little to choose between the *p*-nitrophenyl and methylumbelliferyl *N*-acetyl-β-glucosaminides, since, with the testicular enzyme, the ratio of the respective maximum velocities was 1:0·95.

The Laurence (1957) fluorimeter proved to be particularly suitable for the present kind of study. In agreement with the findings of Mead *et al.* (1955) and Robinson (1956), the fluorimetric estimation of methylumbelliferone was found to be simple and convenient.

Robinson (1956) pointed out that the fluorimetric method permitted the use of very dilute enzyme preparations and that the high dilution would tend to eliminate the effect of interfering substances. Before assaying very dilute preparations, it may be advisable to establish the form of the enzyme activity–concentration curve; it has been shown here that, with a methylumbelliferyl glycoside, this can be done conveniently with a combination of spectrophotometric and fluorimetric techniques.

The fluorimetric method permits the use of low substrate concentrations with the consequent advantage of conserving substrate; however, the advisability of using low substrate concentrations should be investigated, since results presented here show that characteristic properties of an enzyme (e.g. Michaelis constant, pH optimum) may change under these conditions. Further applications of the fluorogenic method for the estimation of glycosidase activity are being investigated.

SUMMARY

1. 4-Methylumbelliferyl *N*-acetyl-β-glucosaminide has been prepared, and its use as a substrate in the assay of *N*-acetyl-β-glucosaminidase has been described.

2. The range and sensitivity of spectrophotometric and fluorimetric estimations of methylumbelliferone gave the procedure advantages over existing methods.

The authors are greatly indebted to Professor R. T. Williams, Dr D. Robinson and Dr J. N. Smith for advice, and use of the spectrofluorimeter. This work was made possible by Research Grants from the Nuffield Foundation and London University.

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Purification and Specificity of Pancreatic Elastase

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(*Received* 23 *March* 1960)

For the determination of the amino acid sequences in proteins, the most useful reagents for the initial breakdown of the molecules are the proteolytic enzymes. Trypsin and chymotrypsin are the most generally used because of their high degree of specificity and because they have different specificities from one another. It would be useful to have other enzymes having complementary specificities which could be used in conjunction with trypsin and chymotrypsin. During work on the structure of insulin (Ryle, Sanger, Smith & Kitai, 1955) it was found that, when insulin was treated with crude pancreatic extracts,

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several bonds that were outside the specificity range of trypsin and chymotrypsin were split. This suggested that one or more other proteolytic enzymes were present and an attempt was made to isolate them and determine their specificities by using the fractions A and B of oxidized insulin as substrates. Evidence was obtained for the presence of an enzyme which splits bonds involving the carboxyl groups of the neutral amino acids with large aliphatic side chains (e.g. leucine and valine). This enzyme appeared to be responsible for many of the splits which were not catalysed by trypsin or chymotrypsin and proved to be identical with elastase. The present paper describes a method for the purification of elastase by using carboxymethylcellulose and describes studies on its specificity with fractions A and B of oxidized insulin.

Elastase was first reported to be present in the pancreas by Balo & Banga (1950). That it was a proteolytic enzyme was indicated by the work of Partridge & Davis (1955) who showed that the dissolution of elastin by elastase resulted in the splitting of peptide bonds. Further evidence was presented by Lewis, Williams & Brink (1956) and Grant & Robbins (1957). The latter workers also showed that although the proteolytic activity of their partially purified elastase was high, its trypsin and chymotrypsin activity as measured against synthetic substrates was low.

MATERIALS

Crystalline α-chymotrypsin and trypsin were obtained from Novo Terapeutisk Laboratorium, Copenhagen, and crystalline carboxypeptidase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Carboxymethylcellulose was prepared by the method described by Ellis & Simpson (1956). Pancreatin batch no. CHS/S 6 was a gift from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Powdered elastin was a gift from Dr S. M. Partridge of Cambridge. This elastin had been prepared by the method of Partridge & Davis (1955) and was essentially free from collagen and carbohydrate.

METHODS

Estimation of elastase activity

Methods for the estimation of elastase activity depend on determining the relative amount of elastin that can be brought into solution. A simplified colorimetric method in which elastin dyed with Congo red is used has been developed. This technique was based on that of Roaf (1908), who used dyed fibrin to measure proteolytic activity.

Powdered elastin (2 g.) was suspended in a saturated aqueous solution (20–30 ml.) of Congo red overnight, and the dyed protein filtered off. The elastin was well washed with water until the washings were clear of dye, and it was then dried by successive washings with acetone and ether.

The colour estimated as $E_{495\ m\mu}$ for completely digested samples was directly proportional to the weight of the sample. The amount of colour corresponding to a given weight of elastin varied slightly from batch to batch. $E_{495\ m\mu}$ for a 0·1% solution of digested dyed elastin was about 1·0.

A sample of the solution to be assayed was added to 3·5 ml. of an elastin suspension in 0·05 M-Na_2CO_3–HCl buffer (pH 8·8) in a 15 ml. centrifuge tube. The weight of elastin in suspension was 4–6 mg./tube. The amount of digestion was estimated by centrifuging down the undissolved elastin and determining $E_{495\ m\mu}$ of the supernatant solution in a spectrophotometer. The undissolved elastin was then resuspended in the supernatant and the digestion allowed to continue. All assays were done at room temperature.

Purification of elastase

Two preparations were used as starting material for purification of the elastase by chromatography on carboxymethylcellulose.

Euglobulin precipitate from pancreatin. The method used was essentially that described by Lewis *et al.* (1956). Pancreatin (23 g.) was extracted by stirring with 250 ml. of ammonium acetate (0·05 M with respect to ammonia) buffer (pH 4·5) for 3 hr. at 5°. The undissolved material was removed in a high-speed refrigerated centrifuge. The sediment was further extracted with 100 ml. of buffer and recentrifuged. The two supernatants were then bulked, and $(NH_4)_2SO_4$ was added to 45% saturation. After 30 min. the precipitate was removed by centrifuging and washed three times with 0·05 M-ammonium acetate buffer containing 45% of $(NH_4)_2SO_4$. The precipitate was then dissolved in 100 ml. of 0·05 M-Na_2CO_3–HCl buffer, pH 8·8, and dialysed against 5 l. of water for 18 hr. at 5° with two changes of water. The resulting euglobulin precipitate was centrifuged down, washed three times with water and freeze-dried.

Crystalline elastase. As the yield of elastase which could be extracted from the pancreatin was low, crystalline elastase was prepared from trypsin (1–300) (Nutritional Biochemicals Corp.) as the starting material, by the method described by Lewis *et al.* (1956). The preparation gave a good yield of needle-shaped crystals, the average yield from 100 g. of trypsin (1–300) being 500 mg. of crystals over five preparations.

Chromatography on carboxymethylcellulose

The material to be fractionated was applied to the column in ammonium acetate buffer of low molarity. Elution was carried out by increasing the NaCl concentration of the developing buffer, and samples (5 ml.) were collected. Protein concentration was estimated from the extinction coefficient at 280 mμ. The molarity of the effluent was determined by reading the resistance of samples (10 ml.) in a conductivity cell (Mullards Ltd., Mullard House, Torrington Place, London, W.C. 1) and relating this to a standard curve. Elastase activity was determined with dyed elastin as described above. The curves shown in the elution diagrams indicate only the presence of elastase. The figures refer to the solution of Congo red-dyed elastin per 0·1 ml. of eluent in 4 ml. of carbonate at an arbitrary time.

The chymotrypsin and trypsin activity was estimated by using the synthetic substrates *N*-acetyl-L-tyrosine ethyl

ester and benzoyl-L-arginine ethyl ester. These estimations were carried out at pH 8 and 25° with an autotitrator and compared with standard amounts of trypsin and chymotrypsin.

Action of elastase on the fractions of oxidized insulin

Digestion of the fractions A and B of oxidized insulin was carried out in an autotitrator (Jacobsen, Leonis, Linderstrøm-Lang & Ottesen, 1957) at pH 8·8 and 25°. The peptides were fractionated by paper ionophoresis (Michl, 1951; Ryle *et al.* 1955) with a potential gradient of 40 v/cm. Buffers at pH 3·5, 6·5 (Naughton, Sanger, Hartley & Shaw, 1960) and 9·1 were used. The latter was prepared by addition of NH_3 to a 2% soln. of $(NH_4)_2CO_3$. The bands were located by dipping narrow guide strips cut from the edge of the paper in a 0·25% soln. of ninhydrin in acetone, and were examined for the presence of arginine, histidine and tyrosine by the colorimetric methods described in Block, Durrum & Zweig (1955). The material from each band was subjected to hydrolysis with 5·7 N-HCl in sealed capillary tubes at 105° for 18 hr., and the amino acids were identified by paper chromatography in butanol–acetic acid–water (4:1:5, v/v) and in phenol–NH_3. *C*-terminal residues of some of the peptides were identified by the use of carboxypeptidase: a carboxypeptidase suspension (2 μl.) and 0·066 M-Na_2HPO_4 (10 μl.) were added to 0·2 ml. of water, and 10 μl. of 0·1 N-NaOH were added to dissolve the carboxypeptidase. This was neutralized by the rapid addition of 10 μl. of 0·1 N-HCl, and the pH of the solution was adjusted to 7·8. 10 μl. of this solution was added to a 0·2 ml. solution of the peptide, and the pH adjusted to 7·8. After incubation (2–6 hr.) of the solution the amino acids released were identified by paper chromatography.

RESULTS

Estimation of elastase activity

In experiments in which the rate of dissolution of elastin was studied with various concentrations of elastase it was found that at low concentrations there was a 'lag phase' during which no elastin was dissolved, and there appeared to be no simple relationship between the amount of elastin dissolved and the concentration of elastase. The procedure cannot be regarded, therefore, as an accurate quantitative method for the estimation of elastase. However, Hall & Czerkawski (1959) and Banga, Balo & Horvath (1959) found similar effects when using different assay procedures.

The colorimetric method was very useful for column chromatography where a large number of samples had to be assayed, as it gave a visual indication of which tubes had elastase activity. The readings of $E_{495\,m\mu}$ gave a rough assay of the amount of elastase.

Experiments were made to ascertain whether agitation of the tube during digestion had any effect on the rate of reaction, but none was observed. This is probably due to the fact that elastase is adsorbed on elastin (Grant & Robbins, 1957), so no shaking is required to produce intimate contact of the enzyme and substrate.

Purification of elastase

Fig. 1 shows the elution curve obtained when the 'euglobulin precipitate' from pancreatin was fractionated on carboxymethylcellulose. A peak containing all the elastase activity was eluted off with 0·2 M-buffer. This peak was assayed for trypsin and chymotrypsin activity against the synthetic substrates benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester. These assays showed the chymotrypsin activity of the peak to be 1·0% of the protein and the tryptic activity less than 0·5%.

The chromatography of crystalline elastase is shown in Fig. 2. As the protein peak had a shoulder on its leading edge which was not elastolytic, chymotrypsin and trypsin assays were done with fractions from the trailing edge of the peak. The chymotrypsin activity was 1–2% of the

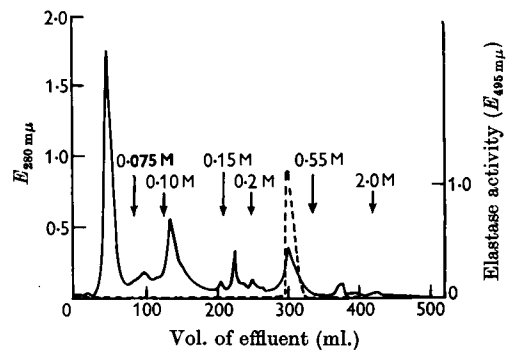

Fig. 1. Chromatography of 'euglobulin precipitate' on carboxymethylcellulose. Euglobulin precipitate (43 mg.) was dissolved in 4·3 ml. of 0·05 M-ammonium acetate buffer (pH 4·5) and applied to the column (10 cm. × 1·5 cm.). The molarity of the buffer was increased in steps by addition as indicated by arrows. —, $E_{280\,m\mu}$; - - -, elastase activity, $E_{495\,m\mu}$.

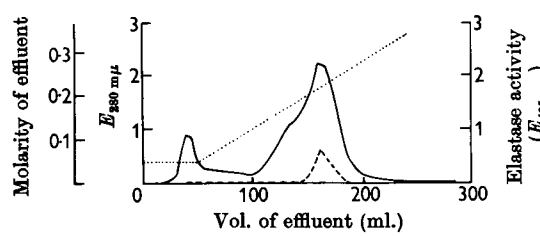

Fig. 2. Chromatography of crystalline elastase on carboxymethylcellulose. Crystalline elastase (100 mg.) was applied in 0·05 M-ammonium acetate buffer (pH 4·6) to the column which was developed with a NaCl gradient. —, $E_{280\,m\mu}$; - - -, elastase activity, $E_{495\,m\mu}$; . . ., molarity of effluent.

protein from the trailing edge whereas the trypsin activity was not detectable, indicating that the trypsin activity of the elastase peak previously prepared from the euglobulin precipitate was probably due to contaminant. 38% of the added protein ($E_{280\ m\mu}$) and approx. 70% of the elastase activity ($E_{495\ m\mu}$) was recovered in the active peak. The material from the trailing edge of this peak appeared to be essentially pure by chromatography and was used in subsequent work. It gave a single peak in the analytical ultracentrifuge.

Reaction of elastase with diisopropyl phosphorofluoridate

The dissolution of elastin in the presence of various concentrations of di*iso*propyl phosphorofluoridate (DFP) is shown in Fig. 3. Complete inhibition was effected by 10^{-4}M-DFP, showing

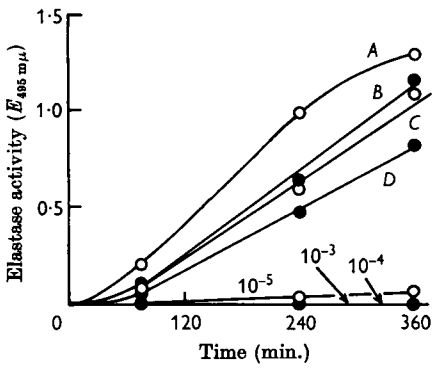

Fig. 3. Inhibition of elastase activity by DFP. Activity was measured by using elastin dyed with Congo red in various concentrations of DFP. Molarities of DFP are: *B*, 10^{-7}; *C*, 10^{-6}; *D*, 10^{-8}. *A*, Control.

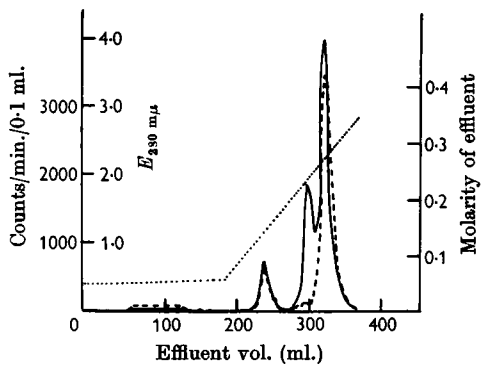

Fig. 4. Chromatography of di*iso*propoxy[^{32}P]phosphinyl derivatives of crystalline elastase on carboxymethylcellulose. Crystalline elastase (100 mg.) was treated with [^{32}P]DFP, dialysed against 0·05M-ammonium acetate buffer (pH 4·5) and applied to the column (40 cm. × 2 cm.). Conditions were as in Fig. 2. —, $E_{280\ m\mu}$; . . ., molarity of effluent; - - -, counts/min./0·1 ml. of effluent.

that elastase, like trypsin and chymotrypsin, is a DFP-sensitive enzyme. Using [^{32}P]DFP, radioactive di*iso*propoxy[^{32}P]phosphinyl-elastase was prepared from crystalline elastase as previously described (Naughton *et al.* 1960). This was subjected to chromatography on carboxymethylcellulose with the result shown in Fig. 4. When the chromatogram is compared with the one from uninhibited elastase shown in Fig. 2, it can be seen that the inactive shoulder in the latter has now been replaced by a separate peak. Two peaks, one showing radioactivity and running in about the same position as untreated elastase and the other non-radioactive, can be seen. The material in the non-radioactive peak was further characterized by the fact that the tubes containing it showed a white precipitate after standing at room temperature or at 0° for about 5 hr. This effect was also noted from the leading edge of uninhibited elastase. The splitting of the shoulder into peaks seems to be due to the fact that elastase has been inhibited by DFP. In four chromatographic separations with uninhibited elastase the inactive peak always appeared as a shoulder, but in every case when di*iso*propoxy[^{32}P]phosphinyl-elastase was used the radioactive elastase and the inactive fraction appeared as distinct peaks. This may be because the di*iso*propoxyphosphinyl group alters the chromatographic rate slightly. No elastase activity was detected after chromatographing di*iso*proxy-[^{32}P]phosphinyl-elastase.

The non-radioactive peak did not react with fraction B of insulin, showing that the contaminant was non-proteolytic. Thomas & Partridge (1960) have found that elastase can be partially activated by cysteine. This is thought to be because the enzyme forms an inactive polymer. Attempts were made to activate the inert peak with cysteine, but as no activity resulted it was concluded that the inactive peak was not caused by an elastase polymer. The small peak which is DFP-sensitive and runs in front of the inactive peak has not been identified.

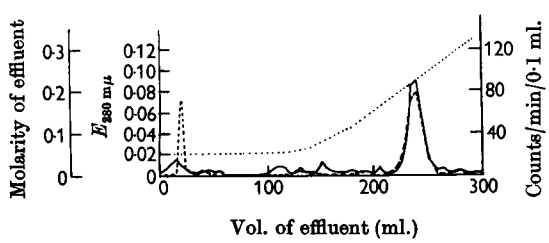

Fig. 5. Chromatography of di*iso*propoxy[^{32}P]phosphinyl derivative of purified elastase on carboxymethylcellulose. Elastase purified by carboxymethylcellulose was allowed to react with [^{32}P]DFP, and a 3 mg. sample was applied to a 2 g. carboxymethylcellulose column as in Fig. 2.

When a sample from the active trailing edge of an elastase chromatogram was inhibited with [^{32}P]DFP and re-run on a 2 g. carboxymethylcellulose column, the elution diagram shown in Fig. 5 was obtained. It can be seen that the protein peak and the radioactive peak parallel one another and there is no trace of an inactive peak running in front. Thus protein from the trailing edge of the elastase peak was not contaminated by the inactive peak.

From the specific radioactivity of the purified di*iso*propoxy[^{32}P]phosphinyl-elastase, it was calculated that the molecular weight of elastase was 28 500, assuming that one mole of DFP reacts with one mole of elastase. The purified di*iso*propoxy-[^{32}P]phosphinyl-elastase was further used to study the amino acid sequence around the reactive serine residue (Naughton *et al.* 1960).

From a nitrogen determination of the purified di*iso*propoxy[^{32}P]phosphinyl-elastase it was found that a 0·1 % solution has $E_{280\ m\mu}$ 1·90.

Action of elastase on fraction B

When the hydrolysis of fraction B by elastase was followed in the auto-titrator there was an initial rapid reaction, which fell off after one equivalent of sodium hydroxide had been added, indicating that one bond was split much more rapidly than the others (Fig. 6). This initial splitting was followed by a much more gradual hydrolysis over a longer period.

Initial rapid reaction. Fraction B (1 μmole) was incubated at 25° with 35 μg. of elastase for 15 min. at pH 8·8. The solution was brought to pH 3 with hydrochloric acid to stop the digestion and dried down in a vacuum desiccator. The hydrolysate was subjected to ionophoresis as a 5 cm. band on a Whatman no. 1 filter paper at pH 3·5 and 40 v/cm. for 1·5 hr. The marker strip showed three bands all moving towards the cathode. Two weak bands moved a distance of 3 and 11 cm. from the origin and the strong band a distance of 6–7 cm. The strong band was a mixture of peptides which could not be separated by ionophoresis at pH 3·5 or 6·5. Ammonium carbonate was found to be a satisfactory volatile buffer which would give a pH high enough to discharge the imidazole ring. The strong band was eluted from the 'pH 3·5' strip and subjected to ionophoresis as a 5 cm. band on Whatman no. 1 paper at pH 9·1, 40 v/cm. for 0·75 hr. Under these conditions two peptide bands were found. The more acidic (BH) gave a positive test for histidine. The slower moving band (BA) gave a positive test for arginine and tyrosine, but a negative one for histidine. The amino acid composition of the bands was determined by paper chromatography. The results, which are shown in Table 1, indicate that peptide BH is derived from the *N*-terminal end of fraction B and BA from the *C*-terminal end. Since peptide BH contains alanine but no tyrosine the splitting must have occurred in the region of the Ala.Leu.Tyr sequence (positions 14–16, Fig. 8).

On treating the peptide BH with carboxypeptidase, leucine and alanine were released. Therefore the rapid split in the B chain of insulin was between leucine and tyrosine residues in positions 15–16. The BA peptide released alanine only under the action of carboxypeptidase, which is consistent with a *C*-terminal sequence of Pro.Lys.Ala.

When a sample of peptide BA was treated with trypsin, a small amount of a peptide having the structure Cy-SO_3H.Gly.Glu.Arg was obtained as well as the anticipated peptide Tyr.Leu.Val.-Cy-SO_3H.Gly.Glu.Arg, indicating that splitting with elastase had also occurred at the Val.-Cy-SO_3H bond and that BA contained two peptides with tyrosine and cysteic acid *N*-terminal residues respectively.

Prolonged digestion of fraction B. Fig. 6 is a record of the alkali uptake when fraction B was digested with elastase. After the initial rapid

Table 1. *Amino acid composition of peptides* BH *and* BA

× ×, × × × and × × × × have the same significance as in Sanger & Tuppy (1951).

Amino acid	Strength in hydrolysate of Peptide BH	Peptide BA
Cysteic acid	× × ×	× ×
Aspartic acid	× × ×	·
Glutamic acid	× × × ×	× × × ×
Histidine	× × × ×	·
Serine	× × ×	× × ×
Alanine	× × ×	× × ×
Tyrosine	·	× ×
Valine	× × ×	× ×
Phenylalanine	× ×	× × ×
Leucine	× × × ×	× × ×
Lysine	·	× × ×
Arginine	·	× × ×
Threonine	·	× ×
Proline	·	× × ×

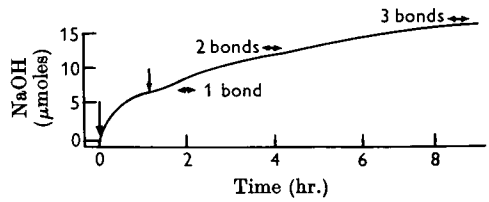

Fig. 6. Alkali uptake when fraction B (9 μmoles in 5·2 ml. of water) was digested with elastase (40 μg. of elastase added at time 0, 80 μg. at 1 hr. as indicated by arrows). Samples were removed at various times; the lines indicating bonds split (↔) have been adjusted to allow for this.

splitting over the first 60 min. there is a gradual slow further splitting. Clearer results were obtained when the isolated BA and BH peptides were hydrolysed separately, and the resulting mixtures fractionated by ionophoresis at pH 3·5. Fig. 7 is an ionophoresis diagram of hydrolysates of fraction B and peptides BA and BH obtained in this way. The structure of the various peptides is shown in Table 2. These were deduced largely from the amino acid composition of hydrolysates of the bands and from the known structure of fraction B. The unchanged BA and BH peptides move at a rate corresponding to band 7. In the ionophoresis diagram of the BH peptide shown, the digestion had not proceeded long enough to produce the peptides due to splitting of the Leu.Cy-SO_3H bond (positions 6–7). The points of cleavage are summarized in Fig. 8.

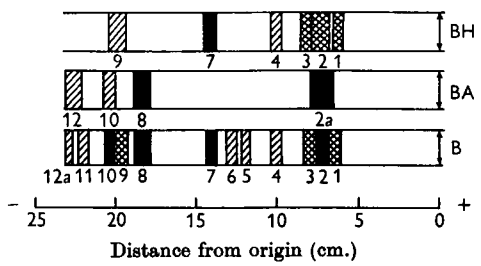

Fig. 7. Ionophoresis diagrams of elastase digests of fraction B and peptides BH and BA. Peptides BH and BA obtained from two μmole samples of fraction B, purified by ionophoresis at pH 3·5 and pH 9·0, and digested with 20 μg. of elastase at pH 8·9 for 24 hr. Ionophoresis pH 3·5, 40 v/cm., 2 hr. (see Table 2).

Action of elastase on fraction A

The reaction of elastase with the A chain of insulin as recorded in the auto-titrator did not show a curve like that for the B chain where one bond was split preferentially. Instead there was a slower reaction which decreased very gradually indicating that a number of bonds were being hydrolysed at equal rates. The peptide pattern obtained from such a hydrolysate gives a complex number of peptides as indicated in Fig. 9. The bands shown in Fig. 9 were eluted and their amino acid composition was determined (Table 3). Band no. 1 was eluted and refractionated by ionophoresis at pH 9, 40 v/cm. for 1 hr. when it gave three bands, 1 A, 1 B, and 1 C. Band A was eluted and refractionated by ionophoresis at pH 6·5, when it gave two bands, A 1 and A 2. Peptides A 2, B and I cannot be

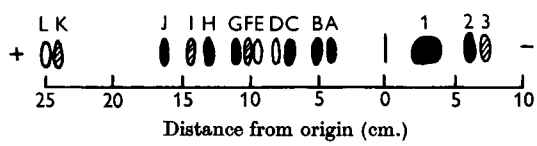

Fig. 9. Ionophoresis diagram of peptides produced by the action of elastase on fraction A. Fraction A (17 mg.) in 2 ml. of 2% $(NH_4)_2CO_3$ buffer (pH 9) treated with 35 μg. of elastase for 18 hr. Ionophoresis on Whatman no. 52 paper, pH 3·5, 35 v/cm., 2 hr. in the apparatus of Gross (1955).

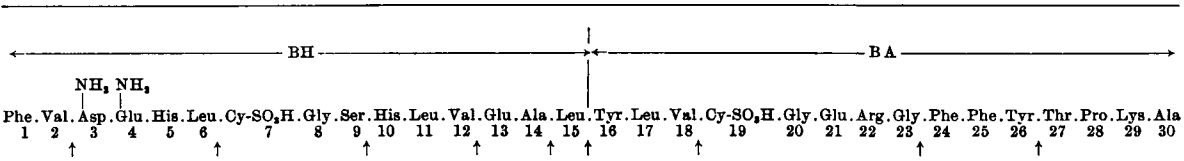

Fig. 8. Specificity of elastase on fraction B.

Table 2. *Peptides obtained from prolonged digestion of fraction* B *with elastase*

Band (Fig. 7)	Probable structure	Remarks
1	Leucine + valine	
2	Glu.Ala + Glu.Ala.Leu	From BH
2*a*	Cy-SO_3H.Gly.Glu.Arg.Gly	From BA yellow ninhydrin colour
3	Val.Glu.Ala.Leu	
4	Phe.Val.Asp-NH_2.Glu-NH_2.His.Leu.Cy-SO_3H.Gly.Ser	Serine liberated with carboxypeptidase
5	Unidentified	Positive histidine reaction
6	Unidentified	Positive arginine and tyrosine reactions
7	Unchanged BH	
8	Phe.Phe.Tyr.Thr.Pro.Lys.Ala	
9	His.Leu.Val.Glu.Ala	Alanine released with carboxypeptidase
10	Tyr.Thr.Pro.Lys.Ala	
11	Phe.Val.Asp-NH_2.Glu-NH_2.His.Leu	
12	Thr.Pro.Lys.Ala	
12*a*	Thr.Pro.Lys.Ala + Asp-NH_2.Glu-NH_2.His.Leu	Time of hydrolysis of BH too short to identify Asp-NH_2.Glu-NH_2.His.Leu

Table 3. *Peptides obtained by digestion of fraction* A *with elastase*

Band (Fig. 9)	Probable structure
1A	Alanine + Leucine
1B	Valine
1C	Tyrosine
2	Gly.Ileu
3	Gly.Ileu.Val
A1	Tyr.Glu.Leu
A2	Tyr.Glu.Leu.Glu.Asp.Tyr.Cy-SO_3H.Asp
B	Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.Cy-SO_3H.Asp
C	Ser.Val.Cy-SO_3H.Ser.Leu
D	Glu.Asp.Tyr.Cy-SO_3H.Asp
E	Unidentified
F	Ser.Val.Cy-SO_3H.Ser.Leu.Tyr.Glu.Leu.Asp
G	Ser.Val.Cy-SO_3H.Ser
H	Gly.Ileu.Val.Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala
I	Gly.Ileu.Val.Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala.Ser.Val.Cy-SO_3H.Ser
J	Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala.Ser
K	Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala.Ser.Val.Cy-SO_3H.Ser
L	Cy-SO_3H.Cy-SO_3H.Ala.Ser.Val.Cy-SO_3H.Ser

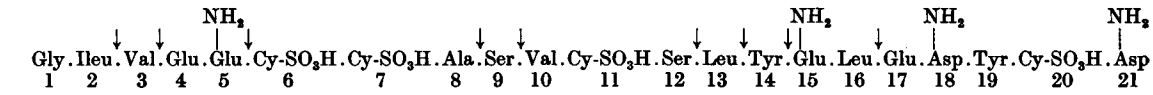

Fig. 10. Specificity of elastase on fraction A.

regarded as being identified with certainty. From the peptides produced it can be seen that the main splits in fraction A are as shown in Fig. 10.

DISCUSSION

Chromatography on carboxymethylcellulose was a useful method of purification of elastase and gave rise to preparations which are probably purer than any previously reported. Owing to the inaccuracy of the assay method, it is not possible to give precise figures for the degree of purification attained, but the results did indicate that there was no great loss of activity on chromatography and that the material from the active peak was more active than the 'crystalline' elastase. The main evidence for purity must, however, rest on the chromatographic results. Preparations of 'crystalline' elastase were shown to be contaminated with two non-elastolytic components which run in front of the active elastase. The slower of these is not separated clearly from the elastase peak but can be seen as a separate peak when the di*iso*propoxyphosphinyl derivative is chromatographed. The material from the trailing edge of the main elastase peak does not contain this contaminant and was considered to be pure elastase; it was used for the specificity studies. It had no detectable tryptic activity as measured on benzyl-L-arginine ethyl ester and only 1–2% activity on *N*-acetyl-L-tyrosine ethyl ester which is probably an inherent property of the elastase rather than due to chymotryptic activity.

The reaction of the elastase with the A and the B chains of insulin showed that the enzyme can attack a wide variety of peptide bonds involving neutral amino acids having aliphatic side chains. The digestion of elastin by elastase can be explained in terms of this specificity as elastin contains a high proportion of amino acid residues possessing hydrocarbon side chains (Newman, 1949; Partridge & Davis, 1955).

Although elastase does not have a narrow specificity it is a potentially useful enzyme for amino acid sequence studies. It has a very different specificity from trypsin and chymotrypsin and may be useful for splitting peptide chains which are not attacked by these two enzymes as well as for further degradation of larger peptides.

SUMMARY

1. Elastase was purified by chromatography on carboxymethylcellulose.

2. With the fractions A and B of oxidized insulin as substrates it was shown that the purified elastase splits bonds adjacent to the neutral amino acids.

We wish to thank Dr B. C. Saunders for a gift of di-*iso*propyl phosphorofluoridate, Dr D. R. Davis for isotopic DFP and Dr S. M. Partridge for pure elastin.

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Ultracentrifugal Studies of Rat, Rabbit and Guinea-Pig Serum Albumins

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(*Received* 13 *May* 1960)

Rat, rabbit and guinea-pig serum albumins have been used in many investigations, but no systematic study of their behaviour in the ultracentrifuge has been reported, except for a short preliminary account of part of the present work (Charlwood, 1959) and a graph showing the dependence on concentration of the sedimentation coefficient of rat albumin at pH 7·5 (Anderson, Canning, Anderson & Shellhamer, 1959). Some measurements have now been completed with the intention of defining the molecular weights of the albumins as accurately as possible, of discovering whether different methods of preparation give essentially the same product and whether the sedimentation coefficients are dependent on pH in the same manner as those of bovine, human and horse albumins (Charlwood & Ens, 1957). In assessing possible differences between preparations, it was necessary to obtain an estimate of the reproducibility of measurements and of the probable magnitudes of errors entering into the various factors concerned. A stock preparation of human albumin, designated A (Charlwood, 1954), was used for many of these experiments.

METHODS

Protein isolation. Some samples of albumin were obtained by electrophoresis of diluted serum in veronal buffer (pH 8·6, *I* 0·1) in the Tiselius apparatus, with automatic control of the compensator, as described by Charlwood (1954) and Warren & Charlwood (1953). Some sera were subjected to preliminary salt fractionation, globulins being mostly removed by saturation with magnesium sulphate followed by filtration (Popják & McCarthy, 1946).

Two organic solvent systems were used for serum fractionation. In the first, 10 vol. of aq. ethanol (96%, v/v, of the alcohol) containing 1% (w/v) of trichloroacetic acid were slowly mixed with 1 vol. of serum at room temperature (Delaville, Delaville & Delaville, 1954). After 30 min., either at room temperature or in the cold (5°), the mixture was centrifuged. The supernatant was dialysed under pressure in the cold against several changes of water. After removal of any precipitate by centrifuging, the albumin solution was dialysed against a suitable buffer. This was veronal (pH 8·6, *I* 0·1) if electrophoresis was to follow, or sodium acetate (pH 4·5, *I* 0·1) if the next stage was to be ultracentrifuging. Solutions not clear at this stage were treated as described below. In some initial experiments the preliminary dialysis against water was omitted, but this proved inadvisable (see Results section). The second method of isolation involving an organic solvent was the same as the previous one, except that the trichloroacetic acid–ethanol system was replaced by 0·4 M-tartaric acid in aq. methanol (90%, v/v, of the alcohol) (Michael, 1958).

When a preparation, made in an organic solvent system, yielded an opalescent aqueous solution which could not be clarified in the ordinary centrifuge, it was often possible to remove the unwanted impurity by centrifuging for 15–30 min. at 39 460 rev./min. in the Spinco preparative rotor SW39. Some electrophoretically separated rat-albumin solutions that contained soluble materials of higher sedimentation coefficients were treated similarly for a period of several hours; this was calculated to bring down the larger molecules but to leave a high proportion of the albumin in the middle of the tubes, whence it could be removed by syringe. This method of fractionation was also applied to one freeze-dried sample of rat albumin that had originally been prepared by the trichloroacetic acid–ethanol method. Many preparations were made on a scale sufficiently large for a comparison to be possible between freeze-dried material and a sample of the same batch which had remained in solution throughout.