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Protein Breakdown in the Brain

SUBCELLULAR DISTRIBUTION AND PROPERTIES OF NEUTRAL AND ACID PROTEINASES

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Protein turnover in brain requires not only synthesis, but also breakdown, as part of the dynamic state. Although considerable information is now available on protein turnover in brain *in vivo* (Waelsch & Lajtha, 1961), the mechanisms relating to breakdown are largely unknown. Previous studies indicated two proteinase families in brain dispersions, one active in the acid region, the other at a physiological pH range (Kies & Schwimmer, 1942; Adams & Smith, 1951; Ansell & Richter, 1954*a*, *b*). Lajtha (1961) has shown that mitochondrial preparations in media buffered at pH 3·8 and pH 7·6 possess the highest endogenous proteolytic activity, followed by lesser activities in the nuclear, microsomal and microsomal-supernatant preparations. The findings indicate the presence of two distinct proteinase systems in brain; however, questions related to their isolation and identification, or the possibility of their associations with specific subcellular organelles similar to those described for liver catheptic activity (lysosomes; de Duve, Berthet & Beaufay, 1959), have not been resolved. In the present study assay procedures were adapted for the study of the distribution of cerebral proteinases in rat-brain subcellular fractions in the presence of native and denatured protein substrates, but, because such subcellular fractions are known to be heterogeneous, further fractionations with a variety of sucrose-gradient techniques were employed. This was an attempt to separate active organelles in order to study possible functional relationships and sites of action and to facilitate the subsequent isolation and study of the various factors participating in the breakdown of cerebral proteins. Conditions for testing proteolytic activity were further explored in the presence of protein substrates with a view to selecting a suitable assay method for the rapid determination of cerebral proteinases. A preliminary report of the differential distribution of cerebral proteinases in mitochondrial subfractions has been given (Marks & Lajtha, 1962).

METHODS

Tissue and method of homogenization. Young adult male albino rats (Sherman strain) weighing about 100 g. were used in all experiments. After exsanguination, brain and in some experiments other tissues, such as liver, kidney, spleen and muscle, were promptly removed, weighed on tared tinfoil, and homogenized at 0° in an all-glass Potter–Elvehjem homogenizer. To ensure reproducibility the pestle was moved five times in each direction vertically during a total time of 1 min. at approx. 2500 rev./min. In a few cases, brain tissue was homogenized in a glass homogenizer fitted with a loose and tight ball pestle (Dounce, Witter, Monty, Pate & Cottono, 1955), or in the Perspex homogenizer of Aldridge, Emery & Street (1960). Subcellular fractions were checked by phase-contrast microscopy. The homogenates were centrifuged in several experiments at

$30000g_{av.}$ for 15 min. to yield a supernatant and precipitate fraction designated 'M1' and 'M2' in the text.

Media for homogenates. The medium employed for extraction and incubation of neutral proteinases was: 0·154M-NaCl in 0·02M-phosphate buffer (prepared with 0·02M-Na_2HPO_4 and adjusted to pH 7·6 with 0·2M-KH_2PO_4), containing Triton X-100 (0·2%), CoA (0·13 mM), ATP (1 mM), phosphocreatine (2 mM) and glutathione (0·5 mM). For experiments in acid conditions, the medium was 0·154M-NaCl in 0·05M-acetate buffer, pH 3·8, containing Triton X-100 (0·2%).

Preparation of protein substrates. Acid-denatured haemoglobin was prepared by a modification of Herriott's (1955) method. A sample (800 mg.) of salt-free haemoglobin (freeze-dried; Mann Research Laboratories) was dissolved in 18 ml. of water, and 0·6N-HCl was added to give pH 1·8. After incubation for 1 hr. at 37°, the pH was restored to the appropriate value with 0·6N-NaOH, with a pH-meter; 4 ml. of buffer was added together with sufficient NaCl after correction for alkali addition to give a final concentration of 0·154M. Final adjustment with water gave a 2·2% (w/v) protein solution. Albumin and other substrates were treated in identical fashion.

Proteins used were from the following sources: α-globulin (bovine) fraction II, γ-globulin (porcine), fibrinogen (bovine), globin and edestin were all from Nutritional Biochemicals Corp.; albumin (bovine) fraction V and casein were from Calbiochem. Inc.

Method for following proteinase activity. Incubations were done at 37° in a gyratory-shaker bath (New Brunswick Scientific Co.) with a reaction mixture that contained 0·5 ml. of medium with and without protein substrate, and 0·5 ml. of tissue preparation diluted with the appropriate buffer to contain 1 mg. of particulate protein. The pH-dependence of proteinases in whole-rat-brain homogenates has been measured by Lajtha (1961) and two pH optima were found, one about pH 3·8 and one about pH 7·8. Tubes were incubated in sets of four for intervals up to 1 hr.; the two tubes serving as controls were fixed with 10% (w/v) trichloroacetic acid at zero-time, the others at the end of the experimental period. After centrifuging, samples of the supernatant were withdrawn for ninhydrin determination with an automatic pipette arrangement: a 'Hirsch–Adams' automatic bi-valve (Clay–Adams Inc.) coupled to a 2 ml. syringe with a male Luer-lock adapter and connected by $\frac{3}{16}$ in. rubber tubing to a 0·5 ml. serological pipette. This arrangement, which can be conveniently attached to a laboratory stand, gave excellent reproducibility for routine determinations in large-scale experiments.

Determination of ninhydrin-positive material extracted by 10% (w/v) trichloroacetic acid was according to the procedures of Moore & Stein (1948). L-Glutamic acid (Calbiochem. Inc.) was used as standard and the results are expressed as μmoles of amino group released/g. of tissue/hr. It is recognized that peptides may give slightly less colour yield with ninhydrin (Moore & Stein, 1951). The proteinase activity was also measured by an alternative method, namely determination of the tyrosine and tryptophan liberated with the Folin–Ciocalteu phenol reagent (Herriott, 1955).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Trichloroacetic acid precipitates were defatted with 1 vol. of acetone and then 2 vol. of chloroform–methanol (2:1, v/v), and the residue was dissolved by heating in 1N-NaOH for 10 min. Bovine albumin (Cohn fraction V) was used as the standard and its N content determined by the micro-Kjeldahl method. An amount of NaOH equivalent to that in the sample taken for determination was added to all standards, since increase in alkali concentration lowered the colour yield measured at 525 mμ.

Procedure of subcellular fractionation. Whole rat brains were homogenized in 0·32M-sucrose and fractionated by methods based on those of Hebb & Whittaker (1958). The total homogenate was centrifuged at 700g for 10 min. to remove debris; with further centrifuging the following were obtained: nuclear fraction (P_1), 1300g for 15 min.; mitochondrial fraction (P_2), 14500g for 20 min.; microsomal fraction, $90000g_{av.}$ for 75 min. The debris and the first two fractions were each washed with 3–4 vol. of 0·32M-sucrose, recentrifuged, and the washings combined. Since these fractions are known to be heterogeneous when examined histologically, further fractionation to decrease contamination was performed.

(*a*) Microsomes were treated by the method of Acs, Neidle & Waelsch (1961) with 0·5% deoxycholate in the presence of Mg^{2+} ions to yield, after centrifuging, a ribosomal pellet and a supernatant containing microsomal membranes.

(*b*) Mitochondria (P_2) were submitted to a series of sucrose gradients based on the procedures of Kuff & Schneider (1954). The method initially adopted was the modification of Whittaker (1959); mitochondria prepared from two rat brains were layered over 0·8M- and 1·2M-sucrose, and centrifuged at 37000 rev./min. ($90000g_{av.}$) for 75 min. in a Spinco 40 rotor. In this manner three subfractions were obtained and are designated 'AP_2', 'BP_2' and 'CP_2' in the text. The second subfractionation was based on the method of Hanzon & Toschi ((1960) and de Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz & Salganicoff (1962). Mitochondria (P_2) obtained from four rat brains were gently dispersed in 0·32M-sucrose with Pasteur pipettes and layered over previously prepared and equilibrated sucrose gradients in the concentration range 0·8–1·4M. These gradients were prepared with 7 ml. volumes of 0·8M-, 1·0M-, 1·2M- and 1·4M-sucrose in cellulose Spinco tubes (1 in. × 3 in.) and left to equilibrate overnight at 0°. Gradient tubes were centrifuged for 2 hr. at 24000 rev./min. ($50000g_{av.}$) in a SW 25-1 Spinco rotor, and the zones designated A, B, C, D and E were removed with fine-tipped 3 ml. Pasteur pipettes. Each fraction was adjusted to 0·32M-sucrose concentration and recentrifuged for 15 min. at 37000 rev./min. ($90000g_{av.}$) in a Spinco 40 rotor. These fractions were checked by phase-contrast microscopy and appeared to contain material similar to that described by de Robertis *et al.* (1962). Fraction E was submitted to further sucrose gradient by layering the fraction dispersed in 1·4M-sucrose in a cellulose tube (0·5 in. × 2 in.) containing 1·2 ml. each of 1·6M-, 1·8M- and 2·0M-sucrose. This yielded, after centrifuging at 37000 rev./min. for 1 hr. ($109000g_{av.}$) in a Spinco 39L rotor, two further fractions, E_1 and E_2.

(*c*) Nuclei were subfractionated by the method of Heald (1959) except that 0·32M- was substituted for 0·25M-sucrose. Nuclei (P_2) from two rat brains were layered over 1·0M-sucrose and centrifuged at 37000 rev./min. for 45 min. ($109000g_{av.}$) in a SW 39L Spinco rotor. The top layer was removed and the residue dispersed in 0·32M-sucrose,

layered over a 1·0–1·4 M-sucrose gradient, and centrifuged at 37000 rev./min. ($109000g_{av.}$) for 80 min. in a SW 39L rotor to yield three layers, R_1, R_2 and R_3. The methods of subfractionation are summarized in Fig. 1.

RESULTS

Time of incubation and the concentration of the enzyme. The appearance of ninhydrin-positive material on incubation of fractions M1 and P_2 extracted by 10% (w/v) trichloroacetic acid is illustrated in Fig. 2. For cerebral proteinases with a pH optimum of 3·8 extracted with aqueous media (fraction M1) activity was linear with time, with a rate of 1780 μmoles of amino group/g. of protein at 1 hr. Since, however, during the same incubation period there was a release of 530 μmoles of amino group/g. of protein in the absence of substrate, this background activity was always determined in order to evaluate proteinase activity determined in the presence of added substrate. Cerebral proteinases in fraction M1 with a pH optimum of 7·6 gave lower activities. Activity was linear with time, yielding a rate of 440 μmoles of amino group/g. of protein/hr. In residue fractions M_2, although the specific activity was considerably lower, the release of ninhydrin-positive material was linear with time with very low activity in the absence of added substrate.

To maintain conditions in which zero-order kinetics apply, it was necessary to use substrate concentrations of 11 mg. of haemoglobin/ml. In its presence the release of amino groups was linear with respect to enzyme concentrations up to 1·0 mg. of protein/ml. The addition of further haemoglobin substrate at these higher enzyme concentrations did not increase the activity. This was interpreted as evidence that activity at high enzyme concentrations was not limited by lack of substrate but that the enzyme extract contained an inhibitor that limited activity at higher concentrations. The proteinase activity in the absence of substrate was also linear with enzyme concentrations up to 1·5 mg. of protein/ml.

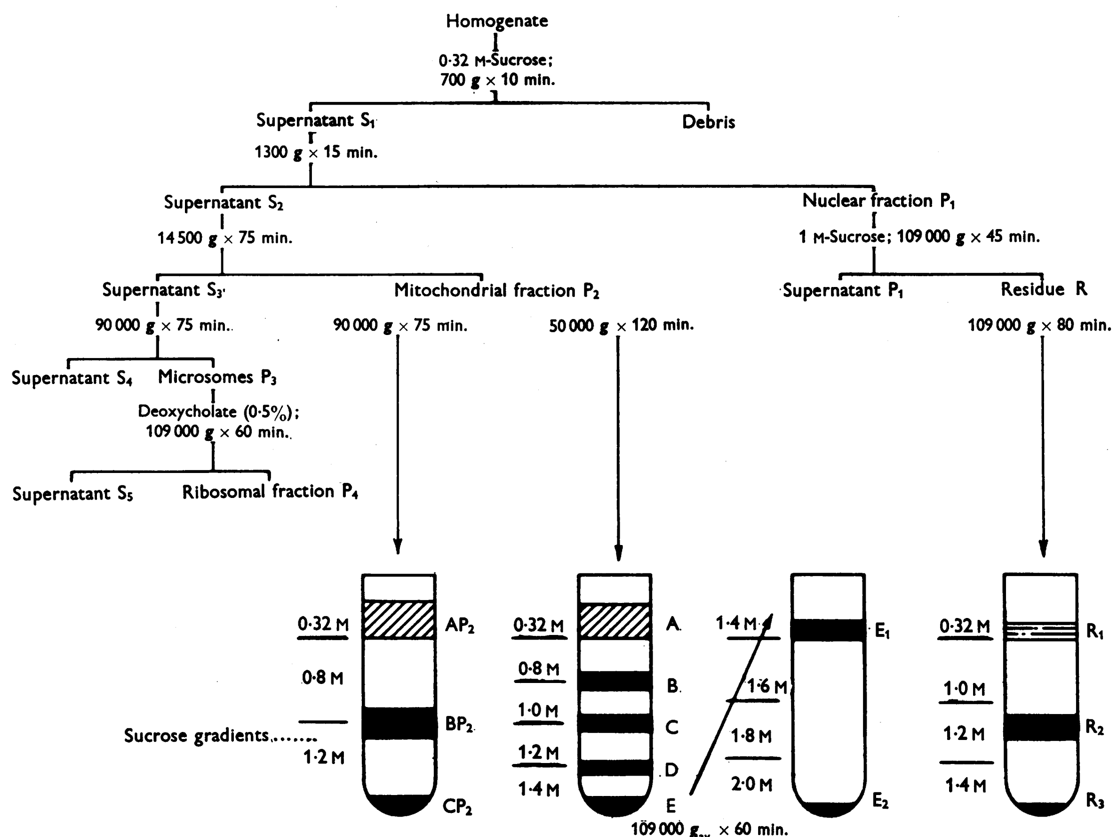

Fig. 1. Fractionation scheme for preparing rat-brain subcellular fractions. Fractions were purified by sucrose-gradient centrifuging to yield subfractions as illustrated. Other details are given in the Methods section.

Activities of proteinases in different tissue homogenates. Neutral and acid proteinases were examined in selected rat tissues for comparison with the enzyme activities in brain tissue under the chosen conditions of assay. The release of ninhydrin-positive material in the absence of substrate, and the specific activities in the presence of haemoglobin and albumin corrected for this background activity, are shown in Tables 1 and 2. Acid-proteinase activity in brain in the presence of haemoglobin was 3-fold the activity of muscle, but only half that of liver; those of spleen and kidney were respectively 10- and 6-fold that of brain (Table 1). Results also show that at pH 3·8 denatured haemoglobin was a better substrate than albumin for spleen, kidney and brain.

The distribution pattern of neutral proteinase in the same tissues showed similarities to that of acid proteinases. In the presence of haemoglobin, brain displayed nearly 3-fold the activity of muscle, but only three-quarters of that of liver; activities in kidney and spleen were respectively 3·5- and 1·5-fold that of brain. The range of specific activities was similar in the presence of albumin, although haemoglobin was a better substrate for spleen, kidney and liver (Table 2). Thus under both acid and neutral conditions the cerebral proteinases were intermediate in activity between those of liver and muscle.

Distribution of proteinase in cerebral particulate fractions. Of the primary brain subcellular fractions tested, mitochondrial fraction P_2 gave the

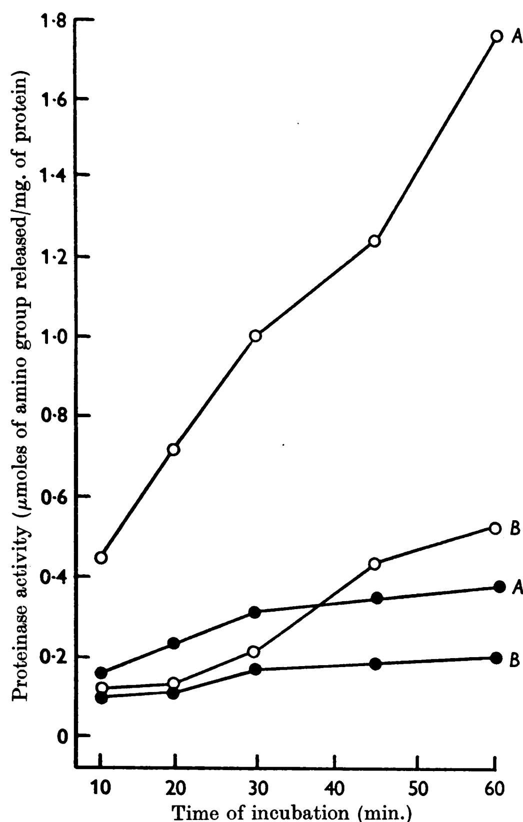

Fig. 2. Appearance of ninhydrin-positive material soluble in 10% (w/v) trichloroacetic acid with change in time of incubation of rat-brain fractions in the presence of acid-denatured haemoglobin at pH 3·8 (*A*) and pH 7·6 (*B*). The conditions of incubation of acid and neutral proteinases are given in Tables 1 and 2 respectively, and the fractions are defined in Fig. 1 and the Methods section. ○, Supernatant fraction M1; ●, mitochondrial fraction P_2.

Table 1. *Acid-proteinase activity in rat-tissue homogenates*

The reaction mixture of 1 ml. was incubated for 1 hr. at 37° in a rotary-shaking water bath and contained 1 mg. of homogenate protein, NaCl (0·154 M), Triton X-100 (0·2%), acetate buffer, pH 3·8 (0·05 M), and 10 mg. of denatured haemoglobin or albumin where indicated. Values are given ±S.D. with the numbers of experiments in parentheses.

	Ninhydrin-positive material released		
	(μmoles of amino group/g. of fresh tissue/hr.)	(μmoles of amino group/g. of protein/hr.)	
		Haemoglobin added*	Albumin added*
Spleen	45±8 (6)	2192±105 (6)	1352 (2)
Kidney	74±10 (6)	1392±90 (6)	852 (2)
Liver	30±10 (6)	583±65 (6)	628 (2)
Brain	19±4 (5)	225±12 (5)	146 (2)
Muscle	13 (2)	62 (2)	0 (2)

* Activity corrected for the release of ninhydrin-positive material released in the absence of substrate.

Table 2. *Neutral-proteinase activity in rat-tissue homogenates*

The reaction mixture of 1 ml. was incubated for 1 hr. at 37° and contained 1 mg. of homogenate protein, NaCl (0·154 M), Triton X-100 (0·2%), phosphate buffer, pH 7·6 (0·02 M), CoA (0·13 mM), ATP (1 mM), glutathione (0·5 mM), and 11 mg. of acid-denatured haemoglobin or albumin. Values are given ±S.D. with the numbers of experiments in parentheses.

	Ninhydrin-positive material released		
	(μmoles of amino group/g. of fresh tissue/hr.)	(μmoles of amino group/g. of protein/hr.)	
		Haemoglobin added*	Albumin added*
Spleen	13±4 (4)	1145±195 (4)	880 (2)
Kidney	35±5 (4)	2770±250 (4)	1190 (2)
Liver	15±4 (4)	1023±50 (4)	610 (2)
Brain	3·2±0·6 (4)	780±47 (4)	795 (2)
Muscle	21±6 (4)	244±12 (4)	264 (2)

* Activity corrected for the release of ninhydrin-positive material released in the absence of substrate.

Table 3. *Distribution of proteinase activity in subcellular fractions of rat brain*

The reaction mixture contained 1 mg. of particulate protein. The conditions of incubation of the acid and neutral proteinases are given in Tables 1 and 2 respectively. Average values are given ±S.D. The fractions are defined in Fig. 1 and the Methods section.

		Ninhydrin-positive material released (μmoles of amino group/g. of protein/hr.)			
		At pH 3·8 (acid proteinase)		At pH 7·6 (neutral proteinase)	
	No. of expts.	No substrate added	Haemoglobin added	No substrate added	Haemoglobin added
Whole homogenate	10	220±25	550±70	130±30	520±80
Nuclear fraction P_1	5	190±19	510±65	110±18	350±12
Mitochondrial fraction P_2	5	350±22	910±80	150±8	450±20
Microsomal fraction P_3	5	230±24	450±30	47±4	250±6
Microsomal supernatant S_4	4	60±5	98±8	95±7	210±10
Ribosomal fraction P_4	4	8±1	15±3	14±2	17±2·5
Ribosomal supernatant S_5	4	93±3	350±25	140±10	240±15

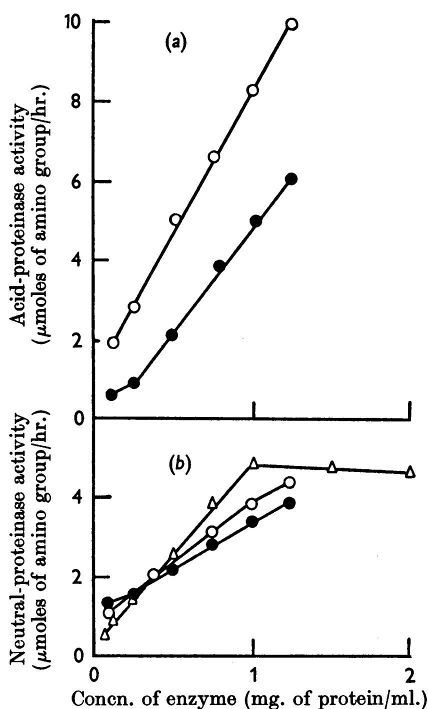

Fig. 3. Appearance of ninhydrin-positive material soluble in 10% (w/v) trichloroacetic acid with change in enzyme concentration on the incubation of rat-brain fractions in the presence of acid-denatured haemoglobin at (*a*) pH 3·8 and (*b*) pH 7·6. The conditions of incubation of acid and neutral proteinases are given in Tables 1 and 2 respectively. The fractions are defined in Fig. 1 and the Methods section. ●, Nuclear fraction P_1; ○, mitochondrial fraction P_2; △, supernatant fraction M1.

highest activity under both neutral and acid conditions. At pH 3·8, in the presence of denatured haemoglobin, activity decreased in the following order: mitochondria, nuclei, microsomes, ribosomal supernatant, microsomal supernatant, ribosomes (Table 3). At pH 7·6 the order of decreasing activity was: mitochondria, nuclei, microsomes, microsomal supernatant, ribosomal supernatant, ribosomes. In the microsomal-supernatant fraction a high activity was also observed initially. With gentle dispersion in the Dounce homogenizer (see the Methods section) activities were considerably lower (Table 3). Homogenization with the Potter–Elvehjem homogenizer with shorter dispersion times also gave lower values for the supernatant fractions, and these milder conditions were adhered to throughout the present study. These results indicated that a significant part of the cerebral proteinases is contained in fragile organelles, some of which may be broken by the usual homogenizing procedures.

To establish optimum assay conditions, the dependence of activity on the time of incubation was studied in a manner similar to that described for aqueous extracts. At pH 3·8 and 7·6 in the presence of substrate, the activities of mitochondrial and nuclear fractions increased linearly with time up to 1 hr. The results for the mitochondrial fraction P_2 are illustrated in Fig. 2.

The same relationship was observed with all mitochondrial and nuclear fractions in the range of enzyme concentrations used in the presence and absence of substrate; examples for fractions P_1 and P_2 in the presence of substrate are included in Fig. 3.

Because of the heterogeneity of brain fractions, the activity previously noted for some of the primary subcellular fractions may have arisen from contamination by other brain particles, e.g. mitochondria. To examine this possibility the microsomal and nuclear fractions were further fractionated. With the microsomes treated by the method of Acs *et al.* (1961) there was very little activity associated with the resulting ribosomal preparations but

higher activity in the ribosomal supernatant (Table 3). Nuclear fraction P_1 was recentrifuged in 1M-sucrose and the residue subfractionated on sucrose gradients of 1·0–1·4M-sucrose. Results summarized in Table 4 show that the residue fraction P_1 contained most of the acid-proteinase and neutral-proteinase activities. This residue fraction contains largely non-nuclear elements (Heald, 1959), and on further subfractionation most of the recovered activity was associated with the heavier particles.

Table 4. *Distribution of acid and neutral proteinases in rat-brain nuclear fractions after separation in 1·0–1·4M-sucrose gradients*

The composition of nuclear fraction P_1 was 12·5 mg. of protein/g. of fresh brain tissue; acid-proteinase activity was 6·3 μmoles of amino group released/g. of fresh tissue/hr. (ninhydrin-positive material released in the absence of substrate was 2·3); neutral-proteinase activity was 2·52 μ-moles of amino group/g. of fresh tissue/hr. (in the absence of substrate, 1·10). Values for recovered proteinase activity are quoted after correction for the release of ninhydrin-positive material in the absence of substrate. The conditions of incubation of the acid and neutral proteinases are given in Tables 1 and 2 respectively. The fractions are defined in Fig. 1 and the Methods section.

Fraction	Protein (% of protein in P_1)	Recovered proteinase activity (%)* At pH 3·8 (acid proteinase)	At pH 7·6 (neutral proteinase)
Supernatant fraction P_1	46	8·6	22·6
Residue fraction R	36	98·3	94
Subfraction R_1	3	7·0	7·5
Subfraction R_2	19	10·2	22·5
Subfraction R_3	12	35·0	52·5

* Supernatant fraction P_1 and residue fraction R are expressed as % recovered from the nuclear fraction P_1; subfractions R_1, R_2 and R_3 are expressed as % recovered from residue fraction R.

Subfractionation of mitochondrial proteinases. It is apparent from a large number of studies that the mitochondrial fractions, which were shown to contain the highest proteinase activity, are a mixture of different cellular particles and cell fragments (Petrushka & Giuditta, 1959; Whittaker, 1959). To study whether cerebral proteinases are associated with a specific organelle, several subfractionations were performed. The results are reported below; the methods for obtaining the fractions are outlined in Fig. 1.

The centrifuging of mitochondrial fraction P_2 in 0·32–1·2M-sucrose gradients yielded three subfractions. The first fraction, AP_2, which contained mostly myelin and glial-cell fragments, yielded the lowest acid-proteinase activity when tested in the presence and absence of haemoglobin. The third fraction, CP_2, which contained mostly mitochondria and which had the densest particles, displayed the highest activity. Fraction BP_2, which contained nerve-ending structures and simple vesicles, was intermediate in activity (Table 5). Comparison of specific activities in the presence of substrate after correction for ninhydrin-positive material released in the absence of substrate revealed that BP_2 displayed nearly 2-fold and CP_2 5-fold the activity of the first fraction.

Neutral proteinases were compared in a similar manner, and results are included in the lower half of Table 5. Activities in general were considerably lower than those of the pH 3·8 preparations. In contrast with acid proteinase, activity was more evenly distributed, with a tendency in all experiments for the highest activity to be in BP_2 and the

Table 5. *Distribution of acid and neutral proteinases in mitochondrial subfractions after separation in 0·8–1·2M-sucrose gradients*

The conditions of incubation of acid and neutral proteinases are given in Tables 1 and 2 respectively. Average values are given ±S.D. The recovery of proteinase activity after correction for material released in the absence of substrate was 84% at pH 3·8 and 133% at pH 7·6. The fractions are defined in Fig. 1 and the Methods section.

Fraction	pH	No. of expts.	Ninhydrin-positive material released (μmoles of amino group/g. of fresh tissue/hr.) No substrate added	Haemoglobin added	Relative specific activity* (CP_2 = 100)
P_2	3·8	10	7·50±1·0	15·50±3·5	—
AP_2	3·8	4	0·14±0·01	0·68±0·03	19
BP_2	3·8	4	0·40±0·01	2·12±0·05	33
CP_2	3·8	4	1·58±0·12	6·07±0·85	100
P_2	7·6	10	2·55±0·50	4·60±1·0	—
AP_2	7·6	4	0·12±0·02	0·85±0·04	135
BP_2	7·6	4	0·77±0·02	1·91±0·08	175
CP_2	7·6	4	0·37±0·10	1·23±0·02	100

* Activity corrected for the release of ninhydrin-positive material in the absence of substrate.

lowest in CP_2, as illustrated in the relative specific activities. The specific activities ran parallel to total activity and indicated, unlike acid proteinase, little purification. This was taken as an indication that probably more than one neutral proteinase is involved and that they are distributed in different fractions. Because of the heterogeneity of neutral proteinases the fractionation procedure was refined by employing a sucrose gradient of greater resolution. In agreement with the previous method of separation, acid-proteinase activity increased with increase in particle density as reflected by the position in the sucrose gradient (Table 6). The differential distribution of acid and neutral proteinases is clearly demonstrated by the ratio in the final column of Table 6. Because of the high activity in fraction E, which consists mainly of mitochondria, it was decided to fractionate E further with sucrose gradients of greater range. With a gradient of 1·4–2·0M-sucrose, fraction E yielded two subfractions designated E_1 and E_2. Results included in Table 6 show the highest acid-proteinase activity in E_2 with complete absence of neutral-proteinase activity. There was a complete absence of background activity in these two fractions.

Since ninhydrin can be expected to react with all free α-amino groups, it was decided to attempt some improvement in specificity by measurement of tyrosine and tryptophan released with the Folin reagent. Measurements of the neutral-proteinase activity of the brain subfractions AP_2, BP_2 and CP_2 respectively (Fig. 1) showed a release of 2·6, 4·2 and 3·0 μmoles of tyrosine/g. of protein/hr. in the absence of substrate, and 9·3, 8·0 and 7·5 in the presence of haemoglobin. The small change produced for each incubation, about 0·005 μmole/mg. of protein/hr., was regarded as lacking sensitivity in relation to the ninhydrin method and was not further employed.

Proteinase activity in the presence of different substrates. In appraising the physiological role of cerebral proteinases consideration must also be given to their action on native protein, although proteins are considered to be more susceptible to proteolysis when denatured (for a recent review see Okunuki, 1961). To examine the substrate specificity of cerebral proteinases, mitochondrial fraction P_2 was incubated in the presence of a variety of native and acid-denatured proteins. At pH 3·8 denatured haemoglobin gave the highest rate of breakdown, followed closely by native globin and haemoglobin; the albumins and untreated casein gave values only about 30% above the rates of ninhydrin-positive material released in the absence of substrate. The addition of untreated α-globulin, the γ-globulins or edestin to the incubation medium did not increase the rate of splitting, and in some instances activity decreased compared with rates in the absence of substrate. The following appeared to inhibit the release of ninhydrin-positive material in the absence of substrate on the basis of tests for significance: edestin ($P < 0·01$), acid-treated α-globulin ($P < 0·01$), untreated γ-globulin ($P < 0·05$). The substrate specificity of the neutral proteinases differed from those active at acidic pH. At pH 7·6 untreated globin gave a rate some 12-fold higher than that previously observed in its absence, and higher than for any substrate tested in the presence of acid proteinases. Fibrinogen, untreated albumin, γ-globulins and casein were without effect.

Table 6. *Distribution of acid and neutral proteinases in mitochondrial subfractions after separation in 0·8–1·4M-sucrose gradients*

The conditions of incubation of acid and neutral proteinases are given in Tables 1 and 2 respectively. Each value is the average of three determinations. Fraction P_2 contained 20·3 mg. of protein/g. of fresh tissue. The recovery of proteinase activity in subfractions A, B, C, D and E was 71% at pH 3·8 and 75% at pH 7·6. The recovery of acid-proteinase activity in subfractions E_1 and E_2 was 61%. The fractions are defined in Fig. 1 and the Methods section.

		Specific activity (μmoles of amino group released/g. of protein/hr.)				
		At pH 3·8 (acid proteinase)		At pH 7·6 (neutral proteinase)		
Subfraction	Protein (% recovered from fraction P_2)	No substrate added	Haemoglobin added	No substrate added	Haemoglobin added	Acid proteinase: neutral proteinase ratio*
A	18	130	120	165	366	0·33
B	16	260	335	144	476	0·70
C	24	134	866	63	349	2·5
D	14	324	1086	96	359	3·0
E	8	619	3841	21	521	7·4
E_1	2·3	0	4230	0	465	9·1
E_2	0·3	0	9670	0	0	—

* Activity corrected for the release of ninhydrin-positive material released in the absence of substrate.

DISCUSSION

Biological role of proteinases in the nervous system. The biological role of proteinases in any tissue, and the nervous system in particular, is not completely clear. It has been suggested that neutral proteinases participate in physiological protein turnover, whereas cathepsins are active mainly under pathological conditions. The possibility that cathepsins participate in some protein metabolism has been discussed (Fruton, 1957; Ansell & Richter, 1954*b*). Cerebral proteinases, as pointed out by Waley & van Heyningen (1962) for lens proteinases, could not play an important role in the breakdown of exogenous protein since these organs are not permeable to proteins to a significant extent. Cathepsins have been generally thought to be excluded from participating in normal protein turnover because their maximal activity is at low pH; however, the fractional activity exhibited near physiological pH may represent a considerable and important aspect of protein metabolism. Moreover, the intracellular pH of specific granules may be lower than commonly supposed, and the optimum pH of catheptic activity may depend on the substrate. The pH optimum of cathepsin D was 3·0 with haemoglobin and 4·2 with albumin (Press, Porter & Cebra, 1960).

At least part of the cathepsins is contained in lysosomes (for reviews see de Duve, 1959; Novikoff, 1961). These particles seem to play an important role in a number of autolytic and necrotic processes and in processes of intracellular digestion.

Although lysosomal particles have not been isolated from nervous tissue (Beaufay, Berleur & Doyen, 1957), the histochemical evidence strongly points to their existence in brain (Novikoff, 1960; Novikoff & Essner, 1962; Ogawa, Mizuno & Okamoto, 1961; Koenig, 1962). In peripheral nerve catheptic activity could be shown histochemically. This activity increased during the first days of Wallerian degeneration, and it may be involved in demyelination. The cathepsins appeared to be constituents of the myelin sheath (Adams & Bayliss, 1961; Adams, 1962).

Neutral proteinases have been found in a number of tissues, including brain (Ansell & Richter, 1954*b*). For full activity near neutral pH, proteinases seemed to require a source of energy in liver and kidney slices (Simpson, 1953; Steinberg & Vaughan, 1956; Steinberg, Vaughan & Anfinsen, 1956), in liver nuclei and mitochondria, and in brain mitochondria (Korner & Tarver, 1957; Penn, 1960). Although these studies indicate the possibility that some pathway of protein breakdown may utilize energy, alternative explanations, such as that energy is required for other reactions and not directly for breakdown, cannot be excluded at present.

An additional role of proteinases may be the modification of existing proteins. By cleavage of a peptide or peptides, enzymes may be converted into an active or inactive form, or protein precursors may be converted. A number of examples for such conversions are known already.

Distribution of proteinases in subcellular fractions. Since the present method measured a family of proteinases, the possibility cannot be excluded that various fractions contained different amounts of certain specific proteinases. In liver, for example, Rademaker & Soons (1957) found that mitochondria contained most of the cathepsin-A and carboxypeptidase activity, whereas the other fractions such as the supernatant contained a higher portion of cathepsin B. Also, differences in the relative distribution of these enzymes between liver and kidney have been reported (Hanson, Hermann & Blech, 1959). The question whether acid proteinases are present in the brain in mitochondria or in lysosomes cannot be decided on the basis of our study. If lysosomes do exist in brain, then our results are consistent with their presence in fractions CP_2 (Table 5) and E (Table 6).

Since lysosomal enzymes are active at low pH, it might be expected that the pH 7·6 proteinases, if they exist as separate proteins, occur at different cellular locations. Our results indicate their presence in highest concentration in mitochondrial subfractions sedimenting in sucrose concentrations of intermediate density (BP_2, Table 5; A and B, Table 6). These fractions contain nerve endings pinched or torn off from their attachment to form isolated nerve-ending particles (Gray & Whittaker, 1960). These fractions contain 5-hydroxytryptamine, choline acetylase, bound acetylcholine (Whittaker, 1959; Johnson & Whittaker, 1962; de Robertis *et al.*, 1962) and substance P (Akira & Kiyoshi, 1962). It is possible that in such particles the neutral proteinases play some role in synaptic function in addition to their more likely role of participating in the metabolism of the proteins of the cell. The low proteinase activity of the ribosomal fraction (Table 3) clearly indicates that the newly formed protein molecule has to be released from its site of synthesis on the ribosome before breakdown at a different site occurs. If the neutral proteinases discussed here take an important part in the metabolism of proteins *in vivo* then protein turnover has to be visualized as synthesis followed by a migration from the site of formation before breakdown takes place. The nuclear fraction P_1 was the second richest in proteinase activity; however, present results support the conclusion that activity arose from contamination, since the major proportion resided in the non-nuclear residue fractions. Dounce & Umana (1962) also concluded from their studies on liver nuclei that intrinsic

proteolytic activity was low, but whenever methods of isolation of nuclei were employed involving mechanical disruption of mitochondria or lysosomes, increased proteolytic activity was found because of contamination of nuclei by proteolytic enzymes from other particles, which also resulted in proteolytic degradation of the nuclear proteins during the isolation procedure. The low intrinsic proteolytic activity of cell nuclei may, however, have significance in cell function, since it is known that release of basic proteins, such as histone, from brain nuclei can affect cell excitability to electrical pulses (Marks, 1961).

Substrate specificity. The results have shown that further fractionation of subcellular elements was accompanied by a fall or even loss of endogenous activity (Tables 5 and 6). This was interpreted as resulting from a separation of the enzyme from its substrate protein, since in most instances activity was almost restored with haemoglobin. The lack of complete recovery of brain proteinases in the particulate fractions, especially acid proteinases, may have occurred as a result of the breakage of fragile organelles during the preparative procedures with subsequent solubilization of the enzymes. The recovery of neutral proteinase was generally higher and in some cases even exceeded that of the starting material (Table 5); this increase in activity may have resulted from separation of inhibitory factors during fractionation. Materials that inhibit proteolytic enzymes are widely distributed in animal tissues; a recent example is the inhibition of purified proteinase from skin by an inhibitory factor obtained in a fairly pure state from serum proteins (Martin, 1961). Similar properties were noted for certain other protein substrates used in the present study which inhibited acid-proteinase activity. It was possible to demonstrate different rates of breakdown by adding a number of native and acid-denatured proteins as substrates. Brain proteinases show differences in substrate specificity compared with other tissues; lens proteinase, for example, split haemoglobin at a much lower rate than it split its endogenous substrate α_2-crystallin (van Heyningen & Waley, 1963).

The different substrate specificities give further evidence that the acid and neutral groups of proteinases are separate enzymes. It is expected that greater differences in substrate specificity among the various enzymes will be found on their further purification.

SUMMARY

1. Optimum conditions for assay of rat-brain proteinases having pH optima of 3·8 and 7·6 were examined with respect to time of incubation and to substrate and enzyme concentrations.

2. Comparison of proteinase activities of several tissue homogenates showed that the activity in brain was less than those in kidney, spleen or liver, but greater than that in muscle.

3. On subcellular fractionation the highest activity of acid and neutral proteinases was in the mitochondrial fraction (P_2), with lower activities in nuclei (P_1), microsomes (P_3), and ribosomal supernatant (S_5).

4. Sucrose-gradient centrifuging of the heterogeneous fraction P_2 to decrease contamination revealed an association of acid proteinases with fractions sedimenting in 1–1·4 M-sucrose and known to contain true mitochondria, whereas neutral proteinases were associated with fractions containing myelin, nerve-ending structures and simple vesicles. Similar purification of P_1 showed only a low activity associated with true nuclei.

5. The subcellular distribution and the substrate specificity of proteinase suggest the presence of two separate enzyme systems. The possible presence of lysosomal particles in brain and the role proteinases may play in this organ are discussed.

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Transfer of the Methyl Group of Methionine to Nucleolar Ribonucleic Acid

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The nucleolus is apparently an important source of cell RNA, although the precise significance of the contribution is not known. Among the nucleolar RNA types described are: 'transfer' RNA (Vincent & Baltus, 1960; Birnstiel & Chipchase, 1963), ribosomal RNA (Edström, Grampp & Schor, 1961; Birnstiel & Chipchase, 1963) and 'messenger'-like RNA ('nucleolar fraction' of Sibatani, de Kloet, Allfrey & Mirsky, 1962; Sirlin, Jacob & Kato, 1962).

Nuclei in the salivary glands of larvae of the chironomid *Smittia* sp. (Diptera) each contain three chromosomes. One chromosome has a nucleolus which surrounds on all sides the nucleolus organizer present in the chromosome. In the nucleolus RNA is first made near to but not within the organizer (Plate 1A), then moves outward, and finally out of the nucleolus (see Figs. 1 and 2 of Sirlin, 1962). The present observations indicate that part of this RNA is 'transfer' RNA. A preliminary account of this work has been published (Sirlin, Jacob & Tandler, 1963).

MATERIAL AND METHODS

Biological material. The stock of *Smittia* was obtained from Professor H. Bauer of the Max Planck-Institut, Tübingen, who proposed (personal communication) to identify the species provisionally as *S. parthenogenetica*.

The actively growing larvae used were in the conventional stage III (Sirlin, Kato & Jones, 1961) which precedes metamorphosis during the fourth developmental instar. The larvae were grown at approx. 9° and were brought to the laboratory about 2 hr. before the experiments began. A diurnal peak of metabolic activity is reached by 2 p.m., and the experimental and control series were arranged in time accordingly.

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