The Reaction Between Elastase and Elastic Tissue

1. THE SUBSTRATE

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The attempted characterization of elastin by observations based on any single property was first criticized some time ago (Hall, 1951). Since then, as part of a co-ordinated project, studies of elastic tissue have been made by both histological and electron-microscopical techniques (Hall, Reed & Tunbridge, 1955); further examination of such tissue has been made by X-ray diffraction methods and preliminary chemical and biochemical observations on the enzymic degradation of elastic fibres have also been reported (Hall, Reed & Tunbridge, 1952; Hall, 1952, 1953). As part of a study of the role of elastin in connective tissue, measurements have been made of those physical properties associated with the physiological function and mechanical stability of the fibres (Wood, 1953, 1954).

Many of these observations have been made on tissue which had been treated with acetic acid to remove the associated collagen (cf. Gross, 1949). Other groups of workers have described different methods for the removal of collagen, employing urea (Stein & Miller, 1938) or alkali (Banga & Schuler, 1953; Lansing, Rosenthal, Alex & Dempsey, 1952). Recently, however, Banga (1953) has put forward the suggestion, based on the observations of Schwarz & Dettmer (1953), that elastin is drastically altered, especially with respect to its internal structure, when treated with such solutions at 100°, during the removal of collagen. In view of this, study of the substrate and the possible effect of the various pretreatments which it may undergo was undertaken before embarking on study of the reaction between the enzyme and substrate. Similarly, it has been considered advisable to keep an open mind regarding the identity of 'elastin'. For this reason, the materials examined are to be regarded as elastic-tissue preparations, the term elastin being used simply as a convenient abbreviation.

MATERIALS AND METHODS

Elastic tissue. Preparations from three sources have been employed: human aorta, ox aorta and ox ligamentum nuchae. The ox tissues were obtained from freshly

slaughtered young beef cattle; otherwise the method of preparation was identical with that described below for human aorta.

Segments of aortic arch were collected at autopsy. Although the age range of the aortae was considerable (20–65 years), only those showing no gross signs of atheromatous degeneration were used. The appearance of even one or two cholesterol plaques beneath the intimal membrane was regarded as sufficient to disqualify the aorta from inclusion in the pool. The accepted tissue was freed from adventitia and the intima stripped away. The remaining media was cut into 5 mm. squares and minced in a power-driven mincer. Stack (1950) has shown that, owing to heating, considerable degradation of connective-tissue proteins can occur during such preparative procedures. To obviate this, the aorta was chilled to -69° with solid CO_2 before and between passages through successively smaller cutting plates. The mince was suspended in 4 vol. of 5% (w/v) NaCl solution and the maceration continued in a top-drive blender. Here again the jar and its contents were thoroughly chilled at frequent intervals during the operation. After filtration and thorough washing, a dried powder was obtained either by freeze-drying or by dehydration with acetone. Excessive drying of these powders might be expected to alter their properties, especially with respect to their resistance to enzymes, and they were therefore kept at 18° over sat. NaCl solution giving relative humidity 75%. The various treatments described below, with the exception of that with urea, did not markedly affect the water regain of the powders, which had an average value of 14.5%. For convenience, therefore, the weights recorded are for tissues equilibrated in this fashion and are referred to as 'air dry'. In the case of the material treated with urea, there was evidence from nitrogen determinations that appreciable amounts of urea remained attached to the protein even after exhaustive washing, and this greatly increased the water uptake.

Considerable difficulty has arisen in the study of the action of elastase on elastic-tissue preparations from various sources, on account of the impossibility of grinding the tissue to uniform particle size, without considerable denaturation due to heating. Fractionation of samples according to size could concentrate tissue components of different types in different size fractions, especially in the case of aorta, where there is evidence of at least two types of elastin-staining element (Benninghoff, 1927). Ground ox aorta was therefore fractionated in standard sieves and treated with elastase. The figures in Table 1 show that, although all the samples have very similar susceptibility to the enzyme, there is a slight increase in activity as the size of particle decreases. This may merely be due to different rates of penetration into particles of varying size, but in order to

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ensure comparable results sets of experiments were confined to one size range, and where possible the smaller sized material was employed. This was considered advisable in view of the observation that in the larger size ranges some of the pieces were compact and dense while others were fibrous and light. These two fractions could be separated in a similar fashion to that suggested by Lansing, Roberts, Ramasarma, Rosenthal & Alex (1951) and the enzyme was 34% more active on the fibrous material than on the dense substance.

Elastase preparations. Elastase was obtained by a method which differed only slightly from the original procedure of Balo & Banga (1950). The small amount of fractionation carried out, however, did appear to have a purifying effect. Dried defatted commercial hog pancreas powder (Viobin, Inc. Montecello, Illinois, U.S.A.) was extracted twice with 5 vol. 0.1M sodium acetate buffer (pH 4.7). Two other commercial samples of pancreas powder (Armour Laboratories, London) were found to be considerably less active as sources of elastase either as bought or after exhaustive solvent extraction. The bulked extracts were brought to 45% ammonium sulphate concentration (w/v) and the precipitate was collected. The precipitate was dissolved in a minimum quantity of water and dialysed in cellophane against running tap water for 24 hr. and then against two changes of 500 vol. of dist. water. A precipitate formed during dialysis, which could be redissolved on the addition of NaCl to 0.5–1% (w/v). The precipitate, carefully dried with acetone at 0°, was used as the typical enzyme preparation. The pH/activity curve for the enzyme was of a similar shape to that reported by Balo & Banga (1950), having a shoulder at pH values below that of the peak.

Protein determination. Hydrolysis was carried out by boiling under reflux in 5 vol. of 5N-HCl for 24 hr. HCl was removed by treatment on columns of Zeo-Karb 226 (Permutit Co. Ltd., London) according to Piez, Tooper & Fosdick (1952). Two-way filter-paper chromatograms were made by the ascending technique on 25 cm. squares. The two solvent systems were *n*-butanol-acetic acid-water (50:10:40, by vol.) and *n*-butanol-pyridine-water (50:10:40, by vol.) adapted from Evans, Evans & Parr (1949). Figures relating to the concentration of the amino acids were obtained by visual assessment of the ninhydrin colours on comparison with known amounts of individual amino acids which had been subjected to the same chromatographic procedure. Protein determinations on the enzyme solutions were made by the method of Lowry, Rosebrough, Farr & Randall (1951), employing a mixed biuret and Folin & Ciocalteu reagent. In the biuret solution the tartrate was replaced by glycerol to facilitate solution of the copper.

Table 1. *The effect of particle size on the activity of elastase on elastin preparations from ox aorta*

Air-dry ox-aorta elastin (50 mg.) suspended in 10 ml. 0.1M sodium glycinate buffer (pH 9.0) 0.4M in respect of NaCl and incubated at 37° for 17 hr. with 0.5 mg. elastase.

Particle size (mm.)	Percentage dissolved
Above 2.5	68.1
1.2–2.5	68.0
0.8–1.2	69.1
0.6–0.8	72.7
Below 0.6	74.6

Protein determinations on the supernatant after the enzyme had been allowed to react were carried out using the biuret reagent of Sols (1947). Reagent (3 ml.) and water (2 ml.) were mixed with 2 ml. of the supernatant. The absorption was read after 15 min. without a filter in the EEL photometer (Evans Electroselenium Ltd., Harlow, Essex). A reading of 25 scale divisions was equivalent to the complete solution of 50 mg. of air-dry protein and the relationship between colour value and protein concentration in the supernatant was linear over the whole of this range.

Polysaccharide determinations. Hexosamine was determined by the method of Boas (1953) on hydrolysates prepared by 6 hr. treatment with 5N-HCl under reflux, and are expressed in terms of D-glucosamine, which was used as standard.

The total reducing substance present in the preparations was determined using the Hagedorn & Jensen method, on the same hydrolysates. Results are expressed as mg. glucose/g. air-dry tissue.

Sulphate was determined using the method of Lugg (1938) for total sulphur, since the non-sulphate sulphur was of negligible amount.

Estimation of the reaction between enzyme and substrate. Except where otherwise stated, 50 mg. of elastin were used in all the measurements of enzyme activity. This amount of elastin was suspended in 9 ml. of buffer in a 25 ml. Erlenmeyer flask. The various effects with different buffers are described by Hall & Gardiner (1955). In the majority of the experiments, the buffer was either 0.1M sodium glycinate in 0.2M-NaCl or 0.1M sodium barbiturate, both at pH 8.7. The reaction was carried out in flasks because the reproducibility of results was dependent on there being a sufficiently large surface area of buffer for the tissue powder to lie in a single layer. To the reaction vessel a solution of enzyme preparation (50–1000 µg. in 1 ml. physiological saline) was added. Since it was observed that shaking may cause inactivation of the enzyme, the mixtures were generally incubated at 37° for 18 hr. without shaking. After incubation, the flasks were immersed in boiling water for 3 min. to destroy the enzyme, the residue was centrifuged down, and the activity measured either by drying and weighing the residue, or more easily by measuring the soluble-protein content of the supernatant.

RESULTS

Treatment with acetic acid

Ligamentum nuchae powder was autoclaved in 50 vol. of 1% (w/v) aqueous acetic acid for 2 hr. at 15 lb./sq.in. In Table 2 are given figures relating to the activity of the enzyme when tested against this substrate. For comparison a similar set of figures are given for the same enzyme on the untreated whole ligament. From these it can be seen that elastase is 2–4 times more active on acetic acid-treated material than on whole tissue. Similar effects can be observed after treatment with acetic acid under a number of conditions, all of which are adequate for the removal of the structurally discernible collagen, as judged by electron-microscopical examination. They range in severity from

boiling with frequent changes of 10 vol. of 1 % acetic acid for periods of up to 5 days, to shaking at 37° for 24 hr. with 50 vol. of 2 % acetic acid. Histological examination of tissue treated by these methods showed, however, that an appreciable amount of amorphous material staining as collagen still remained, especially in human aorta. Relative amino acid analyses for human aorta and ox ligament, both treated with acetic acid, are given in Table 3, and can be compared with similar values calculated from the microbiological assay figures obtained by Neuman (1949) for ligament elastin.

Treatment with urea solution

Stein & Miller (1938) claimed that their preparation of elastin from ox ligamentum nuchae was resistant to solution in boiling 40 % urea solution

Table 2. *The effect of acetic acid treatment on the susceptibility of a ligament preparation to attack by elastase*

Acetic acid treatment as follows: powdered ox ligament (0.6–0.8 mm. particle size) autoclaved for 2 hr. in 50 vol. 1 % (w/v) acetic acid at 15 lb./sq.in. pressure. The conditions of the enzyme reaction were otherwise as in Table 1.

Elastase μg./ml.	Photometer readings (see Methods)	
	Whole tissue	Acetic acid-treated
5	1.5	7.7
10	2.0	9.3
15	2.4	12.0
20	3.2	13.6
25	4.8	14.0
30	5.2	14.4

for periods of up to 5 days. As has been reported earlier (Hall, 1951), this resistance is relative, being a function of the urea/tissue ratio. A period of between 90 and 100 hr. at 100° was required at urea levels above the threshold value for the elastin to pass into solution. With shorter periods of treatment, only small amounts of protein dissolved. Table 4 records the degree of dissolution which occurs with time and the susceptibility of the residue to elastase attack, while the changes in amino acid constitution which occur as urea treatment proceeds can be followed from Table 5.

During urea treatment the pH of the solution rose to a value of 9.8 and ammonia was liberated. The concentration of urea was so large, however, that it was impossible to tell by urea analyses, whether the ammonia came from the breakdown of the urea or from the tissue itself.

Treatment with alkali

The increased susceptibility of elastin which had been treated with urea to elastase attack, and the fact that Banga, Balo & Nowotny (1949) reported an increase in elastase activity of 200 % after alkali treatment of the substrate, could indicate that the substrate was purified during alkali treatment. It has, however, been shown that it is possible to dissolve elastic tissue in 0.1 N-NaOH if the solid is boiled in the alkali for 6 hr. under reflux (Hall *et al.* 1952). Degradation might therefore also occur while the purification proceeded.

Elastic-tissue preparations, which had had the collagen removed by autoclaving with acetic acid as described above, were employed as starting materials in experiments aimed at studying the

Table 3. *Amino acid composition of various elastin preparations*

The figures in columns 2, 3 and 5 were obtained by visual assessment of the ninhydrin colours, those in column 4 by calculation from the microbiological-assay values of Neuman (1949).

Amino acid	Arbitrary units proportional to amino acid concentration			
	Whole human aorta	Acetic acid- treated aorta (autoclaved)	Ligament elastin (Neuman, 1949)	Ox ligament acetic acid-treated (autoclaved)
Alanine	10	10	8	10
Arginine	6	3	—	0
Aspartic acid	6	2	—	1
Glutamic acid	9	5	—	2
Glycine	10	9	10	10
Hydroxyproline	3	2	—	0
Isoleucine	4	3	4	6
Leucine	4	3	6	5
Lysine	8	4	1	3
Methionine	5	1	—	0
Phenylalanine	3	2	5	3
Proline	7	6	8	6
Serine	7	1	2	0
Threonine	4	0	2	0
Tyrosine	2	2	3	1
Valine	7	6	8	7

Table 4. *Loss of weight of elastin preparations at various stages during a purification procedure, and the effect of elastase on them*

The percentages in column 2 are calculated on the original air-dry weight of the tissue. Those in column 3 on the air-dry weight of the individual preparation.

Tissue	Loss in weight during treatment (%)	Loss in weight after treatment with elastase (%)
(1) Whole human aorta	0	46
(2) 1 freed from collagen by acetic acid	16	12
(3) 2 after 24 hr. treatment with 40% urea	28	16
(4) 2 after 48 hr. treatment with 40% urea	43	43
(5) 2 after 72 hr. treatment with 40% urea	60	96.5

Table 5. *Amino acid composition of residues after urea treatment*

Values for amino acid content determined as for Table 3. Because of the retention of urea in the residues after urea treatment, and hence the differing moisture content, the hydrolysates were diluted to equal amino acid content, instead of being compared at similar air-dry weights.

Amino acid	Arbitrary units proportional to amino acid concentration				
	Collagen-free aorta	Days treatment with urea			
		1	2	3	4
Alanine	10	10	9	9	8
Arginine	3	1	0	0	0
Aspartic acid	2	2	1	1	0
Glutamic acid	5	4	2	3	2
Glycine	9	10	8	8	7
Hydroxyproline	2	1	0	0	0
Isoleucine	3	3	1	2	2
Leucine	3	3	2	3	2
Lysine	4	1	1	1	1
Methionine	1	0	0	0	0
Phenylalanine	2	3	1	2	1
Proline	6	8	6	7	6
Serine	1	0	0	0	0
Tyrosine	2	2	1	1	1
Valine	6	8	7	6	6

effect of the period of alkali treatment on the substrate. After autoclaving, the powder was extracted with water until the extract no longer gave a biuret reaction even after tenfold concentration. The resulting powder had the following characteristics expressed in mg./g. of air-dry material: N, 141; SO_4^{2-} , 4.65; moisture, 147; ash, 6.7; polysaccharide, as glucose, 4.2.

Determination of the effect of elastase on a series of elastin preparations which have had various periods of alkali treatment depended on the accurate timing of the process. This was accomplished as follows. A volume of 0.1N-NaOH such as to give a solid:NaOH ratio of 1:20 was boiled under reflux. Momentary removal of the condenser was followed by the introduction of the solid down a polythene chute so that it became rapidly wetted. The solution was returned to the boil within 1 min. and the reaction timed from the recommencement of boiling. At the end of the required period the whole mixture was poured into 500 vol. of ice-cold water,

thus completely arresting the reaction. The residues were then thoroughly washed, dried with acetone and allowed to equilibrate over saturated NaCl solution.

The properties of a series of alkali-treated preparations

A series of alkali-treated substrates was prepared by the method described above, differing only in time of treatment, which increased progressively by 20 min. intervals to 4 hr. Chemical characterization of these included protein, amino acid and polysaccharide and sulphate determinations.

Amino acid composition. Chromatograms of hydrolysates of all the preparations showed no discernible differences in amino acid composition as the period of treatment increased. In all cases the values were similar to those given in Table 3.

Polysaccharide. Determinations of total reducing substance showed a progressive fall from a value of

0.4% during the first 80 min. alkali treatment. Thereafter the content was very low, approx. 0.02% and fluctuated slightly. The hexosamine values are given in Fig. 1. The sulphate content of the preparations dropped within 1 hr. to 40% of its initial value, and after 2 hr. remained constant at values below 23% of this initial figure. The effect of elastase on the alkali-treated substrates was also examined. The results of a typical experiment are given in Fig. 2. From this curve it can be seen that as alkali treatment proceeds, the action of the enzyme at first increases and then falls rapidly. The degree of alkali treatment required for peak action of the enzyme was not always the same for different batches of elastase, but always lay within the range 0-60 min. The sharp drop in activity which occurs after alkali treatment has been permitted to proceed beyond the optimum length of time, is however, not permanent, since if alkali treatment is prolonged beyond 80 min. the curve relating activity to alkali treatment rises again.

Fig. 1. The removal of hexosamine by alkali treatment. The hexosamine content is expressed as a percentage of the air-dry weight of the individual alkali-treated elastic tissue preparations (calculated as glucosamine).

Fig. 2. The effect of elastase on a series of alkali-treated substrates. The substrates were boiled with 0.1N-NaOH for the periods stated. The elastase was allowed to react with 50 mg. of each in 10 ml. 0.1M-Na glycinate buffer for 17 hr. at 37° at a concentration of 25 μ g./ml.

DISCUSSION

One of the main difficulties in assessing the value of analytical work on elastin, as that on other fibrous proteins, is uncertainty as to whether the material has been adequately freed from associated proteins without having been degraded itself. All the chemical methods which have been suggested for the removal of collagen employ heat and require the presence of moderately strong acid or alkali.

Boiling with 1% acetic acid, which does not itself appear to be adequate for the removal of all the 'non-elastin protein' from human aorta must be assumed to have some far-reaching effect since there is a 200% increase in the action of elastase tested against this substrate in comparison with a substrate prepared from whole ox ligament. In the same way, treatment with boiling 0.1N-NaOH must produce a marked change in the substrate, since over the first 90 min. boiling, there is first an increase in susceptibility to elastase, followed by a fall to a very low value. The possibility exists that the residues after acetic acid extraction of human aorta and ox ligament represent two elastins of quite dissimilar amino acid composition. Further treatment of acetic acid-extracted aorta with boiling 40% (w/v) urea solution, however, causes a marked drop in the concentration of polar amino acids and of hydroxyproline, leaving a residue which has an amino acid analysis closer to that of ox ligament which has been treated with acetic acid alone. The removal of these particular amino acids would indicate that the aortic residue after acetic acid treatment contained a fraction similar to collagen in composition but differing from it in its solubility properties. It is interesting to note that Lansing *et al.* (1951) have reported an increase in just these amino acids in human aortic-elastin preparations as the age of the sample increases. Another explanation of the difference between the acetic acid residue from aorta and ligament arises from a consideration of observations made on these tissues by electron-microscopic techniques (Hall *et al.* 1955). In its simplest form, in ox ligament, elastic tissue appears under the electron microscope to be composed mainly of cylindrical fibres which are opaque to the electron beam. If these fibres are treated with elastase the opaque layer is removed, and the fibres no longer appear cylindrical. At the same time an internal fibrillar structure is revealed. This, however, also is susceptible to attack by the enzyme, and ultimately the whole of the tissue passes into solution. Aorta behaves similarly, except that, especially in the lamellar areas of the aortic elastin (Benninghoff, 1927), the fibres do not appear as simple structures of fibrous nature, but as fibres embedded in an amorphous matrix which makes up the body of the lamella. This material is

opaque and is the first part of the tissue to dissolve under the action of elastase, revealing a fibrillar inner structure, as with ligament. The suggestion that 'elastin' consisted of two components has been made before (Hall *et al.* 1952), but the results now reported enable the hypothesis to be expanded. The elastic-tissue preparations from the two sources appear to differ from one another only in the relative concentrations of the amorphous and fibrillar components. Analyses of the alkali-treated substrates can give some indication of the way in which these two components differ from one another. As can be seen from Fig. 2, treatment with alkali for periods of up to 1.5 hr. causes a steady drop in the amount of hexosamine present in the preparation to a minimum value. This minimum value for hexosamine and hence by inference, for polysaccharide, coincides with the period of alkali-treatment which is required for the enzyme susceptibility of the substrate also to reach a minimum. It therefore appears likely that during this period of treatment a fraction of the tissue containing most, but not all, of the polysaccharide present was being dissolved. In the earlier paper in which tentative suggestions regarding the structure of elastin were proposed (Hall *et al.* 1952), it was reported that the whole of the acid liberated by the enzyme was freed during the dissolution of the first half of the protein. It was therefore assumed that the portion of the elastin which was dissolved during this early part of the reaction contained the whole of the polysaccharide. Correlation of elastase susceptibility and the hexosamine content of the alkali-treated substrates indicates that the polysaccharide is not confined to one of the two components of elastic tissue.

A study of the substrate of the elastin-elastase system therefore reveals the following facts. The elastic fibres, as defined by histologists or electron-microscopists, namely the fine fibres and lamellae in aorta and the thick fibres of ligament, do not consist of a single entity. Preparations which have been obtained for chemical studies by alkali or urea treatment are most likely to have had the material containing the majority of the polysaccharide removed. The properties of these preparations will approach those of the whole elastic fibre only in the case of ligament. The relative proportions of the two components will cause the fibrillar material to play a more important role in determining the properties of 'elastin' preparations from this source. All methods at present available for the separation of two components, such as the removal of an amorphous outer layer, are so drastic that it must be assumed that degradation of the fibrils occurs simultaneously. For this reason it will be observed that a series of substrates which have had different periods of alkali treatment has been em-

ployed in the enzyme studies reported in this and the following paper (Hall & Gardiner, 1955). No claim is made for the identity of any particular substrate, and the name elastin is not applicable to a particular entity which can be identified by biochemical and histological methods.

SUMMARY

1. Various methods have been examined for the isolation of an entity, to which the name 'elastin' can be applied.

2. They have been compared by observing the changes produced in the susceptibility of the resulting material to attack by elastase.

3. It is suggested that all existing methods for freeing so-called elastin from associated proteins are either inadequate or so drastic as to commence the degradation of the fundamental unit of elastic tissue itself.

4. For a study of elastic tissue, a series of alkali-treated preparations appear to offer the greatest scope.

5. Further evidence is provided indicating that at least some of the 'elastin' preparations which have been described consist of more than one component, but that in many of these the proportions differ considerably.

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The Reaction Between Elastase and Elastic Tissue

2. PREPARATION AND PROPERTIES OF THE ENZYME

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Balo & Banga (1949a) 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 (1949b) 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 0.1M 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).

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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
0.1M Sodium acetate buffer (pH 4.7) extract—dialysed	{ Solution 3.0 Precipitate 170.0
0.1M Sodium phosphate buffer (pH 8.7) extract—dialysed	{ Solution 2.8 Precipitate 79.4
45% (NH ₄) ₂ SO ₄ precipitate—dialysed	{ Solution 1.64 Precipitate 182