SEQUENTIAL DEGRANULATION OF THE TWO TYPES OF POLYMORPHONUCLEAR LEUKOCYTE GRANULES DURING PHAGOCYTOSIS OF MICROORGANISMS

DOROTHY FORD BAINTON

From the Department of Pathology, University of California at San Francisco, California 94142

ABSTRACT

The sequential discharge of neutrophilic polymorphonuclear leukocyte (PMN) granules—azurophils and specifics—was investigated by electron microscopy and cytochemistry. Thus the enzyme content of PMN phagocytic vacuoles was determined at brief intervals after phagocytosis of bacteria, utilizing peroxidase as a marker enzyme for azurophil granules, and alkaline phosphatase for specifics. At 30 s, approximately half the phagocytic vacuoles were reactive for alkaline phosphatase, whereas none contained peroxidase. Peroxidase-containing vacuoles were rarely seen at 1 min, but by 3 min, vacuoles containing both enzymes were consistently present. Alkaline phosphatase was found in both small and large vacuoles, whereas peroxidase was visible only in large ones. By 10 min, very big phagocytic vacuoles containing considerable amounts of reaction product for both enzymes were evident. These observations indicate that the two types of PMN granules discharge in a sequential manner, specific granules fusing with the vacuole before azurophils. In an earlier paper, we reported that the pH of phagocytic vacuoles drops to 6.5 within 3 min and to ~4 within 7–15 min. Substances known to be present in specific granules (alkaline phosphatase, lysozyme, and lactoferrin) function best at neutral or alkaline pH, whereas most of those contained in azurophil granules (i.e., peroxidase and the lysosomal enzymes) have pH optima in the acid range. Hence the sequence of granule discharge roughly parallels the change in pH, thereby providing optimal conditions for coordinated activity of granule contents.

INTRODUCTION

When microorganisms are engulfed by a polymorphonuclear leukocyte (PMN), the cell degranulates (1–3) and enzymes contained within PMN storage granules (4) are discharged into the phagocytic vacuole (5, 6), where killing and digestion subsequently occur (7–10). Recent reports (11–19) have established that rabbit PMN granules are of two distinct morphologic and chemical types—the azurophil or primary granule and the specific or secondary granule. Azurophil granules represent a special type of primary lysosome in that they contain peroxidase, numerous digestive enzymes, and one-third of the lysozyme of the cell. Specific granules, however, are not lysosomes because they lack digestive enzymes and peroxidase, and are so far known to contain alkaline phosphatase, two-thirds of the lysozyme of the cell, and lactoferrin. Both types of granules have been demonstrated to empty into phagocytic vacuoles, but the sequence of discharge of the two granule types has not been established. Hence the present study was undertaken to determine this sequence of discharge by using cytochemical markers in an
in vivo phagocytic system. Peroxidase was selected as a marker enzyme for azurophil granules, and alkaline phosphatase for specificis. We anticipated that analyzing the enzyme content of phagocytic vacuoles at brief intervals after phagocytosis would indicate whether both granule types empty into phagocytic vacuoles simultaneously, or whether one precedes the other. Preliminary reports of our findings have already been published (20, 21).

MATERIALS AND METHODS

Preparation of Bacteria

Bacteria were grown in trypticase soy broth (Difco Laboratories, Detroit, Mich.) for 18 h at 38°C. Some organisms were heat killed (90°C for 1 h) before use. *Escherichia coli* K12 was obtained from the American Type Culture Collection, Rockville, Md., and *Staphylococcus aureus* from the teaching strains of our Department of Microbiology. *E. coli* E15, an alkaline-phosphatase-negative mutant, was kindly provided by Dr. M. J. Schlesinger, Washington University, St. Louis, Mo.

Production of Exudate

For 2–6 months, New Zealand albino rabbits were given weekly intraperitoneal injections of 1 µg endotoxin (lipopolysaccharide B, *Staphylococcus typhosa* [Difco Laboratories]) in 50 ml isotonic sterile saline.

Experiments on Phagocytosis

4 h after the last endotoxin injection, animals were lightly anesthetized with pentobarbital, and 2 ml of a suspension containing approximately 5 × 10^10 bacteria/ml saline were injected into the peritoneal cavity. Exudate cells were collected by gravity drainage directly into an equal amount of fixative 30 s to 10 min after injection of the bacteria.

Fixation

Cells were fixed in 1.5% distilled glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) with 1% sucrose at 4°C for 10 min to 16 h. The suspension of fixed cells was washed three times in cacodylate-HCl buffer with 7% sucrose and stored overnight in the same buffer at 4°C. Subsequently the preparation was divided into two samples and incubated in the enzyme media described below.

Enzyme Procedures

Cells were incubated either in (a) Graham's and Karnovsky's medium for peroxidase (22) using 3,3'-diaminobenzidine tetrahydrochloride (Nutritional Biochemicals Corporation, Cleveland, Ohio) and H_2O_2, pH 7.6, for 30 min at 25°C; or (b) a modification of Gomori's calcium medium (23) for alkaline phosphatase (13) using β-glycerophosphate (Grade I, Sigma Chemical Co., St. Louis, Mo.), pH 9.2, for 1 h at 37°C. Both media contained 5% sucrose. For alkaline phosphatase, a second step was necessary in which cells were washed three times in 0.1 M propanediol buffer, pH 9, with 7% sucrose, washed quickly once in Michaelis buffer, pH 7.4, plus 7% sucrose, and subsequently incubated in 2% lead nitrate for 10 min at 25°C.

Double Incubations

In some cases, cells were incubated successfully for both marker enzymes. Some were incubated first for peroxidase as described above, washed three times in acetate-Veronal buffer, and then incubated for alkaline phosphatase. Others were incubated first for alkaline phosphatase and then for peroxidase.

Controls

Controls for peroxidase consisted of: (a) incubations in which 3,3'-diaminobenzidine (DAB) or H_2O_2 was omitted, or (b) preincubation of cells in 0.01 M KCN at pH 6.0 for 30 min before adding DAB and H_2O_2, or (c) rinsing of cells in three changes of buffer after incubation in DAB medium and then incubation for 5 min in 0.003 M potassium ferricyanide. In the case of alkaline phosphatase, controls consisted of incubations in which the substrate was omitted or 0.001 M cysteine was added to the medium.

Subsequent Processing

After incubation, cells were washed three times in 0.05 M acetate-Veronal buffer, pH 7.4, with 7% sucrose, packed by centrifugation into blocks; postfixed in either 1% OsO_4 in acetate-Veronal buffer or in ferrocyanide-reduced OsO_4 (24); treated with buffered 0.5% uranyl acetate containing 4% sucrose for 1 h at 22°C; and dehydrated in graded ethanol. Some specimens were dehydrated directly after incubation or after postfixation in OsO_4. Either Araldite or Spurr's mixture was used as the embedding material.

RESULTS

The contents of phagocytic vacuoles were analyzed at brief intervals after the initiation of

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1 The only modification was the use of 0.2 M propanediol buffer (pH 9) substituting for 2% sodium-Veronal buffer.
phagocytosis in vivo for the presence of reaction product for alkaline phosphatase, a marker for specific granules, or for peroxidase, a marker for azurophils. Live or heat-killed *E. coli* or *S. aureus* were used in all experiments.

**Enzyme Localization within Nonphagocytizing Exudate PMN**

**PEROXIDASE:** Reaction product for this enzyme was restricted to the large azurophil granules (Fig. 1) which comprise about 27% of the granule population of exudate PMN. No reaction product was seen in the smaller specific granules nor in any of the other organelles, which are sparse in mature cells.

**ALKALINE PHOSPHATASE:** In the nonphagocytizing cell, no reaction product could be visualized, even in the specific granules, despite clear evidence that they do store this enzyme (15, 16, and Discussion). In addition, the morphologic preservation of organelles was extremely poor in contrast to that obtained with peroxidase, presumably due to the high pH (9.2) of the incubation medium.

**Enzyme Localization in Organisms Used as Test Particles**

**PEROXIDASE:** No reaction product for this enzyme was seen in live or heat-killed *E. coli* K12, *E. coli* E15, or *S. aureus* (see Fig. 8).

**ALKALINE PHOSPHATASE:** No reaction product was seen in live or heat-killed *E. coli* E15 or K12* (see Figs. 2, 3, 9, 10) or in *S. aureus* (see Fig. 4).

**Enzyme Localization within Phagocytic Vacuoles at 30 s, 3 min, or 10 min after Phagocytosis**

**ALKALINE PHOSPHATASE:** Within 30 s, many small phagocytic vacuoles containing test particles were seen, more than 50% containing reaction product for alkaline phosphatase (Fig. 2), indicating that specific granules had discharged their contents even at this early time. After 3 min, many phagocytic vacuoles were considerably larger and most contained extensive deposits of the enzyme, usually outlining the entire wall of the vacuole (Fig. 3). When *S. aureus* was used as the test particle, adjacent vacuoles frequently appeared to be connected by narrow channels filled with reaction product (Fig. 4). Occasionally these channels filled with reaction product were found in continuity with the plasma membrane and, rarely, the entire phagocytic vacuole was connected to the membrane (Fig. 7), demonstrating that discharge of granule enzymes to the exterior of the cell can occur. Within 10 min after the introduction of bacteria, many huge phagocytic vacuoles were present, deposits of reaction product often outlining entire vacuolar walls (Fig. 9) as well as coating the bacterial membranes (Fig. 10). In some cells, a few of the large vacuoles did not contain alkaline phosphatase, and sometimes one cell contained two large vacuoles—one reactive and the other nonreactive (Fig. 10). Generally this situation was noted in cells containing large numbers of ingested microorganisms, suggesting that the cell continued to phagocytize particles after complete degranulation, leaving no granules available for delivery to the newly formed vacuole. Such a phenomenon was previously observed by Hirsch (2). Results were the same with either live or heat-killed bacteria.

**PEROXIDASE:** In a 30 s sample with *E. coli* as the test particle, no reaction product for peroxidase could be found within any of the small phagocytic vacuoles (Fig. 5). After 3 min, however, the phagocytic vacuoles were considerably larger and about 10% contained reaction product for peroxidase (Fig. 6).* At the 10 min interval, large vacuoles filled with peroxidase were frequently observed (Fig. 8) in about 25% of the cells. With *S. aureus*, usually ingested as groups of particles, rare phagocytic vacuoles were found to contain peroxidase after only 30 s. Again,

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2. It should be remarked that *E. coli* K12 can synthesize alkaline phosphatase under specific cultural conditions—i.e., when the medium is depleted of inorganic phosphate (25). Our culture medium contained 2.5 g K2HPO4 in 250 ml H2O. In addition, reaction product for this enzyme has been localized at the fine-structural level in the periplasmic space in other strains of *E. coli* (26).

3. In early studies, cells were also examined at 1- and 2-min intervals, and very few vacuoles were found to be peroxidase reactive. However, a dramatic change occurred at about 3 min, when roughly 10% of the vacuoles contained peroxidase. Hence cells at this time interval were routinely examined thereafter.
Abbreviations

B, bacterium
G, Golgi region
PV, phagocytic vacuole
ag, azurophil granule
c, channels
et, centriole
f, fibrin
g, glycogen
l, lump
m, mitochondrion
n, nucleus
pm, plasma membrane
r, bacterial remnants
sg, specific granule
z, image

All figures are electron micrographs of PMN from rabbit peritoneal exudate. Fig. 1 is a “resting” (non-phagocytizing) cell; the remainder of the figures are phagocytizing cells 30 s to 10 min after the introduction of bacteria and are processed for either peroxidase (Figs. 1, 5, 6, 8) or alkaline phosphatase (Figs. 2–4, 7, 9, 10) or both (Fig. 11).

**Figure 1** Resting PMN from rabbit exudate reacted for peroxidase. The cytoplasm is filled with granules; the smaller, peroxidase-negative specific granules (sg) are more numerous than the dense, peroxidase-positive azurophil granules (ag). A condensed and lobulated (n1 – n3) nucleus and sparse mitochondria (m) are notable features, as well as the islands of glycogen particles (g). Specimen fixed in 1.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) for 10 min, incubated in the peroxidase medium of Graham and Karnovsky for 1 h at 22°C, postfixed in Karnovsky’s ferrocyanide-reduced OsO4, dehydrated in alcohol, and embedded in Spurr’s mixture. Section stained with lead citrate for 30 min. X 10,000.
FIGURE 2  PMN reacted for alkaline phosphatase, 30 s after exposure to live E. coli B12. The cell has phagocytized three bacteria ($B^1 - B^3$) at different stages of ingestion. $B^1$: A microorganism is being engulfed, with the plasma membrane ($pm$) of the PMN invaginating to form the wall of the phagosome. Note the absence of reaction product near $B^1$. A second bacterium ($B^2$) has been internalized, and alkaline phosphatase reaction product is visible in the narrow space between the vacuole membrane and the bacterium (arrows), indicating that the contents of specific granules ($sg$) have been discharged into this phagocytic vacuole. In the lower right portion of the micrograph, a larger phagocytic vacuole ($PV$) contains a bacterium ($B^3$) and some enzyme reaction product. The adjacent specific granules contain no enzyme deposits, nor do the azurophils, which appear completely ($ag$) or partially ($ag'$) extracted. Islands of glycogen appear as empty spaces ($g$), best seen here in the cell above, from a preparation stained en bloc in uranyl acetate. Specimen fixed in glutaraldehyde for 90 min at 4°C, incubated in Gomori's medium for alkaline phosphatase, pH 9.2, for 2 h at 37°C, treated with lead nitrate, postfixed in 1% OsO₄ in acetate-Veronal, stained en bloc in uranyl acetate, embedded in Araldite, and stained for 1 min in lead citrate. $\times$ 24,000.
FIGURE 3  PMN reacted for alkaline phosphatase, 3 min after exposure to live *E. coli* E15. Two phagocytic vacuoles (PV) containing bacteria (B) and clumps of reaction product (arrows) are evident. Note that deposits in PV1 are distributed along the entire vacuolar membrane but are not found in the matrix. A centriole (ce) and nearby Golgi complex (G) are located adjacent to the nucleus (n). Specimen preparation as for Fig. 2. X 29,000.

FIGURE 4  PMN reacted for alkaline phosphatase, 3 min after exposure to live *S. aureus* (B). The phagocytic vacuoles (PV) contain numerous bacteria (B) and extensive deposits of dense reaction product. Adjacent vacuoles are frequently connected by channels (c), some of which (arrow) are apparently continuous with the plasma membrane (pm). Rarely a specific granule appears to contain reaction product (sg'). Most do not (sg). Specimen preparation as for Fig. 2. X 10,000.
results were the same with either live or heat-killed bacteria. Deposit-containing connections to the exterior of the cell were seldom observed.

**DOUBLE INCUBATIONS:** Ideally, examination of the same specimen incubated for both enzymes would be desirable. Some incubations were partially successful because when cells from 10-min specimens were incubated first for peroxidase and subsequently for alkaline phosphatase, both reaction products could be demonstrated within large phagocytic vacuoles (Fig. 11). However, reaction to both enzymes was quite variable and considerably less intense than when the incubations were performed singly. When specimens were incubated first for alkaline phosphatase and then for peroxidase, alkaline phosphatase localization was similar to that in preparations incubated for this enzyme alone. But little or no peroxidase reaction product could be visualized, suggesting that incubation in the alkaline phosphatase medium suppresses peroxidase activity.

**CONTROL INCUBATIONS:** No peroxidase reaction was observed when DAB was omitted. Similarly, when H$_2$O$_2$ was eliminated from the incubation medium, no reaction was usually apparent except for rare extracted azurophil granules and an occasional phagocytic vacuole. The addition of KCN before incubation for peroxidase completely inhibited the reaction. Alkaline phosphatase reaction was inhibited either by adding cysteine to the incubation medium or by omitting the substrate. There was a considerable amount of extraneous, presumably nonspecific, precipitate scattered over the nuclei and cytoplasm, both in the experimental and control incubation specimens.

**DISCUSSION**

Our results establish that reaction product for alkaline phosphatase can be demonstrated within the phagocytic vacuole several minutes before peroxidase reaction product appears. Thus the two types of PMN granules do not empty simultaneously, but rather, sequentially, with specific granules preceding azurophilic. By “sequential” degranulation, we mean that specific granule fusion with the phagocytic vacuole precedes azurophil granule fusion. Data are not available to establish whether or not specific granule-phagocytic vacuole fusion is entirely complete before any azurophilic fuse with the vacuole. The sequence of discharge which we propose was previously suggested by Senda (27), using the light microscope, and by Brederoo and Daems (28), using the electron microscope. Studying phagocytosis in human and guinea pig PMN, respectively, they noted that the smaller granules disappeared before the larger ones. Recently Henson (29) also confirmed our findings concerning the discharge sequence. During his investigation of the interaction of PMN with immune complexes on a nonphagocytizable surface, he observed that alkaline phosphatase was discharged during the initial 5 min of incubation, whereas β-glucuronidase, which is localized in azurophil granules, appeared later.

**Importance of Temporal Changes in the Content of PMN Phagocytic Vacuoles**

Probably the most important consequence of sequential degranulation is that the content of the phagocytic vacuole changes with time, thus permitting contents of the specific granule to function independently. In a previous paper, we studied the time-course of the fall in pH within the phagocytic vacuole. By observing early changes in the color of ingested indicator-stained yeast, we established that intravacuolar acidity drops to pH 6.5 in 3-4 min, and to pH 4 by 7-8 min (30, 31). Therefore the pH of the phagocytic vacuole is near neutral during the first 3 min after phagocytosis, at the time it has acquired the contents of specific granules. These granules are known to contain alkaline phosphatase, lactoferrin, and lysozyme, and all these substances have pH optima in the neutral or alkaline range (see reference 31). Only later (after 3 min), when the pH is rapidly dropping to 4, are azurophil granule contents, most of which have pH optima in the acid range, discharged. These granules contain peroxidase, lysosomal enzymes, lysozyme, and acid mucosubstance (33). Peroxidase, a potent bactericidal and virucidal enzyme, has a pH optimum of 4.5 (see reference 31), while the lysosomal enzymes are largely inactive above pH 4.5. Hence the sequence of granule discharge roughly parallels pH changes, providing conditions which favor sequential granule interaction. As noted in our

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4 Recently collagenase has been localized within the specific granules of rabbit PMN (32) and has been found to have a pH optimum of 7.5.
previous paper (31), another possible consequence of this sequential discharge is that specific granule contents may be digested or altered after entry of the lysosomal enzymes.

**Demonstration of Histochemical Latency of Alkaline Phosphatase in PMN Granules**

The present observations confirm our previous findings indicating that enzymes are not readily demonstrable within mature PMN granules by electron microscopy using metal-salt techniques (15, 19). In this particular case, specific granules contained no reaction product for alkaline phosphatase despite clear biochemical evidence of the presence of this enzyme (16, 17). It was readily found, however, in adjacent phagocytic vacuoles within the same cell. This agrees with our earlier findings in bone marrow—i.e., reaction product for alkaline phosphatase could be demonstrated in immature specific granules with metal-salt techniques during their formation but not in mature specific granules. The mature granules require disruption (e.g., by smearing [15] or by prolonged incubation [19]) for activation. Previously we proposed that during granule maturation, some alteration occurs in the permeability of the granule membrane or in the form of its content, so that the enzymes within cannot be demonstrated in intact granules using methods of the Gomori type. In the phagocytic vacuole, several obvious changes occur: First, the vacuolar membrane becomes a mosaic composed of membrane derived from at least three different sources—the plasma membrane, and membranes of azurophil and specific granules. Next, the contents of the granules appear to become solubilized. Since these two events take place almost simultaneously, we cannot ascertain whether this dramatic emergence of histochemically demonstrable alkaline phosphatase within the phagocytic vacuole but not in adjacent mature specific granules is attributable to one or both factors.

**Extracellular Release of Granule Enzymes**

Our demonstration of the occasional continuity of phagocytic vacuole membranes with the plasma membrane is consistent with the morphologic (2, 34) and biochemical (see references 35 and 36) observations of numerous investigators who have found granule enzymes in supernates of phagocytizing cells. It is noteworthy that in the present work this phenomenon was more evident when clusters of S. aureus were ingested than when a single bacterium (E. coli) was ingested. Many workers have suggested that this extracellular release of granule enzymes may damage host tissue, especially in the case of the proteases (37) and basic cationic proteins, which cause increased vascular permeability (38) and chemotaxis. In contrast, Wright and Malawista (35) have recently speculated that the extracellular release of lysozyme and phagocytin might serve the useful function of amplifying and localizing an inflammatory response as well as neutralizing or killing microorganisms outside the cell. Here we would emphasize that an important factor frequently left untested in such
analyses is the pH of the extracellular medium in vivo with its consequent effect on the activity of the extruded enzymes. This point requires further investigation.

Mechanisms of Degranulation

The mechanism(s) by which specific granules selectively fuse with the newly formed vacuoles before azurophil fusion is unknown. Although it is possible that this initial alkaline phosphatase reactivity may be due to the fact that specific granules outnumber azurophils in each cell by about 2.7 to 1, this explanation seems untenable: If the two types of granules did empty simultaneously, the contents of the phagocytic vacuole would be the same at all time points, and this is clearly not the case at the early intervals. Perhaps

specific granules, in view of their smaller size, move more rapidly than azurophils, enhancing the chance of collision with the vacuole. This possibility, however, remains untested. But some new information is available concerning the differing natures of the membranes of the two granule types. Nachman et al. (39) recently analyzed membranes from both kinds of granules separated by zonal differential centrifugation, and demonstrated distinct variations in their cholesterol-phospholipid ratios and protein components. Moreover, Elsbach and his coworkers have reported that phagocytizing PMN synthesize new membrane lipid (40) and that almost all of this lipid can be found within the membrane of the newly formed phagocytic vacuoles (41). In preliminary experiments on isolated granule fractions, we observed that the two types of granules respond differently to osmotic changes. Specific granules do not rupture or change shape in hypotonic buffer or even in
FIGURE 8  PMN taken from a sample 10 min after exposure to live *S. aureus* and reacted for peroxidase. A large vacuole (PV') filled with enzyme reaction product (arrows) is apparent. No intact bacteria appear within this vacuole, and only a few membranes persist—perhaps representing bacterial remnants (r). Another, smaller phagocytic vacuole (PV) contains a single bacterium, but lacks reaction product for peroxidase. In contrast to the cell in Fig. 9, which has almost completely degranulated, numerous peroxidase-positive azurophil granules (ag) and peroxidase-negative specific granules (sg) are still present in the cytoplasm. The islands of glycogen (g) are partially preserved. A small Golgi complex (G) and centriole (ce) are also visible. Specimen preparation as in Fig. 3, except that the tissue was fixed in glutaraldehyde at 4°C for 16 h. X 26,000.
FIGURE 9  PMN reacted for alkaline phosphatase, 10 min after exposure to live E. coli E15 (B). Observe the large size of one phagocytic vacuole (PV), as well as the alkaline phosphatase deposits (arrows) dramatically outlining vacuolar walls. The cytoplasm is almost devoid of PMN granules, indicating complete cellular degranulation. Several of the intracellular bacteria (B') are in various stages of disintegration; some reaction product can be seen nearby. One bacterium (B') appears to be entering the phagocytic vacuole. Extracellular bacteria (B) are devoid of enzyme reaction product. It is interesting that in the larger phagocytic vacuole, the enzyme seems to outline the entire vacuolar wall, whereas we know that the membrane of this vacuole is a mosaic of plasma membrane plus the membranes of both azurophil and specific granules. In addition, reaction product appears to be sticking to the bacterial membranes, a point better illustrated in Fig. 10. These observations suggest that this enzyme reaction product has a relatively nonspecific affinity for membranes. Specimen preparation as in Fig. 7. × 19,000.
FIGURE 10 PMN reacted for alkaline phosphatase, 10 min after exposure to live E. coli E15 (B). No storage granules can be identified in the cytoplasm, but instead, two large phagocytic vacuoles (PV) are present, both filled with bacteria (B). One phagocytic vacuole (PV') is filled with deposits of alkaline phosphatase, located on the membranes of the vacuole and on those of bacteria (arrows). The adjacent phagocytic vacuole (PV") contains no reaction product; presumably these bacteria were internalized after the supply of specific granules had been exhausted. Specimen preparation as in Fig. 7. × 17,000.

distilled water, whereas azurophills become markedly shrunken and distorted in 0.1 M buffer and rupture easily in distilled water. Considered together, these studies prove that the membranes of each granule type have distinctive properties. It is not yet clear, however, what property or properties of the membrane may account for the initial selective fusion of specific granule and phagosome membranes. Future studies should be pursued to elucidate this possible membrane recognition phenomenon.

Two important unanswered questions regarding the mechanism(s) of degranulation are: (a) Does the process require energy? and (b) Is it dependent upon the presence of microtubules? (as proposed by Malawista and Bodel [42]). Concerning the first question, it is well established that the process of phagocytosis is dependent upon anaerobic glycolysis, with consumption of extracellular glucose and endogenous glycogen, resulting in the production of lactic acid. Thus far, attempts to dissociate phagocytosis and degranulation have failed (43, 44). Indeed, the recent findings of Stossel et al. (45, 46) are consistent with the view that particle uptake and granule interaction with the phagocytic vacuole are closely integrated events. They also observed that once formation of the vacuole had been completed, little further degranulation occurred.

Certain drugs have been reported to partially inhibit degranulation (47, 48), and among them, colchicine (42) is of particular interest. Utilizing the fact that colchicine is known to cause disruption of microtubules, together with their own observation that this drug interferes with
FIGURE 11 PMN 10 min after exposure to E. coli E15, reacted first for peroxidase and then for alkaline phosphatase. In this type of preparation, reaction product for peroxidase appears as in Fig. 8, in the azurophil granules (ag) and as a lump (l) within the phagocytic vacuole (PV). Faint reaction product (lead phosphate) for alkaline phosphatase can also be discerned within the phagocytic vacuoles (arrows) but not in adjacent specific granules (sg). Specimen fixed for 1 h at 4°C in glutaraldehyde, incubated in DAB medium with H₂O₂ for 1 h at 24°C, and incubated in Gomori's medium for alkaline phosphatase for 2 h at 37°C, with subsequent processing as in Fig. 2. X 39,000.

degranulation and formation of digestive vacuoles, Malawista and his co-workers (42, 49) have popularized the concept that microtubules "facilitate the getting-together of lysosomes with vacuoles." Critical to this theory is their interpretation of data indicating that colchicine does not inhibit phagocytosis—that is, it does not alter the internalization of particles. Recently, however, Stossel et al. (45) have developed a method for isolating phagocytic vacuoles which permits measurement of the rate of phagocytosis. They also tested (46) the uptake of particles in the presence of a concentration of cochinic (0.001–0.1 mM) similar to that used by Malawista and Bodel (42), but found that phagocytosis was inhibited. These data clearly indicate that the conclusions of Malawista concerning the role of microtubules in the degranulation phenomenon should be carefully reanalyzed utilizing techniques such as the biochemical assay of phagocytic vacuole contents (45, 46) or the histochemical methods presented in this paper.

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