the best known. The elastolytic effect of the pancreatic extract is, we believe, due to a specific enzyme, which dissolves the elastic fibres. Our experiments prove clearly that on removal of the collagen from the aorta, by repeated extraction with 2 % acetic acid, the remaining elastin can be dissolved by the elastase. On the other hand, the collagen prepared from the tendo Achillis cannot be dissolved by the elastolytic enzyme. It is evident that in the aorta the elastase dissolves the elastin, but not the collagen.

Experiments dealing with the relation of the elastolytic enzyme to trypsin seem to prove that trypsin is not responsible for the dissolution of the aorta. Pure trypsin and chymotrypsin, which have strong proteolytic activity, showed a much smaller elastolytic effect than the pancreatic extract or the purified elastase. This is a proof of the assumption that elastase is not identical with trypsin or chymotrypsin. Further evidence is provided by the fact that the decomposition of the aorta wall takes place by a mechanism different from that of ordinary enzymic hydrolysis of other proteins. With elastase no amino-acids are liberated from the proteins of the aorta wall, though its elastic fibres are entirely dissolved. Therefore the elastolytic enzyme does not

split peptide linkages, but acts in rendering the elastin soluble. It is our belief that possibly the long rod-shaped molecules of elastin are transformed into globular ones without the liberation of amino-acids. Verification of this hypothesis requires further investigation.

The occurrence of an elastolytic enzyme in the human or animal organism has not been shown before. Eijkman (1904) studied the elastin-dissolving activity of bacteria. He prepared, from the lungs of calves, from ligamenta nuchae and from arteries, culture media with which he demonstrated the lysis of elastin as the result of bacterial action. *Bacillus pyocyaneus* showed the highest activity.

SUMMARY

1. Pancreatic extract contains an enzyme which dissolves the elastic fibres of the aorta wall and which is called elastolytic enzyme or elastase.

2. Trypsin prepared according to Kunitz & Northrop (1935–6) has little effect on arteries.

3. The elastase dissolves only the elastin in the aorta wall and leaves the collagen unchanged.

4. Elastase does not liberate amino-acids from the aorta wall, but insoluble elastin is changed into a soluble protein.

5. The pH optimum of the elastase (10·3) is more alkaline than that of trypsin.

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The Reactions of Catalase in the Presence of the Notatin System

By B. CHANCE* (John Simon Guggenheim Memorial Fellow)
Molteno Institute, University of Cambridge

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If hydrogen peroxide (often termed 'bottle hydrogen peroxide' in order to distinguish it from that generated continuously by an oxidizing system) is added to a catalase solution which is concentrated enough for spectroscopic measurements the hydrogen peroxide is all decomposed into oxygen and water in a fraction of a second, thus leaving no time to observe any change in the absorption spectrum of the mixture by ordinary methods. However, by applying the rapid spectrophotometric technique to this reaction it has been possible to detect and to record the absorption spectrum of the catalase-hydrogen peroxide complex which will be referred to as the primary complex (Chance, 1947*a*). If hydrogen peroxide is continuously generated by an oxidizing system, catalase is maintained saturated with hydrogen peroxide for several minutes, and the spectrum of catalase-hydrogen peroxide can be studied by an ordinary spectrophotometric method.

Under these conditions, it has been found that the primary catalase-hydrogen peroxide complex was converted into a new catalase-hydrogen peroxide complex, and that the persistent absorption spectrum which was thus observed belongs to a secondary complex. This complex is catalytically inactive and is analogous to the secondary complex formed by catalase in the presence of alkyl hydrogen peroxides (Chance, 1949*d*). The secondary complex which is also formed in the presence of a solution of 'bottle hydrogen peroxide', is the principal cause of the inactivation of catalase which takes place during the determination of its activity, 'Katalasefähigkeit' (Kat. f.).

If an alcohol and catalase are added to a system composed of an enzyme, catalysing the oxidation of its substrate by means of molecular oxygen which is reduced to hydrogen peroxide, the catalase, instead of decomposing hydrogen peroxide to molecular oxygen and water, uses the peroxide for the oxidation of the alcohol to aldehyde (Keilin & Hartree, 1936). Thus hydrogen peroxide, which is continuously generated when glucose oxidase (or notatin) catalyses the oxidation of glucose to gluconic acid, can be used by catalase for the coupled oxidation of alcohols. A remarkable feature of this reaction is that all the hydrogen peroxide formed in the primary

* Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania.