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Separation of Acid and Neutral Proteinases of Brain

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1. Cerebral proteinases were separated on Sephadex G-100 columns into acid and neutral fractions free from cross-contamination. Acid proteinases were more stable and were purified by additional steps with salt and pH 5·0 precipitations, column chromatography on DEAE- or CM-cellulose and free-flow electrophoresis. 2. The separation made it possible to study the properties of the partially purified enzyme fractions. Some of these properties, such as K_m with selected protein substrates, pH optima and temperature-dependence in the presence and absence of substrates, are described. 3. No requirement for metal ions or added cofactors was demonstrated. Neutral-proteinase activity was more sensitive to inhibition by heavy-metal ions; its activity could be increased by thioglycollate and glutathione, and inhibited by thiol reagents. Neutral and acid proteinases were inhibited by the chymotrypsin inhibitor chloromethyl L-2-phenyl-1-toluene-*p*-sulphonamidoethyl ketone. 4. In the presence of the appropriate synthetic substrates no cathepsin A activity was found, and only trace quantities of cathepsin B or C activities, which were more than 50-fold less than cathepsin D-like activity.

Despite the known existence of proteinases in brain, there have been only limited studies on their distribution and characteristics (Lajtha, 1961; Marks & Lajtha, 1962, 1963*a*; Polyakova & Lishko, 1962; Palladin, Polyakova & Lishko, 1963; Lishko, 1963; Guroff, 1964). Two groups of proteinases have been distinguished on the basis of pH optima (Ansell & Richter, 1954*a*, *b*) and differential distribution in brain particulate fractions (Marks & Lajtha, 1963*b*): one group exhibits maximal action on denatured haemoglobin at pH 3·4–3·8 and the other at pH 7·6. The complete separation of these two groups of enzymes has not been previously reported, and the present study represents an attempt to obtain the separate groups in order to examine their properties and their relationship to protein metabolism in the living brain. A preliminary report of these results has been given (Marks & Lajtha, 1964).

METHODS

Preparation of brain extracts. Young adult male albino rats (Wistar strain) weighing 100–150g. were used. After exsanguination, brains were rapidly removed, weighed and homogenized in an all-glass Potter–Elvehjem homogenizer. Supernatant and precipitate fractions were prepared by centrifugation at $30000g_{av.}$ for 15min.

Acetone-dried powder of rat brain was prepared according to the method of Morton (1955). In other cases, brains were homogenized, suspended in 2vol. of 20mM-phosphate buffer, pH 7·6, and added slowly with stirring to 10vol. of acetone cooled to $-15°$; after filtration the acetone-insoluble material was washed with 3vol. of cold acetone and dried *in vacuo*. Acetone-dried powders could be stored at 0° in the presence of N_2 or *in vacuo*, but the highest activities were obtained if the acetone-dried powders (dried carefully at 0° under a flow of cold air for not more than 3hr.) were extracted immediately after drying.

Methods of following proteinase activity. The methods of incubation and determination of proteinase activity were as described by Marks & Lajtha (1963*b*). Haemoglobin substrate powder (urea-treated) prepared by the method of Anson (1938) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was substituted for acid-denatured haemoglobin; this has the advantage of greater solubility at pH 7·6, but because of increase of background colour on storage it was necessary to prepare fresh substrate for each experiment. Casein (Hammerstein grade; Mann Research Laboratories Inc., New York, N.Y., U.S.A.) was dissolved in distilled water, and the pH was adjusted with HCl to 7·6. The standard reaction mixture (final vol. 1ml.) for the neutral-proteinase assay contained 100–150μg. of enzyme, 30μmoles of tris–HCl buffer, pH 7·6, and 2mg. of denatured haemoglobin or 1mg. of casein. For the acid-proteinase assay the reaction mixture (final vol. 1ml.) contained 15–25μg. of enzyme, 30μmoles of acetate buffer, pH 3·8, or 25μmoles of barbital–acetate buffer, pH 3·4 (Michaelis, 1931), and 2mg. of denatured haemoglobin. Incubations were at 37° for 30min. in a rotary shaking bath, and the reaction was stopped by adding 0·5ml. of 18% (w/v) trichloroacetic acid. With purified preparations neutral

proteinase contained 100 units of activity and acid proteinase 200 units of activity per assay tube. The release of 1mμmole of α-amino group in 30min. at 37° was taken as 1 unit of enzyme activity. To obtain high sensitivity 1ml. of the trichloroacetic acid supernatant was used with the addition of 0·25ml. of 4M-acetate buffer, pH5·5, and then 1ml. of ninhydrin reagent. The method was sufficiently sensitive to detect 3μg. of the glutamic acid (which was used as the standard) or 20 units of activity. In some experiments proteinase activity was measured at 37° in the absence of buffer in a pH-stat (Metrohm) with a continuous recorder by the direct titration of 5ml. of reaction mixture with 0·02N-HCl at pH3·8 for acid proteinase and with 0·01N-NaOH at pH7·6 and 7·8 with a gentle stream of N_2 passed over the surface for neutral proteinase.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin as standard.

Assays with peptides. Cathepsin A activity was determined with N-benzyloxycarbonyl-α-glutamyl-L-tyrosine as substrate by the quantitative ninhydrin method. Cathepsin B was determined with benzoyl-L-arginine amide, and cathepsin C with glycyl-L-tyrosine amide acetate, glycyl-L-phenylalanine amide or L-prolyl-L-tyrosine amide as substrate, by the modified Conway diffusion method of Obrink (1955). The conditions selected for testing cathepsins were the optimum conditions suggested by Fruton (1960). In addition, trypsin was assayed with benzoyl-DL-arginine-β-naphthylamide hydrochloride by the method of Blackwood & Mandl (1961).

Gel filtration. The following Sephadex gels (Pharmacia, Uppsala, Sweden) were used: G-25 (medium grade), G-75 (medium grade), G-100 (140–400 mesh), G-200 (140–400 mesh), the water regains being 2·4, 7·4, 10 and 10ml./g. of dry gel respectively. All gels were soaked in buffer 2 days before use and stored in the cold. For gels stored for long periods, 1–2ml. of butan-1-ol was added as preservative. The distribution coefficient (Gelotte, 1959) was calculated from the formula:

$$K_D = (V_e - V_0)/V_i$$

V_0, the void volume, was determined by noting the appearance of the protein peak as monitored on a continuous-flow ultraviolet cell (Uviscan; Buchler Instruments, Fort Lee, N.J., U.S.A.): V_e was the volume of 20mM-phosphate or 20mM-tris–HCl buffer used to elute the different fractions; V_i, the internal volume, was calculated from the dry weight of gel added and the water-regain value.

Cellulose chromatography. DEAE-cellulose (anion-exchange capacity 0·92m-equiv./g.) and CM-cellulose (cation-exchange capacity 0·7m-equiv./g.) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) columns were prepared according to the methods of Peterson & Sober (1956) and Peterson & Chiazze (1962). The size of the column varied according to the volume of extract and its protein content; generally 100mg. of protein was placed on columns 18cm. × 2·5cm. containing 10g. of DEAE-cellulose powder. Elution was performed with a simple two-chamber device giving an approximate linear gradient. The gradient was measured in a conductivity cell previously standardized with a solution containing NaCl and 20mM-tris–HCl buffer.

Electrophoresis. The final purification step for acid proteinases was by using continuous free-flowing electrophoresis apparatus (Brinkmann Instrument Co., Westbury, N.Y., U.S.A.). Samples purified on DEAE-cellulose columns were concentrated to 1ml. and slowly injected into a glass electrophoresis chamber at a rate of 0·2ml./hr. in the presence of a stream of 50mM-tris–HCl buffer, pH7·6, or 3mM-phosphate buffer, pH6·8, at 2000v and a current of 110–120mA for 5–6hr.; 48 samples were collected and analysed for activity.

Disk-gel electrophoresis of brain fractions. Standard polyacrylamide gel conditions (Ornstein & Davis, 1961) were chosen with running conditions at 300v and 10mA. Gels were stained with 1% (w/v) Buffalo Black in 20% (v/v) acetic acid.

RESULTS

Enzyme extraction. Previous studies have shown differences between the localization of neutral and acid proteinases with a significant proportion of each enzyme being particle-bound (Marks & Lajtha, 1962). A preliminary survey was undertaken to study the extractability of both soluble and particle-bound enzymes from brain homogenates. Since homogenates contain endogenous activity, calculations for enzyme yield were made after correction for release of α-amino groups that occurred in the absence of added substrate. No differences were observed on simple extraction with low-concentration tris–hydrochloric acid or with phosphate buffers. In the presence of Triton X-100 and 0·15M-sodium chloride, conditions previously used for testing proteinase in particulate preparations (Marks & Lajtha, 1963b), there was a large increase in the extraction of acid proteinase (40–60%) without significant increase in the extraction of neutral proteinase. In an attempt to increase yields of neutral proteinase, homogenates were treated with phospholipase and lysolecithin. Snake venom was used as a source of phospholipase; the proteases contained in this preparation were removed by heating for 10 min. at 60°, which was without effect on phospholipase content (Imai & Sato, 1960). The addition of phospholipase (10 mg. to 5 ml. of brain homogenate containing 1·5 g. of tissue, and incubation for 2 hr. at 37°) gave only a slight increase in extractable protein without release of particle-bound proteinase (Table 1). However, homogenates treated with 8mM-lysolecithin by the method of Bauer, Matzelt & Schwarze (1962) resulted in a 3·7-fold rise in extracted protein, with some increase in enzyme yield but with a decrease in specific activity (Table 1).

A number of proteolytic enzymes exist as inactive zymogens that are activated by incubation in the presence of other proteinases native to the tissue (Desnuelle, 1960). Storage of intact brain tissue at 0° was without major effect on enzyme yield; however, incubation of aqueous homogenates led to a pronounced fall in activity (Table 1).

Table 1. *Extraction of cerebral proteinases*

After the preliminary treatments, homogenates were centrifuged at 30000*g* for 1hr. and the supernatants, containing 0·5mg. of protein, were tested for proteinase activity after incubation for 1hr. at 37°. At pH 7·6, the reaction mixture (final vol. 1ml.) contained 20μmoles of tris–HCl buffer and 5mg. of denatured haemoglobin; at pH 3·8, the reaction mixture (final vol. 1ml.) contained 30μmoles of acetate buffer and 2mg. of denatured haemoglobin. Results are expressed as percentages of the activity of the untreated homogenate, which gave at pH 7·6 an increase in acid-soluble α-amino group of 18μmoles, and at pH 3·8 of 35μmoles/g. fresh wt. of tissue/hr. (4) represents treatment before homogenization, and (5)–(7) represent treatments after homogenization but before centrifugation in the medium used in (3).

Treatment	Protein extracted in supernatant (%)	Enzyme yield (%) Acid proteinase	Neutral proteinase
(1) 20mM-Tris–HCl or 20mM-phosphate buffer, pH 7·6	15	37	34
(2) Buffer+NaCl (0·15M)	20	35	42
(3) Buffer+NaCl (0·15M), Triton X-100 (0·2%) and glutathione (0·5mM)	35	63	38
(4) Intact brain kept at 4° for 24hr.	20	26	35
(5) Brain homogenate kept at:			
(*a*) 4° for 24hr.	18	16	15
(*b*) 37° for 1hr.	17	18	30
(*c*) 37° for 24hr.	6	0	0
(6) Snake-venom phospholipase treatment	21	29	19
(7) Lysolecithin (8mM)	56	27	53

Separation of acid- and neutral-proteinase activities in aqueous extracts. Acetone-dried powders of rat brain were extracted by homogenizing with 10vol. of cold 20mM-tris–hydrochloric acid buffer, pH 7·6, and centrifuging at 100000 *g* for 40 min. Separation of the acid- and neutral-proteinase activities was achieved by gel filtration of the clear supernatant on Sephadex G-100 at 2° in 40mM-tris–hydrochloric acid buffer, pH 7·6.

Gel filtration. In long columns (80 cm. × 2·5 cm.) of Sephadex G-100, 45–60% of the neutral proteinase placed on the column emerged in the first 25 ml. of effluent that followed the void volume (peak I in Fig. 1). Acid proteinase appeared at 50–80ml. effluent volume and was accompanied by a pale-straw-coloured or red pigment (peak II in Fig. 1). The distribution coefficient of the proteinase

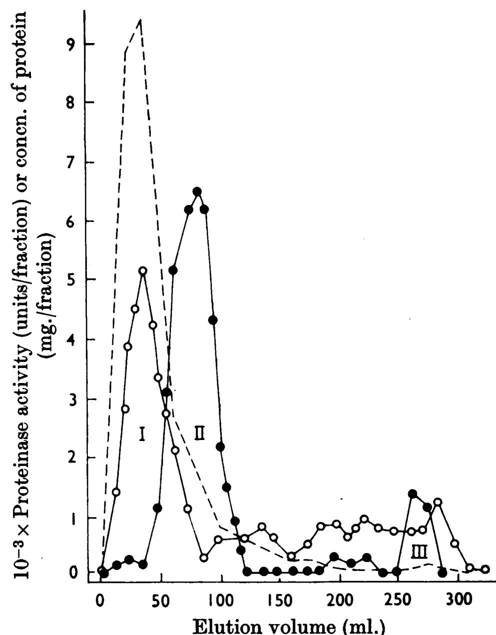
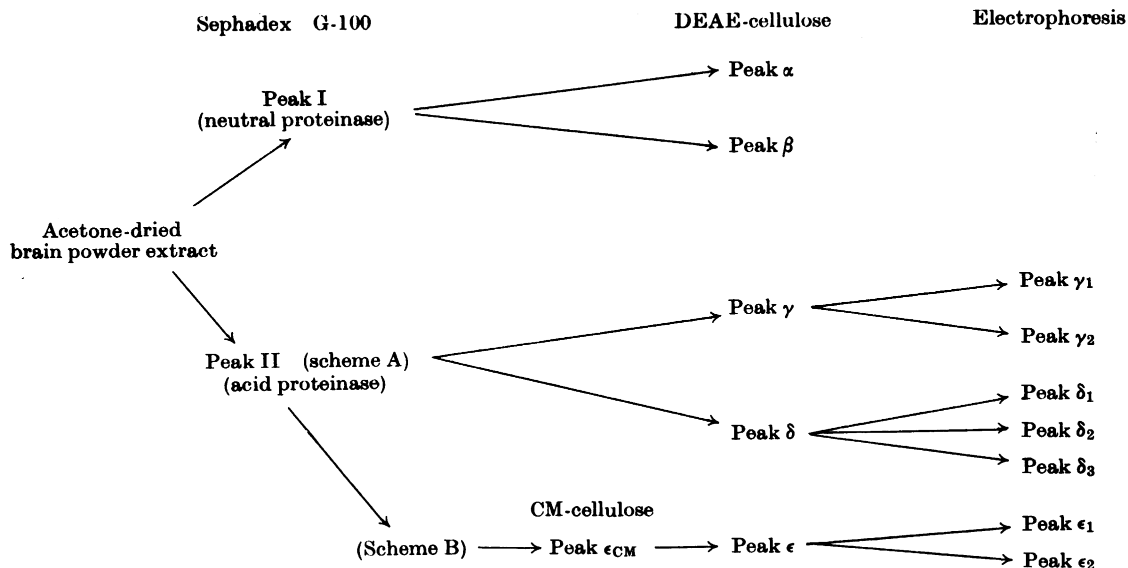


Fig. 1. Separation of cerebral neutral and acid proteinases after passage through a Sephadex G-100 column. Acetone-dried rat-brain powder (2g.) was extracted with 10vol. of tris–HCl buffer, pH 7·6, and centrifuged at 30000*g* for 20min. The supernatant (volume 18ml.) was placed on an 80cm. × 2·5cm. column and eluted with 40mM-tris–HCl buffer, pH 7·6, at a flow rate of 60ml./hr. ○, Neutral proteinase; ●, acid proteinase; the broken line denotes protein. The Roman numerals refer to the peaks described in the text. Standard assay conditions (see the Methods section) were used in this and in the following Figures and Tables unless specified otherwise.

indicates an approximate molecular weight for acid proteinase of 60000 and above 100000 for neutral proteinase. A highly labile third area of activity was frequently observed at 200 ml. effluent volume (peak III in Fig. 1); although this peak represented considerable purification compared with the first two peaks, there was no separation of acid- and neutral-proteinase activities. The first 10 ml. of peak I was completely free of acid proteinase, and the complete peak contained 10% or less of the acid proteinase present in the original extract. Peak II usually contained only traces of neutral proteinase, which could be inactivated by aging the solution for 7 days at 0° at pH 7·6. Acid proteinase was stable under these conditions. A similar separation was achieved with Sephadex G-200, but not with the more strongly cross-linked G-25 and G-75 gels.

Ion-exchange chromatography. Since acid proteinase was stable on storage at 0°, it was possible to obtain a substantial increase in purification with



Scheme 1. Purification of brain proteinases. Fractions were obtained after gel filtration on Sephadex G-100 and ion-exchange cellulose, and then submitted to carrier-free electrophoresis by the method of Hannig (1961).

Table 2. *Purification of acid proteinase*

Extracts and fractions were prepared, as described in the Methods section and illustrated in Scheme 1, from ten rat brains weighing 16·8g. Fractions obtained after DEAE-cellulose chromatography were freeze-dried and then submitted to free-flow electrophoresis by the method of Hannig (1961). Fractions obtained after electrophoresis are illustrated in Fig. 3.

	Volume (ml.)	Protein (mg.)	$10^{-3}\times$ Total units	Sp. activity (units/μg. of protein)
Homogenate	1680	1600	234	0·24
Tris buffer (pH 7·6) extract	36	320	235	0·88
Sephadex peak II	40	65	194	1·90
Precipitation at pH 5·0	25	32·5	180	5·54
$(NH_4)_2SO_4$ (15–60% saturated)	2	17·0	120	7·1
Scheme A				
Peak II on DEAE-cellulose				
Peak γ	1·5	0·8	27·2	34
Peak δ	1·5	1·3	37·9	29
Electrophoresis of peak γ				
Peak γ_1	25	0·2	10·2	51
Peak γ_2	25	0·1	5·8	58
Electrophoresis of peak δ				
Peak δ_1	15	(0·05)	6·0	120
Peak δ_2	25	0·1	6·8	68
Peak δ_3	20	0·1	9·0	90
Scheme B				
Peak ϵ_{CM}	15	8	64	8
Peak ϵ	1·5	3·3	58	17·6
Electrophoresis				
Peak ϵ_1	40	0·2	12·4	62
Peak ϵ_2	25	0·1	8·5	85

additional steps. These methods are summarized in Scheme 1, and the results for the individual steps in Table 2. Scheme A is not described in detail. In scheme B, the more effective method, ten rat brains weighing 18 g. yielded approx. 4 g. of acetone-dried powder, which, after extraction with 40 mM-tris–hydrochloric acid buffer, pH7·6, and gel filtration on Sephadex G-100, yielded about 40 ml.

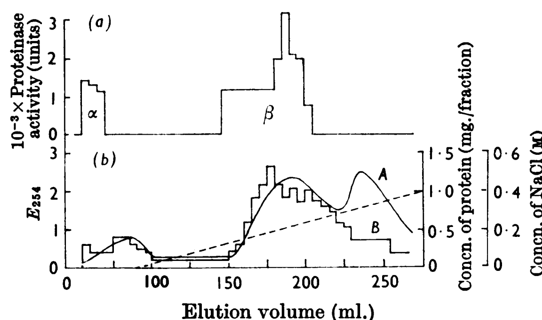


Fig. 2. Purification of neutral proteinase present in Sephadex peak I on a DEAE-cellulose column (20cm. × 2·5cm.). A 30ml. volume of peak I containing 40mg. of protein was placed on the column and eluted with 75ml. of 40mM-tris–HCl buffer, pH7·6, and then a 0–0·5M-NaCl gradient. (*a*) Proteinase activity tested under standard assay conditions. (*b*) Extinction at 254mμ (*A*) and concn. of protein determined by the Folin reagent (*B*); the broken line represents the salt gradient.

of Sephadex peak II (Fig. 1) with a protein concentration of 3–4 mg./ml. This fraction was adjusted to pH5·0 with acetic acid, and after standing 1 hr. at 0° was centrifuged at 30000 *g* and the coloured pigment removed from the supernatant by passage through 10 g. of CM-cellulose contained in a 20 cm. × 2 cm. column. All activity and colour were retained on the column at pH5·5 (10 mM-acetate buffer), but activity with only a trace of colour could be eluted with phosphate buffer, pH6·7, yielding one protein peak (ϵ_{CM}). The acid proteinase was further purified by passage through 10 g. of DEAE-cellulose with phosphate buffer, pH7·6 (peak ε). Protein retained on the column and eluted with 0–0·2M-sodium chloride was active; that eluted with 0·2–0·5M-sodium chloride was inactive. With Sephadex peak I (neutral proteinase) the activity eluted in the first peak (α in Fig. 2) represents 12% of the original activity with maximal purification 20-fold (Table 3). The second peak (β in Fig. 2) eluted with 0·1–0·2M-sodium chloride represents 22% of the activity placed on the column with an 11-fold purification, although in some preparations 30–50-fold enrichment was obtained. Rechromatography of all peaks on DEAE-cellulose resulted in single peaks that emerged at the expected salt gradient.

Electrophoresis. Fractions with the highest activity were pooled and submitted to carrier-free electrophoresis by the method of Hannig (1961). DEAE-cellulose fractions (peaks γ and δ in scheme A) yielded further two or three components having

Table 3. *Separation of acid and neutral proteinases*

The starting material for the purification procedure was 2·2g. of acetone-dried powder prepared from 10g. of rat brain as described in the Methods section. The composition of the extract made with 10vol. of tris–HCl buffer, pH7·6, was 210mg. of protein, 35000 units of neutral proteinase and 240000 units of acid proteinase.

	Yield (%)		Sp. activity (units/μg. of protein)		Purification	
	Neutral proteinase (at pH7·6)	Acid proteinase (at pH3·8)	Neutral proteinase (at pH7·6)	Acid proteinase (at pH3·8)	Neutral proteinase (at pH7·6)	Acid proteinase (at pH3·8)
Acetone-dried powder extract	(100)	(100)	0·17	1·1	(1·5)	(4·5)*
Sephadex G-100						
Peak I	45	0	0·22	0	2·0	—
Peak II	0†	65	0	2·4	—	11
Peak I on DEAE-cellulose						
Peak α	12	0	2	0	18	—
Peak β	22	0	1·2	0	11	—
Peak II on DEAE-cellulose						
Peak γ	0	8	0	12	—	54
Peak δ	0	27	0	24	—	110

* Purification calculated on activity in fresh homogenate.
† Neutral proteinase associated with peak II inactivated by aging for 3 days at pH7·6 and 0°.

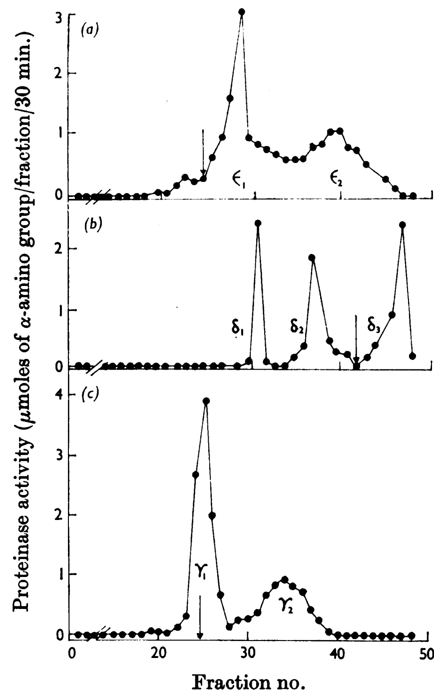


Fig. 3. Distribution patterns of acid proteinase after carrier-free electrophoresis. Enzyme solution was injected at the rate of 0·25ml./hr. in 25mM-tris–HCl buffer, pH 7·6, for the DEAE-cellulose peaks (peaks γ and δ) in (*c*) and (*b*), and in 3mM-phosphate buffer, pH 6·8, for CM-cellulose-purified enzyme (peak ϵ) in (*a*). Conditions were: (*a*) 2000v, 120mA at 2°; (*b*) 2100v, 130mA; (*c*) 2000v, 110mA. The arrows denote points of injection of sample into the electrophoresis chamber.

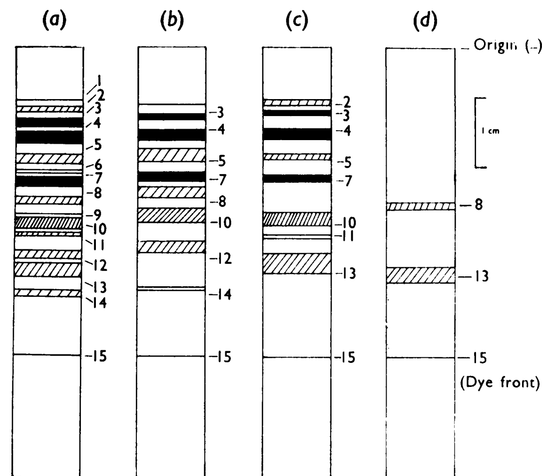


Fig. 4. Disk-gel electrophoresis of brain fractions on polyacrylamide gels (for details see the Methods section): (*a*) brain extract; (*b*) Sephadex peak I; (*c*) Sephadex peak II; (*d*) purified acid proteinase (peak ϵ_1). Shading represents relative intensity of bands.

increased purification (Fig. 3). CM-cellulose fractions (peak ϵ_{CM}) yielded two anodic components. As a further check on homogeneity, selected fractions were analysed by disk-gel electrophoresis. Examples of the typical band pattern obtained are illustrated in Fig. 4. In confirmation of the results for free-flow electrophoresis, it is seen that the number of bands present in the original brain extracts is reduced from approximately 15 to two. With serum samples done in parallel under identical conditions, band 13, which was present in the highest concentration, approximated the position of albumin, and the minor component 8 was in the position of a post-albumin (Ornstein & Davis, 1961).

Properties of purified neutral and acid proteinases. Previous work with proteinases derived from cerebral subcellular fractions (Marks & Lajtha, 1963*b*) showed that a number of proteins were good substrates. In the presence of excess of substrate, enzyme activity was linear with time up to about 30 min. with both purified neutral and acid proteinases. Activity was linear with time up to 30 min. also at lower concentrations of the substrates, as tested with a pH-stat. Enzyme activity under standard assay conditions was also proportional to the concentration of the enzyme. The enzyme used for these studies (DEAE-cellulose peaks β and δ in Scheme 1) gave no increase in ninhydrin-positive material on incubation in the absence of added protein substrate. Similar results were obtained also with Sephadex peaks I and II.

With acid proteinase (peak δ) haemoglobin was a better substrate than globin; maximal action occurred at about 2 mg. of haemoglobin and 2·5 mg. of globin. In 1 ml. of reaction mixture containing 15μg. of protein and 200 units of activity, the Michaelis constant, K_m, derived by Lineweaver–Burk plot was 0·51 mg. ($7{\cdot}6\times10^{-3}$M) for haemoglobin and 0·65 mg. ($1{\cdot}7\times10^{-2}$M) for globin, with maximal velocities respectively of 0·21 and 0·19μ-mole of amino group split in 30 min. (Fig. 5).

For purified neutral-proteinase preparations, maximal action on haemoglobin occurred at 2–3 mg. of haemoglobin and 1–2 mg. of casein in 1 ml. of reaction mixture containing 100μg. of enzyme and 100 units of activity. The K_m calculated for preparations free of endogenous activity by the method of reciprocal plots was 1·76 mg. ($2{\cdot}7\times10^{-2}$M) for haemoglobin and 0·56 ($1{\cdot}6\times10^{-3}$M) for casein, with maximal velocities of 0·1 and 0·12μmole respectively (Fig. 5). Because neutral proteinase was more labile than acid proteinase, the enzyme

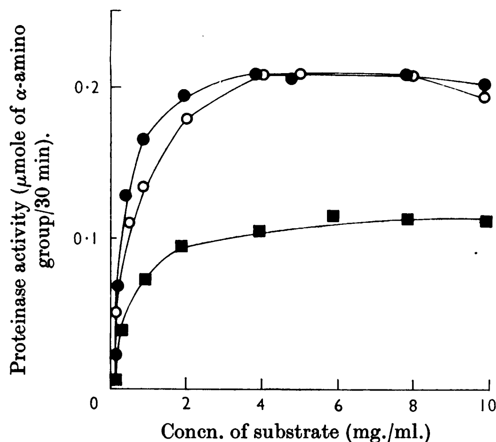


Fig. 5. Comparison of rates of protein breakdown with different substrates. The substrate for neutral proteinase (peak β) was denatured haemoglobin (■); that for acid proteinase (peak δ) was denatured haemoglobin (●), and globin (○).

was used within 3 days of preparation. It showed its greatest stability when stored at pH 5·5 in the cold, and could be kept for 1–2 weeks.

The pH optima of the acid (peak δ) and neutral (peak β) proteinases are shown in Fig. 6: the neutral proteinase is inactive at the optimum pH for the acid enzyme and the acid proteinase shows no activity at neutral pH.

A temperature of 37° was selected for the standard assay conditions to approximate the physiological conditions of protein breakdown in the living brain. However, both acid and neutral proteinases did exhibit higher activities with increase of temperature, with maxima at about 55° [(1) in Table 4], although at these higher temperatures the enzymes rapidly deteriorated when maintained for short periods in the absence of substrate [(2) in Table 4]. At temperatures below 60° the enzymes could be partially protected from thermal inactivation by incubation in the presence of haemoglobin [(3) in Table 4].

Effect of metal ions on proteinase activities. Purified neutral-proteinase preparations showed greater sensitivity to inhibition by metal ions than did acid proteinase (Table 5). Metal ions such as Co^{2+} and Mn^{2+} that are required for optimum brain peptidase activity strongly inhibited neutral proteinase at concentrations that inhibit acid proteinase by 10% or less. An almost complete inhibition was observed with Cu^{2+}, Cd^{2+}, and Zn^{2+}. The metal ions Fe^{2+} and Fe^{3+} were without effect at 0·1 mM. There was some increase in neutral-proteinase activity with Ca^{2+}; this effect varied

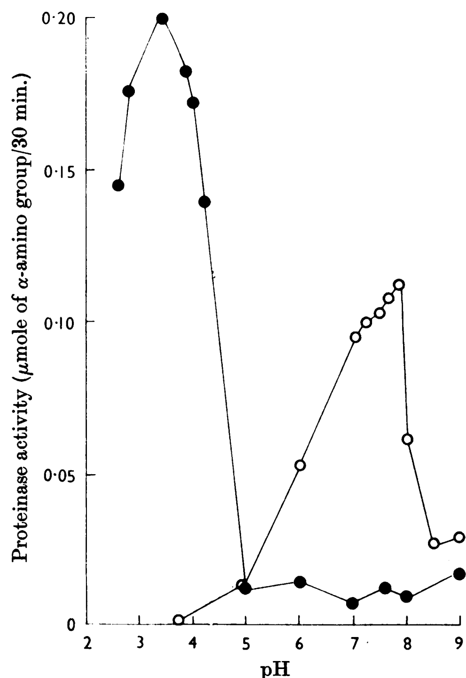


Fig. 6. pH-dependence of protein breakdown. The buffer systems employed were 25 mM-barbital–acetate buffer, pH 2·6–9·0, and 25 mM-tris–HCl buffer, pH 7·2–8·5. ●, Acid proteinase (peak δ); ○, neutral proteinase (peak β).

Table 4. *Temperature optima and temperature sensitivity of cerebral proteinases*

(1) denotes proteinase activity with haemoglobin as substrate, measured after 15 min. at the different temperatures. (2) gives the results for enzyme maintained at these temperatures for 15 min. without substrate, and then incubated with substrate (haemoglobin) for 30 min. at 37°. (3) gives the result for enzyme maintained at the various temperatures for 15 min. in the presence of substrate and then incubated (with substrate) for 30 min. at 37°. Values in (2) and (3) are given after correction for activity during the first 15 min. treatment period. The values are the averages of six determinations agreeing within 10%.

	Proteinase activity (mμmoles/30 min.)					
	Neutral proteinase (at pH 7·6)			Acid proteinase (at pH 3·8)		
Temp.	(1)	(2)	(3)	(1)	(2)	(3)
0°	0	110	100	0	210	240
27	50	110	100	120	230	220
37	100	100	110	240	210	210
45	120	90	120	320	120	200
55	180	60	80	440	10	150
65	0	0	40	150	1	10

Table 5. *Effect of metal ions on purified acid and neutral proteinases*

Acid- and neutral-proteinase activities were determined under standard assay conditions. Results are expressed as percentage changes of activity with added metal ions compared with determinations without additions and run in parallel. The values are the averages of six determinations.

		Percentage change	
Metal ion added	Concn. (mM)	Neutral proteinase (at pH 7·6)	Acid proteinase (at pH 3·8)
Cu^{2+}	0·1	−30	−13
	1·0	−100	−26
Zn^{2+}	0·1	−15	−6
	1·0	−83	−20
Cd^{2+}	0·1	−30	−1
	1·0	−100	−4
Co^{2+}	0·1	+15	−9
	1·0	−100	−8
Mn^{2+}	0·1	0	−11
	1·0	−17	−14
Fe^{2+}	0·1	0	0
	1·0	−19	0
Fe^{3+}	0·1	0	+4
	1·0	−40	−2
Mg^{2+}	0·1	0	0
	1·0	0	0
Ca^{2+}	0·1	0	0
	1·0	+15	−5

with preparations and was in the range 10–25%. No similar effect was observed with Mg^{2+}.

Effect of inhibitors. Neutral proteinase was also more sensitive to the effect of other inhibitors. Activity was inhibited by *p*-chloromercuribenzoate, and markedly decreased by *o*-iodosobenzoate. No major effect on acid or neutral proteinase was observed with EDTA or soya-bean trypsin inhibitor. Increased activity was observed with thioglycollate and low concentrations of reduced glutathione. Ascorbate and the specific chymotrypsin inhibitor chloromethyl L-2-phenyl-1-toluene-*p*-sulphonamidoethyl ketone (TPCK; Schoellmann & Shaw, 1963) caused marked inhibition of both acid and neutral proteinases (Table 6). The inhibition by the latter compound points to the similarity between the active centres of the brain proteinases and chymotrypsin.

Proteinase activities with synthetic substrates. Proteinase assays in the presence of synthetic substrates are less sensitive than with protein substrates (Fruton, 1960); therefore correspondingly

Table 6. *Effect of various compounds on purified acid and neutral proteinases*

Results are expressed as percentage changes from the activity without added inhibitors. The values are the averages of six determinations.

		Percentage change	
	Concn. (mM)	Neutral proteinase (at pH 7·6)	Acid proteinase (at pH 3·8)
p-Chloromercuribenzoate	0·5	−100	−10
o-Iodosobenzoate	1	−60	−10
Chloromethyl L-2-phenyl-1-toluene-*p*-sulphonamidoethyl ketone	1	−40	−70
Ascorbate	5	−48	−10
Thiosulphate	5	−20	−25
Thioglycollate	5	+20	0
Glutathione	0·1	+25	0

larger amounts of enzyme and longer incubation periods were employed. At pH 3·4, the optimum condition for splitting haemoglobin, no activity was detected with synthetic substrates even in the presence of thiols, which are required for a number of proteolytic enzymes. Thus no activity was observed with benzoyl-DL-β-naphthylamide or benzoyl-DL-arginine anilide (trypsin substrates). *N*-Benzyloxycarbonyl-α-glutamyl-L-tyrosine (cathepsin A substrate), benzoyl-L-arginine amide (cathepsin B substrate), glycyl-L-tyrosine amide or glycyl-L-phenylalanine amide (cathepsin C substrates). However, purified acid proteinase tested at pH 5·3 with these substrates in the presence of 2 mM-cysteine revealed some cathepsin B and C activity (Table 7). Purified neutral-proteinase fractions also showed some cathepsin B activity at pH 5·3 and cathepsin C activity with L-prolyl-L-tyrosine amide at pH 7·0 (Table 5). All catheptic activities observed were some 50-fold less than the protein-splitting activity at pH 3·4. In purified preparations catheptic activity was not observed in the absence of cysteine, and it could be inhibited by 1 mM-iodoacetate.

DISCUSSION

Homogeneity. Although acid proteinases could be separated from the enzymes active near neutral pH, it is most likely that each fraction consists of a group of enzymes. The band patterns of the gel electrophoresis also indicate the heterogeneity of the various fractions. The fact that each of the two groups of enzymes (or at least the major portion of these enzymes) can be eluted in a single peak from the Sephadex column shows that a number of properties within a group must be similar. A further

Table 7. *Comparison of acid-proteinase activities in the presence of haemoglobin and synthetic substrates*

Extracts were prepared from ten rat brains which weighed 18g. and yielded 4g. of acetone-dried powder. For determination with denatured haemoglobin (cathepsin D), standard assay conditions were employed at pH 3·4 with barbital–acetate buffer. For determinations with synthetic substrate, enzyme equivalent to 3000–5000 units of cathepsin D was incubated in the presence of 10μmoles of substrate for 2hr. at 37° in 40mM-acetate buffer, pH 5·5 or 5·3. In some experiments for cathepsin C, glycyl-L-phenylalanine amide acetate was substituted as substrate and found to give identical results. Each value is the mean of six determinations agreeing within 10%.

		Activity (μmoles of α-amino group/g. fresh wt. of brain/hr.)		Activity (μmoles of NH_3/g. fresh wt. of brain/hr.)	
Fraction	Concn. of protein (mg./fraction)	Cathepsin D (with haemoglobin at pH 3·4)	Cathepsin A (with *N*-benzyloxy-carbonyl-α-glutamyl-L-tyrosine at pH 5·5)	Cathepsin B (with benzoyl-L-arginine amide at pH 5·3)	Cathepsin C (with glycyl-L-tyrosine amide acetate at pH 5·3)
Acetone-dried powder extract	30	24·8	0	0·41	0·51
Sephadex peak I	9·2	—	0	0·25	0·78 0·70*
Sephadex peak II	5·9	15·8	0	0·22	1·05 0·95*
DEAE-cellulose peak α	0·25	0	0	<0·05	0·10
DEAE-cellulose peak β	1·20	0	0	<0·05	<0·05
DEAE-cellulose peak γ	0·72	2·0	0	0·07	0·06
DEAE-cellulose peak δ	0·42	9·2	0	0·23	0·24

* L-Prolyl-L-tyrosine amide (10μmoles) tested at pH 7·0.

example of similar properties within a group is the inhibition by Cd^{2+} and Co^{2+} of all the neutral enzymes with no significant effect on acidic enzymes. The effect of Co^{2+} on proteinase activity is of interest in view of the fact that brain tissue contains a Co^{2+}-activated dipeptidase (Uzman, Rumley & van den Noort, 1963). The activity of neutral proteinase in the absence of Ca^{2+} and the only slight increase of activity in the presence of this ion indicates that the brain neutral proteinase described by Guroff (1964), which has an absolute requirement for Ca^{2+}, can be only a minor component of our neutral enzyme group. In this connexion, Lewis (1963) reported a proteinase contaminant of pituitary growth-hormone preparation that split haemoglobin at pH 7·0 but like the present brain neutral proteinase did not exhibit a requirement for metal ions or thiols. The method of extraction of brain tissue employed in the present work (as well as other usual extraction methods) leaves a large proportion of cerebral proteins unextracted. It is possible that proteinases with other properties are present in the insoluble fraction not analysed in the present study. New properties of brain proteinases could be further established by the use of other substrates and the addition of possible cofactors.

Acid proteinase. Brain acid proteinase shows similarity to cathepsin D (Press, Porter & Cebra, 1959) that extends to the pH optima with haemoglobin as substrate, the effects of synthetic substrates, metal ions, inhibitors and other compounds, and the finding that it exists in more than one form. Cathepsin D-like activity accounted for over 90% of the haemoglobin-splitting activity, but traces of cathepsins B and C could be detected in the presence of the appropriate synthetic substrate at optimum conditions for testing such enzymes. It is unlikely that cathepsins A, B and C were shed, as observed by Press *et al.* (1959) during procedures for purification of spleen cathepsin D, since no cathepsin A and only low concentrations of cathepsins B and C were observed in the original crude extract. The heterogeneity of cathepsin D is also unlikely to have been due to limited autolysis, since extracts were without endogenous activity. An attempt was made to study the various forms by using a variety of fractionation procedures, since different forms of enzymes could be lost by retention on chromatographic columns or in other ways. The homogeneity of the various forms was further checked with disk-gel electrophoresis. With purification by gel filtration, cellulose chromatography and free-flow electrophoresis, the number of bands detected by disk-gel electrophoresis was reduced from approximately 15 to 2. Band 13 (Fig. 4) appeared in the highest concentration and ran parallel with albumin when compared with serum. It would be expected on the basis of gel filtration that neutral proteinase would have the molecular size equivalent to

globulins and that acid proteinase would correspond to proteins of smaller molecular dimension.

Synthetic substrates. No activity was observed in the presence of synthetic substrates at pH3·4 with and without cysteine, even with 10–25-fold enzyme compared with that used for cathepsin D assays. The absence of activity in the presence of *N*-benzyloxycarbonyl-α-glutamyl-L-tyrosine and cysteine at pH3·4 excludes the presence of catheptic carboxypeptidase in brain extracts (Greenbaum & Sherman, 1962). At pH5·3 and in the presence of cysteine, a small cathepsin B- and C-like activity was found; however, even if allowance is made for a tenfold difference in haemoglobin-splitting ability between brain and spleen (Marks & Lajtha, 1963*b*), the concentrations found in brain are considerably lower than in spleen and in most other tissues. Bouma & Gruber (1964) reported trace quantities of cathepsins B and C in crude brain homogenates (less than 5% of the concentrations in the spleen). The concentration is also comparable with rates found by Lapresle & Webb (1962) for bone marrow; it can be calculated from their data that cathepsin B and C substrates were hydrolysed at a rate of 0·2 and 0·6%/hr./mg. of protein, compared with rates found for brain of 0·1–0·3%/hr./mg. of protein. The ratio of cathepsin D activity to cathepsin B and C activity did not change significantly on separation of peak II into peaks γ and δ or on further purification. No functional role for cathepsins has been accepted, but it is noteworthy that cathepsin B and especially D are associated chiefly with lysosomal fractions (de Duve, Wattiaux & Baudhuin, 1962).

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