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The Reaction Between Elastase and Elastic Tissue

2. PREPARATION AND PROPERTIES OF THE ENZYME

By D. A. HALL* and J. E. GARDINER
Department of Medicine, University of Leeds

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Balo & Banga (1949*a*) first described an enzyme in pancreas capable of causing the total solution of elastin preparations from aorta. Earlier observations on the bacterial digestion of connective tissue (e.g. Ewald, 1890) had demonstrated that enzymes with similar properties were present in a number of micro-organisms. The cultures employed were impure, however, and no attempts were made to isolate the enzymes concerned. Balo & Banga (1950) described the extraction and partial purification of an enzyme from pancreas and suggested the name elastase. Recently, preparations obtained by their method have been employed in attempts to elucidate the structure of elastin (Banga & Schuler, 1953; Lansing, Rosenthal, Alex & Dempsey, 1952; Hall, Reed & Tunbridge, 1952). Balo & Banga (1949*b*) have also shown that there may be a connexion between the activity of elastase and the onset of arteriosclerosis.

MATERIALS AND METHODS

Substrates and sources of enzyme preparations were the same as those used in the preceding paper (Hall, 1955). The active enzyme used in the experiments described below had similar properties to samples kindly supplied by Professor I. Banga, whom the authors would like to thank both for these gifts and for permitting them to see, before publication, a copy of a paper in which she described in greater detail her method of purifying elastase.

Methods. These were essentially the same as those described in the preceding paper. Where purification of the enzyme permitted maximum solution of 50 mg. of substrate at very low enzyme concentrations, it has been found desirable to express the activity of the enzyme in terms of 'specific activity values'. Specific activity is calculated from the 'observed activity' i.e. the biuret value of the supernatant from a system containing 50 mg. of 'elastin' incubated for 17 hr. at 37° in 10 ml. of pH 8·7 buffer, so as to indicate the amount of soluble protein (in mg.) which would be produced by the action of 1 mg. of elastase.

RESULTS

Extraction procedures. The majority of the elastase preparations examined have been obtained by the extraction of defatted pancreas powder with 0·1 M sodium acetate buffer (pH 4·7) as suggested by Balo & Banga (1950). Pilot experiments have shown, however, that extraction at alkaline pH values is quite as effective in removing elastase from the tissue, but removes much inert material as well (Table 1).

* Nuffield Gerontological Research Fellow.

Table 1. *Activity of enzyme at various early stages in the extraction procedure*

Source of enzyme preparation		Specific activity value (see text)
Whole pancreas powder—dialysed		2·9
0·1 M Sodium acetate buffer (pH 4·7) extract—dialysed	Solution	3·0
	Precipitate	170·0
0·1 M Sodium phosphate buffer (pH 8·7) extract—dialysed	Solution	2·8
	Precipitate	79·4
45% $(NH_4)_2SO_4$ precipitate—dialysed	Solution	1·64
	Precipitate	182

Table 2. *Activity of various precipitates derived from fraction A (see text)*

The figures indicate the amount of soluble protein (mg.) obtained from 50 mg. elastin in 10 ml. 0·1 M sodium glycinate buffer pH 8·7.

Method of preparation of enzyme fraction	Enzyme added (μg.)/50 mg. elastin			
	50	100	200	500
Dialysis at 18° (*Pw*)	25·4	37·0	45·0	45·8
Dialysis at 0° (*Pc*)	20·6	34·0	42·0	46·4
Precipitation with acetone (*Pa*)	9·0	15·4	22·8	36·4
Whole 45% $(NH_4)_2SO_4$ precipitate dried with acetone (*Px*)	12·0	20·8	31·0	40·6

Table 3. *The effect of* pH *on the precipitation of elastase*

Treatment of enzyme preparation (see text)	Specific activity value (see text)
Fraction *Px*	160
Precipitate at pH 9·0	530
Solution at pH 9·0	1200
Precipitate at pH 6·0	212
Solution at pH 6·0	1010
Precipitate at pH 5·0	150
Solution at pH 5·0	370

Table 4. *Solution of elastin preparations under the influence of elastase*

Incubation 16 hr. at 37° 50 mg. elastin, 500 μg. elastase in 2·5 ml. 0·1 M sodium glycinate buffer (pH 10·3).

Source of elastin	Percentage dissolved
Human aortic media	86·5
Ox aortic media	45·0
Ox ligamentum nuchae	3·5

Precipitation with ammonium sulphate. Complete precipitation of the enzyme from acetate buffer can be accomplished by adding 45%, by weight, of ammonium sulphate (based on the initial volume of the buffer extract) (Table 1). This material (*A*) has been used as the starting point for the majority of the fractionation procedures described below. The moist precipitate from this stage was dissolved in the minimum quantity of distilled water and dialysed against running tap water for 24 hr. followed by two changes of 500 vol. of distilled water. During dialysis a precipitate formed and was collected and dried with acetone (*Pw*). A further precipitate could be obtained by chilling the contents of the dialysis bag to 0° (*Pc*) and a fine cloudy suspension appeared if the solution were cooled to −15°. This was too small to collect and test, and was combined with the final fraction (*Pa*) which could be precipitated on the addition of 4 vol. of ice-cold acetone. The properties of these various fractions and their relative activity at various enzyme/substrate ratios are given in Table 2.

There was no evidence of any separation of the enzyme into components with different properties by this type of treatment, and it was found possible to redissolve *Pw* by the addition of 1% (w/v) aqueous NaCl, as originally suggested by Balo & Banga (1950). *Pw*, *Pc* and *Pa* were therefore regarded as consisting of essentially similar material and in further experiments were bulked together to give *Px*.

Examination of dialysed fraction Px. The whole dialysed mixture containing both precipitate and solution was brought to pH 9·0 with 2 N-NH_3 and centrifuged. The soluble portion was brought to pH 5·9–6·0 with 10% (v/v) acetic acid and a further precipitate removed by centrifuging, while a final precipitate was obtained on the addition of more acetic acid to pH 5·0. The precipitate obtained at pH 9·0 was washed free of any remaining solution with one-third of the original vol. of 0·02 N-NH_3. The specific activity values of these various fractions are given in Table 3. From this it can be seen that although some active material remains undissolved at pH 9·0, the greater part passes into solution and is still in solution at pH 6·0; however, on bringing the pH to 5·0 there is a marked loss in activity. The solubility of the enzyme, especially at pH 9·0, is dependent on the ionic strength of the solution. Here again separation of enzyme from inactive material appeared to take place, but the enzyme did not appear to contain more than one component.

Sensitivity to pH *of the enzyme–substrate system.* Balo & Banga (1950) published data for pH/activity curves for the enzyme acting on elastic-tissue preparations from aorta. Repetition of their experiments, using, however, sodium glycinate buffer, led to exactly the same results with human aortic material. From these observations it appeared that use of 2·5 ml. of 0·1 N buffer (pH 10·3) was optimum for the dissolution of 50 mg. of elastic tissue under the action of up to 1 mg. of elastase. When attempts were made to treat ox ligament preparations in the same way, it was found that this substrate was completely refractory (Table 4). At lower pH values, however, ox ligament would pass into solution under the action of elastase. A comparison of the pH/activity relationships of the enzyme acting on the two substrates can be made from columns 1, 3 and 5 of Table 5.

Table 5. *Activity of elastase preparations at various* pH *values*

50 mg. elastin. 250 μg. elastase preparation. 2·5 ml. 0·1 M sodium glycinate buffer incubated for 16 hr. at 37°.

	Ox ligament		Human aorta	
Initial pH	Final pH	% dissolved	Final pH	% dissolved
8·15	8·04	25·3	7·37	31
8·52	8·11	37·2	7·37	31·3
8·74	8·45	49·05	7·51	36·6
8·99	8·51	59·6	7·75	45·3
9·29	8·62	66·0	8·05	51·2
9·57	8·75	72·65	8·34	64·0
9·81	8·83	68·5	8·50	72·1
10·03	8·97	46·25	8·71	79·1
10·31	9·55	23·73	8·93	81·2
10·47	9·83	3·93	9·01	84·5
10·60	10·27	4·25	9·05	81·9
10·80	10·57	3·34	9·07	81·0

Table 6. *Acid (m-moles/g.) liberated by elastase from elastin*

Conditions similar to those given in Table 5.

	Elastin	
Initial pH	Ox ligament	Human aorta
8·15	0·09	0·08
8·52	0·09	0·08
8·74	0·19	0·19
8·99	0·25	0·25
9·29	0·36	0·37
9·57	0·55	0·54
9·81	0·50	0·50
10·03	0·46	0·60
10·31	0·43	0·72
10·47	0·40	0·80
10·60	0·13	0·82
10·80	0·04	0·84

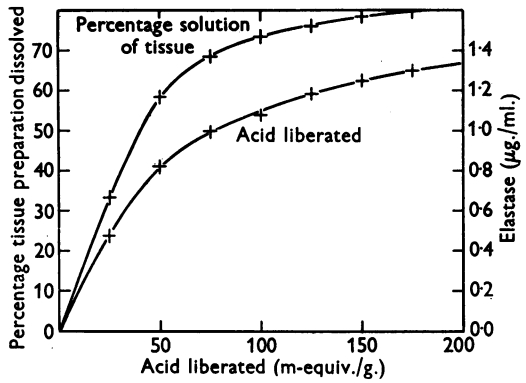

Fig. 1. Comparison of the acid liberated, and the protein dissolved during the action of varying amounts of elastase on a preparation from human aorta. With the exception of the amount of elastase, the conditions were identical in each case: 50 mg. of elastin. 2·5 ml. 0·1 M sodium glycinate buffer, pH 10·3, incubated for 17 hr. at 37°.

Although there appeared to be such a marked difference between the two enzyme–substrate systems, the supernatants from those systems which had been allowed to react under optimum conditions showed considerable similarity, in that the final pH of the buffer solution had fallen to the region of 8·5–9·0. The final figures corresponding to a series of initial pH values are given in the remaining columns of Table 5, and these indicate that large amounts of acid must have been liberated during the course of elastase action.

Increasing the buffer concentration so that the ionic strength of the buffering salt was increased fourfold, and at the same time increasing the volume of buffer to 10 ml., considerably reduced the fall in pH. Curves obtained in this way and showing the pH/activity relationship of the enzyme–substrate systems are identical with those which can be prepared from the figures in the last four columns of Table 5. It can be seen that although there are differences between the shapes of such curves, the optimum values fall between pH 8·7 and 9·0.

Nature, amount and distribution of the liberated acid. Human aorta preparations contained 7·9 mg. sulphate/g. air-dry protein, whereas ox ligament preparations contained 4·65 mg. This sulphate was measured as total sulphur (see preceding paper), but was identified as sulphate after hydrolysis, and in the supernatant after the action of the enzyme, by a variety of procedures. Reaction with $BaCl_2$ in the presence of $KMnO_4$ produced a pink precipitate which could not be decolorized with $K_2C_2O_4$. Solutions also gave a heavy precipitate with benzidine. Whether these observations indicate the liberation of free sulphuric acid or of a sulphate radical still bound to sugar residues, cannot be decided yet, but the amounts liberated from aorta and ligment can be measured by titration of the buffer solution. Fig. 1 demonstrates the relationship between the liberation of acid and the amount of protein passing into solution under the action of

differing amounts of elastase. A comparison of the amounts of acid released from aorta and ligament preparations at various pH values can be obtained from the figures in Table 6. It appears that at initial pH values of above 9·8 there is a rapidly decreasing amount of acid liberated from the substrate in the case of the ligament, whereas the acid liberated from the aorta preparation continues to increase in amount. There is also a corresponding difference in the amount of protein passing into solution over this pH range.

As the tissue passes into solution under the action of elastase, acid is released, but as can be seen from Table 7, the change in pH accompanying the release of acid is complete after the dissolution of only half of the protein.

Table 7. *Release of acid with time of incubation*

250 μg. elastase. 50 mg. elastin. 2·5 ml. pH 9·57 0·1 M sodium glycinate buffer.

Time of incubation (hr.)	Final pH	Amount dissolved (% of sample)
1	8·99	10·45
2	9·02	21·6
4	8·82	23·2
6	8·63	43·6
8	8·33	56·1
12	8·35	72·0
17	8·34	89·1

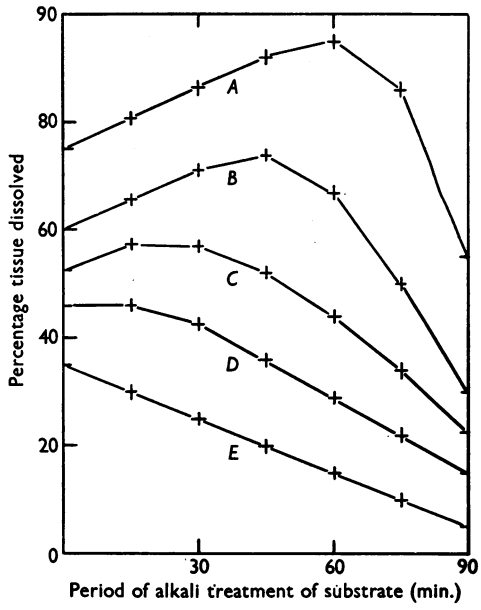

Fig. 2. The effect of alkali treatment on the susceptibility of ox ligament elastic-tissue preparations to attack by elastase. In all cases 50 mg. of each substrate were allowed to react at 37° for 17 hr. with the enzyme in 0·1 M sodium glycinate buffer (pH, 8·7). The concentrations of elastase were as follows: *A*, 100 μg./ml.; *B*, 80; *C*, 60; *D*, 40; *E*, 20.

The activity of the enzyme on alkali-treated substrates. In the preceding paper (Hall, 1955) it is reported that treatment of the collagen-free substrate with boiling alkali causes a marked change in its susceptibility to elastase. In Fig. 2 are given results of a typical series of experiments in which the amount of elastase employed was altered as well as the type of substrate. At concentrations of enzyme lower than those usually employed (250 μg./50 mg. tissue), the peak activity which was reported in the previous paper moves to shorter periods of alkali treatment, whereas at concentration of enzyme above the normal level, the peak moves to periods of longer treatment. The fall to minimum activity occurs at 80–90 min., however, in all cases, and the rise which was previously observed after longer periods in alkali takes place at a constant rate at all elastase concentrations.

The effect of alkali pretreatment of the substrate on the pH *sensitivity of the enzyme.* Examination of the effect of pH on the activity of the enzyme when tested against a series of alkali treated substrates shows an effect of considerable importance. Banga, Balo & Nowotny (1949) showed that alkali treatment caused an increase in the amount of protein dissolved from tissue by elastase. It can be seen from the curves in Fig. 3 that, as alkali treatment

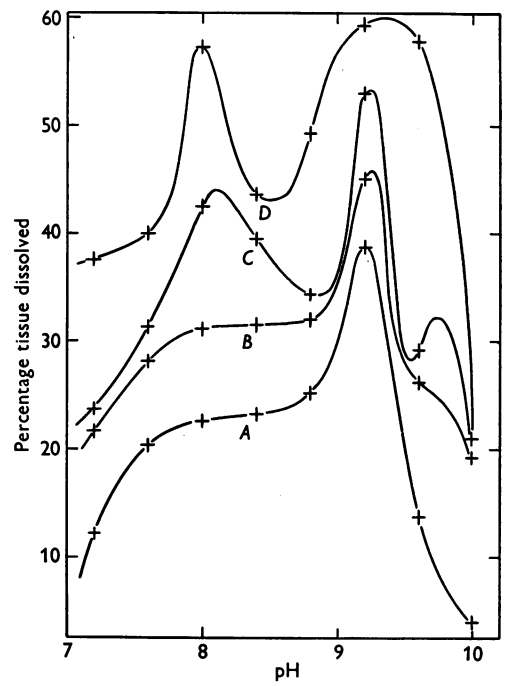

Fig. 3. pH/activity curves for elastase acting on a series of alkali-treated substrates. The substrates concerned have had the following periods (in min.) of alkali treatment: *A*, 0; *B*, 40; *C*, 120; *D*, 240.

Table 8. *The effect of varying the buffering salt on the activity of an elastase preparation*

The buffers were in all cases 0·1 M adapted from Vogel (1943) pH 7·8. The other conditions were as in Table 2, except that 250 μg. of elastase were used.

Period of alkali treatment of substrate (min.) ...	0	60	140	240
Buffer	Absorptiometer readings (see preceding paper)			
Na_2HPO_4–citric acid	1·5	4·5	4·3	7·0
KH_2PO_4–NaOH	3·7	10·2	8·6	12·0
Sodium diethyl barbiturate–HCl	7·4	9·3	7·2	13·3
Sodium glycinate–NaCl	6·6	11·1	5·0	14·4
H_3BO_4–NaOH–NaCl	12·3	13·6	7·8	14·6

proceeds, the degree of activity at the maximum around pH 9·0 increases. The more interesting fact is, however, that the shoulder which with untreated substrates is present in the pH range 7·0–8·0 becomes transformed into a second peak, the height of which also rises with alkali treatment.

The effect of buffering salts on the activity of the enzyme. The main peak and the shoulder of the pH/activity curve do not both lie within the range of maximum buffering capacity of one single simple buffer solution. However, the second peak which appears when the substrate is treated with alkali, lies well within the range of a number of buffer solutions and on the edge of the effective range of the glycine–NaOH buffer which has been employed for most of these experiments. The figures given in Table 8 are activity values of a particular enzyme preparation when tested in various buffers at pH 7·8. From this it can be seen that whereas a number of buffer solutions have a similar effect on the enzyme–substrate reaction, borate buffer enhances the reaction while a buffer containing citrate lowers the activity markedly.

DISCUSSION

Balo & Banga in their early papers on elastase described a number of the properties and the optimum working pH. Since initially their work was wholly concerned with human aorta, they did not observe the apparent resistance of ligament preparations to elastase attack at pH values which appeared optimum for the system when employing human tissue as substrate. The material liberated from the elastin by the action of the enzymes may differ qualitatively from source to source; it is, however, quite apparent that the acid liberated differs quantitatively. Above pH 9·5 the amount of acid liberated from ligament decreases rapidly, whereas that obtainable from human aorta and to a lesser extent from ox aorta continues to rise. Hence there is a marked drop in pH in the case of aorta but not in the case of ligament, at these high pH values. When the enzyme and substrate are suspended in a buffer solution of high pH but low buffering capacity, therefore, there is considerable drop in pH during the reaction with aorta but not with ligament. The pH is lowered sufficiently in the first case to approach the natural optimum of the system, but in the case of the ligament this value is not reached. When adequate buffering salts are present or when the final pH of the system is taken as the operative one, the true optimum of the system is seen to lie in the range 8·7–9·2 and not at 10·3 as stated by Balo & Banga.

When various concentrations of elastase were employed for the reaction with the alkali-treated substrates, the peak of optimum activity moved from zero to a period of 60 min. alkali treatment as the concentration of enzyme was increased. It is known that during this period of alkali treatment constitutional changes take place in the substrate, and it is conceivable that the relative concentrations of two substrates may alter. The movement of the peak could then be explained on the assumption that two enzyme systems exist which are specific for the two substrates, and that as the total enzyme/substrate ratio is altered the system consisting of alkali-treated substrate and its specific enzyme approaches its optimum working conditions. The movement of the peak would then indicate the resultant activity of the two enzymes, one already at its optimum, the other steadily increasing. The rise in activity which occurs after 90 min. must be assumed to be due to main chain degradation owing to hydrolysis of peptide bonds. The linkages which are susceptible to elastase attack are not affected since although the activity rises, the blank, i.e. the degree of solution in buffer alone, is not affected.

Further evidence in favour of there being two elastin components in elastic tissue and two specific elastases, arises from the pH/activity curves. The presence of a shoulder in the original curve of Balo & Banga was always suggestive of the presence of more than one enzyme. When, however, the release of the necessary substrate by the action of alkali causes the shoulder to change into a definite peak, there ceases to be any doubt. The second enzyme, which is the more active on the alkali-treated material, has a range of optimum activity of pH 7·8 to 8·1.

SUMMARY

1. Revised curves relating activity of the enzyme to pH have been obtained for elastase, the optimum pH range for which has been shown to be 8·7–9·2.

2. Although, if adequately buffered, the enzyme is capable of dissolving elastic tissue from three sources at this pH equally efficiently, the products of the reaction are not universally the same.

3. Increasing amounts of sulphuric acid are released from aortic preparations especially above an initial pH of 9·8, smaller amounts are liberated from ox ligament.

4. Evidence is advanced that elastase consists of two enzymes which act on two components of an elastic tissue preparation which is itself dual in nature.

5. The optimum pH of the second enzyme which utilizes alkali-treated elastic tissue as substrate lies in the range 7·8–8·1.

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Observations on the Relationship Between Pteroylglutamic Acid and Nicotinamide Metabolism

By PREMA FATTERPAKER, URMILA MARFATIA and A. SREENIVASAN
Department of Chemical Technology, University of Bombay, India

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Slightly depressed growth and fatty livers have been reported in rats fed moderate amounts of nicotinamide, together with a low protein diet (Handler & Dann, 1942; Handler & Bernheim, 1943). The fatty livers were susceptible to the action of choline, while methionine was found to alleviate the growth inhibition. Since excess of nicotinamide is detoxicated and eliminated as urinary N^1-methylnicotinamide, the observed toxicity of nicotinamide at unphysiological levels of feeding was attributed to forced methylation and consequent depletion of labile methyl reserves (Handler, 1944). In agreement with this, Handler observed no signs of toxicity as a result of ingestion of large quantities of nicotinamide by young rabbits or guinea pigs, neither of which species excrete N^1-methylnicotinamide (Handler, 1944). Production of fatty livers by feeding excessive amounts of glycocyamine, cystine or homocystine have also been reported (du Vigneaud, Chandler, Moyer & Keppel, 1939; du Vigneaud, Chandler, Cohn & Brown, 1940; Stetten & Grail, 1942). More recently, it has been shown that, in the pyridine-fed rat, there is a decreased urinary excretion of creatine owing to a reduction of available methyl groups, pyridine being detoxicated as N-methylpyridinium hydroxide (Dinning, Keith, Parsons & Day, 1950).

The known mediation of pteroylglutamic acid in biological methylations including the methylation of nicotinamide (Fatterpaker, Marfatia & Sreenivasan, 1951, 1952*a*, 1954; Dietrich, Monson & Elvehjem, 1952) suggested the present studies on its influence on the extent of depletion of methyl reserves in rat livers consequent on ingestion of excessive amounts of nicotinamide. The studies included observations on a number of other substances and enzymes expected to be influenced by changes in pteroylglutamic acid or available labile methyl compounds. Pteroylglutamic acid deficiency was induced in the first set of experiments by dietary means, while in the second series aminopterin feeding was used. In the first series, additional groups with choline supplementation were included.

EXPERIMENTAL

Methods

Urinary excretions of creatine (Clark & Thompson, 1949), N^1-methylnicotinamide (Huff & Perlzweig, 1947) and total nicotinic acid (Swaminathan, 1946), liver N (Johnson, 1941),