

Characterization of a Neutral Protease from Lysosomes of Rabbit Polymorphonuclear Leucocytes

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1. The subcellular distribution has been investigated of a protease from rabbit polymorphonuclear leucocytes, obtained from peritoneal exudates. The enzyme, optimally active between pH 7.0 and 7.5, hydrolyses histone but not haemoglobin, sediments almost exclusively with a granule fraction rich in other lysosomal enzymes, and is latent until the granules are disrupted by various means. 2. Enzymic analysis of specific and azurophilic granules separated by zonal centrifugation showed that neutral protease activity was confined to fractions rich in enzymes characteristic of azurophile granules. 3. Recovery of neutral protease activity from subcellular fractions was several times greater than that found in whole cells. This finding was explained by the presence of a potent inhibitor of the enzyme activity in the cytoplasm. 4. The effect of the inhibitor was reversed by increasing ionic strength (up to 2.5M-potassium chloride) and by polyanions such as heparin and dextran sulphate, but not by an uncharged polymer, dextran. 5. The enzyme was also inhibited, to a lesser extent, by 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one, soya-bean trypsin inhibitor and ϵ -amino-hexanoate (ϵ -aminocaproate). 6. The granule fractions failed to hydrolyse artificial substrates for trypsin and chymotrypsin. 7. Partial separation of the enzyme was achieved by Sephadex gel filtration at high ionic strength and by isoelectric focusing. The partially separated, activated enzyme showed an approximately 300-fold increase in specific activity over that in whole cells.

Following earlier suggestions that phagocytes contained packets of 'cytases' which digested foreign material at acid pH (Metchnikoff, 1905; Ehrlich, 1956), Cohn & Hirsch (1960) demonstrated that the granules of polymorphonuclear leucocytes could be considered to be lysosomes. Many of the properties of these granules fitted the known behaviour of lysosomes from rat liver (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) and their enzymic constituents included an acid protease, or cathepsin, but not neutral protease. Cathepsin E has subsequently been partially purified from rabbit bone marrow (Lapresle & Webb, 1960) and from rabbit peritoneal exudate polymorphonuclear leucocytes (Cochrane & Aikin, 1966), and cathepsin D was also partially separated by the latter authors. Extensive investigations of the proteolytic activity of granule fractions of rabbit polymorphonuclear leucocytes on both natural and synthetic substrates

by Wasi, Murray, Macmorine & Movat (1966) confirmed the presence of acid proteases together with an enzyme hydrolysing *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine, an artificial substrate for cathepsin A. They did not, however, find any hydrolysis of benzoyl-L-arginine amide hydrochloride and *N*-acetyl-L-tyrosine ethyl ester, classical substrates for cathepsin B and C respectively. No protease activity was found in the pH range 5-7; moreover, the low activity seen at alkaline pH is of equivocal validity in view of the observations of Umana (1968) on the interference of ribonuclease in assays for neutral proteases by using E_{280} values.

In marked contrast with the apparent absence of neutral protease activity in rabbit polymorphonuclear leucocytes, such activity has been described in human polymorphonuclear leucocytes (Mounter & Atyeh, 1960; Styles & Fraenkel-Conrat, 1968; Janoff & Zeligs, 1968). The latter authors have further characterized, and partially purified, an enzyme capable of degrading orcein-impregnated

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elastin and *N*-benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester, a specific substrate for pancreatic elastase (Janoff & Scherer, 1968; Janoff, 1969). Further, Lazarus, Brown, Daniels & Fullmer (1968a) described a collagenase in human polymorphonuclear-leucocyte lysosomes; this is distinct from the elastolytic activity mentioned above, as shown by ion-exchange chromatography (Janoff & Scherer, 1968).

However, studies by Weissmann & Spilberg (1968) on the degradation of bovine nasal-cartilage protein-polysaccharide by lysosomes from rabbit peritoneal-exudate polymorphonuclear leucocytes revealed the presence of a neutral protease with a substrate specificity distinct from those previously described in human polymorphonuclear leucocytes. We now report the use of calf thymus histone as substrate to confirm the presence of an enzyme in rabbit polymorphonuclear leucocytes active around neutral pH that is incapable of hydrolysing haemoglobin. The subcellular distribution, activation and inhibition of the enzyme are also described. Initial accounts of this work have been previously presented (Davies & Weissmann, 1969; Weissmann & Davies, 1969; Davies, Krakauer & Weissmann, 1970a).

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless otherwise stated. Benzoyl-DL-arginine *p*-nitroanilide, glutaryl L-phenylalanine *p*-nitroanilide and succinyl-L-phenylalanine *p*-nitroanilide were obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; ϵ -aminohexanoate (ϵ -aminocaproate) was from Calbiochem, Los Angeles, Calif., U.S.A.; freeze-dried calf thymus histone, soya-bean trypsin inhibitor, lysozyme (salt-free; 10000 units/ml), trypsin (3 \times crystallized; 180 units/mg) and chymotrypsin (53 units/mg) were from Worthington Biochemicals Corp., Freehold 2, N.J., U.S.A.; 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('tosyl-phenylalanyl chloromethyl ketone'), 1-chloro-3-L-toluene-*p*-sulphonamido-7-aminoheptan-2-one ('tosyl-lysyl chloromethyl ketone') and phenolphthalein glucuronidate were from Sigma Chemical Co., St Louis, Mo., U.S.A.; haemoglobin standardized for protease assay was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; dimethyl sulphoxide was from Matheson, Colman and Bell Inc., East Rutherford, N.J., U.S.A.; sodium hypochlorite N.F. (4-6% NaOCl), heparin sodium U.S.P. and sodium dodecyl sulphate were from Fisher Scientific Co., Fairlawn, N.J., U.S.A.; aetiocholanolone was from Steraloids, Pawling, N.Y., U.S.A.; dextran (clinical grade, mol.wt. 37000-43000), dextran sulphate (sodium salt, 17% S), and sodium deoxycholate were from General Biochemical Inc., Chagrin Falls, Ohio, U.S.A.; chondroitin sulphate (sodium salt) (from shark) was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Another highly purified preparation of chondroitin sulphate was a gift from Dr Lawrence Rosenberg. Triton X-100 and Lubrol PX were gifts from Rohm and Haas Co., Philadelphia 5, Pa., U.S.A.,

and I.C.I. American Inc., Stamford, Conn., U.S.A., respectively. Sephadex G-75 was from Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A. LKB 8101 electrofocusing column (110 ml of capacity), LKB 8121 Gradient Mixer, LKB 10200 Perspex Pump and Ampholine Carrier Ampholites 40%, type 8141, were purchased from LKB-Producter A.B., Bromma, Sweden.

Preparation and subcellular fractionation of polymorphonuclear leucocytes. Rabbit peritoneal-exudate polymorphonuclear leucocytes were induced by injection of 0.1% (w/v) glycogen in iso-osmotic sodium chloride as described by Cohn & Hirsch (1960). Cells were disrupted by passage through a Seitz Millipore filter assembly, but without filter, which contained a fine metal mesh. This procedure, and subsequent differential centrifugation to yield subcellular fractions, was carried out essentially as described by Weissmann, Spilberg & Krakauer (1969). In certain experiments the post-graduate supernatant obtained after centrifugation at 8200g was further centrifuged at 105000g for 2h in the no. 40 head of a Beckman L-2 ultracentrifuge to yield a microsomal pellet.

Separation of azurophilic and specific granules. Separation was carried out by zonal centrifugation using a B XIV head by the method of Baggolini, Hirsch & de Duve (1969). We thank Dr Marco Baggolini for supplying us with these fractions together with details of their peroxidase and alkaline phosphatase activities, determined by the methods of Baggolini *et al.* (1969).

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was used with lysozyme as a standard.

Neutral protease. This activity was measured as the release of acid-soluble arginine or tyrosine from calf thymus histone. Commercial preparations of calf thymus were rendered suitable as a substrate for various proteolytic enzymes by prior precipitation with 10% (w/v) sulphosalicylic acid, which inactivated endogenous neutral protease activity, possibly identical with that described by Furlan, Jericijo & Suhar (1968). Histone treated in this way was insoluble and was used as a suspension in a desired buffer solution. In most instances 0.2 ml vol. of cell fractions and histone (40 mg/ml in 0.1 M-phosphate buffer, pH 7.5) were incubated for 18 h at 37°C. Arginine release was determined by a modified Sakaguchi reaction (Sakaguchi, 1925), which allowed a slow linear development of colour over a period of up to 4 h at 0-2°C. These determinations were carried out on supernatants obtained after termination of assays with a solution containing 5% (w/v) sulphosalicylic acid, 0.01 M-glycine and 0.5% (w/v) 8-hydroxyquinoline. Colour development was initiated by rendering the assay supernatants alkaline with 5% (w/v) sodium hydroxide and adding sodium hypochlorite to a final concentration of 0.5% (w/v). Tyrosine release was determined on supernatants obtained from assays terminated by the addition of 4.7% (w/v) trichloroacetic acid by using Folin-Ciocalteu's reagent, the intensity of blue colour being determined at 700 nm.

Determination of latency of neutral protease. To examine the latency of neutral protease aetiocholanolone was used as an example (Weissmann, 1965) of an agent known to disrupt lysosomal membranes. Portions (1.7 ml) of post-nuclear supernatant (400g for 10 min) were incubated

with aetiocholanolone, dissolved in dimethyl sulphoxide to yield final concentrations of 0.05–0.50 mM for 1 h at 37°C. Suitable controls for the effect of dimethyl sulphoxide and enzyme release in the absence of aetiocholanolone were included in the experimental procedure. At the end of the incubation period all samples were centrifuged at 18000g for 20 min and released neutral protease and β -glucuronidase (as a control lysosomal enzyme) assayed in the supernatant fluid, this activity being expressed as a percentage of enzyme solubilized by Triton X-100.

Effect of heat on neutral protease activity. Loss of neutral protease activity at elevated temperatures was estimated by maintaining samples of polymorphonuclear-leucocyte granule fraction at 60 and 90°C for up to 1 h with subsequent assay of neutral protease activity.

Acid protease. The modification of Anson's (1936) method described by Weissmann *et al.* (1969) was used.

β -Glucuronidase. The method of Talalay, Fishman & Huggins (1946) was used.

Hydrolysis of synthetic substrates. (a) Benzoyl-DL-arginine *p*-nitroanilide. Substrate (2 mM, 0.5 ml), dissolved in 5% (v/v) dimethyl sulphoxide in water, was incubated with 0.5 ml of cell fraction or pure enzyme (trypsin) and 1.0 ml of 0.1 M-sodium phosphate buffer, pH 7.5. The assay was terminated by the addition of 30% (v/v) acetic acid (1.0 ml) after incubation at 37°C for 3 h. Blanks consisted of substrate incubated for 3 h at 37°C without cell fractions, which were added after the termination of the assay. Release of *p*-nitroaniline was measured at 410 nm, with reference to a standard curve constructed from extinction readings at this wavelength on various concentrations of *p*-nitroaniline dissolved in ethanol.

(b) Glutaryl-L-phenylalanine *p*-nitroanilide and succinyl-L-phenylalanine *p*-nitroanilide. The substrates were dissolved at a concentration of 0.5 mg/ml in 0.2 M-sodium phosphate buffer, pH 7.5. Substrate (0.2 ml) was incubated with cell fractions (0.5 ml) or pure enzyme (trypsin or chymotrypsin) for periods of 1–24 h. The assay was terminated by addition of 30% (v/v) acetic acid (0.6 ml) and the *p*-nitroaniline released was measured at 410 nm. Blanks consisted of substrate incubated without cell fractions or pure enzyme, which were added after termination of the assay.

Inhibition and activation of neutral protease. (a) Polymorphonuclear-leucocyte fractions, human plasma and foetal calf serum. Granule fractions (0.1 ml) with neutral protease activity were incubated with and without 0.1 ml of other cell fractions and 0.2 ml of histone for 18 h at 37°C, when release of arginine was measured and expressed as a percentage of the value for granule fraction alone.

(b) ϵ -Aminohexanoate. Inhibition of histone hydrolysis was measured at ϵ -aminohexanoate concentrations ranging from 1.0 mM to 0.1 M. Tyrosine release from histone was measured as these high concentrations of γ -aminohexanoate inhibited the arginine colour reaction.

(c) 1-Chloro-4-phenyl-3-L-toluene-*p*-sulphonamido-butan-2-one and 1-chloro-3-L-toluene-*p*-sulphonamido-7-aminohexan-2-one. The latter was dissolved in 5% (v/v) dimethyl sulphoxide to give assay concentrations ranging from 0.005 to 0.05 mM, whereas the former was dissolved in water to give assay concentrations ranging

from 0.05 to 1.0 mM. The inhibition of histone hydrolysis by trypsin and chymotrypsin by these concentrations of the inhibitors was determined in a similar manner.

(d) Soya-bean trypsin inhibitor. The inhibitor was dissolved in 0.34 M-sucrose and its inhibitory activity determined at concentrations ranging from 1 to 250 μ g/ml. Blanks were constituted by incubating the inhibitor alone with histone.

Arginase activity. Various cell fractions (0.2 ml) were incubated with arginine (0.2 ml, 200 μ g/ml) for 18 h at 37°C, when the assay was terminated with the sulphosalicylic acid-glycine-8-hydroxyquinoline mixture used in the neutral protease assay. The arginine content of supernatants was then determined; blanks were constituted by adding 0.2 ml of arginine solution after termination of the assay.

Influence of ionic strength. The effect was determined of potassium chloride (0.1–2.5 M) on the release of arginine from histone by lysosomal neutral protease. Potassium chloride was included in the buffer used for histone suspension; the highest concentration did not influence the colorimetric determination of arginine.

Effect of naturally occurring polyionic compounds upon neutral protease activity. The effect of heparin, dextran sulphate, chondroitin sulphate and bovine nasal protein-polysaccharide on neutral protease activity was determined by dissolving the polyanions in 0.1 M-sodium phosphate buffer, pH 7.5, at appropriate concentrations for inclusion in the assays. The influence of various polyanions on the arginine colour reaction itself was determined and minor corrections were made when necessary. The influence on neutral protease of the non-charged polymer dextran and the polycation lysozyme activity was also studied in this fashion.

Solubilization of neutral protease. Before purification was attempted it was necessary to determine the most effective means of solubilizing neutral protease and the release of the enzyme from granule fractions by freeze-thawing and a series of detergents was examined. Portions of a granule fraction were continuously stirred overnight at 4°C with the desired concentrations of detergents while another portion was freeze-thawed eight times by alternate immersion in acetone-solid CO₂ and a 37°C water bath. After these procedures the samples were centrifuged at 18000g for 30 min; the supernatants were removed and the pellets resuspended to their original volume. The supernatants and resuspended pellets were assayed for protein, β -glucuronidase and neutral protease activity by the methods described above.

Sephadex gel filtration. Gel filtration was performed on a 60 cm \times 2.5 cm column of Sephadex G-75 equilibrated with 0.1 M-sodium phosphate buffer, pH 7.2, containing 2.5 M-potassium chloride. Granule fraction (5.0 ml) in 0.34 M-sucrose containing 0.1% (v/v) Triton X-100 was made 2.5 M with respect to potassium chloride. After stirring of the granule fraction overnight at 4°C this was applied to the column and 4 ml fractions were collected at a rate of approx. 0.5 ml/min. The E_{280} was taken as a measure of protein content; selected fractions were also assayed for neutral protease activity with histone as a substrate. The column was calibrated with markers of known molecular weight, Dextran Blue 2000 (2×10^6), albumin (65000), cytochrome *c* (12400) and lysozyme (12400).

Isoelectric focusing. A granule fraction, stirred overnight at 4°C in the presence of 0.1% (v/v) Triton X-100, was centrifuged at 23500g for 20 min. The resulting pellet was resuspended to the original volume with 0.34M-sucrose containing 5 mg/ml of heparin (sodium salt), stirred overnight at 4°C, and centrifuged at 23500g for 20 min. The resulting supernatant was dialysed overnight against about 200 vol. of 0.34M-sucrose. This preparation (4ml), containing a total of 4.084 mg of protein, was introduced into the column along with a continuous sucrose density gradient containing 1% carrier ampholites, pH 3-10, by means of the gradient-mixing device connected to a peristaltic pump, following the technique outlined by Haglund (1967).

The focusing was carried out by applying an increasing voltage, from 300 to 600 V, over a period of 48 h, after which fractions (3 ml) were collected by using a peristaltic pump. The fractions were dialysed against 0.34M-sucrose for 48 h and assayed for neutral protease, with histone as substrate, and protein, by using the E_{280} value, corrected for turbidity.

RESULTS

pH-dependence of histone hydrolysis. Detailed analysis of proteolytic activity in the granule fraction of polymorphonuclear leucocytes showed that the release of acid-soluble tyrosine and arginine from histone was maximal at pH 7.3 (Fig. 1). In marked contrast no acid-soluble tyrosine was released from haemoglobin by the granule fraction around neutral pH.

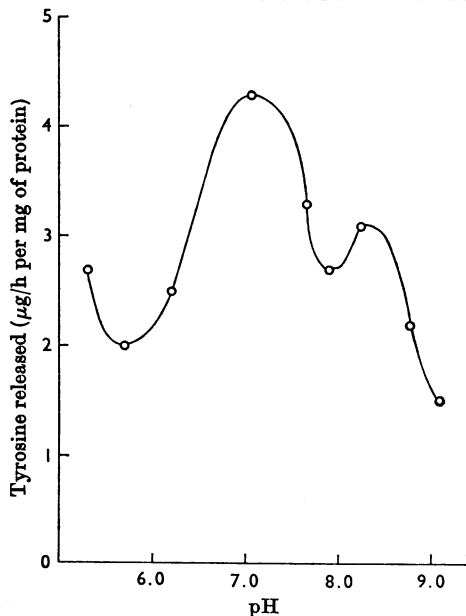


Fig. 1. Dependence on pH of histone hydrolysis by granule fraction of rabbit polymorphonuclear leucocytes. Portions (0.2 ml) of granule fraction were incubated with histone (40 mg/ml) in 0.2M-tris-maleate buffer of various pH values for 18 h at 37°C.

Subcellular distribution of neutral protease activity. Assays of neutral protease and of two lysosomal enzymes (acid cathepsin and β -glucuronidase) in various subcellular fractions of rabbit polymorphonuclear leucocytes demonstrated that neutral protease activity was concentrated in the granule-rich fraction (Table 1). Both tyrosine and arginine release were measured as indicators of neutral protease activity and appear to correspond. When the activity of various lysosomal enzymes found in subcellular fractions was compared with that in whole cells the recovery of acid cathepsin and β -glucuronidase approximated unity, whereas that of neutral protease was increased approximately seven- and twenty-fold for tyrosine- and arginine-reactive material released respectively (Table 2). The activity of neutral protease was so low in non-disrupted cells as to approach the limits of analysis for the two amino acids, possibly explaining the large discrepancy between their percentage recoveries in subcellular fractions. This suggested the presence of an inhibitor of the protease activity within the cell; evidence for this possibility is given below.

Localization of neutral histonase within granule subfractions. When azurophilic and specific granules were separated by zonal centrifugation neutral protease was almost exclusively located in the azurophilic granules (Table 3). This fraction also contained a large amount of peroxidase and little alkaline phosphatase activity, in contrast with the low peroxidase and high alkaline phosphatase activity of the specific granules.

Latency of neutral protease. When aetiocholanolone (0.5 mM) was used to perturb the membranes of lysosomes, it released 35% of the neutral protease and 52% of the β -glucuronidase activity released by 0.1% (v/v) Triton X-100 (Table 4). In contrast, lower concentrations of aetiocholanolone or solvent released only 15% of β -glucuronidase and no neutral protease.

Hydrolysis of artificial substrates by subcellular fractions. In an attempt to define substrate specificity of the protease, a series of artificial substrates of known specificity was examined for their possible hydrolysis by subcellular fractions of polymorphonuclear leucocytes. Neither benzoylarginine *p*-nitroanilide nor succinylphenylalanine *p*-nitroanilide was hydrolysed by cell fractions; control experiments showed satisfactory hydrolysis of these substrates by trypsin and chymotrypsin respectively.

Inhibition of neutral protease. (a) Intracellular inhibitors. The large increase in recovery of neutral protease from subcellular fractions compared to whole cells indicated the presence of an intracellular inhibitor of the enzyme. This possibility was investigated by measuring the neutral protease

Table 1. *Distribution of protein, neutral protease, acid cathepsin and β -glucuronidase in polymorphonuclear-leucocyte subcellular fractions*

The combined recovered specific activities were: for neutral protease, $0.97 \pm 0.19 \mu\text{g}$ of tyrosine/h per mg of protein and $4.46 \pm 1.85 \mu\text{g}$ of arginine/h per mg of protein; for cathepsin, $2.81 \pm 0.87 \mu\text{g}$ of tyrosine/h per mg of protein; for β -glucuronidase, $86.5 \pm 17.2 \mu\text{g}$ of phenolphthalein/h per mg of protein. The numbers of separate experiments are indicated in parentheses. The results are given as percentages, mean \pm s.d.

	Specific activity of fraction		
	Nuclear debris	Granule	Post-granule supernatant
Protein	4.4 ± 0.9 (5)	33.0 ± 5.6 (5)	63.8 ± 5.3 (5)
Neutral protease (histone, pH 7.2)			
Tyrosine	5.6 ± 5.5 (3)	89.6 ± 10.2 (3)	4.6 ± 8.1 (3)
Arginine	1.8 ± 2.3 (5)	97.2 ± 3.1 (5)	0.8 ± 1.8 (5)
Cathepsin (haemoglobin, pH 3.0)	7.0 ± 2.6 (4)	44.0 ± 17.1 (4)	48.5 ± 18.9 (4)
β -Glucuronidase (pH 4.5)	4.6 ± 3.9 (5)	77.4 ± 9.8 (5)	18.0 ± 6.4 (5)

Table 2. *Recovery of neutral protease, acid cathepsin and β -glucuronidase from subcellular fractions of polymorphonuclear leucocytes*

The results are mean \pm s.d. The numbers of separate experiments are indicated in parentheses.

Enzyme	Activity ($\mu\text{g}/\text{h}$ per mg of protein)		Recovery (%)
	Recovered	Whole cells	
Neutral protease (pH 7.2)			
Tyrosine	0.97 ± 0.19 (3)	0.13 ± 0.07 (3)	746
Arginine	4.46 ± 1.85 (5)	0.22 ± 0.27 (5)	2025
Cathepsin (pH 3.0)			
Tyrosine	2.81 ± 0.87 (4)	2.68 ± 1.52 (4)	105
β -Glucuronidase (pH 4.5)			
Phenolphthalein	86.5 ± 17.2 (5)	117.2 ± 49.6 (5)	74

activity of granule fraction mixed with other subcellular fractions. Table 5 shows that the activity of granule fractions was abolished by addition of whole cells or post-granule supernatant. In marked contrast, activity was only slightly inhibited by the nuclear-debris fraction and not at all by the granule wash. In a separate experiment, the inhibitory activity of post-granule supernatants was found to be almost exclusively located in the 100 000g supernatant. The 'microsomal' pellet inhibited neutral protease activity by less than 30%, compared to 97% inhibition by supernatant fractions. Inhibition of neutral protease by various fractions was not due to endogenous arginase activity: incubation of a known quantity of arginine with these fractions for 18 h at 37°C resulted in complete recovery of arginine.

(b) Serum. Both human serum and foetal calf serum completely inhibited the neutral protease activity of granule fractions.

(c) ϵ -Aminohexanoate. Concentrations of ϵ -aminohexanoate from 0.02 to 0.1 M inhibited neutral protease to a maximum of 30% at 0.01 M (Table 6).

Table 3. *Distribution of enzymic activity in azurophilic and specific granules of rabbit peritoneal-exudate polymorphonuclear leucocytes*

Specific activities were: for peroxidase, $\Delta E_{440}/\text{h}$ per mg of protein; for alkaline phosphatase, μmol of substrate hydrolysed/h per mg of protein; for neutral protease, μg of arginine released/h per mg of protein.

	Azurophilic granules	Specific granules
Peroxidase*	15.00	0.955
Alkaline phosphatase*	200	12000
Neutral protease	8.83	0

* Data supplied by Dr Marco Baggiolini.

(d) 1-Chloro-3-L-toluene-*p*-sulphonamido-7-aminohexan-2-one. In the range of concentrations tested (0.05–1.0 mM) no inhibition of neutral protease was observed, whereas trypsin was inhibited almost completely (97%) by the highest concentration used (0.05 mM) (Table 6).

Table 4. Release of neutral protease and β -glucuronidase of rabbit polymorphonuclear leucocytes

All values are expressed as percentage of activity released into the 20000g supernatant by incubation with 0.1% (v/v) Triton X-100 at 37°C for 1 h. For details of experimental procedure see Materials and Methods section.

Concn. of aetiocholanolone (mM)	% of enzyme released	
	β -Glucuronidase	Neutral protease
0	14.4	0
0.01	13.9	0
0.1	16.2	0
0.5	52.2	35.5

Table 5. Inhibition of granule neutral protease, pH 7.2, by subcellular fractions of polymorphonuclear leucocytes

For details of the assay procedure see the Materials and Methods section.

Fraction added (0.1 ml) to granule fraction (0.1 ml)	% of activity added	% of activity remaining
Granules alone	—	100*
Whole cells	0	0
(Post-granule supernatant)	0	0
Nuclear-debris	0	91
Granule	100	176
Granule wash	22	122

* Specific activity: 18.5 μ g of arginine released/h per mg of protein.

(e) 1-Chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one. This agent inhibited neutral protease by 30% at 0.05 mM, the highest concentration used (Table 6). Hydrolysis of histone by chymotrypsin was shown to be inhibited approximately twice as much as neutral protease by this concentration of inhibitor. Problems with solubility of this inhibitor prevented its use at higher concentrations since dimethyl sulphoxide itself inhibits neutral protease when present at concentrations above 2.5% (v/v).

(f) Soya-bean trypsin inhibitor. Concentrations ranging from 0.1 to 250 μ g/ml of inhibitor caused inhibition ranging from 16 to over 60% (Table 6) as compared with 94% inhibition of trypsin (2 μ g/ml) by soya-bean extract (8 μ g/ml).

Effect of heat and storage on neutral protease activity. Sharp decreases in neutral protease activity were seen when granule fractions were

Table 6. Inhibition of granule neutral protease by inhibitors of proteolysis

1-Chloro-3-L-toluene-*p*-sulphonamido-7-aminoheptan-2-one inhibits trypsin 97% at the concentration shown; 7-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one inhibits chymotrypsin 60% at the concentration shown; 8 μ g of soya-bean trypsin inhibitor inhibits trypsin by 94%. For further details see the text.

Inhibitor	Concn.	Inhibition (%)
Post-granule fraction	262 μ g/ml	100
ϵ -aminohexanoate	50 mM	28
1-Chloro-3-L-toluene- <i>p</i> -sulphonamido-7-aminoheptan-2-one	1 mM	0
7-Chloro-4-phenyl-3-L-toluene- <i>p</i> -sulphonamidobutan-2-one	0.05 mM	30
Soya-bean trypsin inhibitor	250 μ g/ml	64

incubated at 60°C, falling to 79 and 50% of control values after 30 and 60 min respectively. Reduction to below 10% of control activity was observed after heating at 90°C for 30 min. No loss of activity was observed after storage of polymorphonuclear-leucocyte granule fractions at -20°C for periods as long as 3 months.

Activation of neutral protease. The presence of both cationic proteins and anionic polysaccharides in polymorphonuclear-leucocyte lysosomes led to studies of the influence of types of these macromolecules on neutral protease activity. Since electrostatic interactions were likely, the effects were also studied of increasing ionic strength of the medium.

Heparin was used as an example of a highly sulphated polysaccharide and dextran was chosen as a representative non-charged polymer. Heparin (Fig. 2) increased neutral protease activity of whole cells lysed by Triton X-100 by at least tenfold; however, dextran had no effect at any concentration investigated. In a separate experiment, dextran sulphate was shown to have an effect on lysed, whole cells similar to that of heparin. In contrast, the less-sulphated chondroitin sulphate (from two different sources) and protein-polysaccharide (from bovine nasal cartilage) had no effect upon neutral protease activity of whole cells.

Lysozyme, containing an excess of eight cationic residues per molecule (Jolles, Jauregui-Addell & Jolles, 1963), was also tested; concentrations of 0.1-5.0 mg failed to influence neutral protease activity in granule fractions.

Increasing ionic strength in the presence of potassium chloride at final assay molarities ranging from 0.1 to 2.5 M resulted in a remarkable increase of

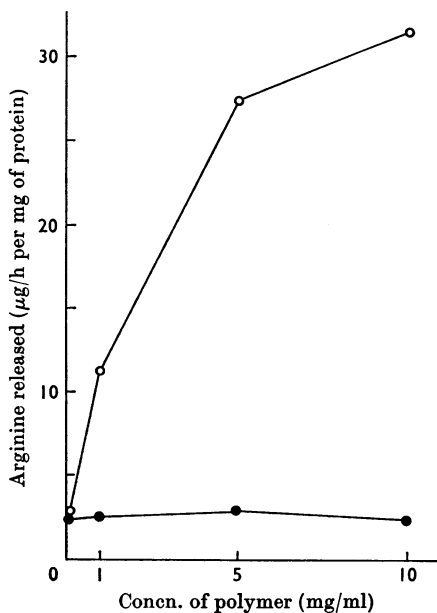


Fig. 2. Effect of an anionic polymer (heparin) and an uncharged polymer (dextran) upon neutral protease activity of lysed polymorphonuclear leucocytes. Assays were performed in 0.1M-sodium phosphate buffer containing appropriate concentrations of heparin (○) or dextran (●). (For details see the Materials and Methods section.)

enzyme activity of whole cells and of nuclear-debris fractions with increasing concentrations of potassium chloride up to 1.0 M (Fig. 3). A further, slight, increase in activity was obtained with 2.5M-potassium chloride (Fig. 3). Such an increase was not found in granule fractions over the wide range of salt concentration tested. In direct contrast, no activity could be detected in post-granule supernatants even at the highest concentrations of potassium chloride. Inhibition of neutral protease activity of granule fractions by post-granule supernatants was almost completely reversed (>90%) in the presence of 2.5M-potassium chloride (Table 7); however, the inhibitory effect of serum remained unaffected.

Sephadex chromatography. A series of procedures were tested for their ability to solubilize neutral protease; these showed that whereas heparin (5 mg/ml) solubilized the greatest amount and highest proportion of neutral protease activity from the granule fraction, potassium chloride and sodium dodecyl sulphate also solubilized moderate amounts of activity. In marked contrast, however, Triton X-100, Lubrol PX and sodium deoxycholate released relatively little activity from the granules. When a 2.5M-potassium chloride extract of granule fraction was obtained as described above and

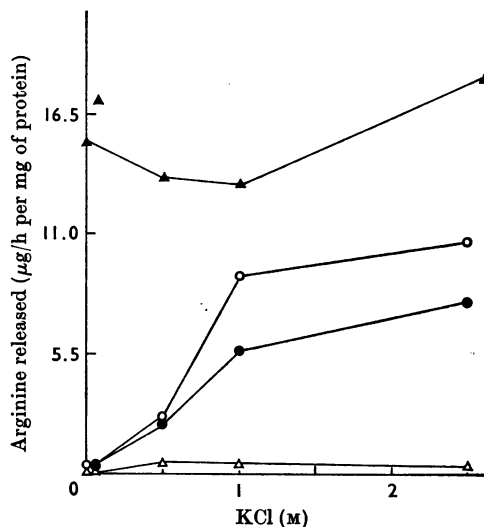


Fig. 3. Influence of salt concentration on neutral protease activity in polymorphonuclear leucocytes and subfractions. Assays were performed in 0.1M-sodium phosphate buffer containing increasing concentrations of potassium chloride. (For details see the Materials and Methods section.) ▲, Granule fraction; ○, nuclear-debris fraction; ●, whole cells; △, post-granule supernatant.

Table 7. Effect of the concentration of potassium chloride on the inhibition of granule-fraction neutral protease activity by post-granule supernatant

Assays were performed in 0.1M-sodium phosphate buffer, pH 7.5, or 0.1M-sodium phosphate buffer, pH 7.5, containing 2.5M-KCl. (For details see the Materials and Methods section.) The percentages of granule-fraction neutral protease activity remaining after mixing with post-granule supernatant in the absence and presence of 2.5M-potassium chloride are indicated by the numbers in parentheses.

	Sp. activity of granule fraction (µg of arginine released/h per mg of protein)	
	No KCl	2.5M-KCl
Granule + 0.34M-sucrose	24.2	44.1
Post-granule supernatant + 0.34M-sucrose	0.3	0.5
Granule + post-granule supernatant	0.3 (1)	40.9 (92)

applied to a Sephadex G-75 column two peaks of neutral protease activity were obtained, the first appearing between bovine serum albumin and cytochrome c; a second peak of low molecular weight appeared much later (Fig. 4). When these peaks were applied to a DEAE-cellulose column no activity could be recovered.

Sephadex chromatography of a 5 mg/ml heparin extract of granule fraction was also attempted.

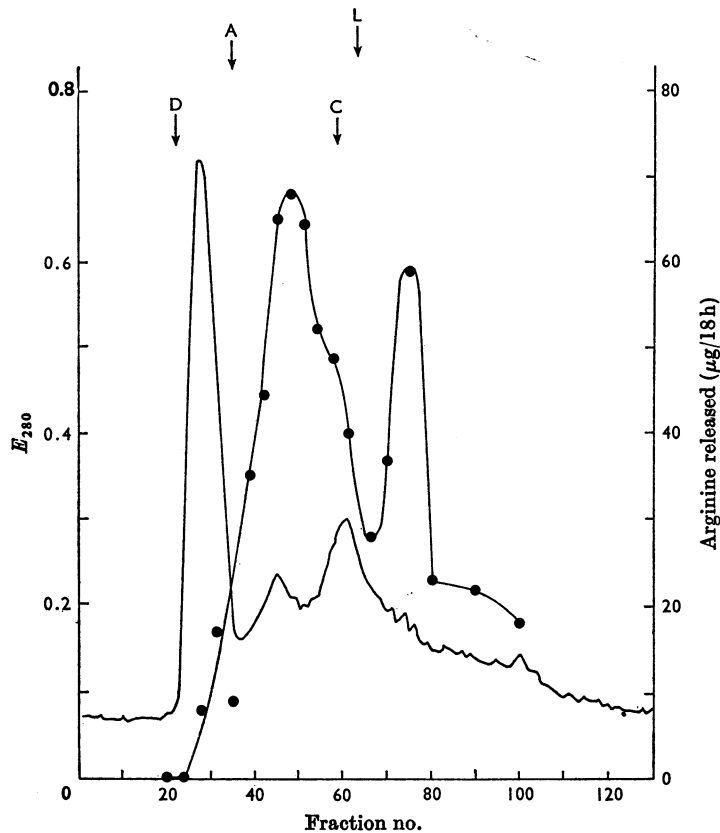


Fig. 4. Sephadex G-75 chromatography of polymorphonuclear-leucocyte granule fraction. Granule fraction (5 ml) was made 2.5M with respect to potassium chloride concentration and applied to a column (2.5 × 60 cm) of Sephadex G-75 equilibrated with 0.1M-sodium phosphate buffer, pH7.2, containing 2.5M-KCl. The column was eluted with this buffer, 60-drop fractions being collected. ●, E_{230} ; —, μg of arginine released from histone/18h. A, Bovine albumin; B, Blue Dextran 2000; L, lysozyme; C, cytochrome *c*.

Elution of such an extract on Sephadex G-75 with 0.1M-sodium phosphate buffer containing heparin (3mg/ml) resulted in all protein being found in the void volume; heparin had evidently formed a macromolecular complex with the protein.

Isoelectric focusing. A two-step procedure was used to solubilize the neutral protease activity from granule fractions. The granules were first exposed to Triton X-100, which solubilized only 4.3% of the neutral protease activity of the fraction while rendering non-sedimentable 43.3% of the protein and 66.4% of the β -glucuronidase activity. After sedimentation of the Triton X-100-disrupted granules, the resulting pellet (which showed a twofold increase in the specific activity of neutral protease over the granule fraction) was resuspended in 0.34M-sucrose containing heparin (5mg/ml). After centrifugation at 8200g for 15min, 48.7% of the neutral protease activity was found to be present in the supernatant, 4ml of which (total

activity of 44.0 μg of arginine released/h) was used for the electrofocusing. Two major peaks of specific activity were found in the acidic pH region, a smaller peak at pH4.2 and the highest peak at pH5.2 (Fig. 5).

The specific activity of neutral protease corresponding to this latter peak was found to be increased by 54.7-fold over the activity of the whole cells (specific activity expressed as μg of arginine/h per mg of protein; at pH5.2, highest peak = 67.3; granules = 3.77; whole cells = 1.26; assayed at optimum heparin concentration). When compared with whole cells assayed in the absence of polyanion, the specific activity was increased 306-fold (Table 2).

DISCUSSION

The use of calf thymus histone as substrate has permitted the detection within rabbit polymorphonuclear leucocytes of a neutral protease that is

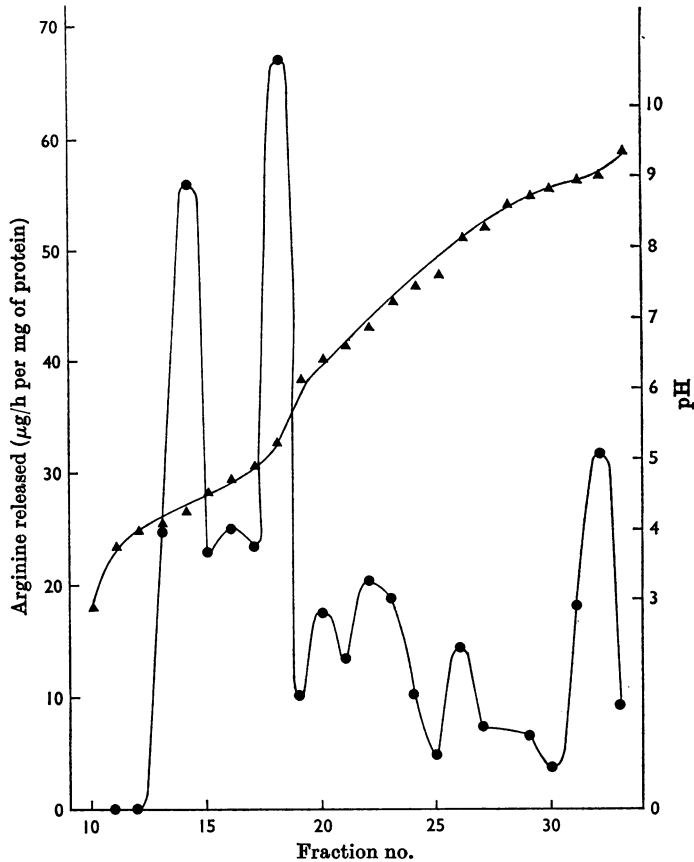


Fig. 5. Isoelectric focusing of neutral protease from rabbit polymorphonuclear-leucocyte granules, in a 3–10 pH gradient. Enzyme activity (●) is expressed as μg of arginine released/h per mg of protein. ▲, pH gradient.

not active upon conventional protein substrates (Cohn & Hirsch, 1960; Wasi *et al.* 1966). The enzyme has been shown to be concentrated in large granule fractions sedimenting between 400g and 8200g. Such fractions contained the highest activities of two other enzymes associated with rabbit polymorphonuclear-leucocyte lysosomes: β -glucuronidase and acid cathepsin (Cohn & Hirsch, 1960; Cochrane & Aikin, 1966). Heterogeneity of polymorphonuclear-leucocyte granules has been demonstrated by both morphological (Bainton & Farquhar, 1966; Wetzel, Horn & Spicer, 1967) and biochemical techniques (Baggiolini *et al.* 1969; Zeya & Spitznagel, 1969). At least three groups of granules have been described, of which the best characterized are the azurophilic and specific granules. These are differentiated on the basis of the time at which they are formed during cellular maturation and the fact that specific granules appear at the convex face of the Golgi complex

whereas azurophilic form at the concave side. Confirming cytochemical and electron microscopical studies by Bainton & Farquhar (1968a,b), Baggiolini *et al.* (1969) separated these granules (and other sub-groups) by zonal centrifugation. They differ considerably in their content of enzymes: acid hydrolases and peroxidase were concentrated in the azurophilic granules whereas alkaline phosphatase was confined to the specific granules; lysozyme was found in both granule types. Our present studies have located neutral protease activity exclusively to the azurophilic granules. The possibility that the absence of activity from specific granules is due to the presence of an inhibitor such as that found in the cytoplasm is rendered unlikely by the fact that incubation of granule fractions in high salt concentrations did not increase enzyme activity significantly. In contrast, inhibitory effects of post-granule supernatants were almost completely reversed in high salt solutions. It is of interest to

find enzymes with widely differing pH optima in the same granule type, since this would raise the question of their functional efficiency at pH values far removed from their optima. There is, however, evidence that acid hydrolases can function at neutral pH: degradation of chick embryonic cartilage by cathepsin D proceeds at pH 7.1 (Weston, Barrett & Dingle, 1969). Finally, although azurophile granules are homogeneous morphologically, they may well differ in their individual enzyme content; no information is available on this point.

The finding of a cytoplasmic inhibitor of neutral lysosomal protease is not confined to the polymorphonuclear leucocyte, since an inhibitor of proteolytic enzymes has also been described in rat liver microsomes and cytoplasm by Kaye & Dabich (1969). The nature and origin of the polymorphonuclear-leucocyte cytoplasmic inhibitor is unknown at present. It may be a product of the cell itself or alternatively it may be endocytosed from plasma, which is known to contain several inhibitors of proteolytic enzymes (Fritz, Trantschold, Haendle & Werle, 1968). Since the cells were obtained from inflammatory exudates, uptake of inhibitor from plasma is quite possible. Moreover, it has been shown that both human plasma and foetal calf serum are potent inhibitors of neutral protease, although results obtained with foetal calf serum should be interpreted with caution in view of its potent arginase activity (Kihara & de la Flor, 1968).

Indeed our localization of the inhibitor to the cytoplasm may be an artifact of our method of preparing subcellular fractions. It is conceivable that if the inhibitor was derived from plasma its true subcellular localization was within secondary lysosomes that were disrupted during preparatory procedures owing to their relative fragility.

Studies on the inhibition of neutral protease showed that post-granule supernatants of polymorphonuclear leucocytes and serum were most effective; lesser degrees of inhibition were found with γ -aminohexanoate, soya-bean trypsin inhibitor and 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamido-butan-2-one. Inhibition by the latter suggests that the enzyme is similar in nature to chymotrypsin (Schoellman & Shaw, 1963). This appears unlikely since the enzyme failed to hydrolyse artificial substrates for chymotrypsin: succinyl- and glutaryl-phenylalanine *p*-nitroanilide. On the same grounds, inhibition of neutral protease by soya-bean trypsin inhibitor does not suggest that it has trypsin-like specificity, since the enzyme failed to hydrolyse benzoylarginine *p*-nitroanilide.

Recently several proteases and peptidases have been located to polymorphonuclear leucocyte lysosomes, and it is important to differentiate these

enzymes from the neutral protease now described. Lazarus *et al.* (1968*a*) and Lazarus, Daniels, Brown, Bladen & Fullmer (1968*b*) described a neutral collagenase within human polymorphonuclear-leucocyte lysosomes, which differs from neutral protease in several respects. In marked contrast to neutral protease the collagenase is not inhibited by serum but inhibited by 0.01M-cysteine and 0.01M-sodium-EDTA (Lazarus *et al.* 1968*b*). As with other mammalian collagenases, granulocyte collagenase cleaves collagen into two distinct fragments. It is, however, noteworthy, that unpurified granulocyte preparations produce multiple digestion products, a process that is inhibited by serum. These findings suggest the presence in human polymorphonuclear-leucocyte granules of a neutral protease or proteases inhibitable by serum that are distinct from collagenase. We have compared the subcellular distribution of neutral protease with several other peptidases found in polymorphonuclear leucocytes (Davies, Krakauer & Weissmann, 1970*b*) and found a distinctly different distribution for each enzyme.

Polymorphonuclear-leucocyte granules contain high concentrations of sulphated anionic polysaccharides (Fedorko & Morse, 1965; Olsson, 1969; Dunn & Spicer, 1969) and cationic proteins (Zeya & Spitznagel, 1963, 1966, 1969; Seegers & Janoff, 1966). The sulphated anionic polysaccharides have been shown to occur in azurophiles of human polymorphonuclear leucocytes together with acid phosphatase (Olsson, 1969). Moreover Zeya & Spitznagel (1969) showed that cationic proteins of rabbit polymorphonuclear-leucocyte granules exist in a distinct granule type, probably azurophiles; indeed Dunn & Spicer (1969) demonstrated by histochemical means the coexistence of anionic polysaccharides and cationic proteins in human polymorphonuclear-leucocyte azurophile granules. Electrostatic interactions of sulphated anionic polysaccharides with other granule components is suggested by the observation of Olsson (1969) that 0.3–0.9M-sodium chloride was required to release chondroitin 4-sulphate, whereas concentrations of 0.3M or less release other granule constituents.

It was therefore of great interest to find that anionic polysaccharides increased the activity of neutral protease by reversing the effect of the cytoplasmic inhibitor. Also pertinent is the observation that, whereas high ionic strength and highly sulphated polyanions did not effect neutral protease activity, they markedly inhibited other lysosomal enzymes such as β -glucuronidase and β -glycerophosphatase (P. Davies, G. A. Rita, K. Krakauer & G. Weissmann, unpublished work). This would suggest that high local concentration of polyanions may inhibit the activity of lysosomal enzymes in primary lysosomes while having no effect on

neutral protease. In secondary lysosomes the situation would be reversed, since the content of primary lysosomes would be much diluted, removing thereby the inhibitory activity of polyanions on acid hydrolases, whereas the introduction of plasma protease inhibitors would drastically decrease or abolish neutral protease activity.

The different effects of potassium chloride on neutral protease activities of whole cells and of granule fractions may be interpreted as being due to the dissociation of an inhibitor-neutral protease complex in whole cells, permitting access of enzyme to substrate. The relative absence of inhibitor in granule fraction would allow expression of activity at low salt concentration. At this time we are unable to estimate what the effect will be of increasing ionic strength on the activity of neutral protease when the enzyme is completely purified; hydrolysis of histone by trypsin and chymotrypsin is increasingly inhibited by salt to the extent of about 60% by 2.5M-potassium chloride. The possibility that the enzyme-inhibitor complex is an ionic one is further supported by the reversal of inhibition by the highly anionic polysaccharides heparin and dextran sulphate, whereas the non-charged polymer dextran had no effect on enzyme activity. Failure of chondroitin sulphate and bovine nasal protein-polysaccharide to influence enzyme activity may be due to their lesser sulphation. A similar explanation may account for the inefficacy of lysozyme which, in addition, can compete with histone as a substrate.

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