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Neutral Proteinases in the Lens

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The enzymes that catalyse the intracellular hydrolysis of proteins are ill characterized. In particular, it is uncertain whether the cathepsins are active in a cell during its lifetime (Fruton, 1960). Nor is it known whether peptidases, also present in all cells, attack the proteins there. Only three peptidases have been highly purified and tested on proteins (leucine aminopeptidase, EC 3.4.1.1, and the pancreatic carboxypeptidases, EC 3 4.2.1 and 3.4.2.2), and they can all attack proteins, although the extent of hydrolysis is very variable. Any enzymes in the lens that catalyse proteolysis are unlikely to be concerned with the breakdown of 'foreign' proteins, as the cells of the lens are enclosed in a capsule which is probably impermeable to proteins. Lens proteinases are thus likely to play a part in protein turnover. Moreover, loss of protein, a common feature of cataract, is presumably a consequence of protein breakdown predominating over protein synthesis.

An important feature of the work described below is that a protein fraction from the lens has been used as substrate. Under these conditions, it is possible to show conclusively that protein breakdown (to amino acids) does take place in lens extracts in neutral solution. A sensitive and general method of detecting the products of proteolysis (the ninhydrin reaction) has been used, and by this method protein breakdown in neutral solution has also been demonstrated with several tissues of the ox and of the rat.

MATERIALS

Tissues. Ox and calf lenses had been stored frozen; rabbit and rat lenses, and the other tissues of the ox and rat, were used fresh.

Buffers. Veronal buffer contained 400 ml. of 0·025 M-diethylbarbituric acid and 14·5 ml. of 0·5 M-sodium diethylbarbiturate in 1725 ml. of solution; CO_2-free water was used; the pH was 7·5, at room temperature. The veronal–Mg^{2+} buffer used in the assays contained 5 ml. of M-$MgCl_2$/l. of veronal buffer.

The 0·2 M-acetate buffer, pH 5·2, used for the removal of protein, contained 3·7 ml. of acetic acid and 50 ml. of N-NaOH in 250 ml.

METHODS

Ninhydrin reaction. The ninhydrin reaction used in this work was carried out by a method rather different from those previously described. Quantitative yields are obtained in the reaction between ninhydrin and most amino acids, when the solvent is a phenol–pyridine mixture (Troll & Cannan, 1953), ethanol (Meyer, 1957), or aq. 2-methoxyethanol (Yemm & Cocking, 1955; Matheson, Tigane & Hanes, 1961), the methods using methoxyethanol being based on the reagent of Moore & Stein (1948, 1954). The reaction is easier to carry out in a predominantly non-aqueous medium, as colour development proceeds more rapidly and the coloured product (Ruhemann's purple) is more stable. Methoxyethanol is a convenient solvent, but its use demands a buffer which is soluble in organic solvents. Since metal ions interfere with the ninhydrin reaction (Meyer, 1957; Jacobs, 1960), the well-known chelating agent, 8-hydroxyquinoline, was chosen as one component of the buffer system; the other component was acetic acid. Both components have pK values in the region of 5, so that they buffer at the pH optimum for the ninhydrin reaction. The procedure described below has proved satisfactory; although it has not been tested on the complete range of amino acids, the colour yield from leucine is quantitative and is high for most other amino acids, but is low (20%) from NH_3 (in the form of ammonium acetate).

Solution A was prepared by adding 8-hydroxyquinoline (3 g.) to methoxyethanol (15 ml.); acetic acid (0·5 ml.) was then added. When the 8-hydroxyquinoline had dissolved, 0·01 M-KCN (0·4 ml.) was added to the solution. Solution B contained ninhydrin (0·15 g.) in methoxyethanol (20 ml.). A portion (1 ml.) of solution B was added to 0·5 ml. of an aqueous solution of an amino acid (0·05–0·5 mM), followed by 1 ml. of solution A. The reaction could be carried out in EEL (Evans Electroselenium Ltd., Halstead, Essex) colorimeter tubes; the contents of the tube were mixed, a glass marble was put on top of the tube, and the tube was heated for 4·25 min. at 100°. After cooling, the solution was diluted with 60% (v/v) ethanol (5 ml.) and read in an EEL colorimeter with a yellow filter (no. 626), or in a spectrophotometer at 570 mμ against a blank which lacked the amino acid. Standards containing 0·04 and 0·1 μmole of leucine were used in the assays.

Preparation of α_1-crystallin and α_2-crystallin. Frozen ox lenses (173 g.) were thawed, and then stirred gently with water (500 ml.) for about 30 min. This procedure extracted material from the cortices of the lenses; the nuclei, which remained intact, weighed about 49 g. The extract was clarified by centrifuging for 20 min. at 10 000g at 2°, and then brought to pH 5·0 by the slow addition of 0·1 N-HCl (98 ml.). The precipitate (α_1-crystallin) was collected by centrifuging, and dissolved in water by the addition of the minimum quantity of saturated $NaHCO_3$; the solution (42 ml.) had pH 7·3; a portion diluted 100-fold had $E_{280} = 1·96$. After removal of α_1-crystallin, the supernatant at pH 5 (vol. 560 ml.) was diluted with ethanol (84 ml.), and the precipitate [α_2-crystallin (François, Rabaey & Wieme, 1955)] collected by centrifuging. The α_2-crystallin was dissolved in aq. $NaHCO_3$, reprecipitated at pH 5 in

13% (v/v) ethanol, and then brought into solution again, dialysed against water, made 2 mM with respect to the acetate buffer described in the Materials section, and precipitated with 4 vol. of chilled acetone. The dry powder was stored frozen. A stock (10%, w/v) solution in the veronal–Mg^{2+} buffer was used.

Method of following proteolysis. The enzymic reaction was carried out, usually at 55°, in veronal–Mg^{2+} buffer. When a substrate was added, this was α_2-crystallin to a concentration of 1% (w/v). Four portions of 0·4 ml. of the reaction mixture were incubated for various times; the tubes withdrawn at the earlier times were stored at −10°, so that protein could be removed from all the tubes at the same time. Acetate buffer (2 ml. of the buffer described above) was added to each tube, and the tubes were then heated at 100° for 3 min. Portions (0·5 ml.) of the filtrate were taken for the ninhydrin reaction.

Assay of leucine aminopeptidase. The enzymic hydrolysis of L-leucylglycine was followed by paper chromatography. The concentration of the substrate was 4 mg./ml. The reaction was carried out in the veronal–Mg^{2+} buffer at 55°; samples (10 μl.) of the reaction mixture were applied at various times to a sheet of Schleicher & Schüll 2043b paper; a drop of 2% (v/v) acetic acid had been applied to the starting line just previously to stop the enzymic reaction when the sample was transferred to the paper. Standards of glycine (0·05–0·15 μmole) were also put on the paper, which was then developed with butan-1-ol–acetic acid–water (40:9:20, by vol.) for 4 hr. The estimations were carried out as described by Bode (1955).

Units. The unit of proteinase is that amount which (under specified conditions) produces 1 μmole of amino acids (leucine equivalent, see Methods section) in 1 ml. of incubation mixture/hr. Specific activity, or purity, is expressed in units/g. of protein; the protein is assumed to have $E_{280} = 1$ for a concentration of 1 mg./ml. The unit of leucine aminopeptidase is that amount which produces 1 μmole of glycine/ml. of assay mixture/hr.

RESULTS

Protein breakdown in lens dispersions before and after fractionation

Proteolysis in dispersions of calf lens continued for 12 days (Fig. 1); the pH was adjusted to 7·4 initially, and had not changed appreciably at the end of the experiment. Bacterial growth was prevented by the addition of chloroform; the effectiveness of this measure was checked by plating out on blood agar.

Proteolysis under the conditions described above is steady but slow. However, 1 hr. sufficed for assay when the α_1-crystallin fraction was the source of the enzyme, and when the reaction was carried out under the conditions (i.e. at 55° in the presence of Mg^{2+} ions) described later. The amounts of amino acids formed were a linear function of time for up to 3 hr. Since the protein in the lens dispersion which serves as substrate might be shed during purification, the assay mixtures contained 1% (w/v) of α_2-crystallin.

In testing the proteolytic activities of the fractions obtained from lens, α_1-crystallin was prepared as described above (see Methods section). The supernatant at pH 5 (this fraction is called β-crystallin) was carefully brought to pH 7·4 (in the cold) by slow addition of 0·1 N-NaOH. The results (Table 1) show that precipitation at pH 5 gave a fraction (α_1-crystallin) with four times the specific activity of the extract, with about 80% recovery of activity. The fraction of lens proteins insoluble in water at pH 7·5 was devoid of activity. An earlier fractionation, in which the assays were carried out at 37°, gave essentially similar results.

Variation of rate of proteolysis with temperature, and thermal stability of the enzyme

Preliminary experiments showed that proteolysis proceeded more rapidly at 55° than at 37°, and further experiments were carried out to study the thermal stability of the enzyme. Table 2 shows that the reaction proceeds most rapidly at 64° (series I) and that the enzyme is fairly stable to heating at 55° before incubation (series III). The rate of reaction at 70° fell off during the assay

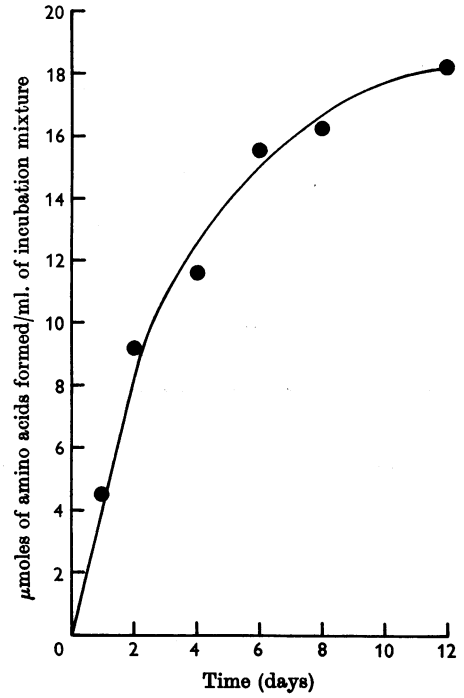

Fig. 1. Proteolysis in calf lens. Calf-lens dispersion (10 g. of lens and 10 g. of water) was dialysed for 24 hr. at 4° against water at pH 7·4. Seven portions of 2 ml. dispersion plus 2 drops of chloroform were incubated at 37° in stoppered tubes for various times and stored frozen until assayed.

Table 1. *Distribution of proteinase activity among protein fractions*

The units of activity are defined in the text. The extract, and the α_1-crystallin, were diluted about 12-fold, and the β-crystallin 4-fold, with veronal–Mg^{2+} buffer. The assay was carried out at 55°.

	Volume (ml.)	Activity (units/ml.)	Total activity (units)	Concn. of protein (g./ml.)	Specific activity (units/g. of protein)
Extract	37·5	2·83	105	0·118	24
α_1-Crystallin	6·3	13·7	86	0·135	102
β-Crystallin	66·0	0	0	0·037	0

Table 2. *Effects of temperature on proteolysis*

In series I and II the final dilution of the stock solution of α_1-crystallin in the assay mixture was 11-fold, and the mixture also contained α_2-crystallin (1%, w/v). In series III a solution of α_1-crystallin (1 ml. of stock soln. diluted with 2 ml. of veronal–Mg^{2+} buffer) was divided into portions of 0·15 ml., and batches of four were heated for various times. Each portion was then diluted with 0·27 ml. of 1·4% (w/v) α_2-crystallin for assay. Different batches of α_1-crystallin were used in the different series of experiments.

Series	Temperature of assay	Activity of α_1-crystallin (units/ml.)
I	36°	3·3
	55	30·9
	64	45·4
	70	21·8
II	55	15·4
	37	1·2
	37*	2·2
	Duration of heating at 55° before assay at 55° (min.)	
III	0	19·0
	15	19·3
	30	19·7
	60	16·9
	120	16·4

* After incubation for 30 min. at 55°.

Table 3. *Effect of metal ions on proteolysis*

Assays were carried out with α_1-crystallin. The final concentration of the salts was 0·01 M; the rate was unaffected when the concentration was 1 mM, in all cases.

Salt	Relative rate (%)	Salt	Relative rate (%)
None	100	$CaCl_2$	172
$FeSO_4$	34	$MnSO_4$	165
$CoCl_2$	41	$MgCl_2$	237
$ZnSO_4$	0		

(30 min.), and the same effect was observed after heating for 30 min. (or longer) at 55° before the addition of α_2-crystallin. Table 2 also shows (series II) that the rate of proteolysis, measured at 37°, was increased twofold by previous heating of the assay mixture for 30 min. at 55°; this effect, which is not observed when the rate is measured at 55°, might be due to inactivation of inhibitors at 55°, or to a thermal denaturation of the substrate if the denatured substrate is more readily hydrolysed. Amino acids are released (from the endogenous substrate) during the heating 'before assay' but the rate given in Table 2 refers to the rate of formation of amino acids after the addition of α_2-crystallin.

Variation of rate of proteolysis with metal ion concentration, ionic strength and pH

The effect of various metal ions on the rate of formation of amino acids from α_1-crystallin is shown in Table 3. The incubations were carried out at 37°, at pH 7·4. The rate was enhanced by the addition of Mg^{2+}, Ca^{2+} or Mn^{2+} ions; the largest increase was obtained with Mg^{2+} ions, which were therefore added to the buffer in assays. In the assays at 55° the usual concentration of $MgCl_2$ was 5 mM; omission of $MgCl_2$ reduces the activity to 35% of the control (Table 4). Removal of Mg^{2+} ions by dialysis against 5 mM-EDTA abolishes most of the activity; subsequent addition of Mg^{2+} ions brings the activity back to nearly half its original value (Table 4).

The effect of NaCl on the rate was examined with α_1-crystallin in veronal–Mg^{2+} buffer made 0·3 M with respect to NaCl; the solution was kept at 4° for 16 hr., then half was dialysed against 200 ml. of veronal–Mg^{2+} for 24 hr. at 4° while the other half was stored frozen. The results (Table 4) show that the rate was lowered in the presence of NaCl, but that removal of the NaCl by dialysis against veronal–Mg^{2+} buffer largely restored the original activity. The rate is also depressed by LiCl, KCl, K_2SO_4, and Na_2SO_4, so that the effect is a general one of the ionic strength of the solution. Attempts to fractionate the enzyme were complicated by this effect, since many methods of fractionation yield solutions containing salts. The rate of hydrolysis of β-lactoglobulin by carboxypeptidase is also markedly lowered by increasing the ionic strength of the solution (Davie, Newman & Wilcox, 1959).

Fig. 2 shows the pH–activity curve of α_1-crystallin measured between pH 6·6 and 8·05 at 55°. The rate was greatest at pH 7·3 and was at least 90 % of maximum between pH 6·9 and 7·6. The proteolytic activity of dispersion of ox-lens cortex was measured at 37° at pH values from 3 to about 8, and at 37° and 55°. There was no evidence of a proteinase in lens with a pH maximum in the acid range (Fig. 3).

Diffusible products of proteolysis

α_1-Crystallin solution (2·5 ml.) plus veronal–Mg^{2+} buffer (37·5 ml.) were incubated at 55° for 7 hr., and then dialysed against 300 ml. of water (and a few drops of chloroform) for 72 hr. at 4°. The diffusate was evaporated to dryness under reduced pressure, and the residue extracted with water (0·3 ml.). The insoluble material was removed by centrifuging, and 5 μl. of the supernatant was applied to Whatman no. 3 paper for electrophoresis. The buffer contained 90 % (w/v) formic acid (125 ml.) and acetic acid (375 ml.) in 2·5 l., and electrophoresis was carried out for 130 min. at 350 v (14 v/cm.) in the apparatus previously described by Cliffe & Waley (1958). The main ninhydrin-positive spots, detected on a guide strip, were contained in a portion of the paper near the cathode, and a piece 8·5 cm. × 4 cm. carrying the spots was cut out and sewn into Schleicher & Schüll 2043b paper, with the long axis at right angles to the long axis of the sheet. Development on the second direction was carried out in butan-1-ol–acetic acid–water (40:9:20, by vol.) for 13 hr. (cf. Richmond & Hartley, 1959). The following amino acids, identified by comparison with markers, were formed: aspartic acid, serine, alanine, valine, arginine, lysine, histidine, glycine, proline, tyrosine, methionine, cystine, leucine or isoleucine or both, and glutamic acid or threonine or both.

Variation of rate of proteolysis with concentration of α_1-crystallin

The stock solution of α_1-crystallin (diluted 100-fold, this solution had $E_{280} = 1{\cdot}96$) was diluted with varying amounts of veronal–Mg^{2+} buffer, and heated at 55° for various times (up to 3·5 hr.). The range of concentrations covered was fivefold.

At the higher concentration of α_1-crystallin (Table 5) the activity tends towards a constant value (i.e. the rate is proportional to the concentration of the enzyme), but the activity falls at lower concentrations of protein (i.e. the rate is not proportional to the concentration of enzyme in the more dilute assay mixtures). This is the expected behaviour when enzyme and substrate are both present in the same solution, i.e. when endogenous substrate is present. This may be shown as follows (cf. Reiner, 1959).

If the solution of α_1-crystallin contains a mg. of enzyme/ml. and b mg. of substrate/ml., and H is the proportion of solution in the reaction mixture,

Table 4. *Reversible inhibition of proteolysis by sodium chloride and by ethylenediaminetetra-acetic acid*

Assays were carried out at 55° in the presence of 1 % (w/v) α_2-crystallin. Expt. 1 relates to the effect of Mg^{2+} ions, and Expt. 2 to the effect of NaCl. The control and the NaCl assay mixtures contained $MgCl_2$.

	Treatment	Relative rate (%)
	None (control)	100
Expt. 1	$MgCl_2$ omitted	35
	Dialysis against 5 mM-EDTA (EDTA, 3·5 mM in incubation mixture)	7
	Dialysis against EDTA, followed by addition of $MgCl_2$ (0·01 M in incubation mixture)	44
Expt. 2	NaCl (0·3 M)	18
	NaCl (0·3 M), followed by dialysis against veronal–Mg^{2+} buffer	70

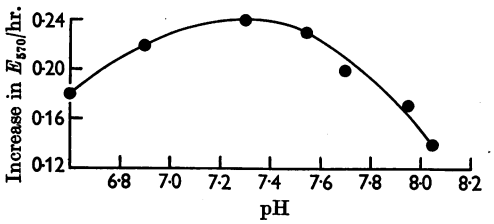

Fig. 2. pH–activity curve of α_1-crystallin at 55°. Protein breakdown was assayed as described in the Methods section. The pH values of the incubation mixtures were measured at room temperature. A stock solution of α_1-crystallin was diluted 1 in 33 with veronal buffer (Gomori, 1955) containing $MgCl_2$ (5 mM) and α_2-crystallin (1 %, w/v).

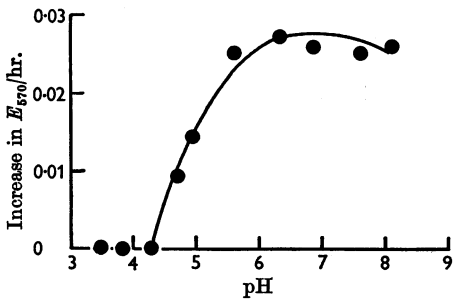

Fig. 3. pH–activity curve at 37° of a dispersion of ox-lens cortex. The dispersion (about 120 mg. of protein/ml.) was dialysed for 48 hr. at 4° against formate buffer (0·01 M; pH 4·5) (Long, 1961) containing 5 ml. of M-$MgCl_2$/l. The pH was adjusted by dropwise addition of N-NaOH or N-HCl, with stirring, in the cold.

Table 5. *Variation of rate of proteolysis with concentration of α_1-crystallin*

The stock solution of α_1-crystallin was diluted tenfold with veronal–Mg^{2+} buffer and from 0·05 to 0·25 ml. of this solution diluted to 0·4 ml. for assay at 55°. The first column (H) refers to the volume of diluted solution of α_1-crystallin/ml. of assay mixture.

H	Concn. of protein (mg./ml.)	Rate (units)	Activity (units/ml.) Expt.	Calc.
0·125	2·45	0·210	16·8	17·6
0·188	3·7	0·410	21·7	22·0
0·25	4·9	0·572	22·9	22·9
0·37	7·26	0·877	23·7	23·6
0·50	9·8	1·208	24·2	24·1
0·625	12·25	1·525	24·4	24·4

then the concentrations of enzyme and substrate in the reaction mixture are aH mg./ml. and bH mg./ml. respectively. If simple Michaelis–Menten kinetics hold (this can, naturally, be only an approximation for such a complex reaction as proteolysis), the rate, v, is given by

$$v = k_2[E]\,[S]/(K_m + [S]) \qquad (1)$$

where k_2 is the rate constant for the decomposition of the enzyme–substrate complex and K_m is the Michaelis constant, and E and S are the enzyme and the substrate respectively. In our case, $[E] = aH$ and $[S] = bH$, and equation (1) becomes

$$v = k_2 abH^2/(K_m + bH) \qquad (2)$$

Only when $H \gg K_m/b$ will the rate, v, become a linear function of H; the straight-line graph of $v = k_2 aH$ is approached asymptotically if H becomes greater than K_m/b. Equation (2) can be written as

$$\frac{H}{v} = \frac{K_m}{k_2 abH} + \frac{1}{k_2 a} \qquad (3)$$

The graph of H/v against $1/h$ is thus linear. Values of 2·54 units for $k_2 a$ and 0·028 for K_m/b were found from the intercept and slope, and were used to calculate the activities shown in the last column of Table 5. The agreement with the observed values for the activity shows that the activity at various concentrations of α_1-crystallin can be quantitatively accounted for if the solution of α_1-crystallin contains both enzyme and substrate. Moreover, by this method, it should be possible to fractionate the preparation and to test the distribution of both the enzyme and the substrate among the fractions. This procedure would be too laborious as a routine, and so experiments were carried out to see whether the rate was proportional to the enzyme concentration in the presence of added substrate. The rate of formation of amino acids was measured after various amounts of α_2-crystallin, itself enzymically inactive, had been added. Fig. 4 shows that the

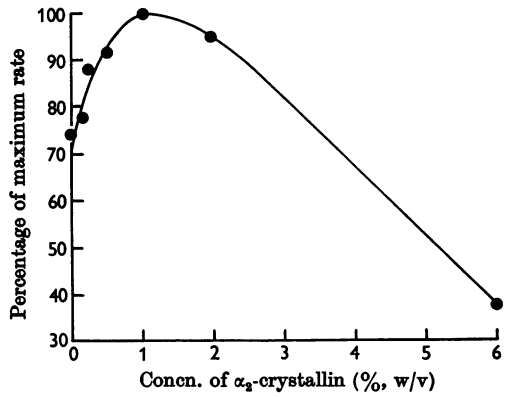

Fig. 4. Effect of concentration of α_2-crystallin on the rate of proteolysis. Each incubation mixture consisted of 0·4 ml. of veronal–Mg^{2+} buffer containing a constant amount of α_1-crystallin (0·014 ml. of stock solution) and varying amounts of α_2-crystallin at final concentrations of between 0·1 and 6·0% (w/v). Incubations were at 55° from 0 to 2 hr. Protein breakdown was assayed as described in the Methods section.

Table 6. *Variation in rate of proteolysis with concentration of α_1-crystallin*

The tubes contained 0·04 ml. of α_2-crystallin, and 0·36 ml. of α_1-crystallin diluted with veronal–Mg^{2+} buffer. Four tubes were set up for each dilution of α_1-crystallin, and incubated at 55° for times which varied from 0, 20, 40 and 60 min. for the lowest dilution to 0, 60, 150 and 240 min. for the highest dilution.

Dilution of stock solution of α_1-crystallin	Rate (units)	Activity (units/ml.)
286	0·045	12·9
143	0·0982	14·0
57·2	0·295	16·8
28·6	0·602	17·2
14·3	1·228	17·5

rate increased somewhat in the presence of 1% (w/v) of α_2-crystallin, but that it decreased at higher concentrations.

The increase in rate with added α_2-crystallin shows that this fraction contains proteins which are hydrolysed by the enzyme in α_1-crystallin; the decrease in rate at higher concentrations of α_2-crystallin suggests that this fraction also contains inhibitors. The complete system (α_1-crystallin and α_2-crystallin) thus contains 'endogenous' substrate (present in the α_1-crystallin fraction), added (exogenous) substrate and, probably, added inhibitors; it is clearly too complex to attempt any kinetic analysis.

The next point was whether the rate in the presence of optimum (1%) α_2-crystallin was proportional to the concentration of enzyme. Table 6

shows that, over a 20-fold range of enzyme concentration, the activity varied by only 26% (over a 4-fold range, at the higher concentrations, the activity varied by only 4%). Under these conditions, the assay is thus satisfactory.

The activities of proteolytic enzymes are commonly tested with casein, denatured haemoglobin or serum albumin as substrates. Although proteolysis occurred with these proteins, the rate was lower than when they were omitted (Table 7). There is thus no evidence that these 'foreign' proteins can serve as substrates; they may be substrates whose turnover rates are less than that of the endogenous substrate in α_1-crystallin, or they may combine with the enzyme but not break down. Only α_2-crystallin increased the rate (Table 7). Earlier experiments with lens proteins, modified by oxidation with performic acid or by reaction with sodium sulphite, gave only materials which decreased the rate of proteolysis; these modifications often render proteins more susceptible to enzymic hydrolysis (Ferrini & Zito, 1961).

Distinction between leucine aminopeptidase and proteinase

Even highly diluted ox-lens extracts catalyse the hydrolysis of L-leucylglycine (Abderhalden & Hanson, 1938), and the leucine aminopeptidase from lens has been purified (Cliffe & Waley, 1958; Fittkau, Glässer & Hanson, 1961*a*). The enzyme that hydrolyses α_1-crystallin was distinguished from the leucine aminopeptidase (the two activities are referred to as proteinase and peptidase) by two experiments, the first employing ethanol precipitation of the crude extract (Patterson, 1959), and the second inhibition by *o*-iodosobenzoate.

In the first experiment, ox lenses (70 g.) were stirred with water (210 ml.) containing $NaHCO_3$ (1 g.) for 30 min. The turbid extract A (240 ml.) was made 0·01 M with respect to $MgCl_2$ and 1 M with respect to NaCl, and cooled ethanol (120 ml.) added. The precipitate was collected by centrifuging at 10000*g* for 20 min. at 0° and extracted with veronal–Mg^{2+} buffer, the extract was dialysed and clarified by centrifuging to give solution B (121 ml.). The supernatant from the first ethanol precipitation was diluted with an equal volume of ethanol, and the precipitate also extracted with veronal–Mg^{2+} buffer, and the extract dialysed and clarified, to give solution C (65 ml.). The three fractions were assayed both for proteinase activity and for peptidase activity (Table 8). The ratio of the two activities was markedly different in the different fractions: proteinase activity was shed, while peptidase activity was enhanced. In a separate experiment, the rate of proteolysis by α_1-crystallin was not increased by the addition of fraction C.

In the second experiment, the results of the reaction with *o*-iodosobenzoate (Table 8) show that the peptidase activity was little affected, but that the proteinase activity was greatly reduced.

Table 7. *Rate of proteolysis in presence of various proteins*

The concentration of α_1-crystallin was 2·1 mg./ml. and the assay tubes were withdrawn at intervals during incubation for 5 hr. at 55°; the rate was constant throughout this time.

Proteins present in reaction mixture	Activity (units/ml.)	Relative activity
α_1-Crystallin	14·1	100
α_1-Crystallin + α_2-crystallin (1%, w/v)	18·9	134
α_1-Crystallin + casein (0·7%, w/v)	1·5	11
α_1-Crystallin + haemoglobin (1%, w/v)	7·0	50
α_1-Crystallin + bovine serum albumin (1%, w/v)	6·6	47

Table 8. *Comparison of proteinase and peptidase activities*

In Expt. 1, the tubes contained 0·037 ml. of fraction A or 0·36 ml. of fractions B and C and 0·04 ml. of 10% (w/v) α_2-crystallin in veronal–Mg^{2+} buffer (total vol. 0·4 ml.) for the assay of proteinase activity. The dilutions of the fractions for the peptidase assay (see Methods section) were 100-fold for A and B and 500-fold for C. Both activities were assayed at 55°.

In Expt. 2, α_1-crystallin solution (0·4 ml.) was diluted to 4·4 ml. with veronal–Mg^{2+} buffer; 0·1 M-*o*-iodosobenzoate (0·02 ml.) was added to 2 ml. of the solution, and the mixture kept for 30 min. at room temperature before assay. Another 2 ml. portion of the solution served as a control.

Expt.	Addition	Fraction	Concn. of protein (g./ml.)	Peptidase activity (units/ml.)	Proteinase activity (units/ml.)	$10^{-3} \times \frac{\text{Peptidase activity}}{\text{Proteinase activity}}$
1	—	A	0·0913	2880	5·6	0·51
	—	B	0·0195	620	0·131	4·74
	—	C	0·0435	7560	0·057	132·6
2	None (control)	—	—	4900	19·6	0·25
	o-Iodosobenzoate	—	—	3920	1·82	2·16

Methods of following proteolysis

The assay with ninhydrin (see Methods section) was used as a routine, but some other methods were also investigated.

Extinction measurements. Measurement of E_{280} after removal of protein was carried out as follows. α_1-Crystallin (1 ml. of stock solution) and 1 ml. of 10% (w/v) α_2-crystallin were diluted with 8 ml. of veronal–Mg^{2+} buffer. Portions (2 ml.) were incubated at 55° for times up to 1 hr.; 3 ml. of 10% (w/v) trichloroacetic acid was added to each portion, and the mixture filtered through Whatman no. 3 paper. The rate of increase in E_{280} of the filtrate was 0·29/hr., and was not appreciably affected by the presence of cysteine (0·01 M) in the reaction mixture. In a parallel experiment, followed by the ninhydrin assay (see Methods section), the rate of increase in E_{570} was 0·3/hr.; when allowance is made for the differing dilutions, the ninhydrin method is 36 times as sensitive as the 'E_{280}' method. In practice, the ninhydrin method is about 10 times as sensitive.

Reaction with 1-fluoro-2,4-dinitrobenzene. The amino acids liberated on proteolysis were estimated by their reaction with 1-fluoro-2,4-dinitrobenzene (Williams & Thorne, 1954). The reaction was carried out either on the filtrate after precipitation of the proteins by 0·2 M-acetate buffer at 100°, or directly on the reaction mixture which was subsequently filtered from the insoluble dinitrophenyl-protein. In either case, E_{420} was measured in acid solution, as the by-product, dinitrophenol, absorbs little radiation under these conditions. This procedure, although generally not so convenient as the reaction with ninhydrin, is useful if the solution to be assayed contains substances which affect the reaction with ninhydrin.

Formol titration. Formol titration on the incubation mixture was used to determine whether there were products (e.g. large polypeptides) which were being precipitated by 0·2 M-acetate at 100° (or adsorbed on the protein precipitate), and which would thus escape estimation by the ninhydrin reaction of the acetate filtrate. α_1-Crystallin (0·2 ml. of stock solution) and α_2-crystallin (1 ml. of 10%, w/v) were diluted to 11·3 ml. with veronal buffer 0·01 M with respect to $MgCl_2$, and the solution was divided into two portions. One was incubated for 5 hr. at 55°, and the other stored frozen. The formol titration was carried out by adding 0·7 ml. of formaldehyde (36%, w/v, brought to pH 8·5) to a 2 ml. portion of solution, and titrating to pH 8·5 with 0·05 N-NaOH (from an Agla syringe). Under these conditions, 1 μmole of leucine required 1·05 μmoles of alkali, and so the results (as in the ninhydrin assay) are effectively in 'leucine equivalents'. The values of the titres on portions of the unincubated sample were 11·0, 11·1, 11·2 Agla divisions, and on the incubated sample 13·2, 13·2, 13·5. The difference of 2·1 Agla divisions (here 2·1 μmoles of NaOH) due to reaction leads to a value of 11·3 units/ml. for the activity of the α_1-crystallin stock solution used; by the ninhydrin method on an acetate filtrate, the activity was found to be 17·2 units/ml. The lower value from the formol titration indicates that some of the amino group released was incompletely titrated (at pH 8·5), and there is clearly no evidence for any extensive formation of amino compounds not present in the acetate filtrate.

Absence of proteinase activity from a subcellular fraction of lens

The capsules of eight fresh ox lenses were ruptured, and the lenses were stirred with 40 ml. of 0·25 M-sucrose for 20 min. at 4°. The dispersion of the cortices was homogenized for 3 min. in a stainless-steel homogenizer of the Potter–Elvehjem type, and the homogenate fractionated by centrifuging at 18500*g* for 1 hr. at 0°. The washed precipitate had little or no proteolytic activity when assayed at 55°.

Comparison of rate of proteolysis in the lens with that in other tissues

Dispersions of tissues were made as soon as possible after death of the animal (a few minutes for the rat and about an hour for the ox). The lenses were weighed and ground in a mortar with a little sand and twice their weight of ice-cold veronal–

Table 9. *Comparison of rate of proteolysis in the lens with that in other tissues*

For details see text. Results are given as a rough guide to the relative rates of proteolysis in the different tissues. Usually only one estimation was done on each tissue (apart from the lens).

Tissue	Animal	Rate (μmoles of amino acid/g. of tissue/hr.) 37°	55°
Lens			
Whole	Calf	0·80	12
Cortex	Ox	0·87	20
Nucleus	Ox	0·41	10
Whole	Rat	1·98	19
Whole	Rabbit	1·06	7·5
Liver	Ox	2·0	3·9
Brain	Ox	3·5	6·2
Spleen	Ox	4·8	7·5
Kidney	Ox	1·5	5·8
Retina	Ox	0·6	1·3
Liver	Rat	8·1	25
Spleen	Rat	12·0	28
Brain	Rat	2·0	4
Kidney	Rat	16·0	38

Mg^{2+} buffer. The other tissues were mopped free of blood, rapidly chilled in ice, and passed through a Fisher mincer ($\frac{1}{16}$ in. mesh grid); the pulp was weighed (about 3 g.) and 2 vol. of ice-cold veronal–Mg^{2+} buffer added. The dispersons of all the tissues were dialysed against 1 l. of buffer at 4° for 24 hr., against fresh buffer for a further 24 hr., and then frozen overnight or longer. Portions (0·4 ml.) of the dialysed dispersions were incubated for various times up to 6 hr. at 37°, and up to 1 hr. at 55°. Protein was removed by boiling with acetate buffer, and the filtered solutions were taken for ninhydrin estimations (see Methods section).

A marked feature of the results (Table 9) is that the rate of protein breakdown by the lens is about 10 times higher at 55° than it is at 37°; increase of temperature had a similar effect on breakdown of α_1-crystallin (Table 2). The rate of proteolysis in the other tissues examined is increased only 2–3 times by raising the temperature from 37° to 55°.

DISCUSSION

Properties of the neutral proteinase in lens

The simplest method of fractionating the soluble proteins of the lens is by precipitation at pH 5; the precipitate is called α_1-crystallin and the protein in solution β-crystallin. Proteinase activity was concentrated in the α_1-crystallin fraction, and this was used as source of enzyme for most of the experiments. When the solution of β-crystallin is made 13 % (v/v) with respect to ethanol, a second protein fraction separates, and this has been called α_2-crystallin (François *et al.* 1955). This fraction, which lacked proteinase activity, was generally used as substrate. The enzyme was relatively stable to heat, and routine assays were carried out at 55°. The pH optimum was 7·3, and the rate was increased by the addition of Mg^{2+} or Mn^{2+} ions, and decreased in the presence of EDTA. The rate of breakdown of protein at 37° in a dialysed dispersion of ox (or calf) lens is about 0·02 %/hr. This rate is increased to 1·0 %/hr. if α_1-crystallin is used and the incubations are carried out at 55° in the presence of Mg^{2+} ions. Most of these properties are shared by the leucine aminopeptidase of the lens (Abderhalden & Hanson, 1938; Hanson & Methfessel, 1958; Methfessel, 1960; Fittkau, Glässer & Hanson, 1961*b*) which is very active in the ox. Leucine aminopeptidase (after purification from pig kidney) hydrolyses several proteins by stepwise removal of amino acid residues from the *N*-terminal end (Hill & Smith, 1958), and so it was an obvious possibility that (lens) leucine aminopeptidase was the enzyme that we were studying. The two enzymes, however, could be distinguished by ethanol precipitation, and by inhibition of the proteinase by *o*-iodosobenzoate; moreover, a purified preparation of the peptidase did not accelerate the proteolysis of α_1-crystallin. Tissues (and the lens is no exception) contain peptidases other than leucine aminopeptidase, but it is not known whether they attack proteins.

A role in protein metabolism is assigned to the enzyme in the lens that catalyses the hydrolysis of phenylalanine ethyl ester (Zeller, Bannerjee & Shoch, 1960; Zeller & Shoch, 1961). Although the hydrolysis of this substrate is catalysed by chymotrypsin, there does not seem to be any cogent reason for assuming that phenylalanine ethyl ester is the substrate for an intracellular proteinase or peptidase (although, of course, it may be).

Proteolysis during prolonged incubation of ox-lens extracts was recorded by Krause (1933), and Fromageot & Prioux (1955) reported that dilute extracts of rat lens could catalyse the hydrolysis of casein in the presence of cysteine, although we have not been able to confirm this result. The extent of proteolysis in ox-lens extracts (Devi, 1961) (as measured by the extinction at 280 mμ of a deproteinized filtrate) reaches a maximum after a given time, and then declines. This result may be attributed to the use of a 'blank' containing no added substrate; hydrolysis of endogenous substrate occurs readily.

Mode of action of lens proteinase

The products formed in the incubation of α_1-crystallin have been separated from material of high molecular weight by dialysis, and shown to be amino acids. Although (as discussed above) there is evidence that the enzyme is distinct from leucine aminopeptidase, this peptidase is present in the α_1-crystallin fraction, and in fact the rate of formation of glycine from leucylglycine is 250 times as rapid as that of amino acids by proteolysis. Thus it is probable that leucine aminopeptidase plays a part in the formation of amino acids from the protein. The rate-determining step may be the conversion of the protein into products, these products then being rapidly broken down to amino acids by leucine aminopeptidase. Further insight into the process awaits the isolation of a preparation of proteinase which lacks aminopeptidase activity.

Metabolic role of lens proteinase

Protein turnover *in vivo* probably occurs, although the rate may vary widely (Simkin, 1959). The problem is complicated by the fact that most tissues contain cells of different types, as well as containing many different proteins. Cultured mammalian cells incorporate labelled amino acids, in the absence of net protein synthesis, at a rate which corresponds to a renewal of 1 % of the protein

present/hr. (Levintow & Eagle, 1961); here there is no complication from cells of different types. The lens is an organ which contains solely epithelial cells, so that its cellular composition has the same homogeneity as has a culture of cells. Protein turnover in the lens probably occurs: the lens, in tissue culture, incorporates amino acids into a fraction which is predominantly protein (Merriam & Kinsey, 1950; Mandel, Dardenne & Lessinger, 1957; Weber, 1959). What is not certain is whether the amino acids were in fact incorporated into protein molecules; Devi, Friel & Lerman (1961) have reported that, in a cell-free system, incorporation was mainly into RNA.

Neutral proteinases in other tissues

Protein turnover presumably involves synthesis and breakdown, both proceeding concurrently. The nature of the stages in protein breakdown are not clear. Ansell & Richter (1954) suggested that the 'neutral proteinase' that they studied in rat-brain extract might play a part in the catabolism of proteins, and the same may be true for the proteinase of lens. 'Neutral proteinases' have been detected also in skeletal muscle (Koskalka & Miller, 1960*a*, *b*), liver (Kuzovleva & Chung-Yen, 1959), lung (Dannenberg & Smith, 1955), lymph node (Stein & Fruton, 1960), adrenal gland (Todd & Trikojus, 1960), skin (Martin & Axelrod, 1958), nerve (Porcellati & Curti, 1960) and erythrocytes (Morrison & Neurath, 1953); and proteolysis occurs in serum (Piez, Oyama, Levintow & Eagle, 1960) and in preparations of histones from calf thymus (Phillips & Johns, 1959). The neutral proteinase in hog pituitary gland resembles the lens proteinase in being precipitated at pH 5 and in being relatively thermostable (Adams & Smith, 1951). Protein breakdown occurs when fragments of mouse liver are incubated (under sterile conditions) (Berenbom, Chang, Betz & Stowell, 1955), in liver slices (Steinberg & Vaughan, 1956) and in liver or brain mitochondria (Penn, 1960). Proteinase activity is also widely distributed amongst subcellular fractions of rat liver (Korner & Tarver, 1957; Hanson, Kleiner & Blech, 1959). It is not clear to what extent (and under what conditions) cathepsins are responsible for protein breakdown. The proteolytic enzyme in ox spleen (and ox kidney) called cathepsin D (Press, Porter & Cebra, 1960; Lapresle & Webb, 1960) is responsible for most of the proteolytic activity of these tissues at pH 4, as gauged by measurements of the extinction at 280 mμ of deproteinized extracts. This enzyme is not active above pH 6·5, and is not an exopeptidase. Leucine aminopeptidase, on the other hand, is not active below pH 6.

Press *et al.* (1960) consider that the cathepsin D in spleen may be acting near its optimum pH (at about 5) *in vivo*. Unless the peptidases of spleen are active at this low pH, the products would be expected to consist mainly of peptides rather than of amino acids. The assay used by Lapresle & Webb (1960) in fact measures the amount of acid-soluble peptides by the biuret reaction. Whatever the situation in the spleen, from the fragmentary evidence generally available about the pH optima of peptidases, proteinases that act in neutral solution would be better able to co-operate with peptidases in the breakdown of proteins into amino acids. We have tested several tissues of the ox and of the rat, and all undergo proteolysis at pH 7·5; the rate is higher at 55° than at 37°. Press *et al.* (1960) did not detect proteolytic activity in neutral solution. There are two factors which may account for this difference. One is that the ninhydrin reaction that we use is a more sensitive measure of peptide-bond splitting than is the extinction at 280 mμ, and the other is that haemoglobin may not serve as a substrate for a 'neutral proteinase'. Certainly, the lens proteinase acted on its endogenous substrate more readily than on haemoglobin, and indeed may not have attacked the haemoglobin at all. Preferential cleavage of endogenous substrate has also been noted for a proteinase from lymph nodes (Stein & Fruton, 1960) and in the enzyme from rat muscle (Koskalka & Miller, 1960*a*, *b*).

The enzymes that catalyse proteolysis at pH 7·5 seem to be of general occurrence, and may constitute a class of their own, the 'neutral proteinases' (Ansell & Richter, 1954). The proteolytic activity of most tissues is much higher at pH 4 than at pH 7·5; in rat liver the neutral proteinase and the cathepsin have been separated (Kuzovleva & Chung-Yen, 1959); the rate–pH profile of the crude extract is simply a consequence of the relatively large amount of the cathepsin.

SUMMARY

1. Dialysed dispersions of animal tissues liberate ninhydrin-positive material on incubation in neutral solution; the rate is 1–16 μmoles of amino compound/g. wet wt. of tissue/hr. at 37°.

2. The system in ox lens is concentrated in that fraction of the soluble proteins which is precipitated at pH 5 (α_1-crystallin). The ninhydrin-positive species formed on incubation of α_1-crystallin are amino acids, i.e. the reaction is proteolysis.

3. Proteolysis (measured by the ninhydrin reaction on boiled filtrates) proceeds considerably more rapidly at 55° than at 37°; the reaction is accelerated by Mg^{2+} ions, and the pH optimum is 7·3.

4. The 'neutral proteinase' in α_1-crystallin acts on endogenous substrate, and on the proteins precipitated with 13 % ethanol at pH 5 (α_2-crystallin).

Proteolysis is retarded, however, by the addition of casein, haemoglobin or serum albumin.

5. The neutral proteinase in lens seems to be distinct from leucine aminopeptidase which is also present in α_1-crystallin. The neutral proteinase, but not the peptidase, is inhibited by *o*-iodosobenzoate, and fractions enriched in peptidase activity show relatively little proteinase activity.

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