A Novel Type of Cytoplasmic Granule in Bovine Neutrophils

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ABSTRACT We obtained cell preparations containing >95% neutrophils from freshly drawn bovine blood. The cells were suspended in sucrose and disrupted in a Dounce homogenizer, and the postnuclear supernate was fractionated by zonal differential sedimentation and by isopycnic equilibration. The subcellular fractions were characterized biochemically by testing for marker enzymes and other constituents known to occur in azurophil and specific granules of other species, and by electrophoretic analysis of extracts of the particulate material. In addition, each fraction was examined by random-sampling electron microscopy.

We found that bovine neutrophils contain in addition to azurophil and specific granules a third type of granule, not known to occur in neutrophils of other species. These novel granules are larger, denser, and considerably more numerous than the two other types. Except for lactoferrin, they lack the characteristic constituents of azurophil granules (peroxidase, acid hydrolases, and neutral proteinases) and of specific granules (vitamin B_{12}-binding protein). Instead, they contain a group of highly cationic proteins not found in the other granules, and they are the exclusive stores of powerful oxygen-independent bactericidal agents.

We studied the fate of the large granules in bovine neutrophils exposed to opsonized particles, the ionophore A 23187, or phorbol myristate acetate. The appearance in the cell-free media of antibacterial activity and of the characteristic highly cationic proteins as revealed by electrophoresis was monitored and compared with the release of azurophil and specific granule markers. In addition, changes of the relative size of the large granule compartment induced by phagocytosis were assessed by morphometry. The results show that exocytosis of the large granules occurs following both phagocytosis and exposure to soluble stimuli. Like the specific granules, the large granules appear to be discharged by true secretion under conditions where the azurophil granules are fully retained.

Biochemical studies of bovine neutrophils were first reported by Hegner (1) who isolated a particulate cytoplasmic fraction enriched in a number of acid hydrolases and alkaline phosphatase. Bovine neutrophils lack lysozyme (2, 3) and have relatively low activities of azurophil granule enzymes. They produce superoxide and H_2O_2 during phagocytosis, but in addition have a high capacity for oxygen-independent killing of several types of bacteria (3–5).

These properties and the predominance of peroxidase-negative granules seen on electron micrographs stimulated our interest in these cells. The results of our subcellular fractionation study show that bovine neutrophils contain a novel type of cytoplasmic storage granules. The novel organelles are larger, denser, and much more numerous than the azurophil and specific granules, the two types that have been found in all mammalian neutrophils studied so far.

MATERIALS AND METHODS

Materials: Reagents used in our work were obtained from the following sources: Zymosan, α-naphthyl acetate, naphthol AS-D acetate and *N*-acetyl-D,L-phenylalanine-β-naphthyl ester was obtained from the Sigma Chemical Co. (St. Louis, MO); *N*-acetyl-D,L-alanine α-naphthyl ester from the Vega-Fox Biochemicals (Tucson, AZ); Cyano[^{57}Co]cobalamin, 370–740 kBq/μg, from The Radiochemical Centre (Amersham, Bucks., UK); Phorbol myristate acetate (PMA) from the Consolidated Midland Corp. (Brewster, NY). Bovine milk lactoferrin was a gift of Dr. B. Senft (University of Giessen, Federal Republic of Germany) and the antiserum to bovine milk lactoferrin was a gift of Dr. P. Masson (International Institute of Cellular and Molecular Pathology, Brussels, Belgium). The phosphate-buffered saline solution (PBS) had the following com-

position: 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4. A calcium-free PBS with the same composition from which $CaCl_2$ was omitted was also used.

Subcellular Fractionation: Neutrophils were purified from fresh bovine blood by differential centrifugation combined with hypotonic lysis of contaminating erythrocytes (3, 6). The isolated cells were washed two to three times with calcium-free PBS. Preparations containing 95% or more neutrophils were used for the fractionation experiments. Before homogenization the cells were pelleted and resuspended at a density of 150×10^6 cells/ml in 0.34 M sucrose, pH 7.4, containing 0.5 mM $MgCl_2$. Addition of $MgCl_2$ was found to minimize nuclear breakage and particle clumping following cell disruption (7). The cells were disrupted in a Dounce homogenizer (Kontes Co., Vineland, NJ) using the tight-fitting B-pestle. 30–60 up-and-down strokes were necessary to break 50–60% of the cells. We obtained a postnuclear supernate by centrifugation of the homogenate at 250 g for 10 min and used it as the sample for subcellular fractionation.

We performed zonal differential sedimentation in a B-XIV titanium rotor (MSE no. 59144) operated by an MSE SS-65 ultracentrifuge (Measuring & Scientific Equipment, Crawley, Sussex, UK). In some experiments, we used the B-XIV volume adapter described by Baggiolini et al. (8). Portions of 10–30 ml of postnuclear supernate were fractionated at 6,500, 8,500, 11,000, or 13,500 rpm for 15 min under the conditions adopted for rabbit (9) and human (10) neutrophils, using sucrose solutions made up in isotonic saline (11). We carried out isopycnic equilibration experiments using a Beaufay rotor as described by Bretz and Baggiolini (10), except that sucrose solutions were made up in isotonic saline instead of water. The fractionation data are presented as in our previous paper (10). All cell fractionation procedures were carried out at 4°C.

Analytical Assays: Peroxidase (10), alkaline phosphatase (12), acid 4-nitrophenyl phosphatase (10), *N*-acetyl-β-glucosaminidase (11), lactate dehydrogenase (11), and protein (13) were determined according to established methods with slight modifications of the assay conditions as shown in Table I. Calcium- and magnesium-dependent ATPase activities were assayed by the method of Gennaro et al. (14) in the presence of 0.017 mM $CaCl_2$ and 1 mM $MgCl_2$, respectively. Inorganic phosphate was determined initially as described by Penney (15) and later by the more sensitive method of Lanzetta et al. (16). Vitamin B_{12}-binding capacity was assayed according to Kane et al. (17) using 1 pmol of ^{57}Co-vitamin B_{12} per assay. Neutral proteinase activity was determined by the method of Levine et al. (18). The incubation mixture contained 0.11 mg [^{3}H]acetyl casein (~150,000 cpm) and the enzyme sample in 400 μl of 0.09 M sodium phosphate buffer, pH 7.5. Lactoferrin was assayed by single radial immunodiffusion (19). For this purpose, portions of the fractions obtained by zonal sedimentation were centrifuged at 100,000 *g* for 35 min and the pellets were extracted with 0.3% cetyl trimethyl ammonium bromide (20) followed by three cycles of freezing and thawing. The extracts were cleared by centrifugation at 8,000 *g* for 10 min and assayed on agarose plates containing an antiserum to bovine milk lactoferrin. Purified bovine lactoferrin served as the standard.

Assays for Bactericidal Activity: *Escherichia coli* (ATCC No. 8739) and *Staphylococcus aureus* (ATCC No. 6538) were grown overnight on BHI agar medium (Difco Laboratories, Detroit, MI) to stationary growth phase and collected in sterile isotonic saline solution. The density of the bacterial suspension was determined by nephelometry at 600 nm, using a standard curve relating optical density to numbers of bacteria, and a stock suspension containing 5–10 $\times 10^7$ organisms per milliliter of sterile PBS was prepared. We centrifuged portions of the neutrophil subcellular fractions at 100,000 *g* for 35 min and the pellets obtained were resuspended in one-twenty-fifth of their original volume in sterile saline. Granules were then lysed by three cycles of freezing and thawing, followed by sonication for 10 s at 100 W (Branson Sonifier, Branson Powers & Co., Danbury, CT). Portions of the lysates were incubated for 30 min at 37°C with 5–10 $\times 10^6$ microorganisms in 10-ml plastic culture tubes (Falcon Plastics, Div. of Becton-Dickinson & Co., Cockeysville, MD) with shaking. The incubation volume was 0.3 ml (2/3 PBS and 1/3 saline) and the pH was 7.4. After incubation, three to four dilutions ($1/10^3$ to $1/10^6$) of the assay mixture were made in saline and 0.1 ml aliquots were plated on BHI agar. Following incubation overnight at 37°C, the numbers of viable bacteria were determined by colony counting.

Gel Electrophoresis: Electrophoretic separation was carried out in 20% polyacrylamide gel slabs at pH 4.3 according to Reisfeld et al. (21). The particulate material present in the fractions obtained by zonal sedimentation was pelleted at 100,000 *g* for 35 min and resuspended in a small volume of saline (one-twenty-fifth the original volume). Portions of these suspensions were extracted by stirring in 0.2 M sodium acetate buffer, pH 4.5, at 0–4°C for 2–3 h, and then centrifuged at 100,000 *g* for 35 min. The clear extracts were submitted to electrophoresis at 15 mA for 14 h. Protein was stained with Coomassie Brillant Blue (22) and proteinase activities were revealed by incubating with α-naphthyl acetate, naphthol AS-D acetate, N-acetyl-D,L-phenylalanine-β-naphthyl ester or N-acetyl-D,L-alanine α-naphthyl ester as substrate (23).

Exocytosis Experiments: Purified bovine neutrophils were suspended in calcium-free PBS at a density of 2.5×10^8 cells per milliliter and kept in ice. Opsonized zymosan, PMA and A 23187 were used as stimuli. Zymosan was opsonized with fresh bovine serum (12 mg/ml) for 30 min at 37°C, washed twice in PBS, and resuspended in PBS at 25 mg/ml. The stimuli (0.2 ml) were added to 1.6 ml of PBS and warmed to 37°C for 5 min. The reaction was then started by adding 0.2 ml of the neutrophil suspension, and was stopped after 5 to 60 min by cooling in ice. In some experiments neutrophils were pretreated with cytochalasin B. The neutrophil suspension (0.2 ml) was mixed with 1.6 ml of prewarmed PBS containing 9 μg of cytochalasin B, and kept at 37°C for 5 min; release was then induced by adding 0.2 ml of opsonized zymosan. Granule constituents and lactate dehydrogenase were determined in the cells and the cell-free media (pellets and supernates after centrifugation at 250 g for 10 min) and in the original neutrophil suspension. Percent release and percent recovery were calculated. In some experiments, done with twice the above cell number, the cell-free media were tested for bactericidal activity or analyzed by electrophoresis. For this purpose, the media obtained after stimulation with PMA or A 23187 were concentrated with an Amicon ultrafiltration unit using a UM 05 Diaflo membrane (Amicon Corp., Lexington, MA). Protein recovery after ultrafiltration was >90%.

Electron Microscopy and Morphometry: Resting or phagocytosing neutrophils were fixed in ice-cold 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h. The samples were then pelleted in small plastic tubes and left overnight at 4–6°C in the above buffer containing 5% (wt/vol) sucrose. After reacting for peroxidase (24), samples were handled according to standard procedures (25). Aliquots of subcellular fractionations obtained by differential sedimentation were processed according to the random-sampling technique of Bretz and Baggiolini and Baudhuin et al. (10, 26). For the morphometrical evaluation of granule release during phagocytosis the blocks were sectioned according to the stratified sampling procedure (27) to assure that the same cell was not cut twice, and negatives were selected randomly as described by Weibel et al. (28). Phagocytosis was quantified by determination of the relative volume densities of intracellular zymosan (points on phagocytosed zymosan over points on cytoplasm and nucleus). Counting was done on micrographs (×2,700) with a photographically superimposed grid. Discharge of the large granules was quantified on micrographs (× 32,000) with a photographically superimposed testing system consisting of bars and points as shown in Fig. 18.

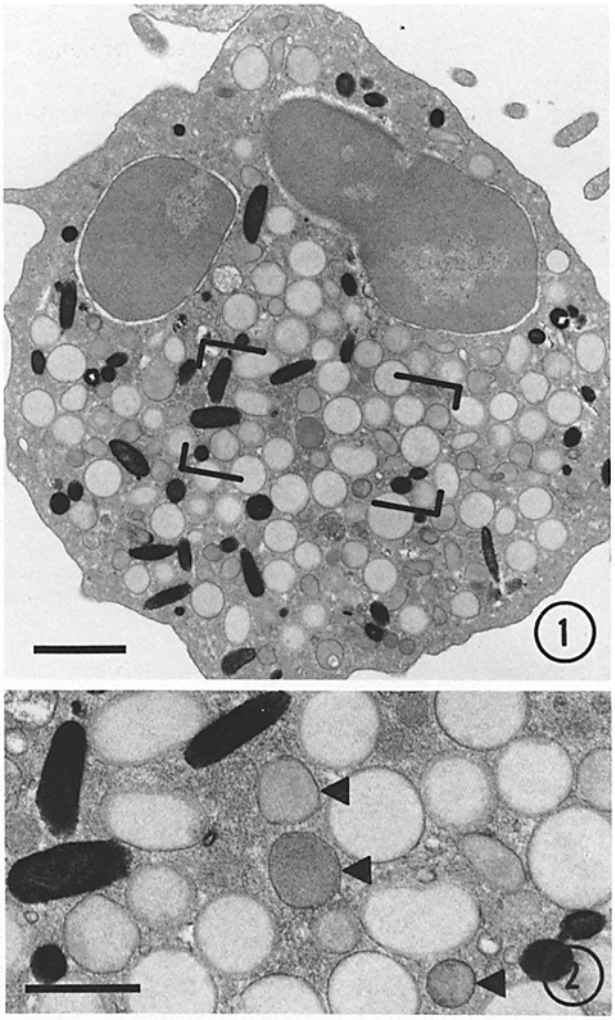
RESULTS

Purification of the Neutrophils

Cells were purified from three to four blood samples obtained from different animals and the preparation yielding the highest percentage of neutrophils was used for subcellular fractionation. The average differential counts (mean percentage ± SD) of the nine preparations used for fractionation experiments were 95.8 ± 2.0 neutrophils, 3.5 ± 1.9 eosinophils, and 0.7 ± 0.6 mononuclear cells.

Morphology of Intact Cells

Insections of bovine blood neutrophils reacted for peroxidase (Fig. 1), three types of cytoplasmic granules are recognized. Peroxidase-positive granules, which by analogy with neutrophils from many other species may be termed azurophil or primary, are present in relatively small numbers. Their profiles are round or elongated. As judged from the compact deposits of reaction product, azurophil granules appear to be rich in peroxidase. Among the peroxidase-negative granules, which are mostly round, two types can be distinguished: large ones (0.4–0.5 μm in diameter) with a pale, very homogenous matrix and small ones (0.15–0.3 μm in diameter) with a more electron-dense content. These morphological differences are reasonably clear, as illustrated by the high-magnification details shown in Fig. 2. However, peroxidase-negative granules with intermediate appearance, i.e., profiles with moderate electron density which are >0.3 μm in diameter, are also seen. Sections of bovine eosinophils, the major contaminants of the purified neutrophil preparations, contain peroxidase-positive granule profiles only. These granules are 0.55–0.8 μm in diameter. Their sedimentation velocity, which is directly proportional to



FIGURES 1 and 2 Fig. 1: Neutrophil from bovine blood, reacted for peroxidase. Specimen obtained from a sample of purified neutrophils prepared for a fractionation experiment. Area reproduced at high magnification in Fig. 2 is indicated. Bar, 1 μm. × 12,000. Fig. 2: High magnification of a portion of the neutrophil shown in Fig. 1 (indicated). Four azurophil granules are seen on the left-hand side. Three small peroxidase-negative granules are indicated by arrow heads. Most of the other profiles represent sections through large peroxidase-negative granules. Bar, 0.5 μm. × 30,000.

the particle volume (29), is therefore expected to be roughly three times greater than that of the largest neutrophil granules.

Subcellular Fractionation

Postnuclear supernates were used as the starting material for zonal differential sedimentation and isopycnic equilibration experiments. The specific activities or contents of the constituents measured are given in Table I. Since in most cases substrate concentration and pH conditions were different, no direct comparison with the corresponding values of human neutrophils (10) can be made. However, we assayed peroxidase in both systems under identical conditions. In bovine neutrophils we found the specific activity to be 15–20% of that of human cells.

Fig. 3 shows the biochemical distribution profiles that were obtained in a zonal differential sedimentation experiment run under conditions assuring the recovery of the large peroxidase-negative granules in the outer half of the gradient. The position of the starting zone is indicated by the profile of the cytosol marker, lactate dehydrogenase. α-Naphthyl acetate esterase has virtually the same distribution. As in rabbit neutrophils (M. Baggiolini, unpublished observations), this nonspecific esterase appears to be a soluble enzyme. Alkaline phosphatase, which is associated with membrane fragments (10) or other light structures (30) in human neutrophils, is slightly resolved from the cytosol enzymes. It is recovered as a sharp peak at the boundary between sample zone and the gradient. The distribution of the Mg^{++}- and the Ca^{++}-dependent ATPases was almost identical (not shown). Peroxidase, which we used to identify the azurophil granules in sections stained cytochemically (Figs. 1 and 2), sediments as a symmetrical peak in the inner half of the gradient. A similar distribution is shown by neutral proteinase activity. In some experiments, significant amounts of peroxidase were recovered against the cushion, most probably reflecting the presence of contaminating eosinophil granules. Vitamin B_{12}-binding protein was assayed as a putative marker of the specific granules since it is an exclusive constituent of these organelles in human neutrophils (31). Its distribution is complex. About one-third of the total is retained in the sample zone and probably represents binding protein released from damaged granules, while the remainder is recovered in a broad peak overlapping with peroxidase. *N*-acetyl-β-glucosaminidase, which in neutrophils of other species is found in the azurophil granules and in the C-particles (9, 10), shows an asymmetrical peak, not dissociated from the starting zone and partly overlapping with peroxidase.

While none of the known granule markers was found in the outer half of the gradient, the cloudy appearance of the fractions collected and the distribution of protein suggested that an important portion of the particulate material, possibly consisting of the pale large granules, was recovered in this zone.

Sedimentation experiments were also run at 6,500, 11,000, and 13,500 rpm. The distributions obtained were consistent with those presented in Fig. 3. However, no significant improvement of the resolution between peroxidase and vitamin B_{12}-binding protein, i.e., between azurophil and the specific granules was obtained.

We also attempted a separation of the three granule populations by isopycnic equilibration. This type of fractionation did not markedly improve the resolution between specific and azurophil granules, as indicated by the equilibration profiles of vitamin B_{12}-binding protein and peroxidase which overlap considerably. As shown in Fig. 4, most of the vitamin B_{12}-binding protein equilibrates in a symmetrical, narrow peak with modal density 1.19–1.20 g/ml. The remainder, probably released from damaged granules (see comments on sedimentation experiments), is spread over the starting zone. Peroxidase has a somewhat lower modal density (1.18–1.19 g/ml). It is slightly but clearly resolved from the vitamin B_{12}-binding protein as indicated by the arrow heads. *N*-acetyl-β-glucosaminidase has the same modal density as peroxidase, but a somewhat broader equilibration profile. The peroxidase profile, on the other hand, is tailing towards higher densities, probably because of contamination by some eosinophil granules. Of particular interest is the distribution of protein. The soluble protein is retained in the starting zone together with the cytosol marker lactate dehydrogenase, while the particulate protein equilibrates as a well-defined peak with modal density >1.20 g/ml. As illustrated in Fig. 4, this protein peak is displaced towards higher density with respect to those of the

TABLE I

Specific Activities or Contents of Enzymes and Nonenzymic Constituents in Postnuclear Supernates of Bovine Neutrophils

Constituent	Assay conditions: Temperature	pH	Substrate concentration	Specific activity* or content
	°C		mM	
Peroxidase	20–22	5.5	0.08	678 ± 63 (8)
N-Acetyl-β-glucosaminidase	20–22	5.0	5.0	7.7 ± 1.4 (6)
Alkaline phosphatase	20–22	9.75	1.25	65.8 ± 12.6 (7)
Acid 4-nitrophenyl phosphatase	37	5.3	5.0	41.7 ± 9.5 (4)
Lactate dehydrogenase	25	7.5	0.4	563 ± 62 (8)
Neutral proteinase	37	7.5	0.275‡	13.7 ± 3.1 (4)
Ca^{2+} ATPase	37	7.0	0.5	4.9 ± 1.5 (4)
Mg^{2+} ATPase	37	7.0	0.5	11.0 ± 2.6 (4)
α-Naphthyl acetate esterase	37	7.0	1.0	24.6 ± 5.1 (3)
Vitamin B_{12}-binding capacity	20–22	7.5	—	35.4 ± 4.1 (8)§
Lactoferrin	20–22	7.5	—	23.8 and 27.2 (2)‖

* Except for neutral proteinase, the specific activity is expressed in mU/mg of protein. 1 U of activity is the amount of enzyme which metabolises 1 μmol of substrate in 1 min. Neutral proteinase activity is expressed as μg of [^{3}H]acetyl casein hydrolyzed to acid-soluble peptides in 1 min. Number of preparations in paretheses.

‡ mg/ml.

§ ng of vitamin B_{12} bound per milligram of protein.

‖ μg/mg of protein.

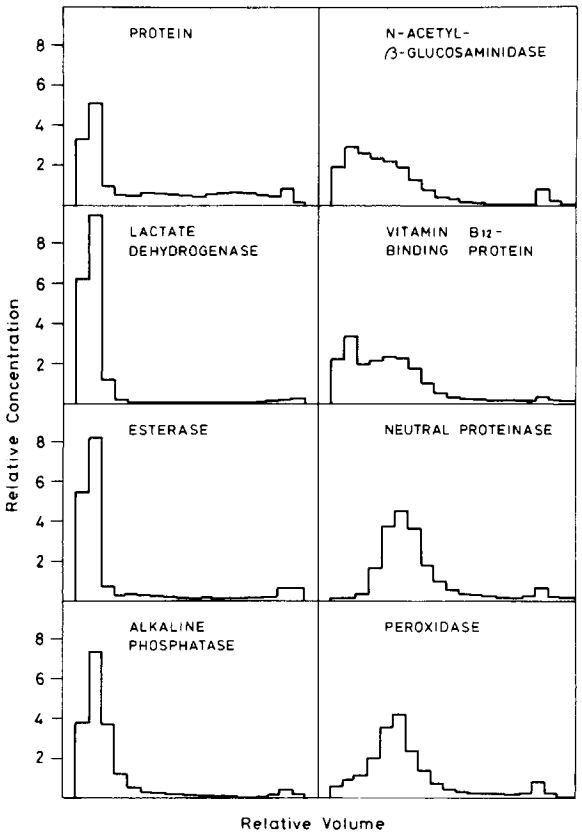


FIGURE 3 Fractionation of subcellular components of bovine neutrophils by zonal sedimentation at 8,500 rpm for 15 min. The graphs are normalized distribution histograms as a function of the volume collected. The radial distance increases from left to right. The ordinate is the concentration in the fraction relative to the concentration that corresponds to uniform distribution throughout the gradient. Percentage recoveries were 88 for protein, 94 for lactate dehydrogenase, 91 for α-naphthyl acetate esterase, 77 for alkaline phosphatase, 82 for *N*-acetyl-β-glucosaminidase, 94 for vitamin B_{12}-binding protein, 174 for neutral proteolytic activity, and 103 for peroxidase.

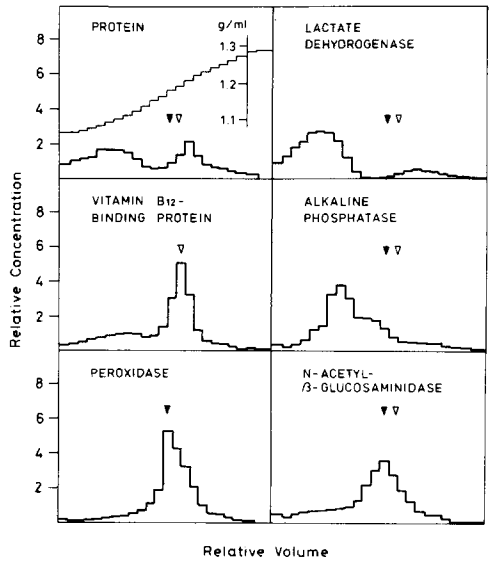


FIGURE 4 Isopycnic equilibration of subcellular components of bovine neutrophils. The histograms are constructed as in Fig. 6. The average density of each fraction is given by the ascending curve in the protein pannel. The black and white arrowheads indicate the peak of peroxidase and vitamin B_{12}-binding protein, respectively. Percentage recoveries were 108 for protein, 91 for vitamin B_{12}-binding protein, 101 for peroxidase, 97 for lactate dehydrogenase, 153 for alkaline phosphatase, and 91 for *N*-acetyl-β-glucosaminidase.

azurophil and specific granule markers, suggesting again the presence of a major subcellular particle population (most likely consisting of the large granules) that accounts for more protein than the azurophil and specific granules together.

Electron Microscopy of Granule Fractions

We employed random-sampling electron microscopy (26) in three sedimentation experiments to examine the ultrastructure of the different types of granules. The results of one such experiment are shown in Figs. 5–10. Fig. 5 represents the

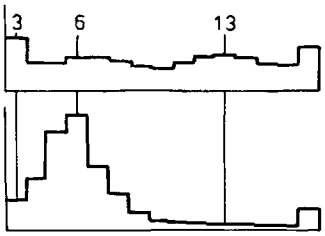


FIGURE 5 Distribution of particulate protein and peroxidase after zonal sedimentation of postnuclear supernate from bovine neutrophils at 8,500 rpm for 15 min. Normalized distribution histograms of the contents of fractions 3 through 17. Fractions 1 and 2 containing the material remaining in the sample zone are omitted. The electron micrographs shown in Figs. 6–8 were prepared from fractions 3, 6, and 13 as described under Materials and Methods.

sedimentation profiles of particle-associated protein and peroxidase. The protein histogram shows two shallow peaks, one corresponding to peroxidase and the other to the zone where the large granules are expected to accumulate. Nearly all fractions were processed and examined in the electron microscope. Figs. 6–8 are random micrographs of the structures present in fractions 3, 6, and 13 as indicated on the histograms of Fig. 5. The lightest material, which just enters the gradient, is shown in Fig. 6. It consists largely of membrane vesicles of various size. Small granulelike structures, some of which are peroxidase-positive, are also seen in fair numbers. In the lower part of the pellicle, just above the filter, these structures are mixed with some soluble protein from the sample zone which forms an electron-dense layer. Clusters or strands of amorphous material, possibly released from broken nuclei or granules are seen here as well as in most other fractions. Fig. 7 shows the particles collected in fraction 6 which contains the highest amount of peroxidase. The micrograph shows mainly round and elongated profiles of peroxidase-negative and peroxidase-positive granules. As clearly seen at high magnification (Fig. 9), the peroxidase-positive, i.e., the azurophil granules, are mostly damaged. Apparently, they become fragile once isolated and tend to break up during preparation for electron microscopy. This we observed in the azurophil granule fractions from all three experiments and, previously, also in similar preparations from human neutrophils (10). The peroxidase that is released from broken azurophil granules appears to adhere to amorphous material and to the surface of peroxidase-negative granules. The peroxidase-negative granules, by contrast, are fully preserved. They have a compact matrix of moderate electron density and a sharp limiting membrane. Their morphology, however, is often blurred by the deposits of peroxidase reaction product. These granules are most likely the particles containing the vitamin B_{12}-binding protein, corresponding to the specific granules of other neutrophils. That the surface peroxidase is not their genuine constituent is obvious from the morphology of intact cells where no oxidized diaminobenzidine is found around peroxidase-negative granules (Figs. 1 and 2). The fast sedimenting fraction 13 (Fig. 8) consists mainly of profiles of large, peroxidase-negative granules. As seen at high magnification (Fig. 10), their matrix is somewhat lighter and less uniform than that of specific granules. In damaged specimens, the matrix is more loose and stains darker. Owing to these features and to their average size, these granules are readily distinguished from the specifics. Centrifugation through sucrose, however, appears to alter their spherical shape which is characteristic in whole cells.

Electrophoretic Analysis of Subcellular Fractions

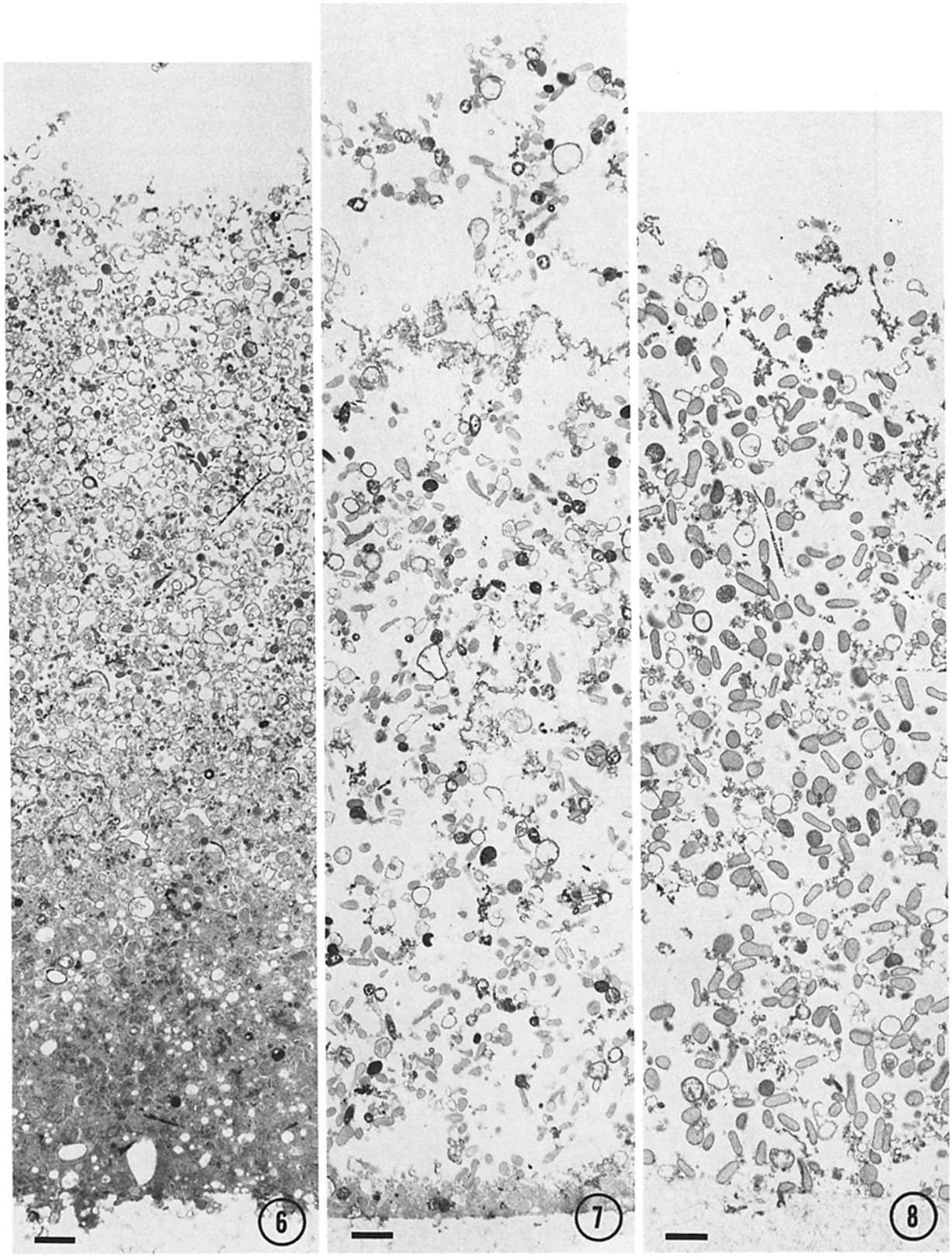
A better characterization of the large granules was obtained by this technique which had already proved very useful in the study of the neutral proteinases of human neutrophils (23). A postnuclear supernate was fractionated by zonal sedimentation as shown in Fig. 11. We know from the results already presented that virtually all azurophil and specific granules are found in the zone covered by the main peak of peroxidase (peroxidase zone), and that the large granules sediment mainly into the outer third of the gradient (large granule zone). The granules present in single fractions were pelleted at high speed and their contents were extracted and separated by gel electrophoresis. Extracts from postnuclear supernates of human and bovine neutrophils were also included for comparison.

A gel stained for protein is shown in Fig. 12. The patterns obtained in the peroxidase zone differ in many respects from those of the large granule zone. In the upper part of the gels, three groups of proteins (A–C) are detectable in both zones. A clear difference is apparent in region B. In the peroxidase zone several poorly resolved bands are seen, while in the large granule zone a cluster of heavy bands is separated from a sharp small band with higher mobility. In region C, a faint band migrates ahead of a major one in the peroxidase zone only. The major difference concerns the most cationic proteins which are only present in the large granule zone. Based on electrophoretic mobility, three groups can be differentiated: D, apparently a doublet; E, a group of at least 3 bands; and F, a prominent single band moving in front.

Gels stained for neutral proteinase activity are represented in Figs. 13–16. Several small molecular weight substrates were used to show different specificities. In accordance with the distributions obtained biochemically (Fig. 3), all the neutral proteinases revealed on the gels are present in the peroxidase (i.e., azurophil granule) zone. An overall picture is obtained with naphthol AS-D acetate (Fig. 13). As the comparison shows, bovine neutrophils contain much fewer proteinase bands than their human counterparts. Staining with more selective substrates, however, clearly indicates that all three types of neutral serine proteinases that are found in human neutrophils (23) are also present in bovine cells. *N*-acetyl-D,L-alanine α-naphthyl ester reveals one band corresponding to elastase (Fig. 14), its phenylalanine analogue detects cathepsin G (Fig. 15), while α-naphthyl acetate (Fig. 16) reveals in addition a third type of proteinase in the upper part of the gel, resembling the proteinase 3 of human neutrophils (32). As in human neutrophils, this proteinase, in contrast to elastase and cathepsin G, is also found in slowly sedimenting organelles which are smaller than azurophil granules. This is shown by the electrophoretic patterns of material from fractions 3 and 5.

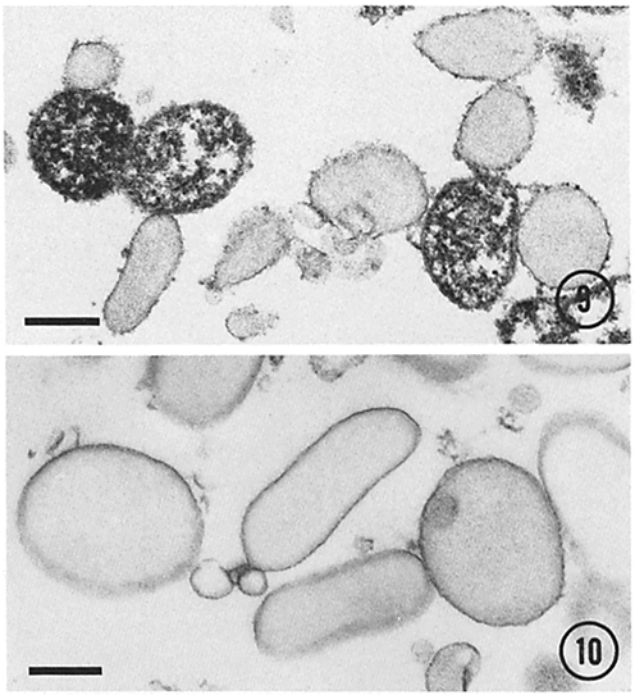
Oxygen-independent Antibacterial Activity

Since the large granules were found to contain several highly cationic proteins (Fig. 12), we looked next at the subcellular distribution of lactoferrin, and of oxygen-independent (i.e., respiratory burst-independent) antibacterial activity using *E. coli* and *S. aureus* as test organisms. The results are shown in Fig. 17. The profiles of neutral proteinase and vitamin B_{12}-binding protein indicate the heavily overlapping distributions of azurophil and specific granules in the upper half of the gradient, a situation similar to that presented in Fig. 3. Lactoferrin has a bimodal distribution with about half of its content coinciding with the above markers and the remainder accumulating in the outer half of the gradient where the large granules are found. On the right-hand side of Fig. 17, profiles of bactericidal activity against *E. coli* and *S. aureus* are pre-



FIGURES 6–8 Survey electron micrographs of filtration samples from fractions 3 (Fig. 6), 6 (Fig. 7), and 13 (Fig. 8) obtained by zonal sedimentation as shown in Fig. 5. The filter face of the pellicles is at the bottom. Bars, 1 μm. × 7,200.

sented. The graphs show the numbers of bacteria still forming colonies after a 30-min incubation with the contents of the particles collected in the gradient fractions (see upper graph for the amount of protein used per assay). The contents of the large granules have a marked antibacterial effect, as indicated by the decrease in the number of viable bacteria by two to three orders of magnitude in the outer half of the gradient. The lower anti-*S. aureus* activity of the last fraction presumably



FIGURES 9 and 10 Fig. 9: High-magnification detail from Fig. 7. Bar, 0.2 μm. × 50,000. Fig. 10: High-magnification detail from Fig. 8. Bar, 0.2 μm. × 50,000.

reflects the contamination with proteins from eosinophil granules and aggregated material. By comparison, the activity detected in other gradient zones is very low. The fact that those fractions which are richest in azurophil and specific granules are virtually inactive indicates that the oxygen-independent antibacterial action of bovine neutrophils is fully associated with the large granules. In particular, this action does not appear to depend on lactoferrin, a constituent of the large granules, which, however, also occurs in fractions containing azurophil and specific granules.

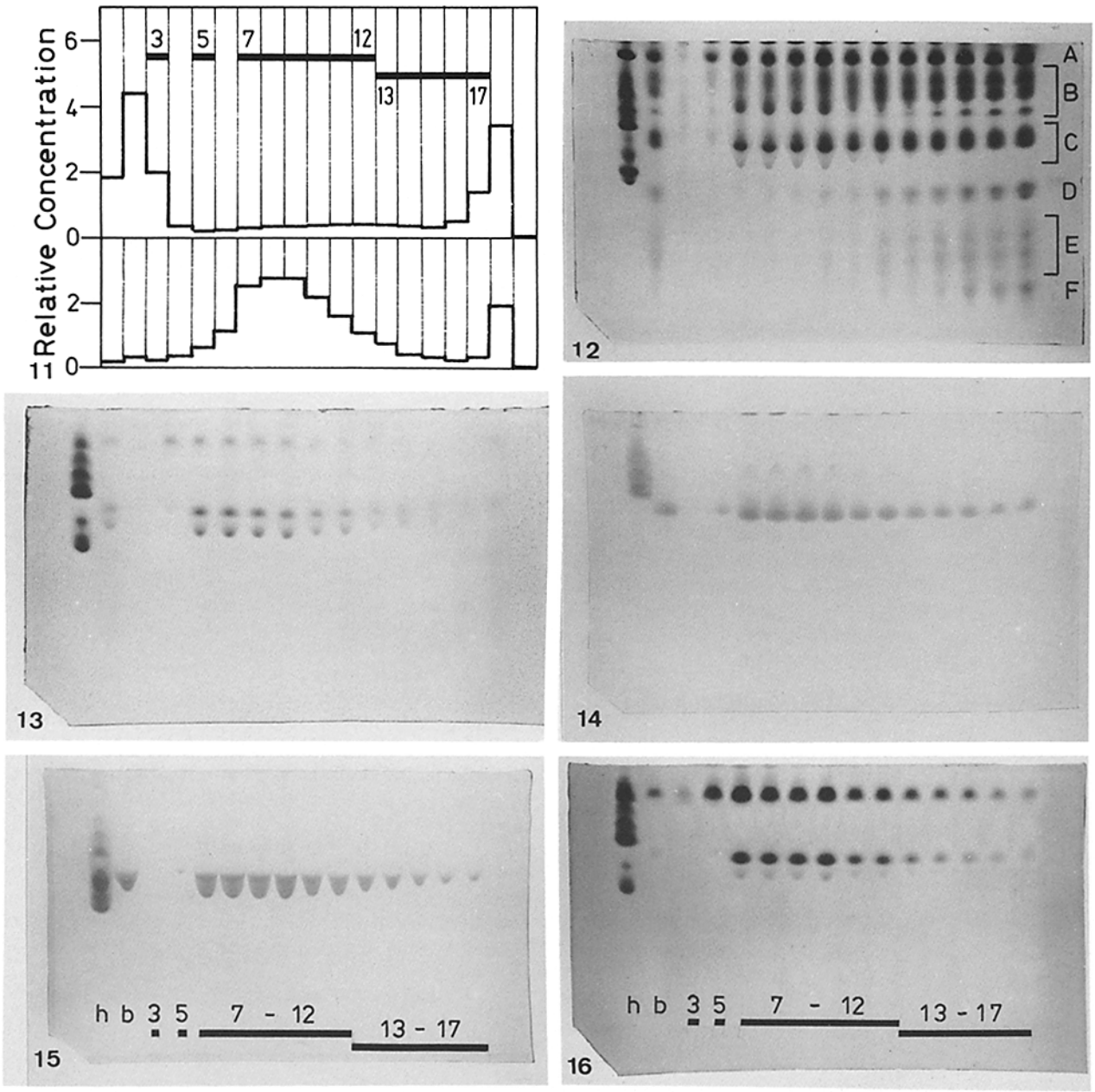
Granule Discharge

We studied release of azurophil and specific granule contents in normal and cytochalasin B-treated cells that were challenged with soluble stimuli or opsonized particles. The results are presented in Fig. 18. In all cases we observed a massive release of the specific granule marker vitamin B_{12}-binding protein. 70–80% of its cellular content were recovered in the incubation medium within 60 min. Release from normal cells which were challenged with PMA or which phagocytosed zymosan increased gradually with time. Pretreatment of the cells with cytochalasin B markedly accelerated this process which was nearly complete within 5 min. By contrast, exocytosis of two constituents of the azurophil granules, *N*-acetyl-β-glucosaminidase and neutral proteinase, was minimal. The percent released into the cell-free media was similar to that of lactate dehydrogenase which was assayed as a measure of cell damage. In another set of experiments (data not shown) up to 50% of the vitamin B_{12}-binding protein was released in 30 min by 1 μM A 23187 or during phagocytosis of opsonized *S. aureus*, while release of myeloperoxidase and of neutral proteinase was <5%. These results show that azurophil and specific granules are discharged by independent mechanisms, as has been reported earlier for human neutrophils (33, 34). In addition this differential release provides further evidence that azurophil and specific granules, which could not be resolved satisfactorily by centrifugation, are separate entities.

Since no selective biochemical marker could be found for the large granules, we determined their discharge by analyzing the cell-free media for the presence of the characteristic highly cationic proteins revealed by electrophoresis and of antibacterial activity. As shown in Fig. 19, the cationic proteins of the large granules become detectable in the media following exposure of the cells to A 23187, PMA, or opsonized zymosan. In another set of experiments, the material actively released by cells that were stimulated with PMA or treated with cytochalasin B and then exposed to opsonized zymosan showed marked antibacterial effects (Table II). Large granule discharge upon phagocytosis was also assessed by morphometry using the test system shown in Fig. 20, which consists of bars and points (circles around left end of bars). The relative numerical density of the large granules was estimated by the ratio between numbers of bars falling on peroxidase-negative granules and numbers of points falling within the cells. Only bars fully enclosed in granule profiles including those touching the granule membrane with their right-hand end were counted. This system was adopted because visual discrimination between the specific granules and the large granules is not possible. Since the specific granules, as shown by their sedimentation behavior, are considerably smaller, they are very unlikely to enclose a bar of the testing system. This system underestimates the relative number of the large granules, but is adequate to quantify changes induced experimentally in comparable samples. The results presented in Table III show that the relative size of the large granule compartment decreased with increasing phagocytic uptake, as estimated by the relative volume density of internalized zymosan. The decrease of the large granule compartment was considerably faster when the cells were pretreated with cytochalasin B, which allows particle attachment but prevents uptake. In this case, the value obtained after 15-min incubation was similar to that following a 60-min phagocytosis period with normal cells.

DISCUSSION

Our study shows that bovine neutrophils contain a novel type of subcellular storage granule. Owing to their large size and high density, these organelles could be resolved from the other subcellular elements by fractionating postnuclear supernates either by sedimentation or isopycnic equilibration. They were differentiated from azurophil and specific granules by biochemical and electrophoretic analysis and by testing for bactericidal activity. The novel granules contain a number of highly cationic proteins that are not found in other subcellular compartments, are the exclusive stores of powerful oxygen-independent bactericidal agents, and lack the enzymes and other constituents usually found in azurophil or specific granules except for lactoferrin. In peroxidase-stained sections of bovine neutrophils, the novel granules may be mistaken for specific granules which also lack peroxidase. The latter, however, sediment much more slowly in sucrose gradients, which allows to establish safe criteria for a discrimination on the basis of profile size. Peroxidase-negative profiles that are >0.35 μm in diameter can be assumed to belong to the novel type. Such profiles are very numerous, indicating that the novel granules constitute the largest storage compartment of bovine neutrophils. A similar conclusion can be drawn from the fractionation experiments where comparatively large proportions of particle-



FIGURES 11–16 Fig. 11: Fractionation of subcellular components from bovine neutrophils by zonal sedimentation at 8,500 rpm for 15 min. Distribution of protein and peroxidase (*upper* and *lower* graph, respectively) are presented as in Fig. 3. The bars on top of the protein histogram indicate fractions which were analyzed by gel electrophoresis. Fractions 3 and 5 as well as a peroxidase zone (fractions 7 to 12) and a large granule zone (fractions 13 to 17) are indicated. Fig. 12: Acrylamide gel electrophoresis of subcellular fractions as indicated in Fig. 11. Equal portions of these fractions were centrifuged at high speed and the respective pellets extracted (see Materials and Methods). Standard samples from postnuclear supernates from human and bovine neutrophils were processed identically. These samples are designated *h* and *b*, respectively, as shown in Figs. 15 and 16. Electrophoresis was carried out at 15 mA for 14 h. The cathode is at the bottom of the gel. Protein was stained with Coomassie Brilliant Blue. Capital letters on the right-hand side indicate zones or bands referred to in the text. Each well contained 35 μl of extract corresponding to the following microgram-amounts of protein (fraction number given in brackets): 75 (*h*), 30 (*b*), 6 (3), 10 (5), 27 (7), 24 (8), 27 (9), 35 (10), 24 (11), 34 (12), 31 (13), 33 (14), 41 (15), 41 (16) and 51 (17). Figs. 13–16: Acrylamide gel electrophoresis of the same samples as in Fig. 12 (50 μl instead of 35 μl of extract per well) run under identical conditions and developed for proteinase activity at neutral pH with the following substrates, naphthol AS-D acetate (Fig. 13), *N*-acetyl-D-L-alanine α-naphthyl ester (Fig. 14), and *N*-acetyl-D,L-phenylalanine β-naphthyl ester (Fig. 15) and α-naphthyl acetate (Fig. 16).

associated protein were recovered in the gradient zone containing the novel organelles.

After having established their identity, it was important to study the fate of the large granules following cell stimulation, and to compare it with that of the azurophil and specific granules. By three independent criteria, the detection of cationic proteins, the assessment of oxygen-independent antibacterial activity, and morphometry, it was firmly established that exocytosis from the large granules occurs during phagocytosis but also following stimulation with PMA and A 23187. Under the same conditions, large proportions of specific granule contents were also released. The similar behavior of the large

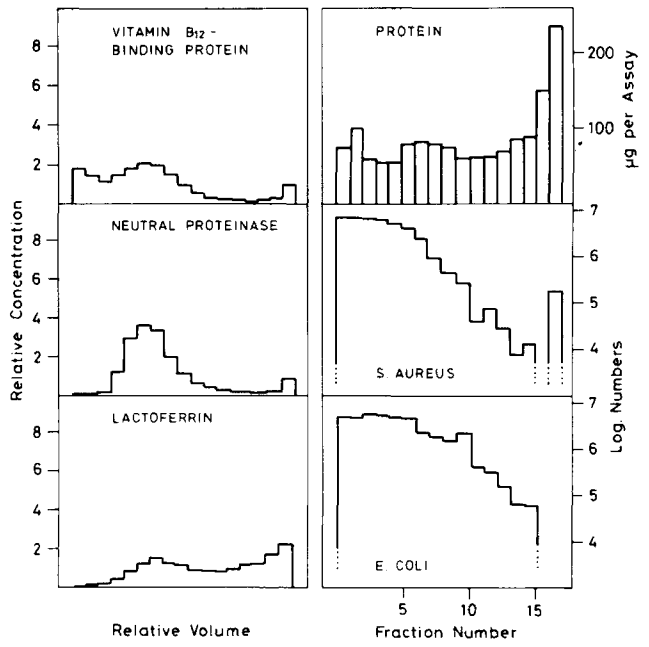


FIGURE 17 Subcellular distribution of oxygen-independent bactericidal activities and lactoferrin in bovine neutrophils. Fractionation by zonal sedimentation at 8,500 rpm for 15 min was performed as described in the legend of Fig. 3. Percentage recoveries were 98 for vitamin B_{12}-binding protein, 142 for neutral proteolytic activity, and 74 for lactoferrin. The two *lower* graphs on the right-hand side show the profiles of bactericidal activity. The number of bacteria remaining viable after 30-min incubation with extracts of the particulate material from equivalent portions of the gradient fractions are presented. The amount of protein added to each bactericidal assay is given in the upper graph. Control values, i.e., bacterial counts (log numbers) obtained after incubation with 100 µg of bovine serum albumin instead of neutrophil material, were 6.8 for *E. coli* and 6.74 for *S. aureus*.

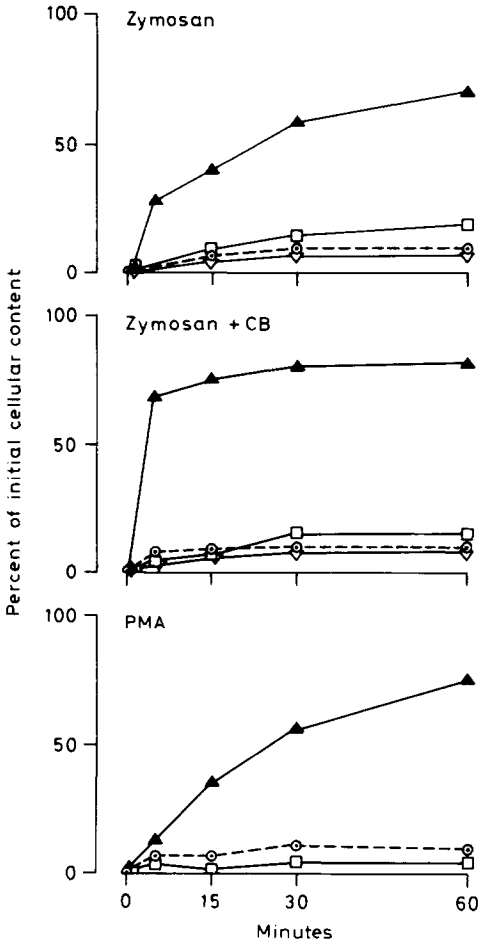


FIGURE 18 Release of vitamin B_{12}-binding protein (▲), neutral proteinase (△), N-acetyl-β-glucosaminidase (□), and lactate dehydrogenase (○) by stimulated bovine neutrophils. Samples of 5×10^7 cells in 2 ml of PBS were exposed to opsonized zymosan (2.5 mg/ml) or PMA (10 ng/ml) or were pretreated with cytochalasin B (CB, 5 µg/ml) and then exposed to opsonized zymosan (2.5 mg/ml). See Materials and Methods for details.

granules and the specific granules was further underlined by the finding that their discharge was markedly accelerated when the cells were pretreated with cytochalasin B. By contrast, release of azurophil granule contents was minimal even from cytochalasin B-treated cells. It thus appears that the large granules are readily mobilized and that their function is not solely related to phagocytosis. The differential discharge of specific and azurophil granules as observed in this study is well documented by results obtained with neutrophils from other species (33, 34).

Subcellular fractionation and biochemical analysis of rabbit heterophil leukocytes (9, 35, 36) and human neutrophils (10, 37, 38) have helped to characterize the azurophil and the specific granules as two storage organelles which, in biochemical terms, differ almost totally from each other (39). Numerous ultrastructural studies combined with cytochemical staining for myeloperoxidase, which selectively reveals the azurophil granules (40, 41), have provided evidence for the presence of two sets of granules as a general feature of neutrophil leukocytes (39, 41). The existence of more than two populations of granules has, however, been proposed repeatedly. Small, phosphatase-positive structures were described as tertiary granules in rabbit heterophils (42, 43), and in human neutrophils a novel storage organelle containing gelatinase has recently been found (44). These structures, however, are much smaller than the common granules and have so far not been identified morphologically. They apparently represent a minor subcellular com-

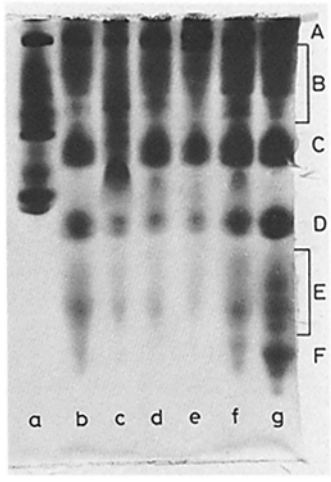


FIGURE 19 Electrophoretic analysis of proteins released from stimulated bovine neutrophils. The cells were incubated for 30 min in the presence of the indicated stimuli and the cell-free media were processed for electrophoresis. Gels were run at pH 4.3 as described under Materials and Methods. The following samples were used (micrograms of protein in brackets): (*a*) extract of total granule fraction from human neutrophils (86.4); (*b*) extract of total granule fraction from bovine neutrophils (63.4); (*c*) postnuclear supernate from bovine neutrophils (43.3); (*d–f*) cell-free media obtained after stimulation of the cells with 1 µM A 23187 (53.9), 2.5 mg/ml zymosan (56.8), or 10 ng/ml PMA (96.7), respectively; and (*g*) extract of bovine large granules purified by zonal sedimentation (115.0). The zones *A–F* correspond to those indicated in the electropherogram of subcellular fractions shown in Fig. 12.

partment. Spitznagel et al. (37) have reported the partial resolution of two populations of azurophil granules from human neutrophils. These data suggest that granules of somewhat different biochemical composition may form at the promyelocyte stage.

The situation in bovine neutrophils is markedly different. Like neutrophils from many other species, they are equipped with two sets of granules with the biochemical properties of azurophil and specific granules. The characteristic feature of the bovine cell, however, is the presence of a third population of granules which are both larger and more numerous than the two other types. These granules account for the largest storage compartment of the bovine neutrophil. Gel electrophoretic analysis of their contents has revealed a variety of components. Up to now we have identified lactoferrin and the antibacterial activity which from what is known from other species (45) may be associated with the most cationic granule proteins. None of the enzymes known to occur in the granules of human neutrophils, the species most thoroughly studied (39), could be detected in the new granules.

So far too little is known about the contents of the large granules to allow an assessment of their role in neutrophil function which presumably differs from that of the azurophil and specific granules. The demonstration that the novel granules contain antibacterial activity, is an important aspect of our study. Evidence from the phagocytosis and exocytosis experiments clearly indicates that the oxygen-independent antibacterial agents present in these granules could function both intracellularly and in the pericellular environment. In all past studies on bacterial killing by granule extracts, the possible contribution of enzymic systems, e.g., myeloperoxidase together with H_2O_2 produced by the bacteria and chloride from the medium, or lytic enzymes, had to be considered (46). The results presented in this report clearly show that the oxygen-independent antimicrobial activity is dissociated from the myeloperoxidase, acid hydrolases, and neutral proteinases, since these enzymes do not occur in the large granules. Finally, the fact that in bovine neutrophils oxygen-independent microbicidal agents of considerable activity are stored in a distinct subcellular compartment and are released in response to various stimuli is evidence for the biological relevance of this host-defense system which does not depend on the respiratory burst.

TABLE II

Release of Antibacterial Activity by Stimulated Bovine Neutrophils

Stimulus*	Neutrophils	μg of released protein tested	No. of colonies × 10^{-6} (percent survival)	
			E. coli	*S. aureus*
None	−	—	4.04	3.40
PMA	−	—	3.77 (100)	3.31 (100)
PMA	+	82‡	2.04 (53)	2.57 (78)
PMA	+	100‡	1.21 (32)	0.43 (13)
PMA	+	176‡	0.11 (3)	0.15 (5)
CB	−	—	4.27 (100)	3.40 (100)
CB + zymosan	+	100‡	2.30 (54)	1.28 (38)
CB + zymosan	+	100‡	2.44 (57)	2.63 (77)
CB + zymosan	+	200‡	1.09 (26)	0.53 (16)

* Neutrophils were incubated for 30 min in the presence of either 10 ng/ml PMA or 2.5 mg/ml opsonized zymosan. *CB*, cytochalasin B-pretreated cells (5 μg/ml for 5 min).

‡ Aliquots of the cell-free media from two separate experiments tested for bactericidal activity. In some cases, the media were concentrated by ultrafiltration (see Materials and Methods).

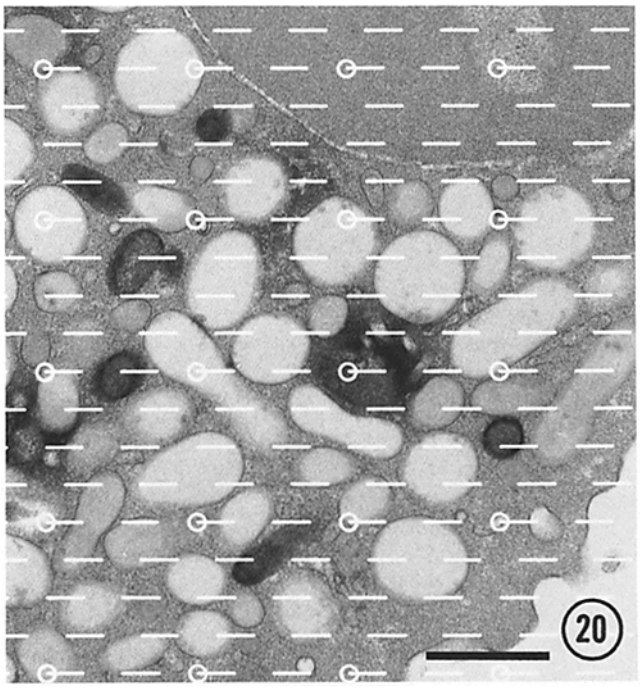


FIGURE 20 Test system for the morphometric evaluation of the relative numerical density of the large granules. Bars are used to evaluate granule numbers and points (circled ends of bars) to evaluate cell volume. Bar, 1 μm. × 32,000.

TABLE III

Morphometric Evaluation of the Discharge of Large Granules during Phagocytosis

Conditions	Time	Relative density	
		Of large granules*	Of phagosome‡
	min		
No addition	15	0.59 ± 0.04	—
Zymosan	15	0.55 ± 0.04 ($P < 0.2$)	0.30 ± 0.02
Cytochalasin B + zymosan	15	0.39 ± 0.04 ($P < 0.001$)	—
No addition	60	0.55 ± 0.04	—
Zymosan	60	0.38 ± 0.04 ($P < 0.001$)	0.67 ± 0.04 ($P < 0.001$)

* Arbitrary density ratios (bar within peroxidase-negative granules over points within cytoplasm and nucleus); mean values ± SE from 30 negatives per group. Statistical comparison with appropriate control according to Student's *t* test, using one-tailed probability tables.

‡ True relative volume densities; mean values from 20 negatives. Statistical comparison between 15- and 60-min phagocytosis according to Student's *t* test, using one-tailed probability tables.

REFERENCES

1. Hegner, D. 1968. Isolierung und Enzymbestand von Granula aus polymorphkernigen Leukozyten des peripheren Rinderblutes. *Hoppe-Seyler's Z. Physiol. Chem.* 349:544–554.
2. Rausch, P. G., and T. G. Moore. 1975. Granule enzymes of polymorphonuclear neutrophils: a phylogenetic comparison. *Blood.* 46:913–919.
3. Gennaro, R., C. Schneider, G. De Nicola, F. Cian, and D. Romeo. 1978. Biochemical properties of bovine granulocytes. *Proc. Soc. Exp. Biol. Med.* 157:342–347.
4. Russell, M. W., and B. Reiter. 1975. Phagocytic deficiency of bovine milk leucocytes: an effect of casein. *J. Reticuloendothel. Soc.* 18:1–13.
5. Ward, P. D. 1976. Interactions between bovine polymorphonuclear leukocytes and staphylococci. *In* Staphylococci and Staphylococcal Diseases. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Gustav Fischer Verlag, Stuttgart. I (Suppl. 5):837–847.
6. Carlson, G. P., and J. J. Kaneko. 1973. Isolation of leukocytes from bovine peripheral blood. *Proc. Soc. Exp. Biol. Med.* 142:853–856.
7. Mottola, C., R. Gennaro, A. Marzullo, and D. Romeo. 1980. Isolation and partial characterization of the plasma membrane of purified bovine neutrophils. *Eur. J. Biochem.* 111:341–346.
8. Baggiolini, M., B. Dewald, and U. Bretz. 1978. A volume adapter for use in a B-XIV zonal rotor. *Anal. Biochem.* 91:123–129.
9. Baggiolini, M., J. G. Hirsch, and C. de Duve. 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* 40:529–541.
10. Bretz, U., and M. Baggiolini. 1974. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. *J. Cell Biol.* 63:251–269.
11. Dewald, B., M. Baggiolini, J. T. Curnutte, and B. M. Babior. 1979. Subcellular localization of the superoxide-forming enzyme in human neutrophils. *J. Clin. Invest.* 63:21–29.
12. Bretz, U., and M. Baggiolini. 1973. Association of the alkaline phosphatase of rabbit polymorphonuclear leukocytes with the membrane of the specific granules. *J. Cell Biol.* 59:696–707.
13. Miller, G. L. 1959. Protein determination for large numbers of samples. *Anal. Chem.* 31:964.
14. Gennaro, R., C. Mottola, C. Schneider, and D. Romeo. 1979. Ca^{2+}-dependent ATPase activity of alveolar macrophage plasma membrane. *Biochim. Biophys. Acta.* 567:238–246.
15. Penney, C. L. 1976. A simple micro-assay for inorganic phosphate. *Anal. Biochem.* 75:201–210.
16. Lanzetta, P. A., L. J. Alvarez, P. S. Reinach, and O. A. Candia. 1979. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* 100:95–97.
17. Kane, S. P., A. V. Hoffbrand, and G. Neale. 1974. Indices of granulocyte activity in inflammatory bowel disease. *Gut.* 15:953–959.
18. Levine, N., V. B. Hatcher, and G. S. Lazarus. 1976. Proteinases of human epidermis; a possible mechanism for polymorphonuclear leukocyte chemotaxis. *Biochim. Biophys. Acta.* 452:458–467.
19. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* 2:235–254.
20. Leffell, M. S., and J. K. Spitznagel. 1972. Association of lactoferrin with lysozyme in granules of human polymorphonuclear leukocytes. *Infect. Immun.* 6:761–765.
21. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (Lond.).* 195:281–283.
22. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
23. Dewald, B., R. Rindler-Ludwig, U. Bretz, and M. Baggiolini. 1975. Subcellular localization and heterogeneity of neutral proteases in neutrophilic polymorphonuclear leukocytes. *J. Exp. Med.* 141:709–723.
24. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291–302.
25. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9:409–414.
26. Baudhuin, P., P. Evrard, and J. Berthet. 1967. Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. *J. Cell Biol.* 32:181–191.
27. Feigenson, M. E., and M. Baggiolini. 1978. Capping of ricin-binding sites does not influence phagocytosis in human polymorphonuclear leukocytes. *Acta Haematol.* 59:144–162.
28. Weibel, E. R., G. S. Kistler, and W. F. Scherle. 1966. Practical stereological methods for morphometric cytology. *J. Cell Biol.* 30:23–38.
29. de Duve, C., J. Berthet, and H. Beaufay. 1959. Gradient centrifugation of cell particles: theory and applications. *Prog. Biochim. Biophys. Chem.* 9:326–369.
30. Rustin, G. J. S., P. D. Wilson, and T. J. Peters. 1978. Localization of human neutrophil alkaline phosphatase in a hitherto unreported organelle. *Clin. Sci. Mol. Med.* 56:11P.
31. Kane, S. P., and T. J. Peters. 1975. Analytical subcellular fractionation of human granulocytes with reference to the localization of vitamin B_{12}-binding proteins. *Clin. Sci. Mol. Med.* 49:171–182.
32. Baggiolini, M., U. Bretz, and B. Dewald. 1978. Subcellular localization of granulocyte enzymes. *In* Neutral Proteases of Human Polymorphonuclear Leukocytes. K. Havemann, and A. Janoff, editors. Urban & Schwarzenberg, Inc., Baltimore-Munich. 3–17.
33. Henson, P. M., M. H. Ginsberg, and D. C. Morrison. 1978. Mechanisms of mediator release by inflammatory cells. *In* Membrane Fusion. G. Poste, and G. L. Nicolson, editors. Elsevier/North-Holland Biomedical Press, New York. 407–508.
34. Weissmann, G., H. M. Korchak, H. D. Perez, J. E. Smolen, I. M. Goldstein, and S. T. Hoffstein. 1979. Leukocytes as secretory organs of inflammation. *Adv. Inflammation Res.* 1:95–112.
35. Baggiolini, M., J. G. Hirsch, and C. de Duve. 1970. Further biochemical and morphological studies of granule fractions from rabbit heterophil leukocytes. *J. Cell Biol.* 45:586–597.
36. Baggiolini, M., C. de Duve, P. L. Masson, and J. F. Heremans. 1970. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J. Exp. Med.* 131:559–570.
37. Spitznagel, J. K., F. G. Dalldorf, M. S. Leffell, J. D. Folds, I. R. H. Welsh, M. H. Cooney, and L. E. Martin. 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. *Lab. Invest.* 30:774–785.
38. Kane, S. P., and T. J. Peters. 1975. Analytical subcellular fractionation of human granulocytes with reference to the localization of vitamin B_{12}-binding proteins. *Clin. Sci. Mol. Med.* 49:171–182.
39. Baggiolini, M. 1980. The neutrophil. *In* Handbook of Inflammation: The Cell Biology of Inflammation. G. Weissmann, editor. Elsevier/North-Holland Biomedical Press, Amsterdam. 2:163–187.
40. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. *J. Cell Biol.* 39:299–317.
41. Bainton, D. F., J. L. Ullyot, and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. *J. Exp. Med.* 134:907–934.
42. Wetzel, B. K., R. G. Horn, and S. S. Spicer. 1967. Fine structural studies on the development of heterophil, eosinophil and basophil granulocytes in rabbit. *Lab Invest.* 16:349–382.
43. Murata, F., and S. S. Spicer. 1973. Morphologic and cytochemical studies of rabbit heterophilic leukocytes. Evidence for tertiary granules. *Lab. Invest.* 29:65–72.
44. Dewald, B., U. Bretz, and M. Baggiolini. 1982. Release of gelatinase from a novel secretory compartment of human neutrophils. *J. Clin. Invest.* 70:518–525.
45. Zeya, H. I., and J. K. Spitznagel. 1968. Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. *J. Exp. Med.* 127:927–941.
46. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin. Hematol.* 12:117–142.