to the conclusion that the factor can catalyse the reduction of soluble material in the leaf extracts. Further work is necessary to trace the paths of hydrogen transport after the reduction of the factor by the illuminated chloroplast and to determine the nature of the group concerned in the oxidation–reduction cycle.

SUMMARY

1. A protein factor from leaves, previously shown to be active in catalysing the reduction of methaemoglobin and metmyoglobin by illuminated chloroplasts, has been purified by fractionation with ammonium sulphate followed by electrophoretic separation of the active material on paper.

2. The purified protein was found to give a single symmetrical boundary in the ultracentrifuge and in the Tiselius electrophoresis apparatus.

3. The molecular weight of the protein, calculated from sedimentation and diffusion measurements, is 19 000.

4. The purified protein is highly active in catalysing the photochemical reduction of metmyoglobin and also stimulates the reduction of cytochrome *c*, cytochrome b_3 and the cytochrome components present in a particulate heart-muscle preparation.

5. The photochemical reduction is inhibited by organic mercury compounds and this inhibition is partially reversed by cysteine.

6. The protein does not catalyse the reduction of haem–proteins when coenzymes served as hydrogen donor. Illuminated chloroplasts were the only effective hydrogen-donating system observed.

7. The chemical nature of the group conferring oxidation–reduction properties to the protein has not yet been determined but neither flavin nor haem could be detected.

8. Metmyoglobin-reducing activity has been observed in extracts of chloroplasts and was shown to represent one-third of the activity extractable from the whole leaf.

In addition to the acknowledgements made in the text the authors wish to acknowledge the help of Mr F. W. Arnold and the late Mr H. Pentelow in the collection of leaf material, and to thank Mr B. E. Boon for his assistance with the ultracentrifuge runs and Mr B. R. Slater for the diffusion measurements. This work formed part of a programme supported by the Agricultural Research Council.

REFERENCES

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Crammer, J. L. (1948). *Nature, Lond.*, **161**, 349.
Davenport, H. E. (1949). *Proc. Roy. Soc.* B, **136**, 281.
Davenport, H. E. & Hill, R. (1955). *Res. comm. 3rd int. Congr. Biochem., Brussels*, p. 103.
Davenport, H. E., Hill, R. & Whatley, F. R. (1952). *Proc. Roy. Soc.* B, **139**, 346.
Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Hill, R. (1936). *Proc. Roy. Soc.* B, **120**, 472.
Hill, R. (1939). *Proc. Roy. Soc.* B, **127**, 192.
Hill, R., Northcote, D. H. & Davenport, H. E. (1953). *Nature, Lond.*, **172**, 948.
Hill, R. & Scarisbrick, R. (1940). *Proc. Roy. Soc.* B, **129**, 328.
Hill, R. & Scarisbrick, R. (1951). *New Phytol.* **50**, 98.
Holt, A. S. (1950). U.S. Atomic Energy Commission Document ORNL-752.
Jagendorf, A. T. (1956). *Arch. Biochem. Biophys.* **62**, 141.
Jencks, W. P., Jetton, M. R. & Durrum, E. L. (1955). *Biochem. J.* **60**, 205.
Kearney, E. B. & Singer, T. P. (1955). *Biochim. biophys. Acta*, **17**, 596.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.
Keilin, J. & Schmidt, K. (1948). *Nature, Lond.*, **162**, 496.
Mackinney, G. (1941). *J. biol. Chem.* **140**, 315.
Margoliash, E. (1952). *Nature, Lond.*, **170**, 1014.
Martin, E. M. & Morton, R. K. (1957). *Biochem. J.* **65**, 404.
Miller, G. L. & Golder, R. H. (1950). *Arch. Biochem.* **29**, 420.
Sörensen, S. P. L. (1912). *Ergebn. Physiol.* **12**, 393.

The Isolation and Properties of a Proteolytic Enzyme, Cathepsin D, from Bovine Spleen

By E. M. Press, R. R. Porter and J. Cebra*
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 29 *July* 1959)

Interest in the fate of protein antigens in an antibody-forming organ has led us to investigate the proteolytic enzymes of the spleen. The intracellular proteases (cathepsins) have been studied in many animal tissues, though none has been fully characterized (see Smith, 1951). Those of the spleen were studied by Anson (1939), using his haemoglobin assay (Anson, 1938) to follow the activity, and an extensive series of papers by Bergmann, Fruton and co-workers (summarized by Fruton, 1957–58) describe many properties of cathepsins A, B and C which have been obtained from both spleen and

* Present address: Rockefeller Institute for Medical Research, New York, U.S.A.

kidney. These three enzymes are distinguished from one another by their different specificities by the use of synthetic peptides as substrates. Our original intention was to attempt a survey of the proteolytic activity of spleen to estimate the number of different cathepsins and the relative amounts present. Hence the non-specific haemoglobin assay was used to follow activity. In fact, one enzyme appears to be responsible for about two-thirds of the proteolytic activity (as judged by haemoglobin assay) of a crude spleen extract. This enzyme has therefore been isolated and as it will not hydrolyse any of the typical substrates of cathepsins A, B and C it has been named cathepsin D. As isolated, it occurs in at least ten forms separable from one another by chromatography and electrophoresis, and present evidence suggests that it is present in the spleen in this complexity. The isolation and properties of cathepsin D will be described in this paper.

A preliminary report of this work has been given (Press & Porter, 1958).

EXPERIMENTAL

Chromatography on cellulose ion-exchangers

Reagents. Sodium borate–phosphate, pH 8·4, 5·25 mM: 2·25 mM-$Na_2B_4O_7$ and 3 mM-NaH_2PO_4; 21 mM: 9 mM-$Na_2B_4O_7$ and 12 mM-NaH_2PO_4; 8·75 mM: 3·75 mM-$Na_2B_4O_7$ and 5 mM-NaH_2PO_4; 13 mM: 5·5 mM-$Na_2B_4O_7$ and 7·5 mM-NaH_2PO_4. Sodium phosphate, pH 3·7, 0·5 M with respect to phosphate; sodium phosphate, pH 6·4, 0·01 M with respect to phosphate; sodium acetate, pH 5·5, 0·01 M, 0·05 M and 0·2 M with respect to acetate. Cellulose was Solka-Flok, B. W. 200 mesh; 2-chlorotriethylamine hydrochloride was obtained from Eastman Kodak Ltd.; monochloroacetic acid was reagent-grade; sodium monochloromethane sulphonate was synthesized as described by Porath (1957).

Diethylaminoethylcellulose. Diethylaminoethylcellulose (DEAE) was prepared as described by Peterson & Sober (1956). Solka-Flok cellulose was graded to remove some of the finer particles by sedimenting in water and discarding the material which did not settle in an hour; this was done three or four times until all the remaining cellulose settled in this time. It was then dried by washing on a Büchner funnel with acetone and finally in an oven at 100°. The 2-chlorotriethylamine hydrochloride was recrystallized by dissolving it in the minimum volume of hot methanol, filtering the hot solution and then allowing it to crystallize at 2°. The yield was greatly increased by the addition of about 10–20% of ethyl acetate at this stage, and the crystals were washed with ethyl acetate and dried. DEAE-chromatography columns were prepared as described by Sober, Gutter, Wyckoff & Peterson (1956) by suspending in 5·25 mM-borate–phosphate buffer, adjusting the pH to 8·4 by addition of a few drops of 5 M-NaH_2PO_4, followed by washing in a sintered-glass filter funnel with about 2 l. of buffer when the pH of the eluate and the buffer were exactly the same. The column was then poured and allowed to settle under 2 ft. of water pressure, the column was washed through with buffer overnight and the pH of the eluate again checked. A piece of filter paper was put on top of the column and then the column allowed just to drain and the protein solution applied to the centre of the paper. When the solution had sunk into the column, it was washed in with a few millilitres of buffer and eluted with buffer under a pressure of about 2 ft. of water. Higher pressures were not used as it was found that the column then packed so tightly that the flow rate was reduced. Concentration-gradient elution was carried out by running a buffer of higher concentration from a reservoir into a mixing vessel of constant volume containing the buffer of lower concentration.

After use, DEAE was recovered by washing on a filter funnel with N-NaOH to remove adsorbed protein and then washing with water to neutral pH.

Carboxymethylcellulose. Carboxymethylcellulose (CM) was also prepared according to the method of Peterson & Sober (1956) with graded Solka-Flok and chloroacetic acid. The CM columns were prepared in the same way as for the DEAE columns by first adjusting the pH with strong buffer and then washing with the buffer to which they were to be equilibrated. After use the CM was recovered by washing with a solution containing 0·25 M-NaCl and 0·25 N-NaOH and then with water to neutrality.

The CM and the DEAE could be dried by washing with ethanol and evaporating in a vacuum desiccator over solid NaOH and conc. H_2SO_4, but this is a slow process and both exchangers can be stored quite satisfactorily in water at 2°.

The flow rate of both DEAE and CM columns was about 50 ml./hr. for columns of cross-section 4·3 cm.^{2} and a pressure of about 2 ft. of water. If the flow rate was less than about 25 ml./hr. the cellulose ion-exchanger was graded by sedimenting in water for an hour and discarding the material which failed to settle. After repeating this two or three times, the flow rate became satisfactory.

Sulphomethylcellulose. Sulphomethylcellulose (SM) was prepared from graded Solka-Flok and sodium monochloromethane sulphonate as described by Porath (1957). The preparation contained a large amount of very fine particles, which were removed by decantation. The exchanger was recovered after use by washing it with 0·1 N-NaOH, followed by water to neutrality.

Phosphate and acetate concentration. Phosphate concentration in the eluate from DEAE columns (Fig. 1*a*) was determined by the method of Martland & Robison (1926). Acetate concentration in the eluate from CM columns, Figs. 3, 4, 6 and 7, was calculated from the formula

$$\log (C_1 - C_v) = \frac{-v}{2{\cdot}3 \times V_0} + \log (C_1 - C_0),$$

where C_1 is the concentration in the reservoir, C_v is the concentration in the mixing vessel when v ml. has run in, V_0 is the volume of the mixing vessel, and C_0 is the original concentration in the mixing vessel.

Electrophoresis

Zone electrophoresis. Zone electrophoresis was performed in a cellulose column as described by Porath (1956) with 0·02 M-sodium acetate, pH 5·6, for 24 hr. at 4–5 v/cm.

Starch-gel electrophoresis. Buffers: 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 9·1, used in the starch gel, contained 4·2 g. of tris, 0·42 g. of ethylenediaminetetra-acetate and 0·32 g. of boric acid in 1 l. of

solution (Aronsson & Grönwall, 1958). The tris buffer for the bridge solution was three times as concentrated as that in the gel. Sodium acetate buffer, pH 5·5, was 0·03 M for the starch gel, and 0·3 M for the bridge solution.

Potato starch (British Drug Houses Ltd.) was hydrolysed in acidic acetone, as described by Smithies (1955), for 2¾ hr. at 38·5°; the reaction was stopped by addition of sodium acetate, the starch filtered and washed with 5 l. of water, followed by 1 l. of borate buffer (0·3 M), pH 8·0, followed by washing with a further 2 l. of water and then washing with acetone and drying at 45° overnight. Amido Black (George T. Gurr Ltd., London, S.W. 6) for staining starch gel: 1·6 % (w/v) in a solvent consisting of methanol–water–acetic acid (50:50:10, by vol.). Wash solution for developing stain: methanol–water–acetic acid (50:50:10, by vol.).

Starch-gel electrophoresis was carried out as described by Smithies (1955) and Poulik & Smithies (1958). Hydrolysed potato starch was used at a concentration of 14 % (w/v); the gel was prepared and poured into a Perspex tray 25 cm. × 4 cm. and 6 mm. deep and allowed to set for 1 hr. or overnight. Electrophoresis in the tris buffer was for 16 hr. at 14 mA and 8–2 v/cm. In acetate buffer the current was 14 mA for 15 hr. at 3–2 v/cm. Calomel electrodes were used and contact was made with the gel through a bridge solution by strips of Whatman no. 3 filter paper soaked in the bridge solution. A horizontal slice of the starch was cut and stained with Amido Black for 2 min. and developed by washing for several hours. Up to three different enzymes could be subjected to electrophoresis in the same gel by inserting pieces of Whatman no. 3 filter paper, soaked in 1 % solution of the enzyme, side by side into a vertical slit cut across the gel. It is possible to recover the protein from the unstained slice of the gel by freezing, thawing and centrifuging the sections containing protein. From the solution squeezed out of the gel in this way, recovery of activity was about 30–50 %.

Methods of enzyme assay

Reagents. Armour Laboratories' bovine haemoglobin enzyme-substrate powder was suspended in water and dialysed at 2° for 4–5 days and then diluted with water to 2·5 % (w/v).

Sodium citrate, 0·4 M with respect to citrate, pH 2·8, gives pH 3·0 in reaction mixture with haemoglobin.

Cysteine hydrochloride (0·07 M) was stored at 2°.

Bovine serum albumin was Armour Laboratories' bovine crystallized plasma albumin.

Trichloroacetic acid (A.R.) was 0·3 M and 0·6 M.

N-Acetyl-DL-phenylalanyl-L-di-iodotyrosine, L-tyrosyl-L-cysteine and L-cysteinyl-L-tyrosine were kindly given by Dr R. Pitt-Rivers.

Benzyloxycarbonyl-L-glutamyl-L-tyrosine and glycyl-L-tyrosine amide acetate were supplied by Mann Research Laboratories. Benzoyl-L-arginine amide was synthesized by Mr A. Hemmings.

Assay with haemoglobin. Proteolytic activity was assayed at pH 3·0 by a modification of Anson's (1938) method. Haemoglobin (2·5 %; 4 ml.) was added to 1 ml. of citrate buffer, 1 ml. of cysteine hydrochloride and 1 ml. of enzyme solution and incubated at 37° for 10 min., then 9 ml. of 0·3 M-trichloroacetic acid was added and the mixture was filtered through Whatman no. 3 paper. The absorption of the filtrate at 280 mμ was measured. Reagent blanks were carried out by adding the trichloroacetic acid before the enzyme solutions. One unit of proteolytic activity is that which will give an extinction of the trichloroacetic acid filtrate at 280 mμ of 1·0 in excess of the blank reading. There is a linear relationship between the amount of enzyme used and the absorption of the trichloroacetic acid filtrate, up to an extinction of 0·3, and all assays were done within this range.

Assay with acid-denatured haemoglobin. Haemoglobin (2·5 %) was incubated at 37° at pH 2·3 in 0·16 M-citric acid for 1 hr. and by addition of N-NaOH the pH was adjusted to that required for the assay. Cysteine hydrochloride was added so that the final solution contained 1·5 % of haemoglobin and was 0·01 M with respect to cysteine hydrochloride. The method of assay was the same as for the routine assay described above.

Assay with acid-denatured albumin as substrate. Bovine serum albumin (1·7 %) was incubated at 37° for 1 hr. at pH 1·8 in 0·33 M-citric acid. N-NaOH was then added to adjust the pH to that required for the assay, and also cysteine hydrochloride to a concentration of 0·01 M. The final solution contained 0·85 % of albumin; 2·5 ml. of this solution was incubated with 1 ml. of enzyme solution at 37° for 10 min. and then 5 ml. of 0·6 M-trichloroacetic acid was added. The mixture was allowed to stand for 1 hr. at 37°, filtered through Whatman no. 3 paper, centrifuged to give a clear solution and the absorption was read at 280 mμ. Reagent blanks were obtained by adding the trichloroacetic acid before the enzyme.

Assays with peptides. Cathepsin A was assayed with benzyloxycarbonyl-L-glutamyl-L-tyrosine, by the micro-titration method of Grassmann-Heyde at pH 5·0, as described by Tallan, Jones & Fruton (1952). A direct-reading pH meter (Electronic Instruments Ltd.) was used to determine the end-point of the titration, since with crude extracts an indicator was not satisfactory. Cathepsin B was assayed with benzoyl-L-arginine amide as substrate by the method of Greenbaum & Fruton at pH 5·0 (1957). Cathepsin C was assayed by measuring the transamidation reaction between glycyl-L-tyrosine amide and hydroxylamine at pH 7·2 (de la Haba, Cammarata & Fruton, 1955).

The assay with the pepsin substrate *N*-acetyl-DL-phenylalanyl-L-di-iodotyrosine was carried out by incubation of 2 mM-substrate with cathepsin D at a concentration of 0·01 mg. of enzyme N/ml. for 20 min. at 37° at pH 2·0, 3·0 and 5·0. The course of hydrolysis was followed by the ninhydrin reaction described by Cocking & Yemm (1954).

For the assays with L-tyrosyl-L-cysteine and L-cysteinyl-L-tyrosine, the substrate at a concentration of 5 mM was incubated with enzyme, concentration 0·03 mg. of enzyme N/ml., at pH 3·6 for 80 min. at 37°. The digest and controls were evaporated and chromatographed on paper with butanol–acetic acid–water (4:1:5, by vol.) in order to detect any tyrosine liberated.

Hydrolysis of B chain of oxidized insulin by cathepsin D

The B chain of oxidized insulin was prepared according to the method of Sanger (1949), from crystalline insulin (Burroughs Wellcome and Co.). Pyridine–acetate buffer, pH 6·5, contained 10 vol. of pyridine, 0·4 vol. of acetic acid and 90 vol. of water. For paper chromatography, the following solvents were used: butanol–acetic acid (5 vol. of water, 4 vol. of butanol and 1 vol. of acetic acid were

shaken together and allowed to settle; the top phase was used to develop the chromatogram); phenol–ammonia (80%, w/v, phenol in water containing 0·01 mM-ethylenediaminetetra-acetate was used to develop the chromatogram in an atmosphere containing ammonia vapour. For staining, 2% (w/v) ninhydrin in aq. 90% ethanol containing 1% of pyridine was used.

A sample (5 mg.) of B chain of oxidized insulin was incubated at pH 3·0 and 37° with 1 unit of enzyme for 2 and 20 hr. and the digestion was stopped by evaporation in a vacuum desiccator. The dry hydrolysate was dissolved in a few drops of water and applied as a thin line 6 cm. long to Whatman no. 4 paper for electrophoresis at pH 6·5 in pyridine–acetate buffer, by the technique of Michl as described by Ryle, Sanger, Smith & Kitai (1955). A potential of 25 v/cm. was applied across the paper for 3 hr. Strips of paper were stained with ninhydrin, and the portions containing the basic and acidic peptides were eluted with 6 N-HCl and hydrolysed in sealed tubes at 105° for 18 hr. The hydrolysates were vacuum-dried several times and chromatographed on Whatman no. 1 paper, butanol–acetic acid and phenol–ammonia solvents being used to identify the component amino acids of these peptides. The neutral peptides were chromatographed on Whatman no. 4 paper with butanol–acetic acid solvent and the peptides thus separated were hydrolysed with 6 N-HCl and chromatographed as for the basic and acidic peptides.

Ultracentrifugal analysis

The buffer was sodium phosphate (I 0·2, pH 6·75). Molecular weights were determined by sedimentation in the ultracentrifuge by the Archibald (1947) procedure as described by Charlwood (1957).

The N-terminal amino acid of the enzyme was determined by the fluorodinitrobenzene method of Sanger (1945), with paper chromatography with 2-methylbutan-2-ol–phthalate solvent to separate the dinitrophenyl (DNP) amino acids, as described by Porter (1957).

The concentration of protein solutions was determined by measuring their absorption at 280 mμ.

RESULTS

Preparation of spleen extracts

Bovine spleens were put into solid CO_2 immediately after removal from the animal and kept frozen until used. They were partially thawed, freed from skin and connective tissue and cut for mincing. A single spleen may yield from 400 to 800 g. of minced tissue. A variety of methods of pretreatment and extraction were tried and these are listed in Table 1. When portions of the same spleen were extracted by the different methods or combinations of these methods the yields of proteolytic activity, as judged by haemoglobin assay, all agreed within experimental error and the yield in the supernatant fluid after centrifuging at 40 000 *g* was the same as in the whole suspension. It appeared therefore that all the proteolytic activity of the spleen is free in the cells or, more probably, is associated with easily ruptured particles such as lysosomes (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). If any of the proteolytic enzymes exist in the cell as inactive zymogens, the activation occurred too rapidly to be observed. The pH optima of these crude extracts, as of purified cathepsin D, lay in the range pH 3·0–4·0 with protein substrates. No evidence of proteolytic activity at neutral or alkaline pH could be detected with the methods of assay used.

Purification of cathepsin D

For routine preparation the minced spleen was extracted by stirring overnight at 2° with twice the volume of 0·1 M-sodium acetate saturated with toluene. The purification procedure for 1 kg. of minced spleen is summarized in Table 2. The yield of proteolytic activity varied considerably from spleen to spleen, 5000–14 000 units of proteolytic activity/kg. being obtained in the course of some 12 preparations.

The crude extract was filtered through four layers of cheese cloth and then centrifuged at 40 000 *g* in a refrigerated continuous-flow Sharples centrifuge. The supernatant was brought to pH 4·6 by adding to 4 vol. of supernatant 1 vol. of acetate buffer (23·4 ml. of acetic acid and 50 ml. of 2 M-sodium acetate made to 1 l. with water). The pH was adjusted to pH 4·6 with acetic acid or sodium hydroxide if necessary. A large precipitate formed, which, after settling for at least 1 hr., was removed by centrifuging (1000 *g*) in a refrigerated bucket centrifuge at 2°. The precipitate was suspended in an equal volume of water and again centrifuged, the supernatants being combined. Between 70 and 90% of the activity was recovered, a sufficiently high figure to suggest that the loss was probably mechanical rather than due to the separation of a distinct enzyme which was insoluble or unstable under these conditions.

Table 1. *Methods of extraction of ox spleen*

All procedures were carried out at 2° except where otherwise stated.

Pretreatment of spleen
Homogenization for 10 min.
Stirring overnight
Acetone-dried powder
Repeated freezing and thawing
Incubation of whole spleen at 20° for 3 hr.
Solvents for extraction
Water
0·15 M-NaCl solution
0·1 M-Citrate buffer, pH 5·9 and pH 7·5
0·1 M-Phosphate buffer, pH 7·0
0·1 M-Sodium acetate
0·1 M-Phosphate buffer, pH 7·5, containing 20% of butanol

Table 2. *Preparation of cathepsin D from 1 kg. of minced spleen*

All procedures were carried out at 2°.

		Volume (ml.)	Total enzyme units/kg. of spleen		Enzyme units/mg. dry wt.
Whole suspension in 0·1 M-sodium acetate		3200	9600		—
Supernatant from centrifuging at 40 000 *g*		2900	8000		0·07
pH 4·6 supernatant		4500	6300		0·2
Precipitate with 90 % saturated ammonium sulphate resuspended and dialysed		400	6000		0·3
Pressure-dialysed solution before chromatography on CM at pH 6·4		70	5900		0·3
Eluate recovered from CM column		120	5750		0·5
Pressure-dialysed solution before chromatography on DEAE at pH 8·4		45	5600		0·5
Eluate recovered from DEAE at pH 8·4		600	3800		3·0
Eluate recovered from second run on DEAE at pH 8·4	α	200	950		10
	β	190	940	Total 3760	10
	γ	200	730		7
	δ	250	1140		5
Eluate recovered from chromatography of β form on CM at pH 5·5	β_1	60	90		21
	β_2	50	200		21
	β_3	90	100	Total 700	19
	β_4	60	200		20
	β_5	75	110		16

Solid ammonium sulphate was added to the combined supernatants at pH 4·6 and 2°, to give a 90 % saturated solution (615 g./l.). The suspension was filtered through Whatman no. 50 paper with suction and the filter cake was resuspended in as small a volume of water as possible and dialysed overnight against running tap water in the cold room. This was primarily a concentration step but some purification was also achieved without loss of activity. Any precipitate remaining was centrifuged at 1000–2000 *g* at 2°, washed with an equal volume of water and the combined supernatants were dialysed under pressure against 0·01 M-sodium phosphate buffer, pH 6·4, for 3 days at 2°. The concentrated solution, adjusted to pH 6·4 with a few drops of acid or alkali if necessary, was run slowly on to a 20 g. CM column (30 cm. × 4·3 cm.²) equilibrated with 0·01 M-phosphate buffer, pH 6·4. Under these conditions the proteolytic activity is not held on the column and was recovered quantitatively in the eluate. Much heavily pigmented inactive material was adsorbed and discarded. The enzymically active eluate was concentrated by pressure dialysis against sodium borate–phosphate buffer (5·25 mM, pH 8·4). The solution (about 50 ml.) was chromatographed on a DEAE column 30 cm. × 4·3 cm.², equilibrated with the same buffer. The remaining pigment was held at the top of the column; some inactive protein was unadsorbed and emerged at the solvent front, followed by the proteolytic activity. A buffer concentration gradient was used with a 250 ml. mixing vessel, and a reservoir containing 21 mM borate–phosphate, pH 8·4. About 70 % of the proteolytic activity was recovered.

Cathepsins B and C, assayed by using their specific substrates, were not eluted under these conditions but could be partially recovered by subsequent step-wise elution with 0·5 M-sodium phosphate buffer, pH 3·7. Only 20 % of cathepsin C and 50 % of cathepsin B originally present were recovered in this procedure, and it is clear that this would be an unsatisfactory method of preparation for these enzymes. The eluate containing cathepsins B and C had about 10 % of the total proteolytic activity, giving an overall loss of 20 %, which is probably accounted for by the loss of the B and C enzymes. Cathepsin A assays on the initial spleen extracts showed only minimal amounts to be present in all preparations and none could be detected after the first stage in purification. No explanation has been found for this discrepancy between our results and those of Fruton & Bergmann (1939).

The cathepsin D fraction was concentrated by pressure dialysis and run again on a DEAE column under similar conditions but with a slower salt gradient. With a column 30 cm. × 4·3 cm.², initial buffer 5·25 mM borate–phosphate, pH 8·4, and a reservoir buffer concentration 21 mM, pH 8·4, the volume of the mixing chamber was increased from 250 ml. to 1 l., thus giving a more gentle gradient. The first DEAE column could be omitted if much lower loads were used but in practice it was found preferable to run again on a second DEAE column, as described, and work with high loads. The elution

diagram is shown in Fig. 1*a*, and it can be seen that there are four enzymically active components, labelled α, β, γ and δ in order of their elution from the column. The recovery of activity from this column is near 100%, suggesting that the losses on the first DEAE column are due to losses of cathepsins B and C. The specific activity of the cathepsin D enzymes varies from preparation to preparation in the range 3–10 units of proteolytic activity/mg. If the activity was below 8 units/mg. the enzymes were run again on DEAE by simple elution chromatography, i.e. at constant salt and pH with 8·75 mM-borate–phosphate buffer, pH 8·4, for the enzymes α, β and γ and 13 mM buffer, pH 8·4, for the δ enzyme. Satisfactory chromatography was obtained under these conditions and gave further purification, as illustrated for the α enzyme in Fig. 2.

It was apparent, however, from starch-gel electrophoresis and ultracentrifuge studies that none of the four enzymes was pure, and chromatography on CM was now used to get further purification. The conditions chosen, which were applicable to all the enzymes, were to put up to 400 units of proteolytic activity on a 25 cm. × 1 cm.² column with an initial buffer concentration of 0·01 M-sodium acetate pH 5·5. After 2 column vol. a gradient was applied with a constant-volume mixing vessel of 250 ml. and a reservoir buffer concentration of 0·05 M-acetate, pH 5·5. After a further 12 column vol. the concentration of the reservoir buffer was changed to 0·2 M-acetate, pH 5·5. The resulting chromatogram with α enzyme is shown in Fig. 3.

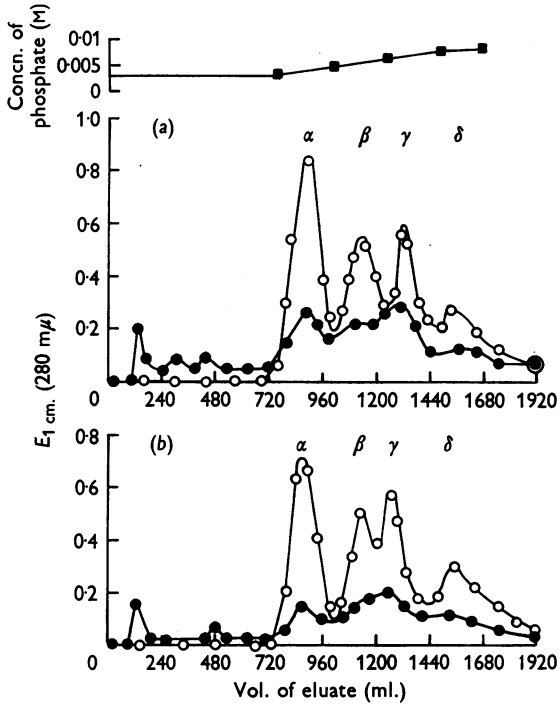

Fig. 1. (*a*) Chromatography of cathepsin D (2000 units of proteolytic activity) in a DEAE column 30 × 4·3 cm.² by gradient elution with sodium borate–phosphate buffer, 5·25–21 mM, pH 8·4. Volume of mixing chamber, 1 l. (*b*) Chromatography on DEAE of a part of the same preparation as in (*a*) (1700 units) which had been left at pH 4·6 and 2° for 1 week before precipitation with ammonium sulphate. ●, Extinction coefficient; ○, units/0·2 ml.; ■, phosphate concentration in eluate.

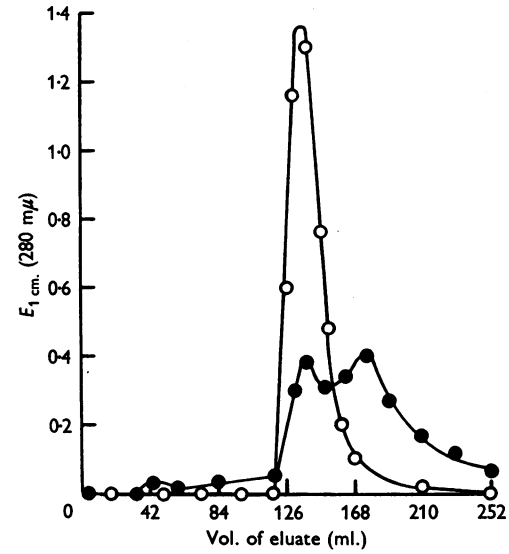

Fig. 2. Rechromatography of α enzyme (150 units) on a DEAE column 30 × 1·5 cm.² at constant buffer concentration and pH (sodium borate–phosphate buffer, 8·75 mM, pH 8·4). ●, Extinction coefficient; ○, units/0·2 ml.

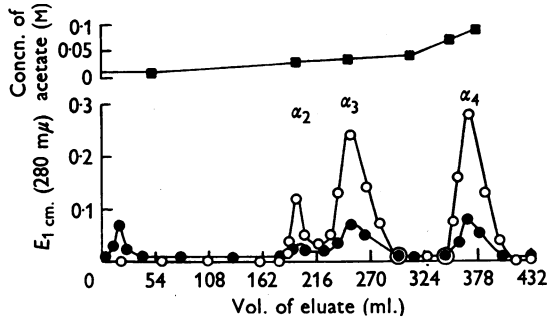

Fig. 3. Chromatography of the α enzyme (150 units) on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, 0·01–0·2 M, pH 5·5. Volume of mixing chamber, 250 ml. ●, Extinction coefficient; ○, units/0·2 ml.; ■, acetate concentration in eluate.

Three active peaks α_2, α_3 and α_4 appear. With β enzyme (Fig. 4) five active peaks are found; with the γ, four peaks, and with the δ, again five. The recovery of proteolytic activity is about 70%. Evidence presented below suggested that the majority of these different forms of cathepsin D were pure.

Evidence for the existence of cathepsin D in at least ten different forms

The validity of the apparent existence of many forms of this enzyme was tested both by further chromatographic studies and by starch-gel electrophoresis (Smithies, 1955). The α, β and γ groups of enzymes ran and ran again at characteristic rates on DEAE columns under constant conditions of salt and pH, as illustrated in Fig. 5. The δ enzyme trails very badly at this concentration but, with 13 mM-borate–phosphate, chromatography on DEAE resolves it into three or four active components. We have not studied these in detail, and so they will be referred to as the δ group. In simple elution chromatography such as this, the same column can be used throughout and technical artifacts are much less likely to arise than in gradient or stepwise elution (see Porter, 1960).

Conditions for simple elution with CM could not be found and, with gradient elution, repeating the running of individual peaks showed the situation to be complex. Some of these second runs are shown in Figs. 6 and 7. α_2 ran again as α_2 and α_4, α_3 ran again as α_3 and α_4, and α_4 gave α_2, α_3 and α_4. This is interpreted as meaning that α_2 is occurring in two positions, some at α_2 and some at α_4, and that α_3 is behaving similarly. The α_4 will be complex, being a mixture of α_2 and α_3 in their slower-running positions. The β enzymes behave somewhat similarly. β_1 runs again correctly but β_2 gives rise to β_2 and β_4, and β_3 to β_3 and β_5. β_4 in turn gives rise to β_2 and β_4 and β_5 to β_3 and β_5. Similar behaviour is found in the γ and δ group enzymes. For some reason the two and three groups of enzymes each give rise to two zones on the column. The slower of these zones run together as α_4 in the α group and separately as β_4 and β_5 in the β group and also in the γ group as γ_4 and γ_5.

Thus there appear to be two genuinely distinct α enzymes, α_2 and α_3, three β enzymes, β_1, β_2 and β_3, two γ enzymes, γ_2 and γ_3, and three or more of the δ group of enzymes, though the last-named have not been examined in as great detail. A

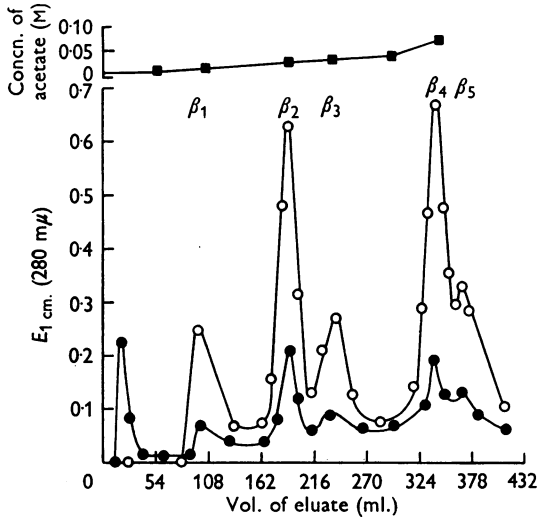

Fig. 4. Chromatography of the β enzyme (370 units) on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, 0·01–0·2 M, pH 5·5. Volume of mixing chamber, 250 ml. ●, Extinction coefficient; ○, units/0·2 ml. ■, acetate concentration in eluate.

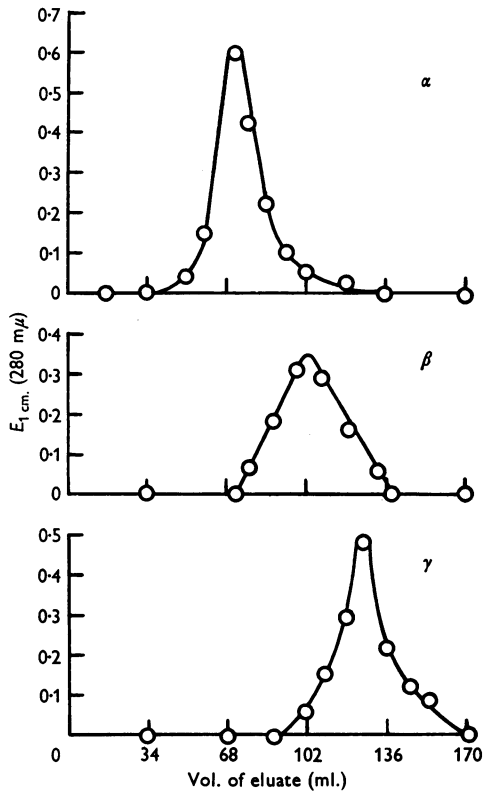

Fig. 5. Rechromatography of α, β and γ enzymes on a DEAE column 23 × 1 cm.² at constant buffer concentration and pH (sodium borate–phosphate buffer, 8·75 mM, pH 8·4). α eluted at R (defined by Martin & Synge, 1941) 0·32, β eluted at R 0·22, γ eluted at R 0·18. ○, Units/0·2 ml.

diagrammatic representation of these results is given in Fig. 8.

The behaviour of these enzymes was now studied in starch-gel electrophoresis and it proved possible to correlate many of the chromatographic results with those obtained with this quite different technique. Thus when purified enzymes are run in starch gel at pH 9·1 (Fig. 9) the most rapidly eluted α enzymes have the lowest mobility, β and γ enzymes move farther but are indistinguishable and

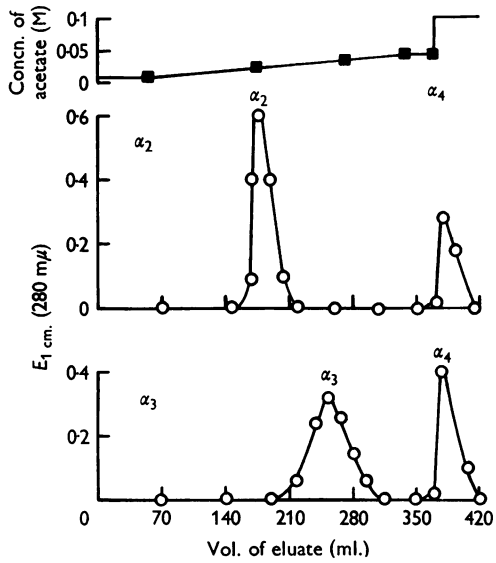

Fig. 6. Rechromatography of α_2 (136 units) and α_3 (155 units) enzymes on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, pH 5·5, 0·01–0·05 M, followed by a step to 0·1 M. Vol. of mixing chamber, 250 ml. ○, Units/0·2 ml.; ■, acetate concentration in eluate.

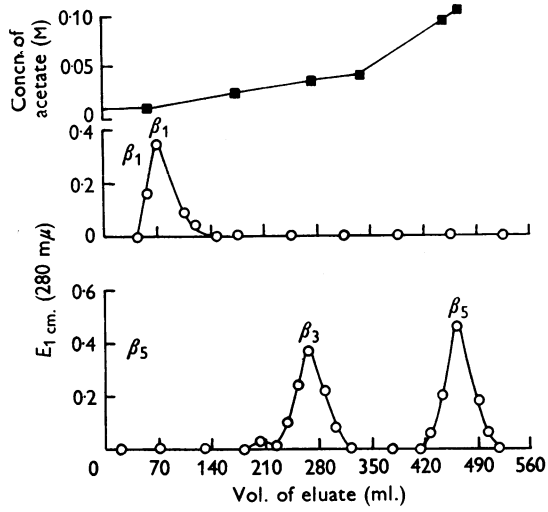

Fig. 7. Rechromatography of β_1 (136 units) and β_5 (250 units) enzymes on a CM column 25 × 1 cm.² by gradient elution with sodium acetate buffer, 0·01–0·2 M, pH 5·5. Vol. of mixing chamber, 250 ml. ○, Units/0·2 ml.; ■, acetate concentration in eluate.

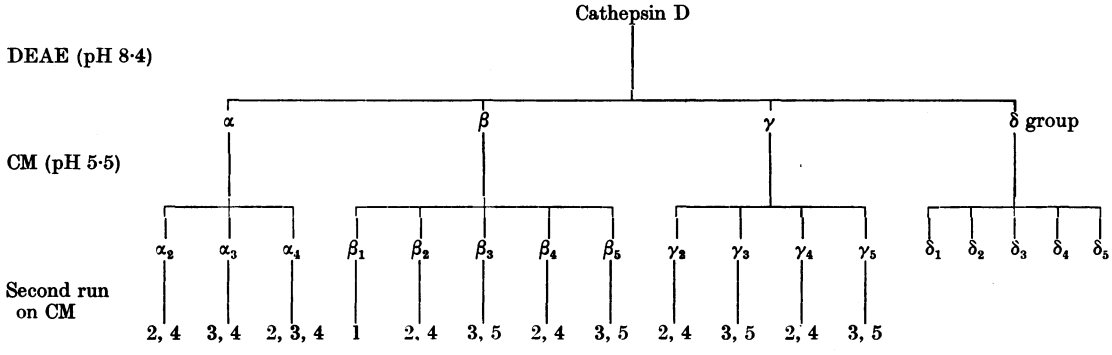

Fig. 8. Results of chromatography and rechromatography on CM of the α, β, γ and δ enzymes.

the δ group gives three components, the slowest one moving at the same rate as the β and γ. Apparently the δ enzyme is a mixture of several enzymes, as was shown by chromatography on DEAE with 13 mM-borate–phosphate buffer, but the β, γ and slowest δ components are not separable by electrophoresis under the conditions used.

At pH 5·5 (Fig. 10) α_2 and α_3 moved at the relative rates expected from chromatography on CM at pH 5·5, and α_4 proves to be a mixture of α_2 and α_3 as second running on the columns suggested. Similarly with the β enzymes (Fig. 11), 1, 2 and 3 move as expected, 4 runs as 2, and 5 runs as 3. There is therefore no evidence of the double zoning on starch-gel electrophoresis as found in chromatography. If the β enzymes are subjected to electrophoresis at pH 9·1 then they all have the same mobility, in agreement with the chromatographic results. Similarly all the α enzymes have the same mobility at pH 9·1 and are distinct from β enzymes in electrophoresis at the same pH. Thus the starch-gel electrophoresis results agree with the chromatographic results but the double zoning does not occur and the difference between β and γ enzymes at pH 8·4 can be picked out by chromatography but not by electrophoresis.

It therefore appears that cathepsin D can be separated into four or more groups differing in charge at pH 8·4 and that each group can be further subdivided into two or three components differing in their charge at pH 5·5.

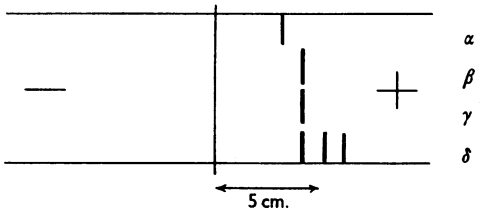

Fig. 9. Starch-gel electrophoresis of cathepsin D enzymes in 35 mM-tris buffer, pH 9·1; 8–2 v/cm. for 16 hr.

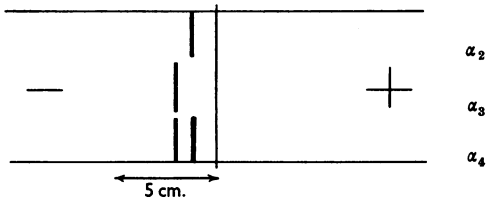

Fig. 10. Starch-gel electrophoresis of α enzymes in 30 mM-sodium acetate buffer, pH 5·5; 3–2 v/cm. for 16 hr.

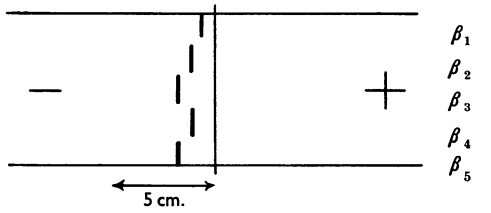

Fig. 11. Starch-gel electrophoresis of β enzymes in 30 mM-sodium acetate buffer, pH 5·5; 3–2 v/cm. for 16 hr.

Anomalous behaviour of cathepsin D on carboxymethylcellulose at pH 5·5

No satisfactory explanation has been found for the double-zoning phenomena of peaks 2 and 3 of the α, β and γ group of enzymes when run on CM at pH 5·5. At this pH the carboxyl groups of the ion-exchanger are not fully ionized and the percentage ionization is influenced by the salt concentration of the buffer (Peterson & Sober, 1956). Further, this pH is close to the isoelectric point of the protein, which appears to be about pH 6·0. Either of these phenomena might contribute to the anomalous behaviour, but electrophoresis in starch gel as well as chromatography has shown that satisfactory fractionation is dependent on working between pH 5·0 and 6·0. Alternative techniques, such as zone electrophoresis on cellulose columns and chromatography on the ion-exchange resin IRC 50, were tried but without success. Sulphomethylcellulose (Porath, 1957) was prepared, as the more strongly acidic groups are fully ionized at pH 5·5, but similar results to those on CM at the same pH were obtained. At pH 5·0 on SM, satisfactory resolution of the different enzyme peaks was not obtained. Use of other buffers such as citrate or phosphate, instead of acetate, did not affect the chromatographic behaviour, so a careful study was made of the effect of varying the pH between 5·5 and 6·0 with the CM columns. It was found that at pH 5·75 α_2 and β_2 would run as single peaks but that the α_3 and β_3 still gave a second peak in the α_4 and β_5 positions respectively, though less than when running at pH 5·5. At higher pH values some of the enzymes were not retarded on the column. For preparative purposes therefore pH 5·75 appears to be the optimum condition and in the most recent work it has been used for routine preparations.

Purity of cathepsin D fractions

Of the many forms of cathepsin D described only five have been subjected to detailed investigation of their purity and these have been taken principally from the α and β groups. The specific activity of these enzymes was found to be the same, 21 units of proteolytic activity/mg., within the error of the assay.

In starch-gel electrophoresis the enzymes gave only a single band in the two buffers used. As the

high resolving power of this technique is believed to depend on both charge and size (Smithies, 1955), it was expected that these enzymes would appear also to be monodisperse when examined in the ultracentrifuge. This was confirmed for α_3 and β_2. The $S_{20,w}$ was 3·3 and the molecular weight determined by the Archibald procedure was found to be 58 000. The two enzymes were indistinguishable by these criteria.

N-terminal amino acids were estimated by the fluorodinitrobenzene method and both enzymes (α_3 and β_2) gave *N*-terminal glycine, about 0·8 mole/mole of enzyme. There is presumably 1 mole of terminal glycine and the low figure found may arise because of the difficulty of correcting for the loss during hydrolytic liberation of this rather labile dinitrophenyl amino acid. Small amounts (less than 0·1 mole/mole) of terminal glutamic acid were also detectable but whether they arose from a contaminant or as an artifact of the estimation is not known.

Properties of cathepsin D

The properties of several of the α, β, γ and δ enzymes were studied and no differences between them could be found. The results reported are therefore believed to be applicable to all forms of the enzyme.

pH *optima*. The substrates were bovine haemoglobin and bovine serum albumin, both denatured with acid before use, and citrate buffer was used from pH 2·0 to 8·0. The results are given in Fig. 12. With haemoglobin as substrate the optimum is at pH 3·0, and with serum albumin at pH 4·2. As judged by this technique, the hydrolysis of serum albumin is only about one-fortieth of that of haemoglobin. The assay is a measure of the production of peptides soluble in 0·6 M-trichloroacetic acid and containing aromatic amino acids. It is unlikely to be directly related to the number of peptide bonds broken. However, the difference in susceptibility of the two substrates is great, so that serum albumin is clearly relatively resistant to hydrolysis by cathepsin D.

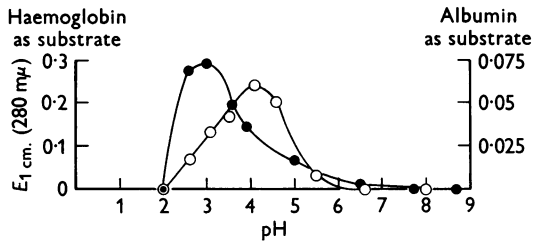

Fig. 12. pH optima of cathepsin D. ●, 0·002 mg./ml. of enzyme; acid-denatured haemoglobin (1·3%) as substrate; ○, 0·02 mg./ml. of enzyme; acid-denatured albumin (0·6%) as substrate.

Activation of cathepsin D. The haemoglobin activity of the crude spleen extract was increased about 20% by addition of cysteine to 0·01 M in the reaction mixture. The assay of pure cathepsin D was not, however, affected by the presence of 0·01 M-cysteine, 1 mM-iodoacetamide, 0·1 mM-*p*-chloromercuribenzoate or 1·5 mM-ethylenediaminetetra-acetate in the reaction mixture. It is therefore unlikely that sulphydryl groups play any role in the catalytic activity of the enzyme. The activation by cysteine, observed with the crude enzyme, is lost on the first CM column (Table 2). The disappearance of sulphydryl activation occurs at a step where no proteolytic activity is lost and is presumably therefore not due to the separation of a different sulphydryl-dependent enzyme. It may be that the cysteine is protecting an essential group of cathepsin D against partial inactivation by impurities but that this group is not itself a sulphydryl group.

Cathepsin D was incubated with di*iso*propyl phosphorofluoridate at 100 × mol.prop. of enzyme at pH 3·1 and pH 7·4 for 20 min. at 37° but its activity was unaffected.

Stability. Cathepsin D is heat-labile, all activity being lost after heating at neutral pH at 60° for 40 min. This in contrast to cathepsin C, which is stable under these conditions (Tallan *et al.* 1952). The influence of pH on the stability of the enzyme at 37° was measured and the results are given in Fig. 13. The activity was rapidly lost below pH 2·5 but was unchanged after 2 hr. at pH 6·5. The difference between the stability and pH optimum curves (say at pH 2·5 and 3·5) suggests that instability was due to acid denaturation rather than autolysis, though both may contribute. The loss of activity in acid solutions appears to be irreversible.

Specificity. Six peptides have been tested as substrates for cathepsin D: the substrate for cathepsin A and pepsin, benzyloxycarbonyl-L-glutamyl-L-tyrosine; the substrate for cathepsin B, benzoyl-L-arginine amide; the substrate for cathepsin C,

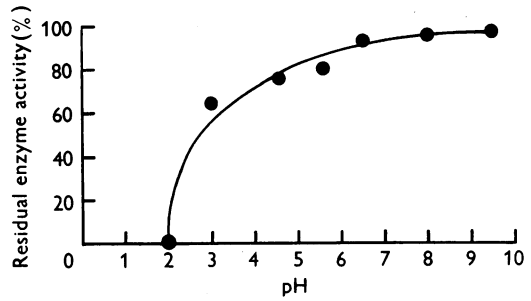

Fig. 13. pH stability of cathepsin D at 37°. ●, Percentage of original enzyme activity recovered after 2 hr. at 37° at different pH values.

glycyl-L-tyrosineamide; the pepsin substrates *N*-acetyl-DL-phenylalanyl-L-di-iodotyrosine, L-tyrosyl-L-cysteine and L-cysteinyl-L-tyrosine. No evidence of hydrolysis could be detected in any of these peptides.

In view of the failure to detect cathepsin A with certainty in the crude spleen extracts, the hydrolysis of benzyloxycarbonyl-L-glutamyl-L-tyrosine was investigated in some detail and the results are summarized in Table 3. At ten times the amount of crude extract used by Fruton & Bergmann some evidence of slight hydrolysis of the substrate was found, but it was still too close to the blank values to be certain that it was significant. Pure cathepsin D did not hydrolyse this substrate. The crystalline enzymes, pepsin and carboxypeptidase, in parallel experiments hydrolysed the peptide and the presence of free tyrosine in the hydrolysis products was confirmed by paper chromatography. It is clear that cathepsin D is unrelated to cathepsin A and the status of the latter enzyme is uncertain.

In view of the failure to find a synthetic peptide substrate for cathepsin D its action on the B chain of oxidized insulin was examined. Incubation was at 37° for 2 and 24 hr. with an enzyme: substrate ratio of 1/100 by wt. After 2 hr. the following peptides were found: 1, $Phe^{1}...Leu^{15}$; 2, $Tyr^{16}...Phe^{24}$; 3, $Tyr^{16}...Phe^{25}$; 4, $Phe^{25}...Ala^{30}$; 5, $Tyr^{26}...Ala^{30}$. [For definition of these abbreviations see the *Biochemical Journal* (1953), **55**, 5.]

After 24 hr. peptides 1, 4 and 5 were still present but peptides 2 and 3 had lost their *N*-terminal tyrosine. In addition the dipeptide Ala^{14}–Leu^{15} and free tyrosine were found. The peptide Phe^{1}–Glu^{13}, present in the 24 hr. hydrolysate, was only just separable from Phe^{1}–Leu^{15} under our conditions.

No difference in the specificities of the different forms of cathepsin D could be found. The results with the B chain are summarized in Fig. 14 and it can be seen that the specificity, though more restricted, is similar to that of pepsin, in spite of the failure of cathepsin D to hydrolyse any of the pepsin substrates tested.

Table 3. *Hydrolysis of benzyloxycarbonyl-L-glutamyl-L-tyrosine at* 37°

Enzyme	Protein N (mg./ml.)	Hydrolysis (%)	Time (hr.)
Crude spleen extract (Fruton & Bergmann, 1939)	0·25	53	2
Crude spleen extract	2·50	10	2
Pure cathepsin D	0·12	None	20
Pepsin	1·4	80	19
Carboxypeptidase	0·07	80	16

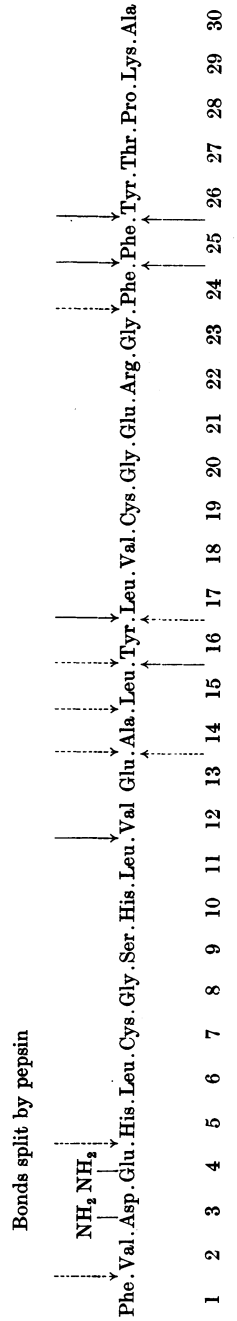

Fig. 14. A comparison of the digestion of the B chain of oxidized insulin by pepsin in 0·01 N-HCl (Sanger & Tuppy, 1951) and by cathepsin D in 0·04 M-acetic acid, pH 3·0. Bonds marked with unbroken arrows split rapidly; broken arrows indicate bonds that split more slowly.

DISCUSSION

The purification procedure described for the isolation of cathepsin D is tedious, in spite of the use of chromatographic procedures with a high resolving power and is in contrast with the one- or two-step methods which are successful for other proteins such as insulin (Porter, 1953) and chymotrypsinogen (Hirs, 1953) by similar techniques. It is noteworthy that several proteins will move as one component on DEAE even when simple elution at constant pH and salt concentrations is used (see, for example, the chromatogram of the α enzyme in Fig. 2). From the technical aspect the chromatography appeared to be entirely satisfactory: the enzyme had a finite partition coefficient and there was no question of an abrupt change of the coefficient from ∞ to 0 with change of pH and salt concentration such as Tiselius (1954) had suggested on theoretical grounds. The separations on ion-exchange columns are primarily, though not entirely, dependent on charge differences but attempts to make use of other properties such as solubility, in salt fractionation and liquid–liquid chromatography, were unsuccessful.

There seems little doubt that cathepsin D is the principal proteolytic enzyme of ox spleen as judged by the haemoglobin assay. More than 90% of the activity of a spleen mince could be extracted and about two-thirds of this activity was characterized as cathepsin D. In ox kidney, the proteolytic activity is only about two-thirds of that of the spleen, but again most of the activity is attributable to an enzyme which resembles cathepsin D closely and which may be identical with it (Cebra & Press, unpublished work). The kidney enzyme shows the same complexity as that of the spleen, making it improbable that this multiplicity of form is related to immunological function, since the kidney takes no part in antibody production. Cathepsins B and C account for some of the remaining activity but the presence of other uncharacterized proteolytic enzymes cannot be excluded, though in our work conclusive evidence for the existence of cathepsin A could not be obtained. It is probable that cathepsin D is identical with the enzyme partially purified by Anson (1939). Calculation of the relation of our different enzyme units suggests that the preparation which he obtained was already one-third pure. Cathepsin D may also be related to an enzyme obtained from ox lung (Dannenberg & Smith, 1955), which has a specificity similar to pepsin on the B chain of oxidized insulin but which will not hydrolyse peptide substrates for pepsin.

The low pH optimum on protein substrates (pH 3·0 for haemoglobin and pH 4·2 for serum albumin) is in agreement with the results of previous work on the proteases of the spleen (Anson, 1939), and in other tissues such as pituitary (Adams & Smith, 1951), brain (Kies & Schwimmer, 1942) and erythrocyte stroma (Morrison & Neurath, 1953). However, in both pituitary and stroma a second protease with an optimum at pH 7·0–8·0 was also found and, in one of the earliest reports on the spleen proteases (Hedin, 1904), it was stated that hydrolysis of fibrinogen could be demonstrated in both acetic acid and sodium carbonate solutions. We have been unable to find any significant activity with the haemoglobin assay, at neutral or alkaline pH, whatever method of extraction was used. The pH–activity curves of crude extracts followed closely those of the purified enzymes. A similar low pH optimum has been reported for another spleen enzyme, deoxyribonuclease (Koerner & Sinsheimer, 1957). As the cytoplasmic pH of cells such as monocytes and neutrophils lies in the range pH 4·7–5·5 (Sprick, 1956), and there is a possibility of local variation of pH in different cell structures, it seems probable that these enzymes may function near to their optimum pH.

The most surprising finding has been the large number of different forms in which cathepsin D has been isolated. The reproducibility of the results and the correlation between the quite different techniques of chromatography and electrophoresis in starch gel suggests that there is a genuine difference between the various enzymes. As the enzymes appear to be identical except for their charge at about pH 5·5 and about pH 8·4 the difference would be explicable by the presence of varying amounts of weak acidic and basic groups. For example, the α, β, γ and δ groups of enzymes may contain differing amounts of carboxylic or phenolic groups which are ionized above pH 8·0 but which are unionized at pH 5·5. Similarly, β_1, β_2 and β_3 enzymes may contain different amounts of a weak base such as an imidazole group which is ionized below pH 6·0 but unionized above pH 8·0.

Several possible explanations of this multiplicity of forms were considered. There is a marked variation in the total proteolytic activity of different spleens and in the relative content of the different forms of cathepsin D. Confusion due to the presence of varying amounts of extraneous material, such as blood, which cannot easily be washed out of a spleen, was eliminated by finding that the proteolytic activity of volumes of whole ox blood similar to the volume of the spleen was negligible. More likely was the production of different active forms by limited autolysis during extraction and purification. To test this, one spleen was divided into two portions immediately after mincing. One part was prepared as rapidly as possible and the other was allowed to stand at 2° for 1 week at

pH 4·6, when it was prepared independently. Figs. 1*a* and *b* show that the chromatograms on a DEAE column are identical within the limits of the method. In another experiment, one part of a spleen was prepared directly and another converted into an acetone-dried powder and stored at 2° before extraction. Again the same qualitative and quantitative results were obtained. Further, incubation of partially purified enzymes under a variety of conditions did not affect their chromatographic behaviour on DEAE and CM columns. If autolysis is responsible for the complexity found then it must occur very rapidly and not go beyond a predefined limit in each spleen. This seems improbable.

Another possible explanation is that a single enzyme protein was associated with differing amounts of an impurity or with differing impurities. However, the final products behaved as single components as judged by chromatography and starch-gel electrophoresis under different conditions. They appeared to be monodisperse in the ultracentrifuge and had only one *N*-terminal amino acid in approximately molar ratio, and all had the same specific enzymic activity. This would not exclude the possibility that the differences were due to small charged molecules bound to the protein but, if so, the binding must be exceptionally firm, as it was unaffected by different purification procedures, including ion-exchange chromatography, which would be expected to remove them. Specific binding of small molecules as firmly as this would itself probably imply structural differences between the different forms.

It seems probable therefore that cathepsin D does exist in ox spleen (and probably in ox kidney) in at least ten different molecular forms. Similar findings with other enzymes have been reported (see Markert & Møller, 1959 for references and also Aqvist & Anfinsen, 1959) and it has been suggested, from the work with haptoglobins (Smithies & Walker, 1955) and haemoglobin (Itano, 1957), that this complexity is a reflexion of genetic variations in individual animals. It remains to be investigated whether this is true of cathepsin D.

SUMMARY

1. A proteolytic enzyme has been isolated from bovine spleen, which accounts for two-thirds of the total proteolytic activity of the crude mince. It is present in at least ten forms which differ from one to another in their charge at pH 8·4 or 5·5.

2. The specific activity of the different forms of the enzyme is the same and their purity has been established by starch-gel electrophoresis at pH 9·1 and 5·5, by ultracentrifugal analysis and determination of *N*-terminal amino acid, which is found to be glycine.

3. Cathepsin D has a pH optimum of 3·0 with acid-denatured haemoglobin and 4·2 with acid-denatured albumin. It will not hydrolyse the synthetic substrates described by Fruton (1957–58) for cathepsins A, B and C nor will it hydrolyse the pepsin substrates *N*-acetyl-DL-phenylalanyl-L-diiodotyrosine, L-cysteinyl-L-tyrosine or L-tyrosyl-L-cysteine. The specificity of action on the B chain of oxidized insulin is similar to that of pepsin but more restricted. No difference in specificity could be detected between the different forms of the enzyme.

4. The proteolytic activity of cathepsin D is not affected by cysteine, iodoacetamide, *p*-chloromercuribenzoate, ethylenediaminetetra-acetate or di-*iso*propyl phosphorofluoridate.

5. Cathepsin D is heat-labile and activity is rapidly lost below pH 2·5 at room temperature.

We wish to thank Dr P. A. Charlwood for determining the sedimentation constant and molecular weight of cathepsin D, also Dr R. Pitt-Rivers for giving the three synthetic substrates for pepsin, and Mr A. Hemmings for synthesis of benzoyl-L-arginine amide and sodium monochloromethane sulphonate. We also wish to thank Mr A. Allen for technical assistance.

REFERENCES

Adams, E. & Smith, E. L. (1951). *J. biol. Chem.* **191**, 651.
Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Anson, M. L. (1939). *J. gen. Physiol.* **23**, 695.
Aqvist, S. E. G. & Anfinsen, C. B. (1959). *J. biol. Chem.* **234**, 1112.
Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.
Aronsson, T. & Grönwall, A. (1958). *Scand. J. clin. Lab. Invest.* **10**, 348.
Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.
Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, xii.
Dannenberg, A. M. & Smith, E. L. (1955). *J. biol. Chem.* **215**, 55.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
de la Haba, G., Cammarata, P. S. & Fruton, J. S. (1955). *Methods in Enzymology*, vol. 2, p. 64. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Fruton, J. S. (1957–58). *Harvey Lect.* **51**, 64.
Fruton, J. S. & Bergmann, M. (1939). *J. biol. Chem.* **130**, 19.
Greenbaum, L. M. & Fruton, J. S. (1957). *J. biol. Chem.* **226**, 173.
Hedin, S. G. (1904). *J. Physiol.* **30**, 155.
Hirs, C. H. W. (1953). *J. biol. Chem.* **205**, 93.
Itano, H. A. (1957). *Advanc. Protein Chem.* **12**, 215.
Kies, M. W. & Schwimmer, S. (1942). *J. biol. Chem.* **145**, 685.
Koerner, J. F. & Sinsheimer, R. L. (1957). *J. biol. Chem.* **228**, 1039.
Markert, C. L. & Møller, F. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 753.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.

Martland, M. & Robison, R. (1926). *Biochem. J.* **20**, 847.
Morrison, W. L. & Neurath, H. (1953). *J. biol. Chem.* **200**, 39.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
Porath, J. (1957). *Ark. Kemi*, **11**, 97.
Porter, R. R. (1953). *Biochem. J.* **53**, 320.
Porter, R. R. (1957). *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Porter, R. R. (1960). *Biochemical Handbook*. Ed. by Long, C. London: E. & F. N. Spon Ltd. (In the Press.)
Poulik, M. D. & Smithies, O. (1958). *Biochem. J.* **68**, 636.
Press, E. M. & Porter, R. R. (1958). *Abstr. 4th int. Congr. Biochem., Vienna*, 4–39.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1949). *Biochem. J.* **44**, 126.
Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 481.
Smith, E. L. (1951). *The Enzymes*, vol. 1, pt. 2, p. 793. Ed. by Summer, J. B. & Myrbäck, K. New York: Academic Press Inc.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Smithies, O. & Walker, N. F. (1955). *Nature, Lond.*, **176**, 1265.
Sober, H. A., Gutter, F. J., Wyckoff, M. M. & Peterson, E. A. (1956). *J. Amer. chem. Soc.* **78**, 756.
Sprick, M. G. (1956). *Amer. Rev. Tuberc.* **74**, 552.
Tallan, H. H., Jones, M. E. & Fruton, J. S. (1952). *J. biol. Chem.* **194**, 793.
Tiselius, A. (1954). *Ark. Kemi*, **7**, 443.

Acylative Decarboxylation with Particular Reference to the Acetylative Decarboxylation of the 2:4-Dinitrophenyl Derivatives of some Amino Acids

By J. C. CRAWHALL and R. W. E. WATTS
The Medical Unit, St Bartholomew's Hospital, London, E.C. 1

(*Received* 1 *July* 1959)

In studies on glycine metabolism in normal human subjects (Watts & Crawhall, 1959) and in patients with hyperoxaluria (Watts, Scowen & Crawhall, 1958) we needed to degrade 2:4-dinitrophenylglycine to yield the ^{13}C-labelled carboxyl-group carbon atom selectively as carbon dioxide for mass spectrometric analysis. We attempted to achieve this by oxidative means but were unable to limit the reaction to the carboxyl group. However, base-catalysed acetylative decarboxylation was satisfactory, and the present paper describes the procedure together with the result of an attempt to identify the reaction product other than carbon dioxide. The application of this reaction to the decarboxylation of hippuric acid and of some other dinitrophenyl-amino acids has also been studied. A small amount of carbon dioxide is formed when acetic anhydride and a basic catalyst are heated together in the absence of any other reactant, and we have investigated the time course of this reaction and the relative amounts of carbon dioxide which are evolved when different catalysts are used in order to reduce this to a minimum. The apparent acetylative decarboxylation of the dinitrophenyl derivatives of secondary amino acids is of some theoretical interest, for it is difficult to see how such compounds can form the intramolecular ring structures which have been postulated for the intermediates in this type of reaction (see, for example, Cornforth & Elliott, 1950). The rates of carbon dioxide evolution from dinitrophenylsarcosine and dinitrophenylglycine are not appreciably different and the possible significance of this observation will be discussed.

The acetylative decarboxylation reactions which we have observed may be considered to have an analogy with the biological synthesis of δ-aminolaevulic acid from glycine and succinyl-coenzyme A (cf. Gibson, Laver & Neuberger, 1958; and Kikuchi, Kumar, Talmage & Shemin, 1958). We therefore sought evidence for the succinylative decarboxylation of dinitrophenylglycine and of the related compound glyoxylic acid-2:4-dinitrophenylhydrazone. The latter reaction was of some further interest in view of the possibility of reducing the theoretical product (4-oxoglutaraldehyde-2:4-dinitrophenylhydrazone) to δ-aminolaevulic acid.

EXPERIMENTAL

Melting points were determined by the standard method, except where otherwise stated, and are uncorrected. Micro-analyses were performed by Weiler and Strauss, Oxford. [1-^{13}C]Glycine was synthesized as described previously (Watts & Crawhall, 1959).

Dinitrophenyl derivatives. The dinitrophenyl (DNP) derivatives of alanine, serine, cystine, phenylalanine, tyrosine, leucine and valine were a generous gift from Dr D. F. Elliott. DNP-sarcosine, DNP-proline, DNP-α-amino*iso*butyric acid, DNP-aspartic acid and DNP-[1-^{13}C]glycine were prepared by dissolving the amino acid in