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Neutral Proteinases in the Lens

2. PARTIAL PURIFICATION AND PROPERTIES*

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The term 'neutral proteinase' denotes a widely occurring class of proteolytic enzymes that are most active at about pH 7.5. This property distinguishes neutral proteinases from cathepsins, as cathepsins are defined as having pH optima of about 4 (Fruton, 1960). Waley & van Heyningen (1962*a*) used as source of enzyme the fraction of the soluble proteins of ox lens that is precipitated at pH 5 (α_1 -crystallin). This fraction contains both the proteinase and proteins that serve as substrates. The enzyme has now been purified; the 'endogenous' substrate has been shed, and this permits a comparison of substrates. Moreover, the proteinase has been largely separated from the leucine aminopeptidase in lens. Part of these results have been published in a symposium (van Heyningen & Waley, 1962; Waley & van Heyningen, 1962*b*).

METHODS

Veronal buffer. This contained 400 ml. of 0.025 M-diethylbarbituric acid, 14.5 ml. of 0.5 M-sodium diethylbarbiturate and 8.6 ml. of M-MgCl₂ in 1725 ml. of solution; CO₂-free water was used; pH was 7.5 at room temperature.

Assays. The proteinase was assayed with α_2 -crystallin (1%, w/v) as substrate in veronal-Mg²⁺ buffer at 55° (Waley & van Heyningen, 1962*a*). Four samples were usually taken, after incubation for 0, 1, 2 and 3 hr. The extent of reaction (followed by the ninhydrin reaction) was proportional to the time for at least 3 hr. under these

conditions. The unit of proteinase is that amount which produces 1 μ mole of amino acid (leucine equivalent) in 1 ml. of incubation mixture/hr. Leucine aminopeptidase was assayed with L-leucylglycine as substrate, the enzymic hydrolysis being followed by paper chromatography. The unit of peptidase is that amount which produces 1 μ mole of glycine/ml. of assay mixture/hr. (Waley & van Heyningen, 1962*a*).

Gel-filtration. This was carried out on 2.7 cm. $\times$ 15 cm. long columns of Sephadex G 25 (medium grade; Pharmacia, Uppsala, Sweden) in the cold room; the columns were equilibrated and eluted with the veronal-Mg²⁺ buffer. The concentration of protein in the fractions was estimated by *E* at 280 m μ , read against the veronal buffer, which scarcely absorbs at this wavelength; *E*_{1 cm.}^{1%} 10 was assumed for all protein fractions. For α_2 -crystallin, calibration by the biuret reaction (Gornall, Bardawill & David, 1949) with bovine serum albumin as standard gives *E*_{1 cm.}^{1%} 9.3.

Diethylaminoethylcellulose. Diethylaminoethyl (DEAE)-cellulose (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was graded by repeatedly allowing it to settle in water for about 20 min. and removing the supernatant. The DEAE-cellulose was washed on a sintered-disk funnel with 0.5 N-NaOH, and then with water; it was converted into the chloride form by washing with N-HCl and then washed with water. Finally, the DEAE-cellulose was equilibrated with the veronal-Mg²⁺ buffer, pH 7.5, and then packed into a column, 2.25 cm. $\times$ 12 cm. long. Chromatography was carried out in the cold room (at about 6°); the rate of elution was 1–2 ml./min. with a pressure of about 80 cm. water.

Hydroxyapatite. This was prepared by the method of Tiselius, Hjerten & Levin (1956) and a column about 1.7 cm. $\times$ 7 cm. long used. Before use it was washed with 50 ml. of M-phosphate, pH 7.4, prepared from NaH₂PO₄.

* Part 1: Waley & van Heyningen (1962*a*).

2H₂O (78 g., 0.5 mole) and N-KOH (40 ml.), made up to 500 ml. The other buffers used for chromatography were made by dilution of this M-phosphate buffer. The column was then washed with 250 ml. of 1 mM-phosphate buffer. The same sequence of operations was used to regenerate the columns after chromatography. The concentration of phosphate in the eluate was measured by the method of Fiske & Subbarow (1925).

The rate of elution (in the cold room) varied on different occasions, from 0.4 ml./min. to less than 0.1 ml./min., at an applied pressure of about 80 cm. water. Elution was hastened by applying a pressure of 2.5 cm. Hg with a bicycle pump, with the apparatus described by Edwards (1960).

RESULTS

Purification of lens proteinase by fractional precipitation and extraction. All the experiments to be described on the purification of the proteinase started from α_1 -crystallin, prepared as described by Waley & van Heyningen (1962*a*). The methods tried first were unsuccessful, and thus are mentioned only briefly here.

Adsorption on calcium phosphate gel seemed promising; the proteinase was more readily adsorbed on the gel, and less readily eluted from it, than the bulk of the protein. However, no reproducible method was found for eluting the enzyme from the gel and even in the most successful experiments the recovery of activity was poor. Fractional precipitation with acetone was tested thoroughly. The enzyme was stable at the low temperatures used, as the overall recovery of activity from all the fractions was good, but there was appreciable activity in each fraction, and no one fraction was significantly enriched in enzyme. Whether the fractional precipitation was carried out at pH 6.1 or at pH 6.7, the most active fraction had a specific activity that was only 1.2 times that of the original solution.

Fractional precipitation with ammonium sulphate gave results rather similar to those obtained by precipitation with acetone; there was some activity in each fraction. Better results were obtained by fractional extraction of an ammonium

sulphate precipitate (Table 1). The protein was precipitated from 2.4M-ammonium sulphate at pH 7, and the precipitate was extracted by successively more dilute solutions of the salt. Each fraction was desalted by gel-filtration before assay; owing to the efficiency of the gel-filtration the ammonium sulphate was completely removed and did not interfere with the ninhydrin reaction used in the assay. About 75% of the protein was recovered after gel-filtration, which took only about 30 min. The most active fraction was obtained by extraction with 1.2M-ammonium sulphate; about half the activity was recovered in this fraction, which was 2.4 times enriched in enzyme.

Purification of lens proteinase by chromatography. The degree of purification achieved by the ammonium sulphate extraction was not high, but this procedure was sometimes used as a preliminary to chromatography on DEAE-cellulose (Cl⁻ form). All the protein in α_1 -crystallin was adsorbed from veronal-Mg²⁺ buffer; stepwise elution was carried out with sodium chloride in veronal-Mg²⁺ buffer (0.1, 0.2 and 0.3M-sodium chloride). The results are shown in Fig. 1. With either α_1 -crystallin (Fig. 1*a*) or the (desalted) 1.2M-ammonium sulphate extract (Fig. 1*b*) the bulk of the protein (peak B) was eluted by the 0.2M-sodium chloride, but the activity was eluted by 0.3M-sodium chloride (Fig. 1*a*, peak C). The presence of the salt in the eluate interfered with the assay (Waley & van Heyningen, 1962*a*) and so pooled fractions were desalted by gel-filtration. The specific activity was increased four- to eight-fold by chromatography on DEAE-cellulose, but recovery of activity was only about 15%.

The fractions were also assayed with L-leucylglycine for peptidase activity (Fig. 1*b*). Nearly all the peptidase was eluted by 0.2M-sodium chloride, so that chromatography on DEAE-cellulose largely separates the peptidase from the proteinase. This chromatographic separation confirms our earlier conclusion (based on less direct evidence) that the proteinase is distinct from the peptidase.

Table 1. *Fractional extraction by ammonium sulphate*

α_1 -Crystallin soln. (about 20%, w/v; 4 ml.) was diluted with 5.8 ml. of veronal-Mg²⁺ buffer; 1.8 ml. of the mixture was withdrawn, and the remainder was treated with 12 ml. of 4M-(NH₄)₂SO₄ soln., pH 7, with stirring, and then centrifuged at 10 000g at 0° for 30 min. Three-quarters of the precipitate was extracted successively with 1.6, 1.2 and 1.0M-(NH₄)₂SO₄ soln. Each extraction was carried out with 5 ml. of (NH₄)₂SO₄ soln. with stirring for 20 min. at 4°. Each extract was desalted by gel-filtration before assay.

Fraction	Volume (ml.)	Activity (units/ml.)	Total activity (units)	Concn. of protein (mg./ml.)	Specific activity (units/g. of protein)
α_1 -Crystallin	6	6.09	36.5	67.7	90
1.6M-(NH ₄) ₂ SO ₄	12.4	0.033	0.41	0.384	80
1.2M-(NH ₄) ₂ SO ₄	17	1.08	18.3	4.95	218
1.0M-(NH ₄) ₂ SO ₄	21	0.353	7.41	8.23	43

The solution that contained the enzyme (Fig. 1*b*, peak C) was dilute (concentration of protein was about 0.2 mg./ml.), and such dilute solutions lost most of their proteolytic activity on storage. The activity could, however, be preserved if the solution was saturated with ammonium sulphate (at pH 7.5) and stored at 4°. This step was therefore interposed before the next method of purification, which was chromatography on hydroxyapatite. The fractional extraction with ammonium sulphate, gel-filtration and chromatography on DEAE-cellulose were carried out as described in Fig. 1*(b)*, but starting from 13.3 ml. of stock solution of α_1 -crystallin. Ammonium sulphate (23.5 g.) and saturated aqueous sodium hydrogen carbonate (3.2 ml.) were added to the pooled fractions (peak C) eluted by 0.3M-sodium chloride (29 ml.) and the mixture was stirred for 2 hr. and then stored for 48 hr. at 4°. The precipitate was collected by filtration on a glass-fibre paper and extracted (at 4°) with 2.5 ml. of 1 mM-disodium hydrogen phosphate. The extract was clarified by centrifuging and the solution of protein freed from ammonium sulphate by gel-filtration. On chromatography on hydroxyapatite at pH 7.5 all the

protein was adsorbed, and stepwise elution with phosphate buffer (pH 7.5) gave a series of peaks (Fig. 2). The concentration of phosphate in the eluate was lower than that applied to the column; apparently the hydroxyapatite is capable of binding appreciable amounts of PO_4^{3-} ion. The fractions, pooled on the basis of their protein content (from E_{280}), were submitted to gel-filtration to remove PO_4^{3-} ion before assay (marked inhibition is caused by even quite low concentrations of phosphate). The last fraction (G) was the most active; its specific activity was 5000 units/g. of protein, which represents a 70-fold purification. Unfortunately, the overall recovery of activity (from α_1 -crystallin) was only about 1%.

Of the two methods of chromatography, greater losses were encountered on the DEAE-cellulose than on the hydroxyapatite, and this suggested that it might be better to start by chromatography on hydroxyapatite.

Direct chromatography of α_1 -crystallin on hydroxyapatite proved to be such an efficient process that even the ammonium sulphate extraction was omitted. Most of the protein (Fig. 3, peak F) was eluted at relatively low concentrations

Fig. 1. Chromatography on DEAE-cellulose. (a) α_1 -Crystallin (2 ml.) and veronal-Mg²⁺ (40 ml.) were clarified by centrifuging and applied to DEAE-cellulose. The column was washed with 30 ml. of buffer and then eluted successively with portions (75 ml.) of 0.1, 0.2 and 0.3M-NaCl in veronal-Mg²⁺ buffer. The continuous line gives E_{280} and the broken line gives the proteinase activity on pooled fractions after gel-filtration. (b) A portion (16 ml.) of a mixture of α_1 -crystallin (6.9 ml.) and veronal-Mg²⁺ (10 ml.) was treated with 24 ml. of 4M- $(\text{NH}_4)_2\text{SO}_4$; the precipitate was collected by centrifuging (see Table 1) and extracted first with 10 ml. of 1.6M- $(\text{NH}_4)_2\text{SO}_4$, then with 10 ml. of 1.2M- $(\text{NH}_4)_2\text{SO}_4$. The latter extract was passed through Sephadex G25, stored frozen and, on the next day, was fractionated on DEAE-cellulose as described above. The solid blocks give the peptidase activity.

of phosphate, and contained some of the activity. The remaining activity was eluted later (peak G), and the enzyme in this peak was about 30-fold purified (Table 2, Expt. 1). This single-stage process provided the source of the enzyme for many of the experiments described below. The specific activity (average from seven experiments) was about 2000 units/g. of protein. Subsequent chromatography on DEAE-cellulose raised the specific activity to 5000–7000 units/g. of protein (Table 1, Expt. 2); this represents a 70–100-fold purification over the α_1 -crystallin, or about a 300-fold purification over lens extracts.

Peptidase activity in the purified proteinase. The purified proteinase (Table 2, Expt. 2) was assayed with leucylglycine for peptidase activity. The glycine liberated was estimated by measuring the maximum intensity of the spots (Waley, 1957; Cliffe & Waley, 1958); this is a more sensitive method of assay than that previously used (Waley & van Heyningen, 1962a), and was required because of the low activity of the peptidase. Even so, the rate was barely measurable; 0.2 μ mole of

glycine/ml. of incubation mixture was liberated in 3 hr.; thus the peptidase activity is 0.067 unit/ml., or 0.67 unit in the 10 ml. of C' obtained. Since the peptidase activity of α_1 -crystallin is 4900 units/ml. (Waley & van Heyningen, 1962a), the recovery of peptidase in fraction C' is only 0.67/10 $\times$ 4900, or of the order of 0.001%; i.e. 99.999% of the peptidase originally in α_1 -crystallin has been shed. These values assume that the peptidase activity in C' is due to leucine aminopeptidase, but it is possible that the proteinase itself might attack leucylglycine. Although the peptidase activity of fraction C' is low when compared with that of α_1 -crystallin, it is not so very much less than the proteinase activity of fraction C' (Table 3).

The activity of the purified proteinase towards several synthetic substrates was tested; 0.05 ml. of fraction C (from DEAE-cellulose, Expt. 2, Table 2; 0.3 M-sodium chloride eluate, not submitted to gel-filtration) was added to 1–3 mg. of the following compounds (enough potassium hydrogen carbonate was also mixed with the two first-named compounds to give a solution finally at pH 7.4): poly-(α -L-glutamic acid), α -L-glutamyl-L-tyrosine, phenylalanyl-L-phenylalanine amide, L-leucyl-

Fig. 2. Chromatography on hydroxyapatite of fraction obtained by treatment with $(\text{NH}_4)_2\text{SO}_4$ and then chromatography on DEAE-cellulose (see text). This fraction (9.2 ml.; E_{280} 0.455; specific activity 1050 units/g. of protein) was applied to hydroxyapatite and eluted with portions (20 ml.) of phosphate buffer, pH 7.5. All the protein was adsorbed and no protein was eluted with 20 ml. of 0.05 M-phosphate. The histogram line gives E_{280} ; ●, concentration of phosphate.

Fig. 3. Chromatography of α_1 -crystallin on hydroxyapatite. For details see text and Table 3; the histogram line and ● have the same meanings as in Fig. 2.

Table 2. *Purification of the proteinase by chromatography*

In Expt. 1 (Fig. 3) 1 ml. of a stock solution of α_1 -crystallin was diluted with 3 ml. of water, and the mixture was clarified by centrifuging. One portion (0.4 ml.) was diluted to 2 ml. for assay and measurement of E_{280} ; another portion (3.1 ml.) was applied to a 1.7 cm. $\times$ 7 cm. long column of hydroxyapatite; stepwise elution was carried out with portions (20 ml.) of 0.15, 0.2, 0.4 and 0.8 M-phosphate. The pooled fractions representing the protein peaks (G and F) were passed through Sephadex G25 (to give fractions G' and F') before assay. In Expt. 2, 13 ml. of a stock solution of α_1 -crystallin and 39 ml. of water were clarified by centrifuging, and 40 ml. was applied to a 4 cm. $\times$ 7 cm. long column of hydroxyapatite. Stepwise elution was carried out with portions (200 ml.) of 0.15, 0.20 and 0.40 M-phosphate. The fractions eluted between 525 and 562 ml. were combined, treated with 31.5 g. of $(\text{NH}_4)_2\text{SO}_4$ and 4.3 ml. of saturated NaHCO_3 with stirring at 4°, and dialysed against stirred saturated $(\text{NH}_4)_2\text{SO}_4$, pH 7.4, overnight in the cold room. The precipitate was collected by centrifuging for 3 hr. at 10 000g at 2°, dissolved in 4 ml. of veronal-Mg²⁺ buffer and passed through Sephadex G25. The pooled fractions containing the protein (11.2 ml., E_{280} 1.68) were applied to DEAE-cellulose (2.25 cm. $\times$ 12 cm. long) at 4°. The column was washed with 35 ml. of veronal-Mg²⁺ buffer, and then with 75 ml. of 0.3 M-NaCl in veronal-Mg²⁺ buffer. The pooled fractions containing protein eluted by the 0.3 M-NaCl were passed through Sephadex G25 to give fraction C'.

Expt. no.	Fraction	Volume (ml.)	Activity (units/ml.)	Total activity (units)	Concn. of protein (mg./ml.)	Specific activity (units/g. of protein)
1	α_1 -Crystallin	0.775	13.8	10.7	182	76.9
	F'	20	0.084	1.68	4.17	20.2
	G'	11.4	0.353	4.03	0.127	2780
2	α_1 -Crystallin	10	17.6	176	10.35	85.1
	C' (DEAE-cellulose)	10	0.248	2.48	0.044	5640

Table 3. *Specific activities of proteinase and peptidase*

Specific activities of the proteinase and the peptidase are in units/g. of protein.

	α_1 -Crystallin	C'
Proteinase	85	5 640
Peptidase	24 500	1 500

glycylglycine, L-lysyl-L-lysine dihydrochloride, L-tyrosyl-L-leucine, L-alanylglycine, glycyl-L-leucine, glycyl-L-tyrosine, L-tyrosine ethyl ester, L-histidyl-L-histidine and glycylglycine. The mixtures were kept at 55° for 3 hr., and examined by paper chromatography. No signs of the formation of products were seen, except for some hydrolysis (probably non-enzymic) of tyrosine ester.

Comparison of substrates. The source of the proteinase that was used by Waley & van Heyningen (1962a), α_1 -crystallin, contained substrate. Different proteins could not be satisfactorily compared as substrates, because they had to compete with the endogenous substrate in α_1 -crystallin. Since the rate of proteolysis in dilute solutions of α_1 -crystallin was raised by the addition of α_2 -crystallin (the 13 % ethanol pH 5 precipitate) (Fig. 4), α_2 -crystallin was chosen as standard substrate for the experiments described in this paper.

Since the α_1 -crystallin contains substrates for the lens proteinase, it would be expected that these would be found in some of the fractions obtained by chromatography (Fig. 4). The main peak (Fig. 1a, B) from DEAE-cellulose did contain

substrates for the proteinase, but the proteins in peak A were not attacked (Table 4). The rate of proteolysis was lower on proteins in B than on α_2 -crystallin when tested on a range of substrate concentrations (Fig. 5); some proteolysis was also observed in the absence of added substrate. There is a marked difference of rate with fraction B and α_2 -crystallin as substrates. This suggested that perhaps one component of α_2 -crystallin might be particularly readily attacked, and α_2 -crystallin was therefore fractionated further (cf. Fromageot, Sirchis, Petit & Prioux, 1956). The subfraction derived from α_2 -crystallin (whose preparation is described in Table 4) was, however, attacked at the same rate as α_2 -crystallin itself.

The supernatant after precipitation of α_1 -crystallin at pH 5 is called β_1 -crystallin (Fig. 4, B). Although this fraction must contain proteins which serve as substrates, since it is the source of α_2 -crystallin, it does not itself serve as such. Moreover, on addition of β_1 -crystallin to an incubation mixture containing α_2 -crystallin, the breakdown of the α_2 -crystallin is completely inhibited. This is a striking demonstration of the complexity of the effects operating in the breakdown of mixtures of proteins. Although β_1 -crystallin acts as an inhibitor when the purified proteinase is being studied, it is less effective in lens dispersions, since proteolysis occurs in unfractionated lens dispersions (Waley & van Heyningen, 1962a). Here the proteins in β_1 -crystallin that are potential inhibitors must be interacting instead with other proteins in the α_1 -crystallin fraction; these latter have presumably

Fig. 4. Scheme of fractionation to show preparation of substrates. Fractions shown enclosed by lines have been tested as substrates.

Table 4. *Action of purified proteinase on various substrates*

Rates are given relative to the rate with α_2 -crystallin as substrate; concentrations of the substrates were 1% (w/v). For an outline of how the substrates were prepared, see Fig. 4. The fraction called β_1 was obtained from a portion of the supernatant after precipitation of α_1 -crystallin at pH 5 (after neutralization with NaHCO_3) by dialysis, and precipitation with acetone. The fraction called β_2 was similarly obtained from a portion of the supernatant at pH 5, made 13% (w/v) in ethanol, after precipitation of α_2 -crystallin. Fractions A and B were obtained by pooling the tubes from chromatography on DEAE-cellulose, dialysis and either precipitation with acetone or freeze-drying. The subfraction of α_2 -crystallin was prepared by adding $(\text{NH}_4)_2\text{SO}_4$ (4 g.) to α_2 -crystallin (1 g.) in water (20 ml.) containing 0.35 ml. of saturated NaHCO_3 . The precipitate was collected by centrifuging, and the supernatant (17 ml., E_{280} 15) dialysed against water for 72 hr. Acetate buffer (Waley & van Heyningen, 1962*a*) (2 ml.) and chilled acetone (180 ml.) were then added, and the precipitate (150 mg.) was collected by filtration and dissolved in 1.5 ml. of veronal- Mg^{2+} buffer.

Substrate	Relative rate (%)	Substrate	Relative rate (%)
α_2	100	Fractionated α_2	103
β_1	0	A	0
β_1 and α_2	0	B	40
β_2	37	Serum albumin	8
		Haemoglobin	15

been shed from the purified proteinase. After the precipitation of α_2 -crystallin, the supernatant contains proteins that may act as substrates (Table 4, β_2).

The results so far have been concerned with lens proteins as substrates. The proteinase does also attack other proteins: albumin and haemoglobin are substrates (Table 4).

Fig. 5. Variation of rate of reaction (with purified proteinase) with concentration of substrates. O, Fraction B; ●, α_2 -crystallin.

Variation of rate of proteolysis with temperature and ionic strength. Two characteristic properties of the crude proteinase were the high temperature coefficient of the rate, and the marked inhibition by added salts (Waley & van Heyningen, 1962*a*). Both these properties are also shown by the purified proteinase: the rate at 37° was 8.2% of that at 55°, and the presence of 0.33M-sodium chloride caused about 90% inhibition. The activity in peak F from the hydroxyapatite (Fig. 3) was also inhibited by salts (about 60% by 0.33M-sodium chloride).

Action of inhibitors on the proteinase. The crude proteinase (in α_1 -crystallin) is inhibited by 1 mM-*o*-iodosobenzoate (Waley & van Heyningen, 1962a), and some other inhibitors that are likely to react with thiol groups are also effective (Table 5). These include Cu^{2+} ions, *N*-ethylmaleimide and GSSG. The *N*-ethylmaleimide also inhibited the purified proteinase, as did *p*-chloromercuribenzoate (Table 5). Taken together, these results suggest that one or more thiol groups may be necessary for the activity of the proteinase. The concentrations of the reagents needed to inhibit the purified enzyme were greater than might have been expected from the results with the crude enzyme; it seems probable that they reacted fairly slowly with the dilute solution (about 0.1 mg./ml.) of the purified enzyme. Thus the reaction of α_1 -crystallin (about 24 mg./ml.) with *N*-ethylmaleimide was incomplete after 30 min. The reaction was followed by the decrease in E_{300} of the reagent (Roberts & Rouser, 1958).

Products formed by the purified proteinase. The usual substrate, α_2 -crystallin, although devoid of proteinase activity, contained peptidase activity. Assay with L-leucylglycine showed that, at a concentration of 1%, w/v (the concentration at which α_2 -crystallin was used as a substrate), the peptidase activity amounted to 340 units/ml. of incubation mixture. That this activity was high enough to prevent the accumulation of appreciable concentrations of any small peptides that might be formed by the action of the proteinase was confirmed by the

action of trypsin on α_2 -crystallin. Trypsin normally degrades proteins to form peptides, but the main products when α_2 -crystallin was the substrate were amino acids. However, when the α_2 -crystallin was first boiled to destroy the endogenous peptidase, the main products were peptides.

α_2 -Crystallin (0.2 ml. of 10%, w/v) was heated to 100° for 15 min., diluted to 1 ml. with 0.4M-potassium hydrogen carbonate soln. and trypsin (5 mg.; Armour, containing about 50% of magnesium sulphate) in water (1 ml.) was added. After incubation for 1 hr. at 37° the products were examined by paper electrophoresis at pH 4 (Cliffe & Waley, 1958). This method is quite sensitive, as the positions occupied by amino acids are such that most of the peptides liberated are likely to be detected. The samples were applied to the middle of the paper, and electrophoresis for 3 hr. at 10v/cm. showed a series of spots travelling towards the cathode, occupying positions between the neutral and basic amino acids. When unboiled α_2 -crystallin was the substrate, the four main spots were in the positions of the basic amino acids (unresolved), the neutral amino acids (unresolved), glutamic acid and aspartic acid.

These exploratory experiments with trypsin serve to show that for any intermediates to be seen the substrate (α_2 -crystallin) must be boiled, and that electrophoresis at pH 4 could be used to detect peptides. Unfortunately, the purified proteinase (fraction C', Expt. 2, Table 2) acted rather slowly on boiled α_2 -crystallin: the increase in ninhydrin colour after 3 hr. at 55° amounted to only 0.1 μ mole of amino compounds/ml. of incubation mixture, or about 0.7 μ mole in the incubation mixture, which had previously been heated for 15 min. at 100° and 6.5 ml. of fraction C'. The mixture was then dialysed for 72 hr. at 4° against 60 ml. of water (and a few drops of chloroform); the outer fluid was stirred. The diffusate was concentrated to a volume of about 1 ml. in a rotary evaporator, and acidified with 0.1 ml. of 8M-acetic acid. The amino compounds were then separated from the veronal buffer by adsorption on a column (3.5 cm. long $\times$ 0.4 cm. diam.) of Dowex 50 (H^+ form; X 4) (Harris, Tigane & Hanes, 1961). The column was washed with 0.5 ml. of 0.5M-acetic acid, and then with 1 ml. of water. The amino compounds were eluted with 2N-triethylamine (redistilled) in 20% (v/v) acetone, and the eluate was dried *in vacuo*. As a control, 0.7 ml. of 10% (w/v) α_2 -crystallin was boiled, diluted with 6.5 ml. of veronal-Mg²⁺ buffer, incubated at 55° for 3 hr. and used as described above.

The products from the enzymic reaction and the control were dissolved in 20 μ l. of water, and samples (5 μ l.) examined by electrophoresis at pH 4

Table 5. *Inhibition of proteolytic activity*

Rates are given relative to controls without inhibitors. Experiments with α_1 -crystallin were carried out with about 2% (w/v) of α_1 -crystallin and no added substrate. Experiments with the purified proteinase were carried out in the sample described in Table 2 (Expt. 2); portions of 0.36 ml. of G' and 0.04 ml. of inhibitor were left for 15 min. at room temperature, and then 0.04 ml. of 10% (w/v) α_2 -crystallin was added before incubation at 55°.

Enzyme	Inhibitor	Concn. (mm)	Inhibition (%)
α_1 -Crystallin	<i>N</i> -Ethylmaleimide	1.5	100
		0.4	100
		0.04	39
		0.008	20
	GSSG	2.5	50*
Purified proteinase	<i>N</i> -Ethylmaleimide	1.3	87
		0.13	0
	<i>p</i> -Chloromercuribenzoate	0.50	100
		0.05	18
		0.01	0

* The mixture (α_1 -crystallin + GSSG) was passed through Sephadex G 25 before assay; most of the uncombined GSSG was thus removed before assay.

for 2.7 hr. at 12v/cm. In both mixtures, the strongest spots were in the positions occupied by amino acids; but the enzymic sample showed also about eight faint spots that were absent from the control sample. Thus the amino acids are mainly contaminants in the (boiled) α_2 -crystallin (even traces of contaminants would show up after the solutions have been concentrated so much), but the other spots, which are taken to be peptides, arise by the action of the proteinase.

Attempted purification of neutral proteinase from rat liver and from ox spleen. The presence of neutral proteinases in these (and other) tissues was shown by Waley & van Heyningen (1962a), and attempts have now been made to purify the proteinases by the procedures used for the enzyme from lens. The proteinase activity in the extracts of both tissues was precipitated at pH 5; little or no activity was found in the pH 5 supernatant, but although much protein was shed, the specific activity was not appreciably raised and much activity was lost.

On one occasion, the enzyme from the pH 5 precipitate of rat liver was successfully purified on hydroxyapatite. This result could not subsequently be repeated; the peak G (Fig. 3) was present, but contained little or no activity. Protein is precipitated during gel-filtration, and this precipitate may contain the enzyme, which is thus lost from solution. If the purified enzyme from rat liver is precipitated from solutions of low ionic strength, but is less active in solutions of high ionic strength, its isolation and assay will be difficult.

Our results with extracts of ox spleen were based on assays with ninhydrin on deproteinized filtrates. When E_{280} (of deproteinized filtrates) was used to measure proteolysis, Press, Porter & Cebra (1960) could detect no activity at pH 7.5; their enzyme (cathepsin D) had a pH optimum about 3.5. We have compared (by the ninhydrin method) the rates of proteolysis, in extracts of ox spleen, at pH 3.5 and pH 7.5; the initial rate (at 37°) at pH 3.5 is of the order of 10 times the rate at pH 7.5. Haemoglobin (acid-denatured) was used as substrate, but the difference between the rates at pH 3.5 and at pH 7.5 is much the same in the absence of added substrate. After precipitation at pH 4.6, nearly all of the cathepsin D is in the supernatant (Press *et al.* 1960). When the pH 4.6 precipitate is used as source of enzyme the rate of proteolysis at pH 3.5 is about the same as at pH 7.5. Therefore the relative rates of proteolysis at the two pH values depend on whether the unfractionated extract or the pH 4.6 precipitate is used. This observation suggests that different enzymes are responsible for proteolysis at pH 3.5 and pH 7.5.

We conclude that the commonest method of assaying proteinases (E_{280} of deproteinized filtrates

after incubation for 10 min. at 37°) is unsuitable for neutral proteinases, and that although most of the potential proteolytic activity of ox spleen is due to cathepsin D, the neutral proteinase is responsible for the activity at pH 7.5.

DISCUSSION

Purification of the lens proteinase. The main properties observed for the lens proteinase that has been purified are that it is precipitated at pH 5, and relatively strongly adsorbed on both DEAE-cellulose and hydroxyapatite. The high affinity for the anion-exchange resin may be correlated with the high affinity for the hydroxyapatite, if the binding groups are $-\text{NH}(\text{C}_2\text{H}_5)_2$ in the former and Ca^{2+} ions (Tiselius *et al.* 1956) in the latter. If the proteinase is (like α_2 -crystallin as a whole) iso-electric at pH 5, it will have a net negative charge at pH 7.5, the pH at which chromatography was carried out, and this is consistent with its affinity for the positively charged group of the chromatographic adsorbents. Conversely, most of the proteinase activity and also most of the protein in α_1 -crystallin were unadsorbed on carboxymethyl-cellulose (Na^+ form) at pH 5.8.

Although the specific activity of the purified proteinase is about 300 times that of lens extracts, the extent to which the proteinase could be further purified is unknown. The specific activity of the purified enzyme (Table 2, Expt. 2), about 5.6 μ -moles of amino acid/mg. of enzyme protein/hr. at 55°, may be compared with the rates of other proteolytic reactions in the same units, but at 37°: the (initial) rate with carboxypeptidase on α_2 -crystallin is about 20, and with trypsin on boiled α_2 -crystallin about 2. Our present preparations thus have specific activities not very different from that of other proteinases of known purity.

Mode of action of lens proteinase. There are two classes of proteolytic enzymes: those (exopeptidases) that attack only the *N*-terminal or *C*-terminal peptide bond and split off amino acid residues one at a time, and those (endopeptidases) whose range of attack is governed mainly by the nature, rather than the position, of the amino acid residue. Exopeptidases can be further subdivided into aminopeptidases and carboxypeptidases. Several peptidases are present in lens (Hanson, 1961; Spector, 1962), but there is no evidence that they attack proteins. Although pancreatic carboxypeptidase does attack α_2 -crystallin, it liberates principally one amino acid (serine), and, moreover, the lens does not hydrolyse benzyloxycarbonyl-glycylphenylalanine (Hanson & Methfessel, 1958), a substrate for (pancreatic) carboxypeptidase.

Direct investigation of the mode of action of the lens proteinase was handicapped by the presence of

the leucine aminopeptidase; this was highly active both in the crude enzyme (α_1 -crystallin) and in the usual substrate (α_2 -crystallin). Even the purified proteinase contained appreciable peptidase activity. Nevertheless, the purified proteinase probably liberated peptides from a boiled sample of α_2 -crystallin, and it seems probable that the lens proteinase is an endopeptidase. Nothing is known of its 'side-chain' specificity. The neutral proteinase from semen attacks *N*-acetyltyrosine ethyl ester (Lundquist, Thorsteinsson & Buus, 1955), but this substrate was barely attacked by α_1 -crystallin.

Several substances that react with thiol groups inactivated the lens proteinase, and so it may belong to the class of 'thiol enzymes'; other proteolytic enzymes in this category include ficin (Liener, 1961), papain (Smith, 1961), probably cathepsins B and C, and perhaps the neutral proteinase from rat liver (Kuzovleva & Chung-Yen, 1959).

The investigations have been carried out with the lens proteinase which is eluted from hydroxyapatite after the bulk of the protein (Fig. 3, G). There is also proteolytic activity in the main peak (F); both enzymes are inhibited by salts, and thus may be isoenzymes.

Metabolic role of neutral proteinases. The paucity of information about how proteins are catabolized has led to the view that '... the intracellular breakdown of proteins is still at least as obscure as their synthesis' (Raacke, 1961). Our work suggests that proteins are broken down to amino acids *in vivo* by the co-operation of a neutral proteinase (probably an endopeptidase) and an exopeptidase (an aminopeptidase). This mechanism offers certain advantages. The endopeptidase would be expected to cleave a polypeptide chain at several points, and at each point a fresh susceptible bond would be generated for the exopeptidase to act on. If an endopeptidase were to act on its own, the rate would be expected to fall off as the number of susceptible bonds decreased. An exopeptidase on its own would be handicapped because only a small proportion of the peptide bonds would be susceptible (i.e. would be terminal). When the two types of enzyme act together the dwindling number of bonds available to the endopeptidase is compensated for by the increasing number available to the exopeptidase. Here, then, is a possible mechanism for protein breakdown *in vivo*.

Even less is known about the reasons for the breakdown of proteins. A tentative suggestion is that proteins slowly undergo irreversible denaturation under physiological conditions, and that the denatured protein (which is assumed to be no longer functional) is then hydrolysed to amino acids by the mechanism outlined above. This idea is consistent with the observations that denatured

proteins are more susceptible to proteolysis than native proteins. If the rates of turnover of enzymes could be compared with their rates of denaturation under physiological conditions, it might be possible to test this hypothesis. Whether or not this is the reason for protein breakdown, protein breakdown *in vivo* will affect the activities of enzymes in cells, and thus may govern metabolic patterns. For, although the rates of enzymic reactions *in vivo* usually depend on the concentrations of substrates, they will also depend on the concentrations of enzymes.

SUMMARY

1. The neutral proteinase from ox lens has been partially purified; the main steps are precipitation at pH 5, and chromatography on hydroxyapatite. Chromatography on diethylaminoethylcellulose was also used and this separated the proteinase from leucine aminopeptidase.

2. The proteinase is probably an endopeptidase and is inhibited by compounds that react with thiol groups. The rate of proteolysis is decreased by increasing the ionic strength.

3. The proteinase acts on different fractions of lens proteins at widely differing rates and some fractions contain inhibitors. The best substrate is α_2 -crystallin (the proteins precipitated with 13% ethanol at pH 5). Serum albumin and haemoglobin are also attacked.

4. Some attempts to purify the neutral proteinase from rat liver, and from ox spleen, are recorded.

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Direct Evidence for the Hexose Monophosphate Pathway of Glucose Metabolism in Skin

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Barron, Meyer & Miller (1948) first suggested the possibility of the existence of the hexose monophosphate pathway in rat skin. This hypothesis was supported by Hershey & Mendle (1954), who demonstrated the presence of 6-phosphogluconate dehydrogenase in human skin. Glucose 6-phosphate dehydrogenase has also been found in skin by Hershey, Lewis, Murphy & Schiff (1960).

By comparing the rates at which C-1 and C-6 of glucose were converted into carbon dioxide, Freinkel (1960) also concluded that the hexose monophosphate pathway operates in human skin. Using a similar technique, Gilbert (1962) was unable to demonstrate the hexose monophosphate pathway in cattle skin: he attributed this difference to species variation. However, the observations of Freinkel (1960) were confirmed by Pomerantz & Asbornsen (1961) with rat skin. These authors interpret their findings with caution, however, bearing in mind the fact that not all of the carbon atoms of glucose are converted into carbon dioxide (Wood, 1955) and the uncertainty concerning the fate of the triose phosphate (Katz & Wood, 1960). The evidence for the existence of the hexose monophosphate pathway in skin is thus conflicting and indirect. It therefore seemed desirable to demonstrate in skin the presence of some of the intermediates involved in the hexose monophosphate pathway.

MATERIALS AND METHODS

Preparation of skin extracts. Slices of guinea-pig-ear skin were cut by hand from the dorsum of the ear of the adult animal after cervical fracture. Skin slices weighing between 5 and 10 mg. were labelled with ^{32}P by incubating at 37° for 4 hr. in 0.5 ml. of Krebs-Ringer phosphate (Krebs, 1933) containing $[\text{P}^{32}\text{O}_4]^{3-}$ (approx. $100\text{ }\mu\text{C}/\text{ml.}$), glucose (0.02 M) and streptomycin sulphate ($50\text{ }\mu\text{g.}/\text{ml.}$). In some experiments, ^{14}C was also incorporated by adding uniformly labelled glucose [$\text{U-}^{14}\text{C}$]glucose ($3.6\text{ }\mu\text{C}/\mu\text{mole}$ of glucose) to the medium: for reasons of economy, the volume of medium used in these experiments was 0.1 ml.

After incubation the skin was frozen in liquid nitrogen and extracted in a glass tissue-grinder with ten times its weight of cold 10% (w/v) trichloroacetic acid. To the clear supernatant obtained after centrifuging, EDTA (approx. 5 mg.) and sufficient ammonia to make the solution alkaline were added. This extract was used for chromatography.

Chromatographic methods. For chromatography of sugar phosphates, Whatman no. 1 chromatography paper was soaked for 30 min. in a 0.2% solution of EDTA adjusted to pH 8.5 with sodium hydroxide. After washing in six changes of water, the sheets were hung up to dry at room temperature. For chromatography of sugars, untreated Whatman no. 1 paper was used.

Descending chromatograms were run in the following solvents at 27° : I, propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) for 40 hr.; II, 2-methylpropan-2-ol-pyridic acid-water (20:1:5, v/w/v) for 30 hr.; III, phenol-water (4:1, w/v) for 24 hr.; IV, butan-1-ol-propionic acid-