

SUBCELLULAR LOCALIZATION AND HETEROGENEITY OF NEUTRAL PROTEASES IN NEUTROPHILIC POLYMORPHONUCLEAR LEUKOCYTES

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The possibility that the digestive enzymes of phagocytes may be noxious to both the endocytosed microorganisms and the host was already anticipated by Metchnikoff when he postulated the presence of cytases in phagocytes and of a mechanism to prevent them from attacking the phagocyte itself (1). For some time, acid proteinases such as cathepsin D and E were thought to be mainly responsible for tissue damage induced by polymorphonuclear leukocytes (PMNs)¹ (2-5). More recently, however, human PMNs were found to be very rich in proteolytic enzymes with optimum activity at neutral pH, which are capable of degrading a wide variety of tissue components, such as the vascular basement membrane (6, 7), the elastic lamina of the arterial wall (7, 8), structure proteins of lung tissue (9), cartilage proteoglycans (10-13), and native collagen (14-16). Two kinds of neutral proteases which are very likely to attack some of the above mentioned structures, elastases and collagenases, have been isolated from human PMNs during the last 2 yr (17-19). A further group of enzymes with chymotrypsin-like activity has also been characterized (20). The complex pathogenetic implications of neutral proteases in inflammation have been reviewed recently by Janoff (21).

In the present work, the subcellular localization of elastase-like activity and of enzymes that hydrolyze casein and histone (22) at neutral pH has been investigated, both in human and in rabbit PMNs, by isopycnic equilibration according to the methods established in previous studies (23-25). In addition, the heterogeneity of the proteases has been studied by different staining procedures after acrylamide gel electrophoresis of extracts of single density equilibration fractions.

Materials and Methods

Fractionation Techniques. Human PMNs from pooled buffy coats of donor blood were purified, homogenized, and fractionated by isopycnic equilibration in Beaufay's rotor as described by Bretz and Baggiolini (25). Rabbit PMNs were obtained from glycogen-induced peritoneal exudates, homogenized, and fractionated by differential centrifugation to give a postnuclear (4,000 g-min)

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¹Abbreviation used in this paper: PMNs, polymorphonuclear leukocytes.

supernate as previously described (23). The postnuclear supernate was then subjected to isopycnic equilibration under the conditions used for human PMN preparations (25).

Presentation of the Fractionation Results. The raw data were converted to normalized distribution histograms according to the principles described by Beaufay et al. (26) and plotted as relative concentration of component vs. relative volume collected. Average distributions from different experiments were calculated with the help of a new computer program which performs the following operations: The sets of histograms to be averaged are first aligned along the abscissa in order to correct for possible variations in total volume. The histograms of one component which has been assayed in all experiments (in the present case, peroxidase) are taken as leads. They are integrated over 50 identical volume intervals, and then shifted toward each other to the point of maximum overlapping. Upon alignment, each single histogram is integrated over a fixed number of identical volume intervals (usually 25, which closely corresponds to the number of fractions originally collected). The standardized histograms of the single components from each experiment are then averaged by calculating the mean and the corresponding standard deviation of the relative concentration values of each standard fraction. Calculations and plotting were performed on a Siemens 4004-35-64 K computer (Siemens Corp., Medical Industrial Div., Iselin, N. J.).

Biochemical Assays. Protein was determined either according to the method used by Miller (27) or by the automated method described by Leighton et al. (28). Peroxidase, alkaline phosphatase, lysozyme, and the glycosidases were determined in all fractionation experiments as described by Bretz and Baggiolini (25).

Elastase-like activity (subsequently termed elastase) was determined by one of two methods: (a) By measuring the rate of hydrolysis of *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester, as described by Janoff and Basch (29). Sample aliquots of 0.05–0.1 ml were added to 3.0 ml of a mixture containing 4 mM substrate, 2 mM Tris buffer adjusted to pH 8.0 with HCl, 0.05 M KCl, and 0.05% Triton X-100. Measurements were made at 25°C by pH Stat titration using an automatic titrator, Type TTT 1 (Radiometer Inc., Copenhagen, Denmark). (b) By determining the rate of liberation of *p*-nitrophenol from *N*-tert-butoxycarbonyl-L-alanine *p*-nitrophenyl ester, according to Visser and Blout (30). Briefly, 20 μ l of 10 mM solution of substrate in methanol were added to an assay mixture containing 0.45 M sodium phosphate pH 6.5, 0.09% Triton X-100, and the sample in a final vol of 1 ml. Increase in absorbance at 347.5 nm was followed for 3–5 min at 25°C in a Zeiss PM 4 spectrophotometer (Carl Zeiss, Ober Kochen, West Germany) equipped with a W+W recorder (W. and W. Electronics Ltd., Basel, Switzerland).

Neutral protease activities on casein or histone were determined as follows: Before use, casein was denatured by heating at 100°C for 15 min, and histone was precipitated with 10% sulfosalicylic acid (22). Assay mixtures contained in a total vol of 0.8 ml 0.5% of either casein or histone, the sample, 0.01% digitonin, and 0.05 M phosphate buffer pH 7.5 (or pH 6.0 when indicated). Incubations were carried out at 37°C in a shaking bath for 60 or 120 min with casein, and for 20 or 60 min with histone as substrate. In each case the reaction was terminated by the addition of 0.2 ml of cold 25% TCA. After standing in ice for 30 min and centrifugation, the acid-soluble hydrolysis products in the supernate were estimated with fluorescamine (31). A 0.1 ml aliquot of the TCA supernate was mixed with 3.0 ml of 0.2 M sodium borate buffer pH 8.5, followed by rapid addition with vigorous stirring of 1 ml fluorescamine solution (15 mg dissolved in 100 ml acetone). Fluorescence was read at 475 nm with excitation set at 390 nm in a Hitachi-Perkin-Elmer fluorescence spectrometer model 203 (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). L-Leucyl-L-leucine served as standard.

Electrophoretic Techniques. Electrophoresis was carried out in 20% polyacrylamide gel slabs at pH 4.3 under the conditions described by Reisfeld et al. (32), except that to both the separating and the stacking gels, as well as to the anode buffer, 0.05% Triton X-100 and 0.01% digitonin were added. Samples from fractionations of human PMNs were prepared for electrophoresis as follows. Aliquots of 0.5 ml of the gradient fractions were diluted with 0.2 M sucrose and centrifuged in a MSE SS-65 ultracentrifuge (Measuring and Scientific Equipment Inc., Crawley, Sussex, England) at approximately 3×10^6 g-min (40,000 rpm for 30 min in a titanium angle rotor no. 59593) to sediment the particulate material. The pellets were resuspended in a small vol (0.1–0.4 ml) of isotonic saline. Protein was determined in 3- μ l aliquots of the suspensions, and then adjusted, in each sample, to a concentration of 1.6 mg/ml after the following compounds (final concentration) had also been added: Triton X-100 (0.3%), digitonin (0.03%), glycerol (10%), and NaCl (0.48 M). Samples from fractionations of rabbit PMNs were treated in the same way, except that protein was not determined

in the resuspended pellets, but was adjusted to 2 mg/ml on the basis of the protein content of the gradient fractions before centrifugation. In this case, the final concentration of NaCl in the suspensions that were subjected to electrophoresis was 0.15 M. Electrophoresis was performed in a slab apparatus of the type described by Studier (33). Sample aliquots of 20 μ l, corresponding approximately to 30 μ g of protein were applied to each sample well. Electrophoresis was allowed to proceed for 14 h at 20 mA with samples from human PMNs, and for 5 h at 30 mA with samples from rabbit PMNs. Gels were stained for protein with Coomassie Brilliant Blue according to Laemmli (34). Esterolytic activities were demonstrated with naphthol AS-D acetate (35) and with *N*-acetyl-D,L-alanine α -naphthyl ester (36) as substrates. After preincubation in two changes of 0.1 M phosphate buffer, pH 7.0, the gels were transferred to the appropriate substrate solutions which were prepared as follows: (a) 30 mg of naphthol AS-D acetate dissolved in 3 ml of *N,N*-dimethylformamide and 20 mg of Fast Blue RR dissolved in 2 ml of *N,N*-dimethylformamide were added separately with rapid mixing to 60 ml of 0.1 M phosphate buffer, pH 7.0, containing 20% *N,N*-dimethylformamide. (b) 30 mg of *N*-acetyl-D,L-alanine α -naphthyl ester dissolved in 3 ml acetone and 20 mg of Fast Blue RR dissolved in 2 ml acetone were added to 60 ml of 0.1 M phosphate buffer, pH 7.0. Incubations were carried out at 37°C until defined bands appeared, and then were stopped by placing the gels in a 7% solution of acetic acid.

Results

Biochemical Assays. Complete studies on the dependence of the assayed protease activities on pH, incubation time, and amount of added sample were carried out only with subcellular preparations from human PMNs. The results (Fig. 1) show that the proteases assayed have optimum activity at pH values around neutrality, and that substrate hydrolysis is proportional to the amount of sample over a wide range. The conditions established for human PMN preparations were also used for the assays of rabbit proteases.

Specific Activity of the Neutral Proteases. The mean values \pm SD are given

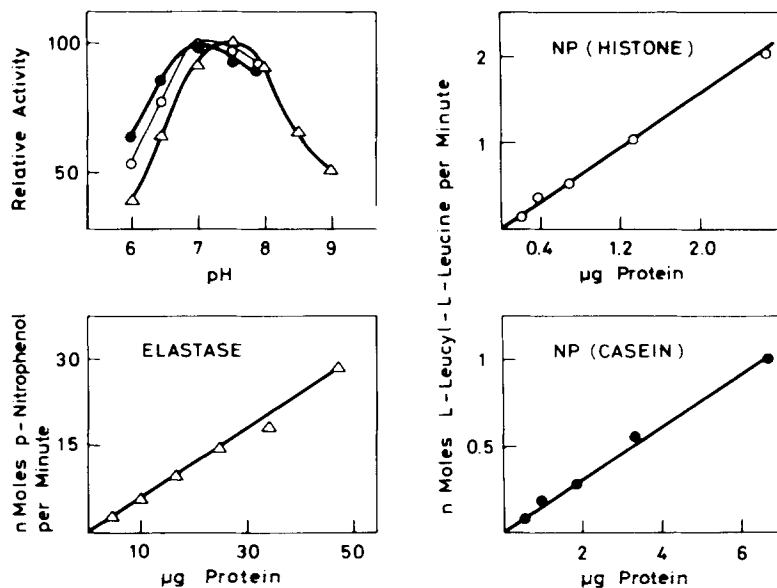


FIG. 1. Assay conditions for neutral proteases (NP) from human PMNs. Hydrolysis of histone (O), casein (●), and *N*-tert-butoxy-carbonyl-L-alanine *p*-nitrophenyl ester (Δ) as a function of pH and amount of PMN protein added. Dependence on pH was determined in the following buffers: 0.05 M sodium phosphate, pH 6.0–8.0; and 0.1 M Tris-HCl, pH 7.5–9.0.

in Table I. Under the conditions used, postnuclear supernates from human PMNs have about 10–30 times greater proteolytic activity than corresponding preparations from the rabbit. When measured in postnuclear supernates, the activity of elastase was about 60% of that obtained in corresponding granule preparations. In accord with this, the addition of the soluble fraction was found to lower the elastase activity of isolated granules. This suggests the presence of a soluble elastase inhibitor in PMNs. A similar observation has been reported by Janoff and Blondin (37). Because of this effect of cytosol, the elastase recoveries in the fractionation experiments were calculated on the basis of the activity of a 250,000 *g*-min pellet of the starting material.

Fractionation Results. Fig. 2 shows the distribution histograms obtained

TABLE I
Specific Activity of Neutral Proteases in Postnuclear Supernates from Human and Rabbit PMNs

Enzyme	Specific activity	
	Human PMNs	Rabbit PMNs
Elastase	402.9 ± 84.2 (5)	52.4 ± 19.2 (3)
NP (casein)	95.6 ± 24.2 (3)	3.1 ± 1.0 (3)
NP (histone)	395.4 ± 58.4 (3)	15.7 (2)

Values are mean specific activities ± SD in milliunits per milligram of protein. One unit of activity is defined as the amount of enzyme that forms 1 μmol of product per minute, i.e., for elastase 1 μmol of *p*-nitrophenol and for the two other neutral proteases (NP) TCA soluble peptides equivalent to 1 μmol of L-leucyl-L-leucine. Number of experiments are given in brackets.

upon isopycnic equilibration of the components of postnuclear supernates of human PMNs. Three separate experiments in which the neutral proteases were measured were averaged with five former experiments of this type selected on the basis of optimal recovery. Two of these experiments were reported in a previous paper (25). The close similarity of the averaged distribution histograms with those of single experiments (25), and the small standard deviations obtained upon averaging underline the remarkable reproducibility of the isopycnic fractionations. Such results have been commented on in detail in a previous paper (25) so that we can limit ourselves to the interpretation of the histograms of the neutral proteases. Elastase and the neutral proteases hydrolyzing casein and histone have very similar, sharp, and unimodal isopycnic equilibration profiles which almost perfectly overlap that of peroxidase. This indicates that they are exclusive constituents of the azurophil granules. There is almost total overlapping between the neutral proteases and the lysosomal glycosidases. However, the distribution profiles of these two classes of hydrolases show a minor, but clear, difference in the upper half of the gradient where the glycosidases exhibit a small, broad peak suggesting the presence of a minor population of particles of lysosomal character (25). The neutral proteases have virtually no activity in this

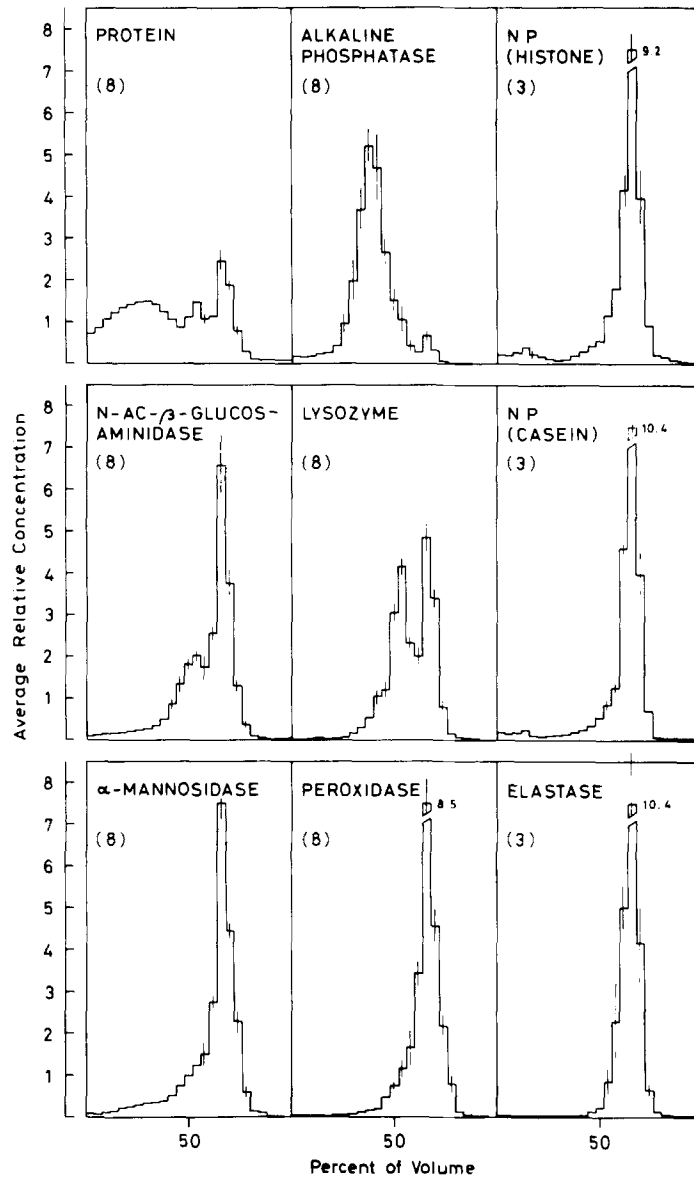


FIG. 2. Subcellular distribution of neutral proteases in human PMNs. Graphs represent averaged volume distributions obtained upon isopycnic equilibration. Density increases from left to right. Ordinate shows concentration in fraction relative to concentration corresponding to uniform distribution throughout the gradient. Vertical lines on histograms represent standard deviation (shown only if greater than 0.2). Number of experiments is given in brackets. Percentage recoveries were 101 ± 10 for protein, 91 ± 10 for peroxidase, 96 ± 14 for lysozyme, 87 ± 14 for alkaline phosphatase, 91 ± 12 for *N*-acetyl- β -glucosaminidase, 90 ± 11 for α -mannosidase, 92 ± 11 and 85 ± 26 for neutral proteases (NP) with histone and casein as substrate, respectively, and 125 ± 23 for elastase. Alignment of the single experiments before averaging (see Materials and Methods) was done on the basis of the distribution of peroxidase. The average abscissa shifting of the single experiments corresponded to 1.7% of the total volume.

zone of the gradient. Because of their distribution, they are, alongside peroxidase, seemingly perfect marker enzymes for the azurophil granules.

Using Sephadex gel filtration, Rindler-Ludwig et al. (38) have resolved two main peaks of proteolytic activity against casein from granule extracts of human PMNs. It was subsequently found that these two fractions have pH optima of 6.0 and 7.4, respectively, and differ in their susceptibility to EDTA (39). In two fractionation experiments we have, therefore, assayed for casein hydrolysis at both pH values, in the presence and absence of EDTA. The relative distribution profiles obtained were identical with those of casein hydrolysis at pH 7.5 shown in Fig. 2, thus suggesting that these different forms of neutral proteases have identical subcellular localization.

The results obtained by isopycnic fractionation of postnuclear supernates from rabbit PMNs are shown in Fig. 3. The similarity between the equilibration profiles of peroxidase and those of the neutral proteases seen in fractionations of human PMN homogenates essentially holds true in these experiments also. Even with the sensitive fluorescence assay used, the measurement of casein or histone hydrolysis in rabbit PMN fractions posed some problems because of the low enzyme activity present (see Table I). The resulting inaccuracies are likely to be the cause of the minor profile irregularities in the upper part of the gradient. Despite these uncertainties, the fractionation results presented support the conclusions reached in our former studies that the azurophil granules of human PMNs have a very similar enzymic equipment as those of the rabbit (24, 25).

Electrophoretic Analysis of Subcellular Fractions. In order to further characterize the granule-bound neutral proteases, granule-rich fractions from isopycnic equilibration experiments were extracted and analyzed by acrylamide gel electrophoresis. Two substrates were used for the demonstration of neutral proteolytic activities. The first, *N*-acetyl-D,L-alanine α -naphthyl ester, has been reported to specifically stain for elastase activity (17, 36). The second, naphthol AS-D acetate, is a substrate of esterases as well as of PMN proteases (38). Upon electrophoresis of granule extracts, this substrate produces patterns identical with those obtained with naphthol AS-D chloroacetate which has been shown to be a protease substrate (20, 40).

Figs. 5 *a-c* show the pattern obtained with fractions from human PMNs. The samples applied to the gels are extracts of the eight consecutive fractions indicated in the histograms of Fig. 4 *a*. This range covers the equilibration zones of both azurophil and specific granules. A sample of a corresponding extract of the starting material of the isopycnic fractionation (II) was also run for comparison. In the gels which were stained for esterolytic activity (Figs. 5 *b* and *c*), several reactive bands are seen. Maximum intensity was always found in those fractions which showed maximum activities of elastase, the latter being represented in Fig. 4 *a* as a marker for the azurophil granules. The staining patterns of these fractions correspond to that obtained with the starting material. These results demonstrate that human PMNs contain a considerable number of single enzymes with proteolytic activity at neutral pH, which are all confined to the azurophil granules.

Protein staining (Fig. 5 *a*) reveals additional nonesterolytic bands. On the basis of these patterns, we have marked 10 clearly distinguishable zones (A-J) on the

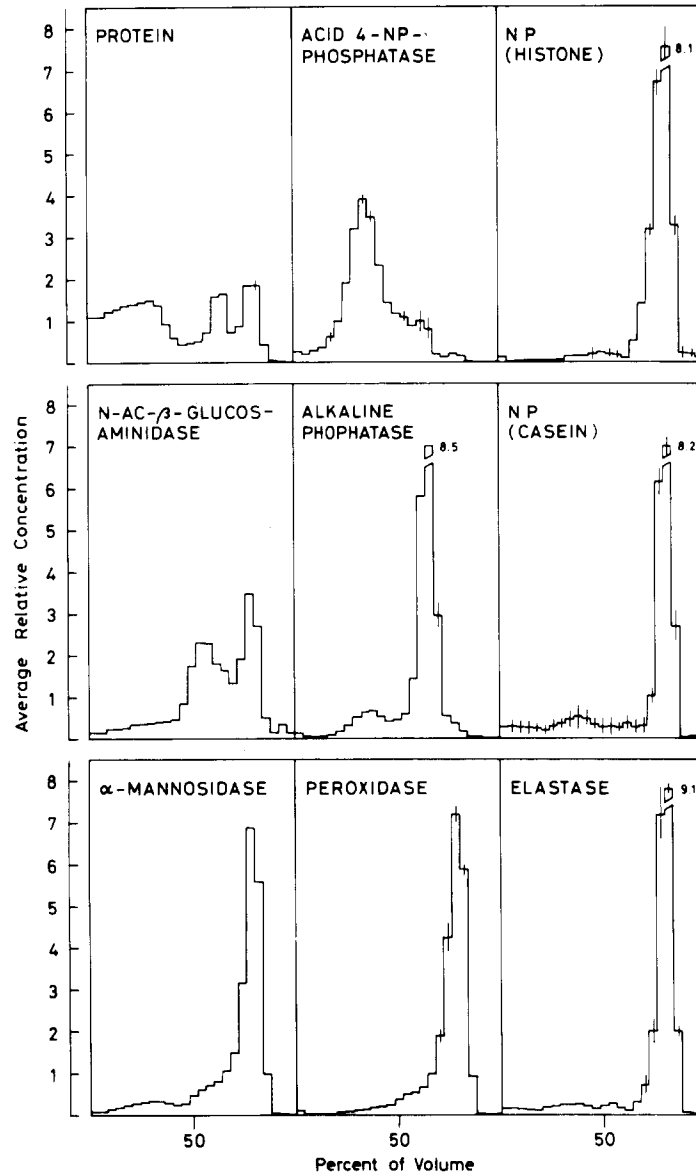


FIG. 3. Subcellular distribution of neutral proteases in rabbit PMNs. Graphs are explained in the legend to Fig. 2. Mean percentage recoveries from two experiments were 100 for protein, 107 for peroxidase, 102 for alkaline phosphatase, 87 for acid 4-nitrophenyl-phosphatase, 128 for *N*-acetyl- β -glucosaminidase, 108 for α -mannosidase, 80 and 122 for neutral proteases (NP) with histone and casein as substrate, respectively, and 108 for elastase. The average abscissa shifting of the two experiments corresponded to 1% of the total volume.

gels. Staining for elastase activity with *N*-acetyl-D,L-alanine α -naphthyl ester as substrate (Fig. 5 *b*) produces a group of three major bands (D, E, and F), which are likely to correspond to the three elastase isoenzymes recently demonstrated by Janoff (17), and by Sweetman and Ornstein (36) using a similar technique. In

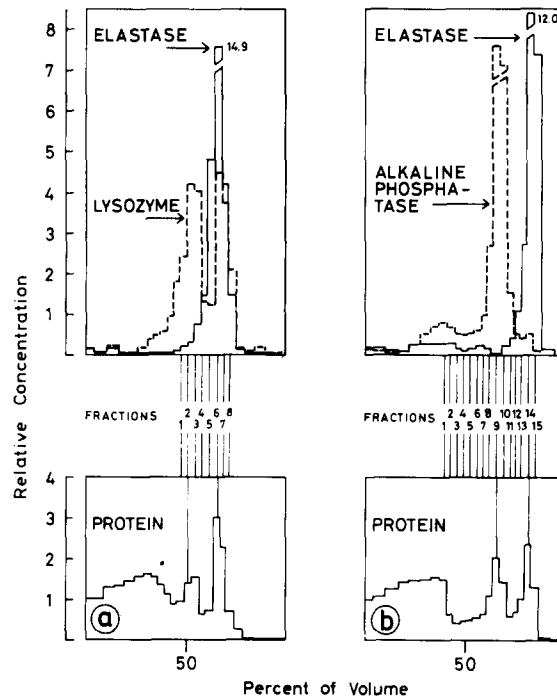


FIG. 4. Isopycnic equilibration of subcellular components of human (a), and rabbit (b) PMNs. Graphs show normalized distribution histograms from single experiments as a function of the volume collected. Elastase shown as a marker for the azurophil granules, and lysozyme (a) or alkaline phosphatase (b) as a marker for the specific granules. The two granule populations are also reflected by the protein histograms.

addition, our gels clearly show a few less prominent bands, one between D and E, and a possible doublet between E and F. The slowly migrating band close to the origin only appears when detergents are incorporated into the gel. This has also been reported by others (17, 36). All bands with elastolytic activity are also visible in those gels which were stained with the second substrate, naphthol AS-D acetate (Fig. 5 c). These gels show four additional bands, three prominent, highly cationic bands, G, I, and J, and the slowly moving band C. These highly cationic proteins, one migrating slightly behind, and two in front of lysozyme, are likely to correspond to three chymotrypsin-like enzymes which have been recently purified from granule preparations of human PMNs (20).

A comparison of the two enzyme activity stainings with the protein patterns (Fig. 5 a) shows that the elastase activities between D and F and the highly cationic bands G, I, and J all correspond to well-defined protein bands, thus indicating that the neutral proteases of human PMNs account for an important portion of the basic proteins of the azurophil granules.

Completely different protein patterns are obtained with extracts of the fractions containing the specific granules (Fig. 5 a, nos. 1-3). Four bands are clearly seen, a weak one barely entering the separating gel (A), a very strong one in zone B, one band in zone C, and lysozyme (H) which is present in all extracts tested. As seen in the figure, lysozymes from both azurophil and specific granules

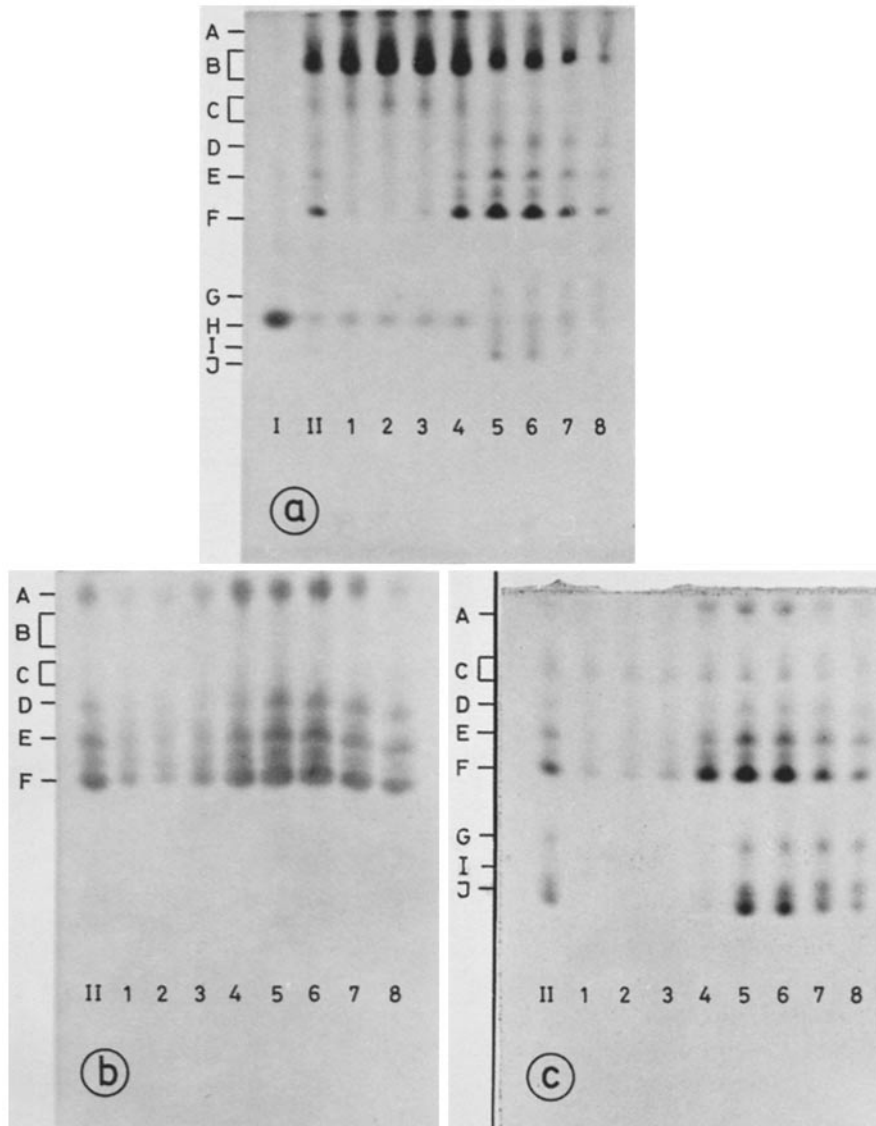


FIG. 5. Acrylamide gel electrophoresis of extracts of fractions obtained by isopycnic equilibration of subcellular components of human PMNs. Gel *a* was stained for protein, and gels *b* and *c* for protease activity, using as substrates *N*-acetyl-D,L-alanine α -naphthyl ester and naphthol AS-D acetate, respectively. Numbers at the bottom designate extracts from single fractions, and correspond to the fraction numbers of the histograms of Fig. 4 *a*. Two reference samples are shown: (I) purified lysozyme from human PMNs, and (II) an extract of the postnuclear supernate which was used as the starting material for the isopycnic fractionation. Capital letters on the left-hand side indicate zones or single bands as mentioned in the text. Cathode is at the bottom of the gels. Electrophoresis was carried out for 14 h at 20 mA.

have identical electrophoretic mobilities, which suggests that the same protein may be stored in both types of granules.

The results of a similar experiment performed with granule-rich fractions from rabbit PMNs are presented in Figs. 6 *a-c*. The samples applied to the gels are

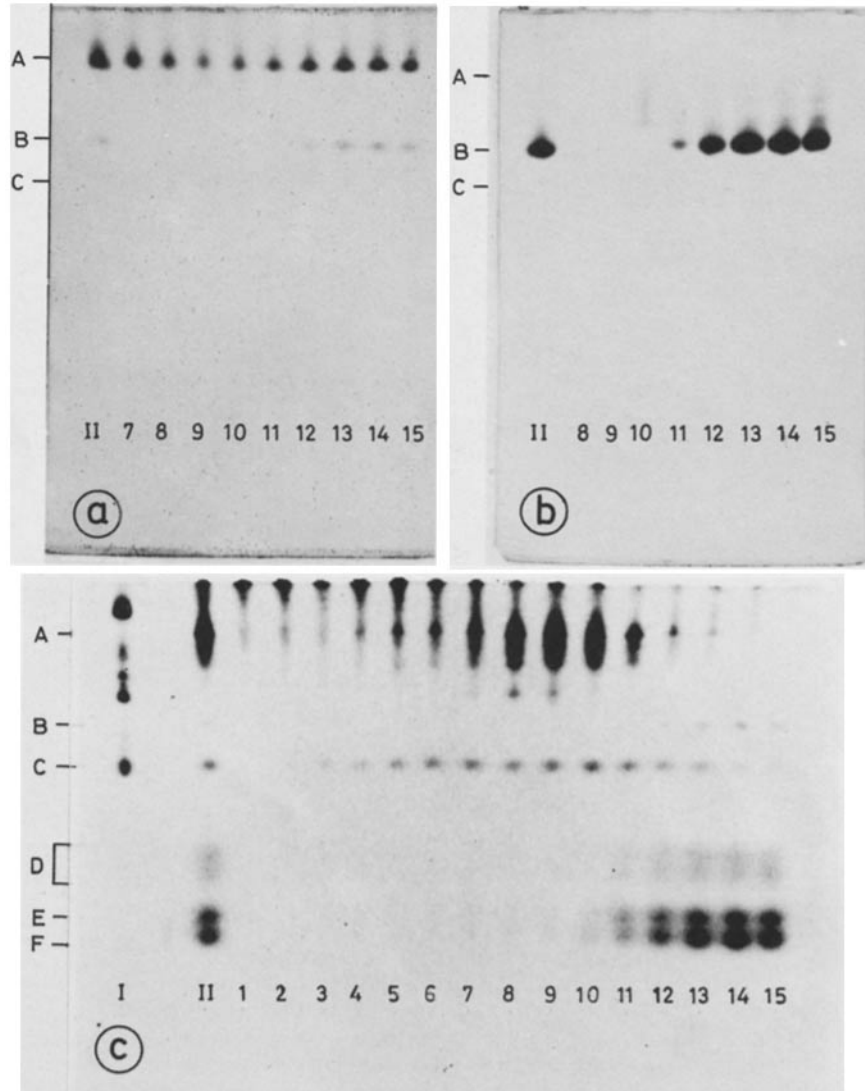


FIG. 6. Acrylamide gel electrophoresis of extracts of fractions obtained by isopycnic equilibration of subcellular components of rabbit PMNs. Gels *a* and *b* were stained for protease activity, using as substrates naphthol AS-D acetate, and *N*-acetyl-D,L-alanine α -naphthyl ester, respectively, and gel *c* was stained for protein. Numbers at the bottom designate extracts from single fractions, and correspond to the fraction numbers of the histograms of Fig. 4 *b*. Extracts of postnuclear supernates from human (I) and rabbit (II) PMNs were used as reference samples. Gel zones or single bands are marked on the left with capital letters as in Fig. 5. Cathode is at the bottom of the gels. Electrophoresis was carried out for 5 h at 30 mA.

extracts of the fractions designated 1-15 in the histograms of Fig. 4 *b*. A striking difference between these electrophoretic patterns and those obtained with human PMNs are immediately evident. Activity staining with *N*-acetyl-D,L-alanine α -naphthyl ester as substrate (Fig. 6 *b*) reveals only one major elastase band and 3 or 4 very minor, more slowly migrating components which all belong exclusively to the azurophil granules (fractions 12-15). The major elastase band can also be demonstrated in the same fractions and at the same localization by using naphthol AS-D acetate as substrate, but here it appears as a comparatively weak band. In addition, the naphthol AS-D acetate zymogram shows a slow-moving band (A) in the extracts of all fractions tested. This activity is equally prominent both above and below the fractions with highest specific granule contents (nos. 9 and 10), and, therefore, does not parallel the distribution of any of the neutral proteases determined biochemically.

The protein pattern seen in Fig. 6 *c* furthermore shows that the very fast migrating proteins (zones D, E, and F) are also localized exclusively in the azurophil granules and that they appear to represent major constituents of these particles. Two groups of these proteins can be distinguished, the strongly staining bands E and F in front and two slightly more slowly moving, less prominent bands in zone D. The most striking feature of the cationic proteins of rabbit PMNs is their fast rate of migration, especially when compared to the most cationic proteins of the human sample (I) which (under the conditions used) migrate similarly to lysozyme in zone C.

Discussion

Elastase and neutral proteases acting on histone and casein were found to be localized exclusively in azurophil granules both in human and rabbit PMNs. Electrophoretic analysis of extracts of azurophil granules from human PMNs revealed up to 10 distinct proteins with esterolytic activity, which are likely to account for the protease activity determined biochemically. In rabbit PMNs, the number of electrophoretically detectable proteases is considerably smaller. In both species, the most prominent bands of the zymograms show elastase activity. Elastase appears to be a major component of the azurophil granules of PMNs as has also been pointed out by Janoff (17). Human PMN elastase has been purified by Janoff (17) as well as by Ohlsson and Olsson (19), and has been shown to consist of three isoenzymes. Ohlsson and Olsson (19) have found that these three elastases have mol wt between 33,000 and 36,000, similar amino acid composition except for arginine, and identical antigenic properties. On electrophoresis in polyacrylamide gel, the three isoenzymes described by Janoff (17) show mobilities and staining intensities with *N*-acetyl-D,L-alanine α -naphthyl ester similar to those of the three major elastase bands found in the present work (Fig. 5 *b*: D, E, and F).

On the basis of the electrophoretic analysis of granule contents reported in this paper, it is possible to directly compare some of the properties of the most cationic PMN proteins of both species studied. Cationic proteins with strong bactericidal activity *in vitro* were first described in rabbit PMNs by Hirsch (41), and subsequently studied by Zeya and Spitznagel (42). In confirmation of earlier data (43), our results clearly show that these proteins are confined entirely to the

azurophil granules of rabbit PMNs. These highly cationic proteins are characterized by their very fast migration rate in polyacrylamide gel electrophoresis, moving well ahead of lysozyme, as well as by their apparent lack of protease activity (Fig. 6). The most strongly cationic proteins of human PMNs were also found to be localized exclusively in the azurophil granules. However, they show a similar migration rate as lysozyme in gel electrophoresis (Fig. 5) and, therefore, may be considerably less basic than rabbit cationic proteins. Furthermore, all three bands, two migrating in front of and one slightly behind lysozyme, display esterolytic activity as demonstrated by their staining with naphthol AS-D acetate. Rindler-Ludwig and Braunsteiner (20) recently have characterized three cationic proteins from human PMN granules with very similar electrophoretic behavior. These proteins were found to have chymotrypsin-like activity. Olsson and Venge (44, 45) using agarose gel electrophoresis demonstrated the presence of seven cationic proteins exhibiting faster mobility towards the cathode than does lysozyme in granule extracts of human leukocytes. Five of these components were obtained in pure form and characterized by determination of their molecular weights, amino acid compositions, and antigenic properties.

Despite the potentially important role of neutral PMN proteases in pathological processes involving tissue destruction, the subcellular localization of these enzymes has not been thoroughly studied in the past. Working with human PMNs, Folds et al. (46) found enzymes that hydrolyze denatured hemoglobin and benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester at pH 7.2 and 6.5, respectively, in particles containing β -glucuronidase and acid β -glycerophosphatase. These particles were subsequently identified as azurophil granules by the same group (47). Analyzing azurophil and specific granules obtained from PMNs by rate zonal centrifugation (48), Davies et al. (22) found that neutral histone hydrolase was localized in azurophil granules only. These findings are in complete agreement with our results which show that not only the proteolytic activities assayed biochemically with three different substrates, but also the proteases visualized by staining with two esterase substrates after electrophoretic separation are wholly confined to the azurophil granules.

However, one neutral protease, collagenase, which we were not equipped to assay in these experiments, has been shown by Robertson et al. (49) to be localized in the specific granules of rabbit PMNs. So far, this is the only protease reported to occur in specific granules. In view of the data now available on the subcellular localization of all the other neutral PMN proteases investigated, the finding on collagenase requires confirmation.

Summary

The subcellular localization of elastase and of neutral proteases hydrolyzing histone and casein was determined in human and rabbit polymorphonuclear leukocytes using fractionation by isopycnic centrifugation. Granule-rich fractions obtained by this technique were extracted and analyzed by acrylamide gel electrophoresis, and proteolytic activity on the gels was demonstrated by staining with either *N*-acetyl-D,L-alanine α -naphthyl ester or naphthol AS-D acetate as substrate. In both species, all neutral proteases assayed were found to be

localized exclusively in the azurophil granules. Specific activities were about 10–30 times higher in human than in rabbit preparations. In extracts of human azurophil granules up to 10 proteins exhibiting esterolytic activity could be demonstrated after electrophoretic separation. Three major and two or three minor components of these esterases were shown to possess elastase activity. Similar zymograms prepared with extracts from rabbit azurophil granules revealed only one major elastase band. The electrophoretic analysis further showed that the most strongly cationic proteins of both human and rabbit PMNs were also confined to the azurophil granules.

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