

# A Novel Intracellular Compartment with Unusual Secretory Properties in Human Neutrophils

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**Abstract.** Human neutrophils contain a novel intracellular compartment that is distinct from the previously characterized azurophil and specific granules. This compartment is distinguished by the presence of cytochemically detectable alkaline phosphatase activity. The alkaline phosphatase-containing compartments are short rod-shaped organelles that rapidly undergo a dramatic reorganization upon cell stimulation with either

a chemoattractant or an active phorbol ester. Biochemical analysis shows that in unstimulated neutrophils the majority of the alkaline phosphatase activity is intracellular, but after stimulation essentially all of this activity becomes associated with the cell surface. The exocytotic pathway is unusual in that these small organelles fuse to form elongated tubular structures before their association with the plasmalemma.

NEUTROPHILS represent the first line of defense against invading microorganisms and are important mediators of the acute inflammatory response. These cells contain a potent array of microbicidal mechanisms including the ability to generate toxic oxygen species (i.e., superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen) (for reviews see Badwey et al., 1986; Curnutte and Babior, 1987). Other portions of this armory reside in the cytoplasmic granules which are a hallmark of this cell type. The azurophil granule contains myeloperoxidase which acts in concert with hydrogen peroxide for microbicidal activity (e.g., Weiss et al., 1982). Other polypeptides important in oxygen-independent microbicidal activity are also present in granules. These include the defensins (Ganz et al., 1985) and cationic proteins (e.g., Weiss et al., 1978; Modrzakowski and Spitznagel, 1979). The azurophil granule also contains the typical array of lysosomal enzymes (e.g., Bainton et al., 1971). In addition, neutrophils also possess a so-called specific granule which has its own set of unique markers, including lactoferrin and vitamin B-12 binding protein (e.g., Borregaard, 1988). The specific granule is also the intracellular site of a neutrophil-specific b cytochrome (Jesaitis et al., 1990), the complement receptor CR3 (Bainton et al., 1987) as well as certain extracellular matrix receptors (Yoon et al., 1987; Singer et al., 1989). These proteins can be translocated to the cell surface in a stimulus-dependent manner and are important in the process of cell activation and adhesion. Studies on the structural basis of formation of the azurophil and specific granules have been carried out during neutrophil development in bone marrow (e.g., Bainton and Farquhar, 1968; Ackerman, 1968; Spicer and Hardin, 1969).

Alkaline phosphatase (AlkPase)<sup>1</sup> activity has often been employed as a marker enzyme for the plasmalemma in cell fractionation studies of human neutrophils (e.g., Dewald et al., 1982; Borregaard et al., 1983; Todd et al., 1984; Parkos et al., 1985; Ohno et al., 1985; Krause and Lew, 1987; O'Flaherty et al., 1990). However, it has been shown that this activity can be upregulated to the cell surface during neutrophil stimulation and that unstimulated neutrophils have an unidentified intracellular pool of alkaline phosphatase which can be detected after lysis of cells with detergent (Borregaard et al., 1987).

We now show that the majority of AlkPase activity in these cells resides in a unique compartment which is distinct from the azurophil and specific granules. We further show that this compartment can be mobilized by stimulation with either a chemotactic peptide or PMA and that during such stimulation these organelles undergo a rapid and dramatic alteration in their structure; furthermore, the subsequent secretory event occurs by an unusual pathway. A preliminary account of portions of this work has appeared in abstract form (Kobayashi, T., and J. M. Robinson. 1990. *J. Histochem. Cytochem.* 38:1043).

## Materials and Methods

### Reagents

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): agarose (type XI), DMSO, dextran, *N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine, Histopaque 1083, HBSS, poly-L-lysine, *p*-nitrophenylphosphate, *p*-nitrophenol,  $\beta$ -glycerophosphate, levamisole, saponin, sodium caseinate, PMA, *N*-Tris [hydroxymethyl] methyl-3-aminopropane-sulfonic acid (TAPS), Tricine, and Triton X-100. Glutaraldehyde (25%

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1. *Abbreviations used in this paper:* AlkPase, alkaline phosphatase; DSSA, diazonium salt of sulfonilic acid.

aqueous) and sodium cacodylate were obtained from Polysciences, Inc. (Warrington, PA). Osmium tetroxide was purchased from Stevens Metallurgical, Corp. (New York, NY). Gelatin (granular, 100 bloom), phosphotungstic acid, and potassium ferrocyanide were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Cerium chloride was from Alfa, Morton Thiokol, Inc. (Danvers, MA). Heparin was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ). Rabbit antihuman lactoferrin (IgG fraction) was obtained from Cappel, Organon Teknika Corp. (West Chester, PA). Protein A-gold (10-nm diameter) was purchased from Janssen Life Sciences (Piscataway, NJ). Forvar (powder) and ethylene dichloride were obtained from Ladd Research Industries, Inc. (Burlington, VT). Epon 812 was supplied by Balzers Union (Hudson, NH). All other reagents were of the highest grade available.

## Preparation of Cells

**Human Neutrophils.** Human neutrophils were obtained from healthy adult males. Whole blood was drawn into plastic syringes (cells were kept in plastic tubes throughout). Different procedures for isolation of neutrophils were compared. Coagulation was prevented by heparin (20 U/ml) or acid citrate dextran. Leukocytes were separated from erythrocytes by sedimentation ( $1 \times g$ ) in 6% dextran (mol wt  $\sim 450,000$ ) in HBSS without  $Ca^{++}$  or  $Mg^{++}$  at either 4°C or 22°C. After dextran sedimentation the leukocyte-rich upper phase was centrifuged at 150 g for 10 min at 4°C (cells were maintained at 4°C until used for experiments; no more than 15 min elapsed between final isolation step and initiation of experiments). Residual erythrocytes in the initial pellet were lysed by treatment with a small volume of distilled water for 15–20 s and then quickly brought to isotonicity with buffer. Cells were washed an additional time and then used with no further purification, or alternatively they were further enriched by centrifugation through Histopaque (cell viability was at least 95% as judged by trypan blue exclusion and 98% neutrophils by differential counting). The resulting pellet was washed once in HBSS before use.

**Guinea Pig Neutrophils.** Male Hartley guinea pigs (300–350 g) (Hartman; Sprague-Dawley, Inc., Indianapolis, IN) were maintained on chow pellets and water ad libitum. Elicited neutrophils were prepared as described previously (Robinson et al., 1982) after intraperitoneal injection of 30 ml of sterile 12% sodium caseinate in isotonic saline. Preparations contained  $\geq 90\%$  neutrophils with viabilities always  $\geq 90\%$ . The animals used in these studies were maintained in an AAALAC facility in accordance with the guidelines for care and use of laboratory animals of The Ohio State University and the National Research Councils.

## Cell Stimulation

Human neutrophils were stimulated by treatment with the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) or by PMA. The latter compound is known to be an activator of protein kinase C (Nishizuka, 1986). Stock solutions of the stimuli were prepared in DMSO and stored at  $-20^\circ\text{C}$  until needed. These compounds were diluted with DMSO so that the final concentration of solvent in the assays was 0.25% (vol/vol) in all cases.

Cells were stimulated as indicated for periods of 1, 3, 5, 10, or 15 min at 37°C. Cells at varying numbers ( $1 \times 10^6$ – $2 \times 10^7$ ) were treated with fMLP ( $10^{-7}$  M) or PMA (50 ng/ml). After stimulation neutrophils were prepared, as described below, for cytochemical or biochemical analysis. Unstimulated cells were those that were maintained at 4°C until fixed or assayed. In other cases cells were incubated with DMSO (0.25% vol/vol) for the same time periods as the stimuli.

## Fixation

Human neutrophils (unstimulated, stimulated, or DMSO treated) were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) containing 5% sucrose for 5 min at 4°C; they were then layered over round glass cover slips (13-mm diameter) that had been coated with poly-L-lysine (0.1%). The cells were fixed for an additional 10 min during which time they could adhere to the poly-L-lysine. After fixation the cover slip preparations were washed three to four times in cacodylate buffer. Unstimulated guinea pig neutrophils were fixed and washed in the same manner. In some experiments cells were fixed for 15 min (in this same fixative) in suspension. These cells were washed in cacodylate buffer by centrifugation. In each case (cover-slip-attached or in suspension) cells were then processed for cytochemistry or biochemistry.

## Cytochemistry

Cytochemical localization of AlkPase was achieved by incubating fixed and washed cells in a cerium-containing reaction medium, which was a modification of the procedure that we have described previously (Robinson and Karnovsky, 1983; Robinson, 1985). This medium contained 50 mM tricine, 100 mM TAPS, 2 mM  $CeCl_2$ , 2 mM  $MgSO_4$ , 0.006% Triton X-100, 0.004% saponin, 5% sucrose, and 2 mM substrate. Either  $\beta$ -glycerophosphate or *p*-nitrophenylphosphate were suitable substrates for the cytochemical reaction. The reaction medium was pH 9.3. The cytochemical incubations were done with cells attached to poly-L-lysine-coated cover slips or with cells in suspension for 1 h at 37°C with constant agitation. In some experiments the coverslip preparations were overlaid with agarose (4%) before the cytochemical reaction.

Control cytochemical incubations consisted of (a) omission of substrate from the reaction mixture; (b) omission of  $Mg^{++}$  from the reaction mixture; and (c) inclusion of levamisole (1 mM) in the complete reaction mixture. Levamisole is an inhibitor of AlkPase (e.g., Cyboron and Wuthier, 1981; Robinson, 1985). In other cases cells were incubated in a cytochemical medium containing substrate but lacking detergents necessary for permeabilization of these cells.

The cytochemical localization of acid phosphatase was also determined in unstimulated neutrophils. The cytochemical reaction was carried out with a cerium-based procedure as described previously (Robinson, 1985).

## Microscopy

After the various cytochemical incubations, cells were postfixed with 2%  $OsO_4$  in 0.1 M cacodylate buffer (pH 7.4) containing 1.5% potassium ferrocyanide (Robinson et al., 1982). Alternatively, the  $OsO_4$  postfixation step was omitted. Cells were then dehydrated in a graded series of ethanol and subsequently embedded in Epon 812.

Conventional "thin" sections ( $\sim 0.075 \mu\text{m}$  in thickness) were cut with a diamond knife and observed with a Philips 300 electron microscope operated at an accelerating voltage of 60 kV. "Thick" sections ( $\sim 0.75 \mu\text{m}$  in thickness) were also cut with a diamond knife. These sections were observed in a Philips 300 operated at 80 kV or in a Philips CM-12 operated at 120 kV. Thick sections were usually coated with a thin layer of evaporated carbon (carbon coating served to dissipate heat created by the electron beam) before observation.

## Morphometry

Electron micrographs of thick sections, taken through the centers of cells, were prepared at a standard microscope magnification (10,000 $\times$ ). These negatives were subsequently printed at a standard enlargement factor (2.5 $\times$ ). Granules containing reaction product for AlkPase were traced onto tracing paper with a drafting pen (0.18-mm tip). The images of the tracings were captured with a video camera by a Tracor Northern 8502 image analysis computer (Tracor Northern, Inc., Middleton, WI). Images of these tracings were analyzed by the Tracor Northern system to determine (a) maximum projection distance, (b) minimum projection distance, and (c) width for each of the traced organelles. Ten micrographs of neutrophils from each of three individuals were analyzed, resulting in the measurement of  $\sim 3,000$  structures.

## Immunocytochemistry

Cells were prepared for the cytochemical localization of AlkPase as indicated above; after the reaction, cells in suspension were washed and embedded by centrifugation into 2% agarose (low temperature gelling). The solidified agarose was cut into small pieces and then infiltrated with 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.2) (e.g., Tokuyasu, 1980; Griffiths et al., 1984). The agarose blocks were then mounted on specimen pins and frozen in liquid nitrogen. Ultrathin cryosections were cut with a Reichert Ultracut E equipped with a FC 4D cryounit. The instrument settings were such that the knife was maintained at  $-90^\circ\text{C}$ , the specimen was  $-80^\circ\text{C}$ , and the chamber was  $-120^\circ\text{C}$ . Sections (80–90 nm in thickness) were collected on formvar-coated nickel grids that had also been coated with evaporated carbon. The grids with sections were incubated with 0.5% BSA and 0.1% gelatin in PBS for 1–2 h at 22°C. They were then incubated in 20 mM glycine in PBS for 10 min followed by four quick rinses in buffer. The sections were subsequently incubated with rabbit antihuman lactoferrin (1 mg/ml) for 30 min. Control incubations consisted of nonimmune serum or omission of this step. The grids were then rinsed in buffer four times over 20 min. Detection of the primary antibody binding sites was achieved with

a 30-min incubation in protein A-gold (10 nm) that was diluted 1:50 with PBS over that supplied by the manufacturer. The grids were rinsed several times in buffer and then postfixed in 2% glutaraldehyde in PBS for 10 min. They were then washed in PBS followed by distilled water. The sections were negatively stained with aqueous 1% phosphotungstic acid at pH 7.0 for 10 min, blotted with filter paper, air dried, and examined with the electron microscope.

## Biochemistry

Neutrophils were purified with Histopaque before biochemical measurement of AlkPase activity in order to minimize contribution to the assay by other cell types that may have been present. Unstimulated cells or those that had been treated with fMLP ( $10^{-7}$  M) for 15 min at 37°C were fixed in suspension with 2% glutaraldehyde and washed, as for the cytochemical experiments (see above). Fixed cells were used for the biochemical experiments in order to make these results more comparable to the cytochemical data. In other experiments, fresh, unfixed cells were used for the measurement of AlkPase activity.

Two different strategies were employed in order to assess the proportion of the AlkPase activity that was on the cell surface as compared to that which was intracellular. In one case, cells were assayed in the absence or presence of Triton X-100 (0.1%). In the second case, cells were treated with the diazonium salt of sulfanilic acid (DSSA) as described previously (DePierre and Karnovsky, 1974; Badwey and Robinson, 1991). The DSSA can react with proteins on the cell surface under certain conditions without penetrating into the cytoplasm. Thus, it serves as a general enzyme inhibitor which does not cross cell membranes. Cells were treated with DSSA (4 mM in PBS) for 30 min at 37°C and then centrifuged. The DSSA reaction was stopped by washing cells with BSA (final concentration of 30 mg/ml) in PBS and incubating for 20 min at 4°C.

Cells that had been incubated in the presence or absence of DSSA were then assayed for the detection of AlkPase activity with *p*-nitrophenylphosphate as substrate by spectrophotometrically monitoring the formation of *p*-nitrophenol at 410 nm; the buffer system employed for this assay was the same as that used for the cytochemical studies except cerium was omitted. The reaction was initiated by addition of substrate and was allowed to proceed for 30 min at 37°C. The reaction was stopped by transferring tubes to an ice-water bath; the cellular material was then removed by rapid centrifugation. The resulting supernatant was read at 410 nm. Reference blanks for each experimental condition consisted of the assay medium, with cellular material but lacking substrate. Cell number was determined by direct counting with a hemocytometer. Standard curves for the product were prepared with *p*-nitrophenol in the assay buffer.

## Results

### Analysis of Cytochemical Localization of Alkaline Phosphatase Activity in Thin Sections

Human neutrophils contain the enzyme alkaline phosphatase which can be readily observed in cells incubated for the detection of this activity with cerium as the capture metal. In thin sections ( $\sim 0.075$   $\mu$ m in thickness) of unstimulated cells the intracellular reaction product was localized in small cytoplasmic structures which appeared as rod shaped or rounded structures depending upon their orientation in the plane of section (Fig. 1). Unstimulated cells displayed little reaction product on the cell surface under these conditions.

### Analysis of Cytochemical Localization of Alkaline Phosphatase Activity in Thick Sections

Localization of AlkPase activity in 0.75- $\mu$ m thick sections of unstimulated cells led to a much better appreciation and characterization of the morphological properties of these structures and of their distribution. The alkaline phosphatase-positive compartments (Alk compartment) are typically rod-shaped, although more circular profiles are also observed (Fig. 2 *a*). There was no apparent difference in the distribution of cytochemically detectable AlkPase with any

of the neutrophil isolation procedures employed. The cytochemical reaction for AlkPase in the Alk compartment was specific since (*a*) it was dependent upon the presence of substrate (Fig. 2 *b*); (*b*) it was inhibited by levamisole (Fig. 2 *c*); (*c*) it was dependent upon the presence of  $Mg^{++}$  in the medium (data not shown); and (*d*) it was dependent upon an alkaline pH. In this latter regard an entirely different granule population was detected when this same type of cytochemical reaction was carried out at an acidic pH (i.e., pH 5.0) (Fig. 3). Reaction product for detection of AlkPase within cells was restricted to the AlkPase-positive compartment and Golgi and was not detected in other organelles.

### Morphometric Analysis of Alkaline Phosphatase-containing Compartment

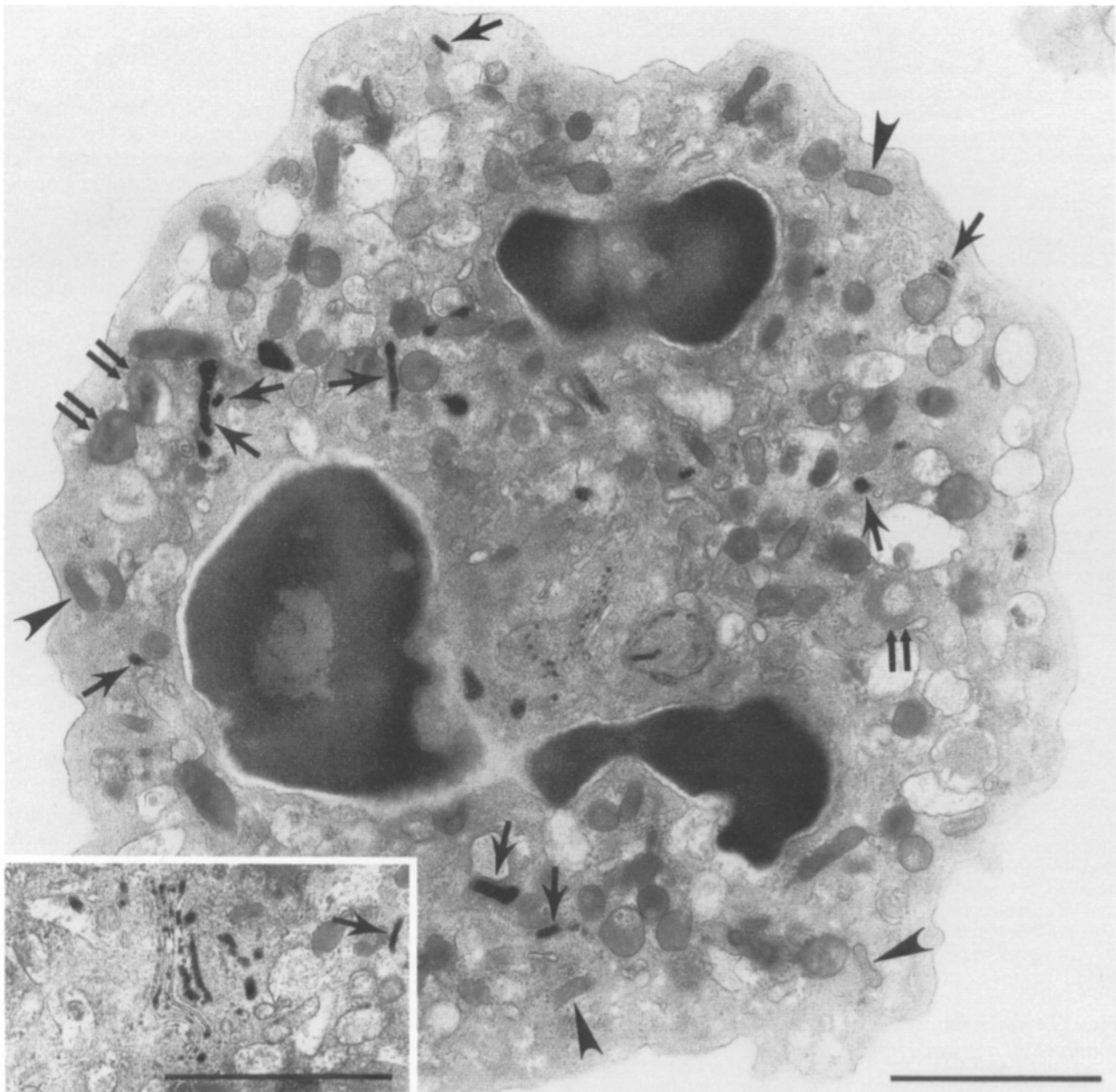
Computer-assisted morphometric analysis of these structures in thick sections was very useful in establishing a morphological description of this unique organelle. These results show that they have an average maximum projection distance of 0.234  $\mu$ m, a minimum projection distance of 0.107  $\mu$ m, and a width of 0.105  $\mu$ m in unstimulated cells. Since the Alk compartments are not all in the same orientation, a variety of sizes are observed in sectioned material. The maximum projection distance distribution of these profiles is shown in Fig. 4.

### Reorganization of Alkaline Phosphatase-containing Compartment during Neutrophil Stimulation

The Alk compartments do not appear to have a preferential cytoplasmic distribution in unstimulated cells (e.g., Fig. 2 *a*). There is, however, a rapid reorganization of this compartment upon fMLP stimulation. After 1–3 min stimulation these cells undergo a series of morphological transformations which include change in cell shape. Concomitant with changes in cell shape is a dramatic reorganization of the Alk compartment. Within 1–3 min these structures have become aggregated, to a large extent, at the cell center (Fig. 5 *a*). Many of the structures are in the process of fusion while many have increased in length at this point. There is a progressive change in the Alk compartment over the next few minutes (Fig. 5 *b*).

As is the case with fMLP stimulation, treatment of neutrophils with PMA leads to dramatic alterations in the organization of the Alk compartment. Within 1–3 min after PMA addition, there is an aggregation of AlkPase-positive structures at the cell center (Fig. 5 *c*). Many of these structures are in the process of fusion by this time. There is a continuous change in the morphology of this compartment with time (Fig. 5 *d*). At later points (e.g., 10–15 min), the distribution of AlkPase in PMA-treated cells differs from fMLP-treated cells in that there are more numerous AlkPase-positive vacuoles in PMA-stimulated cells (data not shown).

The extent to which Alk compartments fuse during the early stages of cell stimulation can be perhaps best visualized by the three-dimensional appearance of these images as stereo-pairs. Fig. 6 is a stereo-pair of micrographs of a neutrophil that was stimulated with PMA for 1 min before fixation, showing the transformation of Alk compartments into elongated tubular structures. Similar results are obtained after fMLP stimulation. It should be noted that there is an apparent decrease in the number of individual AlkPase-positive



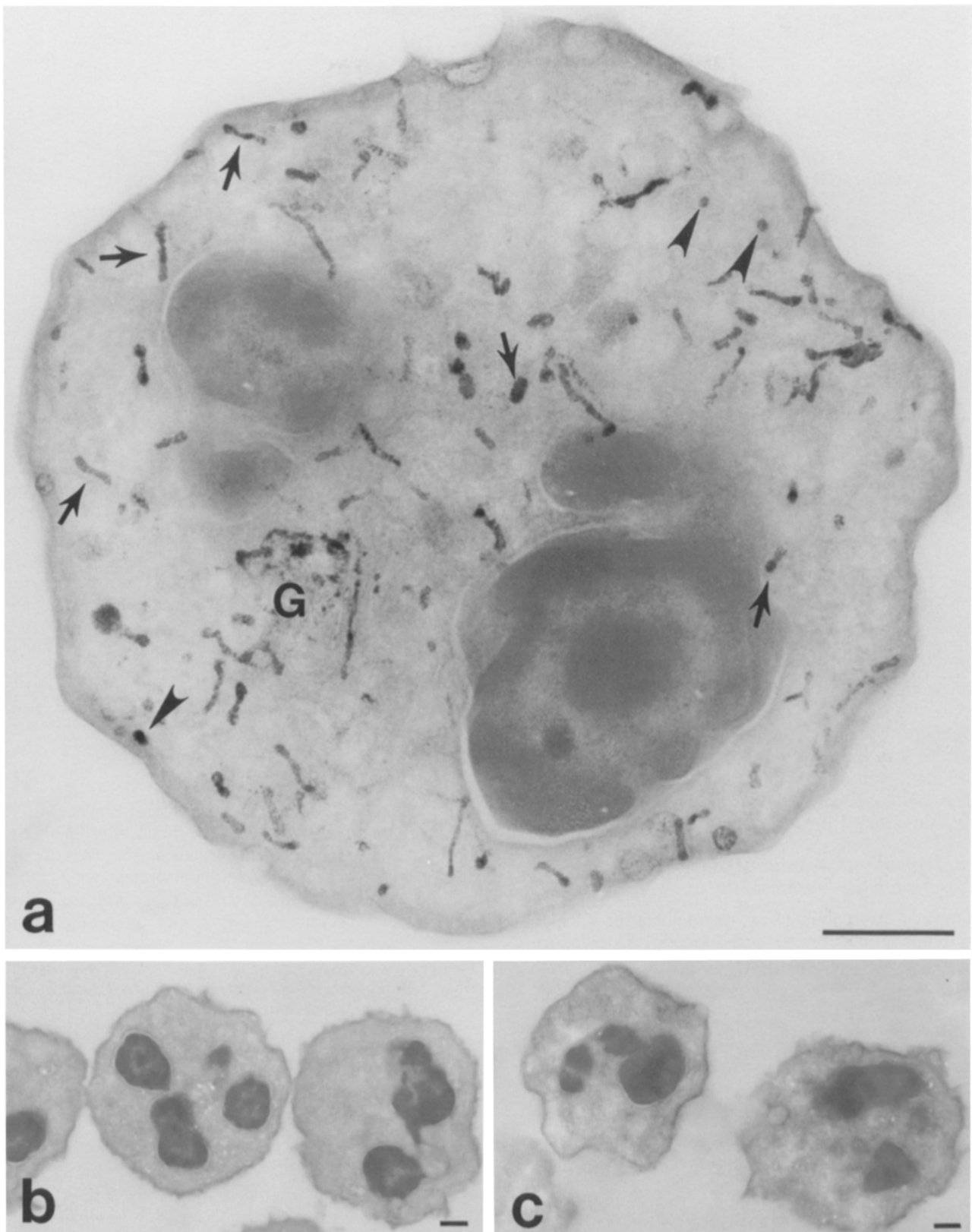
**Figure 1.** Electron micrograph of a "thin" section of an unstimulated neutrophil that was incubated for the cytochemical detection of AlkPase activity. AlkPase activity was found primarily in an intracellular granule type (*single arrows*). These were typically slender rod-shaped organelles, although they were sectioned in a variety of other orientations. There was typically reaction product in the Golgi complex as well. Other neutrophil granules lack demonstrable AlkPase activity; the azurophil granule (*double arrows*) and specific granules (*arrowheads*) do not contain reaction product. Inset shows a slightly higher magnification of a neutrophil Golgi from another cell with reaction product demonstrating the presence of AlkPase activity and the close relationship between the Golgi and Alk compartments (*arrow*). Bars, 1  $\mu\text{m}$ .

structures during cell stimulation; this is consistent with the occurrence of granule-granule fusion.

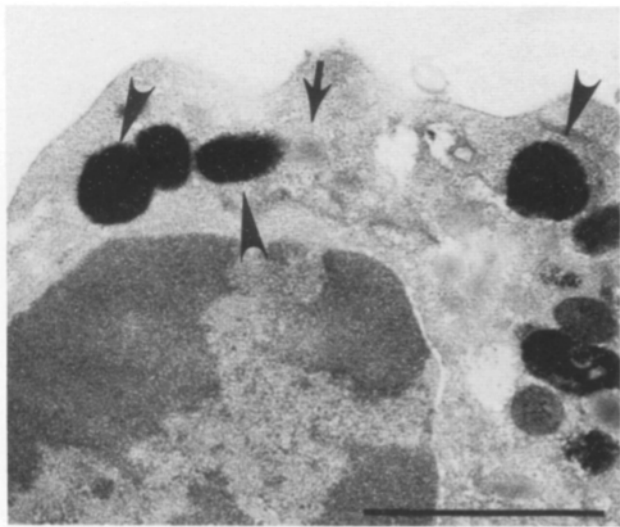
This AlkPase-containing compartment becomes increasingly tubular and by 15 min the intracellular staining is found almost exclusively in tubular organelles or in rounded granules which are lined up in rows like beads on a string (Fig. 7). These latter structures may result from glutaraldehyde-induced breakup of the tubular membrane compartments. We and others have demonstrated glutaraldehyde-induced fragmentation of other tubular structures in different cell types

(Robinson et al., 1985; Heuser, 1989). In any case there is an essentially complete morphological transformation of the Alk compartment within 15 min of fMLP stimulation. It should be emphasized that while there is a consistent pattern with regard to the formation of these tubules in stimulated cells there is morphological variation as indicated in Fig. 7.

Exocytosis of the Alk compartment occurs at discrete points rather than over the entire cell surface (Fig. 7). This may be a consequence of the morphological transformation of this compartment. Fusion of a tubular structure at a single



**Figure 2.** Electron micrographs of “thick” sections of unstimulated neutrophils. (a) Cell that was incubated for the cytochemical detection of AlkPase activity. AlkPase activity was found primarily in slender rod-shaped organelles (*arrows*). These structures were sectioned in a variety of orientations including cross-sections (*arrowheads*). Reaction product was also evident in the Golgi complex (*G*). (b) Control incubation in which unstimulated neutrophils were reacted in medium lacking substrate. Note the absence of reaction product under these conditions. (c) Control incubation in which the AlkPase inhibitor levamisole (1 mM) was present along with the complete cytochemical medium. Note the lack of reaction under these conditions. Bars, 1  $\mu\text{m}$ .



**Figure 3.** Electron micrograph of a thin section of an unstimulated human neutrophil that was incubated for the cytochemical detection of acid phosphatase activity. Acid phosphatase was present in the large azurophil granules (arrowheads). Smaller granules (arrow) lack this activity. Bar, 1  $\mu\text{m}$ .

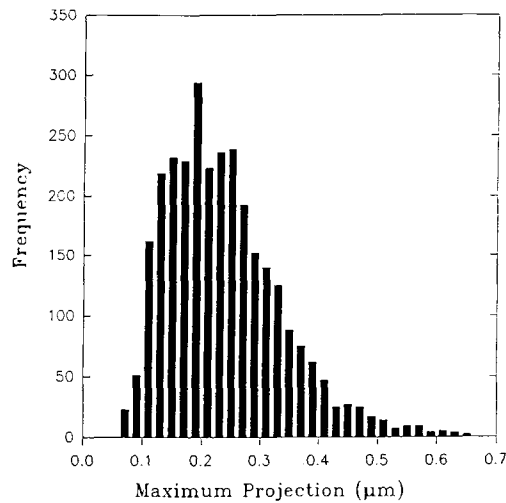
area on the plasmalemma would mean that portions of these tubules deeper in the cell would be in continuity with the extracellular space. That this is the case was demonstrated in experiments in which the cytochemical medium lacked permeabilization agents. Under these conditions intracellular accumulation of reaction product would only occur in those structures that are in continuity with the outside (see below). It should also be mentioned that the Alk compartment appears to be very sensitive to modification. Cells incubated at 37°C for 15 min with DMSO (0.25% vol/vol) display alterations in this compartment though not as dramatically as in cells stimulated with fMLP or PMA (data not shown).

#### **Localization of Intracellular Alkaline Phosphatase in Nonpermeabilized Neutrophils**

Unstimulated neutrophils have a requirement for cell permeabilization for the cytochemical detection of intracellular AlkPase activity. Incubation of these cells in cytochemical medium lacking permeabilization agents results in the absence of AlkPase staining (Fig. 8 a). In contrast, AlkPase activity was readily demonstrated in cells that had been stimulated with fMLP for 15 min (Fig. 8 b). Under these conditions reaction product was usually found in tubular structures similar to those observed in cells which had been stimulated with fMLP and then reacted in the complete cytochemical medium containing permeabilization agents (e.g., Fig. 2 a). These results show that tubular AlkPase-containing structures which form during fMLP stimulation fuse with the plasmalemma and are in continuity with the cell surface.

#### **Immunocytochemical Localization of Lactoferrin in Ultrathin Cryosections: Absence of Colocalization with Alkaline Phosphatase**

Unstimulated neutrophils that had been reacted for the cytochemical localization of AlkPase were prepared for ultracytomicrotomy. Binding sites for antilactoferrin were read-



**Figure 4.** Histogram of the frequency distribution of the maximum projection distance of AlkPase-containing structures in unstimulated neutrophils. This graphically illustrates that the AlkPase-containing compartments from unstimulated cells are relatively small and uniform; 75% of the structures are between 0.11–0.33  $\mu\text{m}$  in length (maximum projection distance).

ily demonstrated in an abundant granule type, the specific granule. Other structures containing reaction product denoting the localization of AlkPase activity were not labeled with gold particles (Fig. 9). These results along with the other cytochemical and morphological findings indicate that AlkPase is not a constituent of the specific granule.

#### **Biochemical Analysis of the Cellular Distribution of Alkaline Phosphatase Activity in Fixed and Living Cells**

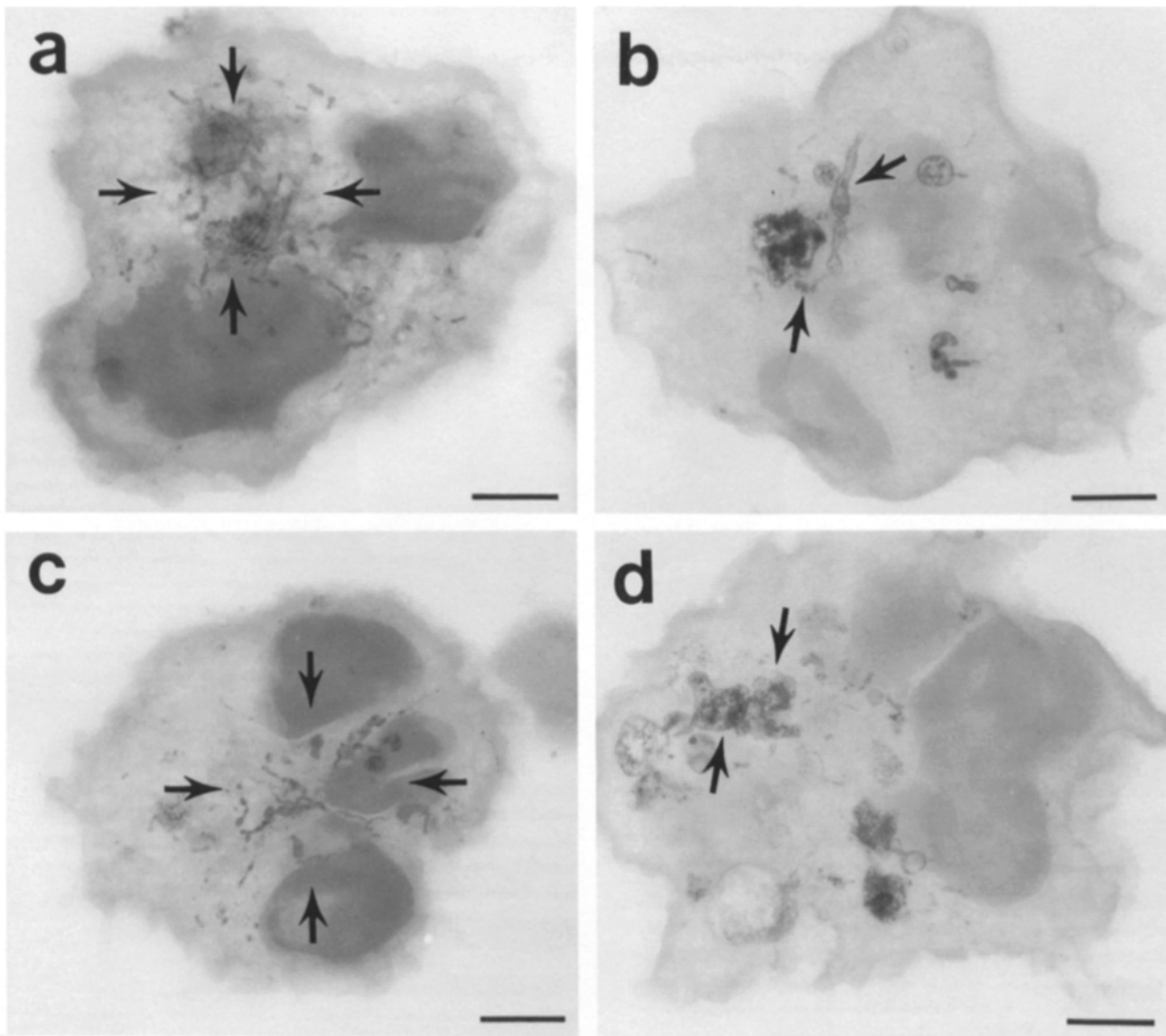
The distribution of AlkPase in unstimulated versus fMLP-stimulated neutrophils was studied by biochemical methods for comparison with the cytochemical data. The levels of cell surface AlkPase were measured in nonpermeabilized cells, while the total activity (inside and outside) was measured in the presence of Triton X-100. Under these conditions

**Table I. Effect of fMLP Stimulation on AlkPase Localization in Neutrophils: Glutaraldehyde-fixed Neutrophils**

Treatment	Activity*	Total activity
	nmol/30 min/1 $\times 10^6$ cells	%
Nonstimulated		
+ Triton	9.9 $\pm$ 2.0 (n = 9)	100
– Triton	3.2 $\pm$ 0.7 (n = 9)	32
Stimulated (fMLP 10 <sup>-7</sup> M)‡		
+ Triton	9.9 $\pm$ 0.6 (n = 3)	100
– Triton	9.2 $\pm$ 0.6 (n = 3)	92

\* Activity for fixed neutrophils was measured as the generation of *p*-nitrophenol from the substrate *p*-nitrophenylphosphate at 410 nm.

‡ Cells were stimulated with fMLP (10<sup>-7</sup>M) for 15 min at 37°C. Other conditions are given in Materials and Methods.

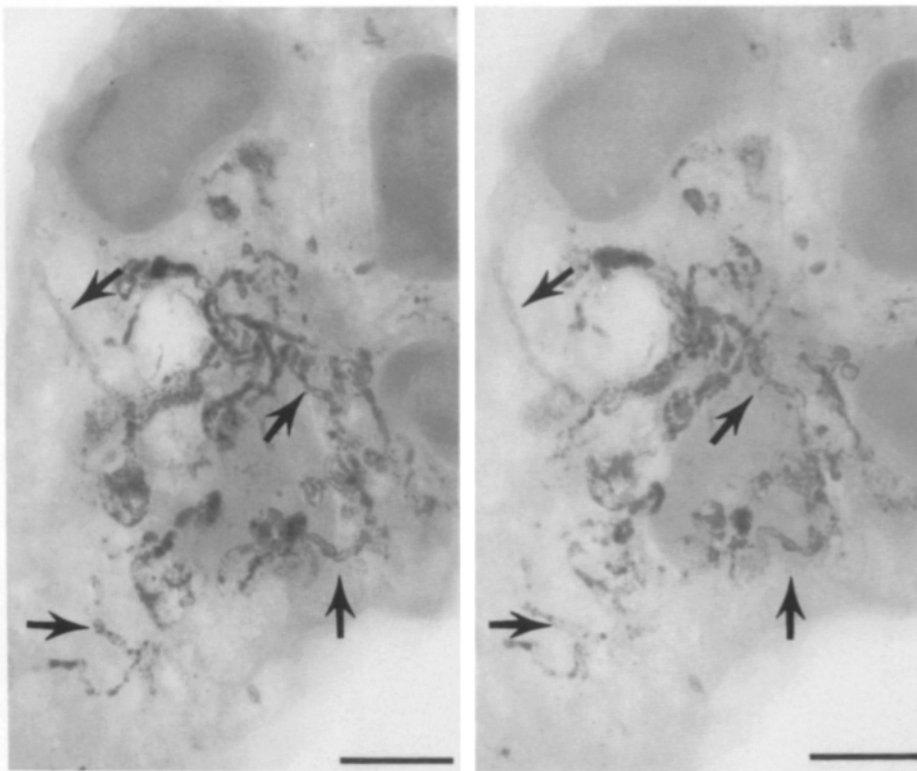


**Figure 5.** Electron micrographs of “thick” sections of neutrophils. (a and c) Cells that were stimulated with fMLP or PMA, respectively, for 1 min before fixation. AlkPase activity was largely present in structures that have aggregated toward the cell center (arrows). Note that several of the structures have increased in length compared to those in unstimulated cells. (b and d) Cells that were stimulated with fMLP or PMA for 5 and 3 min, respectively, before fixation. By this time point the morphology of the AlkPase compartment has become further modified (arrows). Bars, 1  $\mu\text{m}$ .

$\sim 30\%$  of the activity was associated with the cell surface in unstimulated cells, while these levels rose to  $\sim 90\%$  in fMLP-treated neutrophils (Table I). These results show that there are two pools of AlkPase activity in unstimulated neutrophils: one pool is associated with the cell surface which is in continuity with the extracellular medium, while the second is intracellular and is not in contact with the extracellular space. Furthermore, the intracellular pool can be mobilized and become associated with the cell surface upon stimulation with fMLP.

The effects of the nonpenetrating inhibitor DSSA on AlkPase activity were also investigated. DSSA reacts with a variety of proteins and is a general inhibitor of those enzymes with which it comes in contact. In fMLP-stimulated cells

DSSA inhibited  $\sim 80\%$  of the AlkPase in the presence or absence of Triton. In unstimulated neutrophils, on the other hand, only  $\sim 15\%$  of the total activity was inhibited in the absence of Triton (Table II). These results substantiate the previous findings, namely, that in unstimulated neutrophils the majority of the AlkPase activity is intracellular. Furthermore, the intracellular activity is mobilized and becomes associated with the cell surface upon stimulation with fMLP and can then be inhibited by DSSA. The activity of AlkPase and its distribution in fresh, unfixed cells were also determined. As expected, the activity was greater in unfixed cells than in fixed ones (Table III). The distribution of AlkPase (i.e., percent inside vs. outside) was, however, the same in unfixed and fixed cells. In addition, use of DSSA indicates



**Figure 6.** Stereo-pair electron micrographs of a "thick" section of a neutrophil that was stimulated with PMA for 1 min before fixation. The three-dimensional appearance of the reaction product better illustrates the formation of elongated tubular AlkPase-containing structures (arrows) after cell stimulation. In this stereo-pair the micrographs were taken with goniometer tilt angles of +30° (left) and -30° (right). Bar, 1  $\mu$ m.

that ~70% of the AlkPase activity is intracellular in fresh, unstimulated cells (Table III).

#### **Cytochemical Localization of Alkaline Phosphatase in Guinea Pig Neutrophils**

The cytochemical localization of AlkPase was also determined in unstimulated guinea pig neutrophils for comparison with the human neutrophil. The guinea pig cell is known to have high levels of an ecto-alkaline phosphatase as well as a population of AlkPase-positive granules (Robinson,

1985; Badwey and Robinson, 1991). Analysis of thin and thick sections illustrates the presence of cell surface and granule AlkPase activity (Fig. 10). Moreover, these results show that the cytochemical medium employed in the present study can readily detect ecto-AlkPase as well as granule-associated activity.

#### **Discussion**

We show that human neutrophils contain a previously uncharacterized intracellular compartment with unusual secretory properties. In these studies we have utilized AlkPase activity as a convenient marker for this organelle, since its presence and location can be readily demonstrated by both

**Table II. Inhibitory Effect of the Diazonium Salt of Sulfanilic Acid on AlkPase Activity of Stimulated and Nonstimulated Neutrophils: Glutaraldehyde-fixed Neutrophils**

Treatment	Activity*	Total activity‡	Inhibition
	nmol/30 min/1 $\times$ 10 <sup>6</sup> cells	%	%
Nonstimulated			
+ Triton	8.0 $\pm$ 1.1 (n = 3)	81	19
Stimulated (fMLP 10 <sup>-7</sup> M)§			
+ Triton	2.1 $\pm$ 0.1 (n = 3)	21	79
- Triton	2.1 $\pm$ 0.7 (n = 3)	21	79

\* Activity for fixed neutrophils was measured as the generation of *p*-nitrophenol from the substrate *p*-nitrophenylphosphate at 410 nm.

‡ Percent total activity (remaining after DSSA inhibition) was calculated using the appropriate 100% values from Table I.

§ Cells were stimulated with fMLP(10<sup>-7</sup> M) for 15 min at 37°C before fixation. Other conditions are given in Materials and Methods.

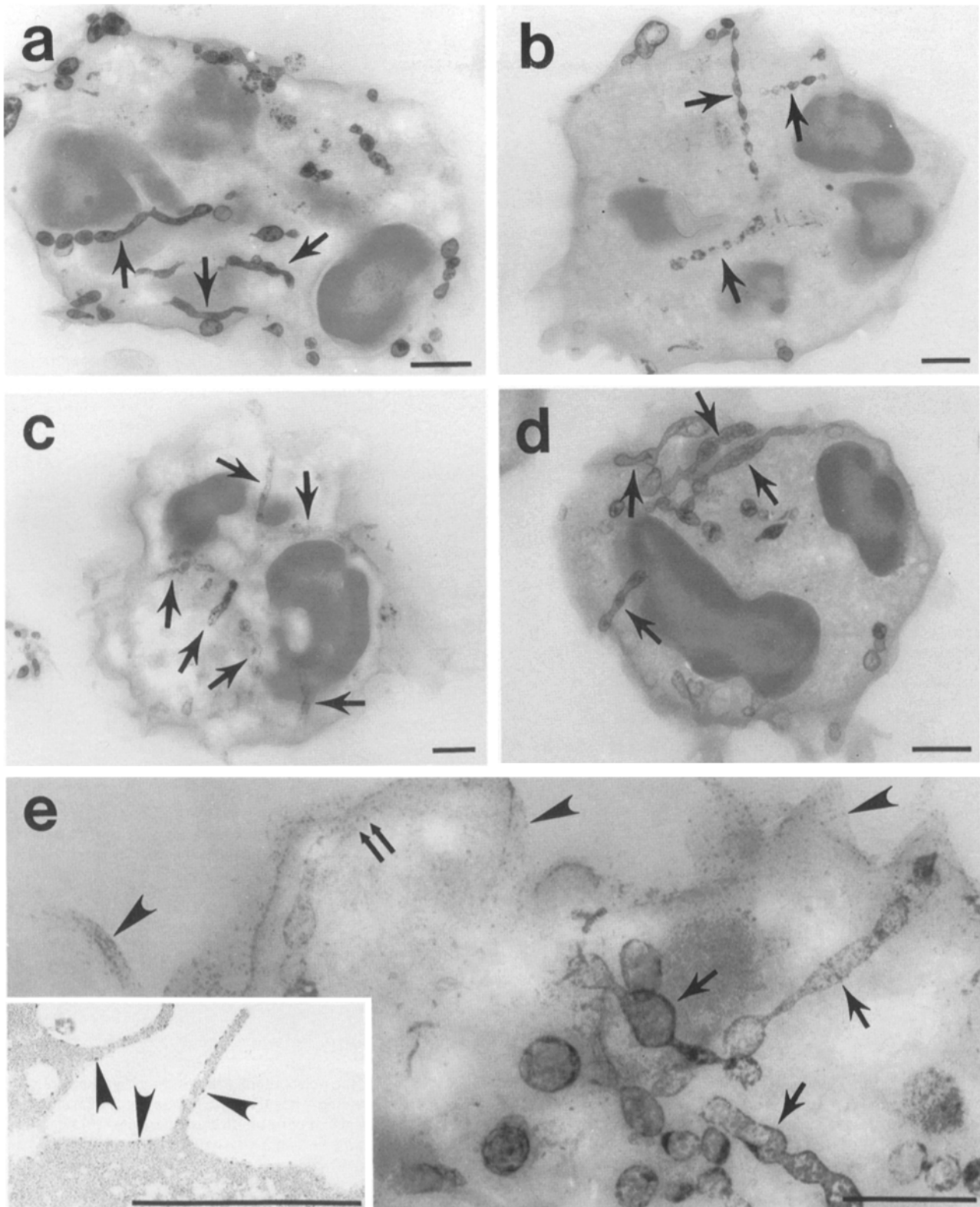
**Table III. Localization of AlkPase and the Effect of the Diazonium Salt of Sulfanilic Acid: Unfixed Neutrophils**

Treatment	Activity	Total activity	Inhibition
	nmol/30 min/1 $\times$ 10 <sup>6</sup> cells	%	%
Without DSSA			
+ Triton	68.3 $\pm$ 11.4 (n = 3)	100	-
- Triton	21.7 $\pm$ 4.9 (n = 3)	32	-
With DSSA‡			
+ Triton	53.3 $\pm$ 15.2 (n = 3)	78	22

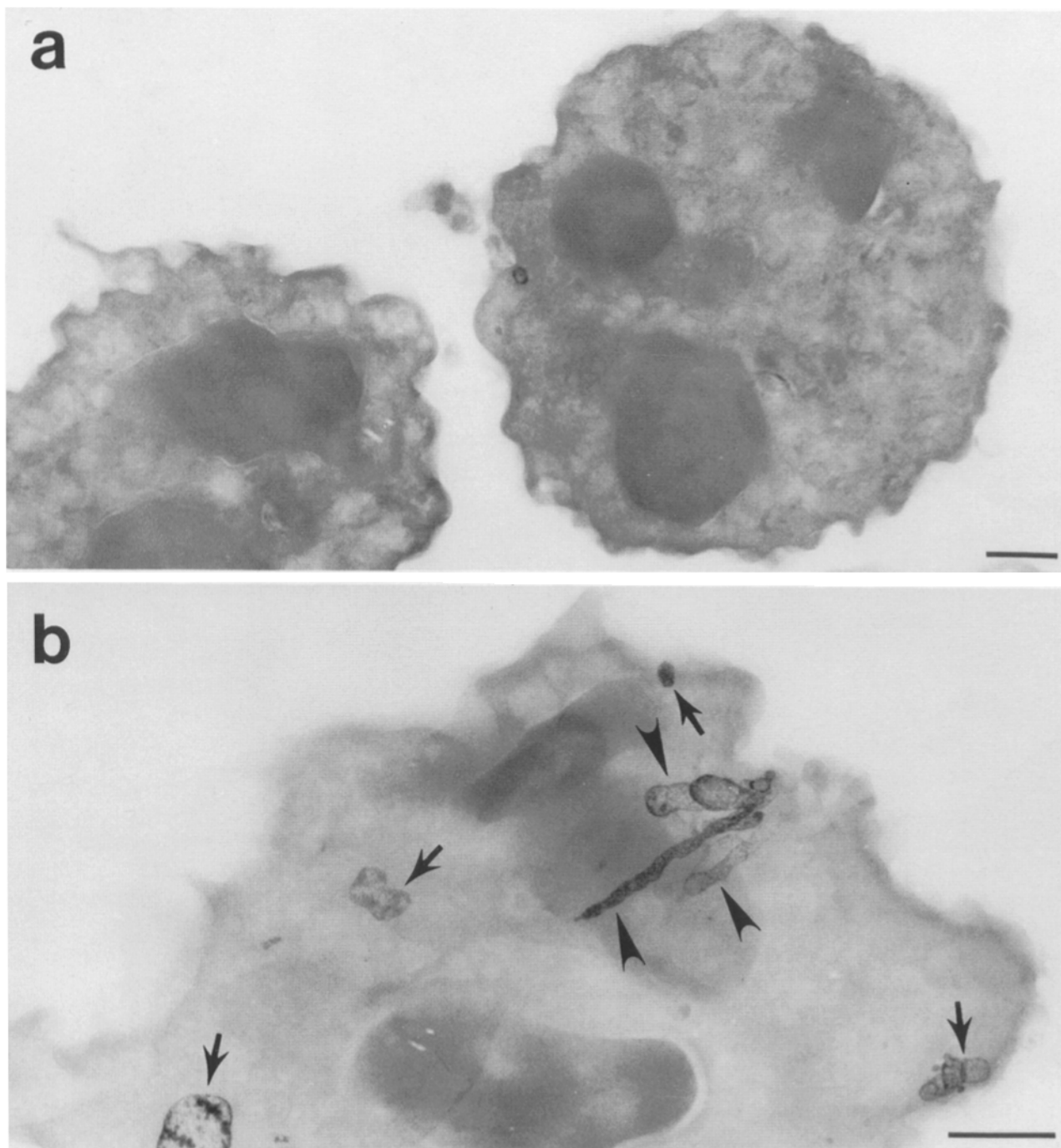
\* Activity for unfixed neutrophils was measured as the generation of *p*-nitrophenol from the substrate *p*-nitrophenylphosphate at 410 nm.

‡ Cells were treated with DSSA before determination of AlkPase activity in the presence of Triton.





**Figure 7.** Gallery of “thick” section electron micrographs of neutrophils that were stimulated with fMLP for 15 min before fixation. At this time point the intracellular AlkPase activity was present in tubular structures (arrows). (a–e). In some cases these tubules were more vesicular in appearance (see b). At higher magnification (e) cell surface reaction was evident. This cell surface reaction has a punctate distribution (arrowheads) in thick sections (e) and thin sections (e, inset). Fusion of these tubular structures with the plasmalemma appears to occur at focal points (e, double arrows) rather than over the entire tubule. Bars, 1  $\mu$ m.

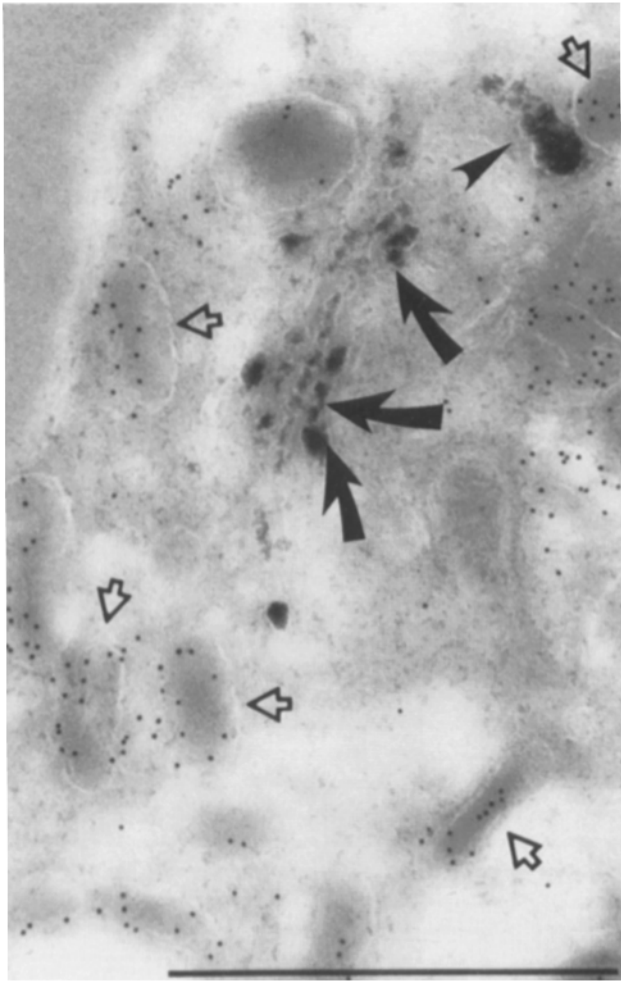


**Figure 8.** Electron micrographs of “thick” sections of neutrophils that were incubated for the detection of AlkPase in the absence of cell permeabilization. (a) Unstimulated neutrophils illustrating the lack of granule or Golgi staining when these cells were not permeabilized. (b) A neutrophil that was incubated with fMLP for 15 min before fixation. Even in the absence of permeabilization there are several structures that are positively stained for AlkPase activity. This indicates that these structures are in continuity with the extracellular space. The morphology of these structures ranges from tubular (*arrowheads*) to vesicular (*arrows*). Bars, 1  $\mu\text{m}$ .

biochemical and cytochemical methods. This combined approach has distinct advantages over either methodology employed alone.

AlkPase activity is visualized cytochemically at the ultrastructural level in conventional thin sections as being a relatively low abundance compartment that is morphologically distinct from the more well characterized azurophil and

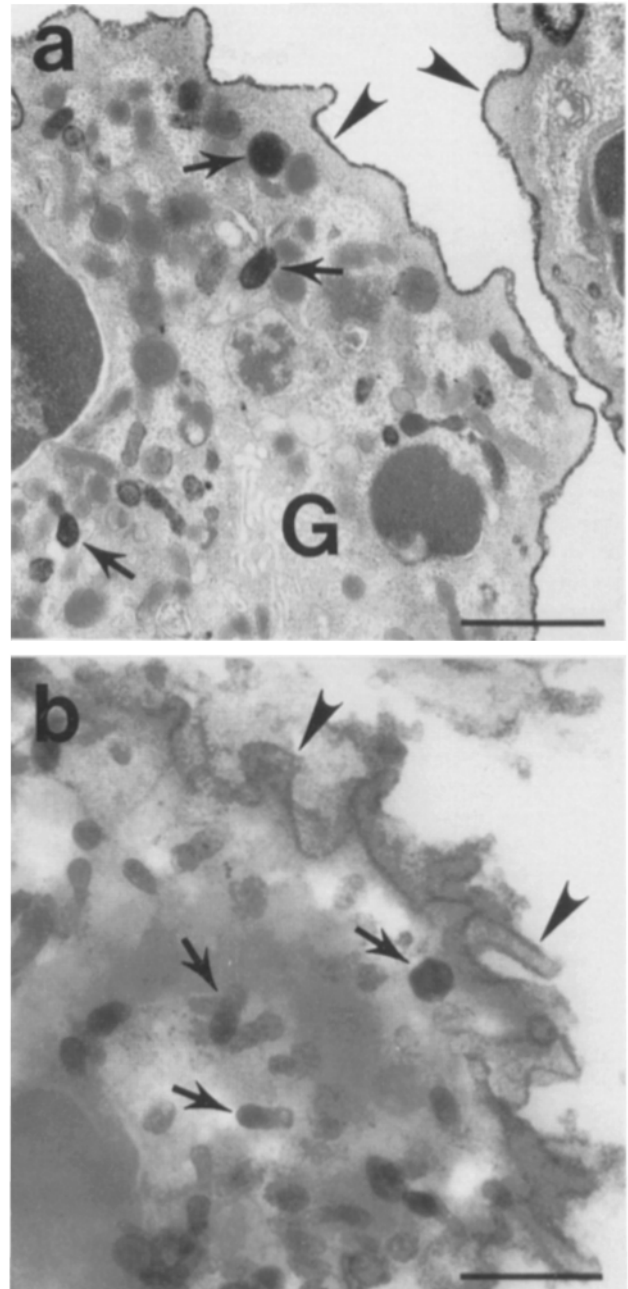
specific granules which typify neutrophils. A much better appreciation of the Alk compartment was obtained when thick sections were examined in the electron microscope. Use of these preparations enables us to carry out a morphometric study on a large number of structures so that a more detailed morphological description of native Alk compartment from unstimulated cells is obtained. This is important



**Figure 9.** Ultrathin cryosection of an unstimulated neutrophil that was incubated for the cytochemical demonstration of AlkPase activity and subsequently for the immunocytochemical localization of lactoferrin. AlkPase activity was detected in the Golgi (**bold arrows**) and in AlkPase-positive structures (**arrowheads**). Colloidal gold particles indicating the localization of lactoferrin were found over another population of granules (**open arrows**). There was no evidence for colocalization of AlkPase and lactoferrin. Bar, 1  $\mu$ m.

in understanding their structure, since they undergo a profound modification upon cell stimulation.

The behavior of the Alk compartment following treatment of neutrophils with an appropriate stimulus (e.g., fMLP; PMA) is very dramatic and unusual. The initial event is an aggregation of Alk compartments in centers of cells. This aggregation is rapid, since it was observed within 1 min after stimulation (this time [1 min] is defined as the interval between addition of a stimulus and initiation of cell fixation). During this same time period the AlkPase-containing compartments increase in length. This increase in length most probably reflects the fusion of individual Alk compartments. During this same time there is an apparent decrease in the number of individual Alk compartments, which is consistent with fusion. The AlkPase-containing compartment becomes further modified with longer times of stimulation. This is particularly evident with fMLP as the stimulus. After 15 min exposure to fMLP the intracellular AlkPase activity is, for



**Figure 10.** Unstimulated guinea pig neutrophils that were incubated for the cytochemical detection of AlkPase. (a) Thin section preparation that illustrates abundant reaction on the cell surface of adjacent cells (**arrowheads**). In addition, intracellular reaction was present in a population of granules (**arrows**), but was lacking in the Golgi (**G**) in mature cells. (b) Thick section preparation showing cell surface (**arrowheads**) and granule (**arrows**) staining. Bars, 1  $\mu$ m.

the most part, restricted to a few elongated tubular structures that are relatively linear in organization. This latter point suggests a close association between these structures and the microtubule system. We are presently conducting experiments to test this hypothesis. PMA-treated cells differ from fMLP-treated cells in that there are more numerous AlkPase-positive vacuoles in the PMA-treated neutrophils. Vacuoles containing cytochemically detectable AlkPase activity could arise by fusion of Alk compartments with PMA-induced phago-

some-like structures or by internalization of plasmalemma containing molecules of AlkPase. Such PMA-induced internalization of neutrophil surface membrane is known to occur (Robinson et al., 1987).

At later times after fMLP stimulation (15 min was the maximum time employed in this study) there is evidence for fusion of these elongated tubular structures and increased amounts of AlkPase on the cell surface proper or in continuity with it. We observed a punctate distribution of cell surface AlkPase in cytochemical experiments. It is not known, at present, if this accurately reflects the distribution of this enzyme on the cell surface or whether it represents a limitation of this technique. It is conceivable that this is the localization pattern on the cell surface; AlkPase belongs to the class of proteins that are anchored to the plasmalemma via covalent linkages to glycosyl-phosphatidylinositol (Low, 1987). It may be that this distribution reflects microheterogeneity in the neutrophil plasmalemma with regard to phosphatidylinositol groups and thus the distribution of AlkPase. Precedence for clustering of glycosyl-phosphatidylinositol proteins within the plasmalemma comes from recent work of Rothberg et al. (1990a) on the membrane receptor for 5-methyltetrahydrofolate. Moreover, they report that the maintenance of this receptor in clusters depends on the presence of cholesterol in the membrane (Rothberg et al., 1990b). It will be of interest to compare this localization of AlkPase to that of other glycosyl-phosphatidylinositol-linked proteins in stimulated human neutrophils.

Biochemical experiments directed toward determining the subcellular distribution of AlkPase are in agreement with the cytochemical results. Measurement of AlkPase in unstimulated cells with a medium lacking Triton X-100 results in detection of only ~30% of the total activity on the cell surface. Inclusion of Triton X-100 in the assay medium leads to the detection of 100% of the activity. These results show that the majority of AlkPase in unstimulated neutrophils is intracellular. On the other hand, when cells are stimulated for 15 min with fMLP and then prepared for measurement of AlkPase activity there is no difference between samples incubated in the presence or absence of Triton X-100. This shows that the intracellular pool of AlkPase has become associated with the cell surface in a stimulus-dependent manner. Our results support those of Borregard et al. (1987) with regard to the distribution of AlkPase in stimulated and unstimulated neutrophils. However, cytochemical results indicate that this is a complex issue since significant amounts of AlkPase can be in continuity with the extracellular space (and thus detectable as a cell surface activity in a biochemical assay), yet not be on the cell surface proper. This is due to the pattern of exocytosis observed in which an elongated tubular structure fuses with the plasmalemma and is in continuity with the extracellular medium even though most of the structure remains inside the cell.

Other biochemical measurements in which cells were treated with DSSA, a nonpenetrating general inhibitor of enzyme function, further support the existence of a large intracellular pool of AlkPase in neutrophils. Unstimulated cells that were incubated with DSSA and assayed for total activity (with Triton X-100) retained ~85% of the total activity, indicating that most of the AlkPase is intracellular and thus not accessible to DSSA. In cells stimulated for 15 min with

fMLP, on the other hand, only ~20% of the total activity remained after DSSA treatment. In this case fMLP stimulation induced upregulation of AlkPase such that it was accessible to the nonpenetrating DSSA and thus inhibitable.

The uniqueness of the Alk compartment and the distinction of these structures from the azurophil and specific granules was demonstrated in several ways. The morphological attributes of Alk compartments as well as their abundance are distinctive from the other granule types. Alk compartments undergo dramatic alteration and become essentially completely continuous with the extracellular space after stimulation with fMLP; this is not the case for specific or azurophil granules. Markers for the Alk compartment (AlkPase), the specific granule (lactoferrin), and the azurophil granule (acid phosphatase) do not colocalize. Mature circulating neutrophils, as used in this study, typically display AlkPase activity in the Golgi complex. The formation of azurophil and specific granules is known to be completed by this stage of neutrophil development. This observation suggests that Alk compartments form late during neutrophil differentiation and may contain residual AlkPase activity in the Golgi in mature cells; alternatively, these structures may continue to form in mature cells. Studies on differentiating bone marrow neutrophils will be required to answer this interesting question.

A major point raised by this study is why the Alk compartment and its unusual behavior in stimulated neutrophils have not been previously documented. As mentioned in the introduction of this paper most investigators employ AlkPase activity as a marker for the plasmalemma in cell fractionation studies. Based on the results of the present study as well as those of Borregard et al. (1987), the usefulness of this activity as a plasma membrane marker for unstimulated neutrophils is highly questionable. The Alk compartments would appear to have the same buoyant density as plasma membrane vesicles during centrifugation and thus are readily separated from the morphologically distinct azurophil and specific granules. Another important consideration is that Alk compartments are particularly sensitive to upregulation; therefore, if cells are partially activated during isolation it may lead to a high cell surface-associated distribution. In any case, alternative markers should be employed for detecting plasma membranes instead of AlkPase.

Cytological and cytochemical studies of AlkPase distribution in human neutrophils have been conducted previously. Bainton et al. (1971) detected AlkPase at the ultrastructural level in the myelocyte stage of neutrophil development but were unable to demonstrate this activity in mature cells. They attributed this activity in myelocytes to the specific granules. Wilson et al. (1983) reported the cytochemical localization of AlkPase in a large tubular structure in human neutrophils which they referred to as the phosphasome. Both of these studies employed lead-based metal capture cytochemical methods rather than the more sensitive cerium-based method as employed in this study. In addition, in the present study we used very mild permeabilization conditions which greatly facilitate the cytochemical localization of intracellular AlkPase. Thus, the methodological improvements used in this study probably account for our ability to identify positively the AlkPase-containing structures. Our morphometric data as well as the analysis of the behavior of the Alk compartment during cell stimulation strongly suggest that the

structure previously referred to as the phosphasome corresponds to the AlkPase-containing structures we observe in stimulated cells (see Fig. 1 in Wilson et al., 1983).

Improved cytochemical methods and the use of thick sections for EM in conjunction with biochemical measurements allow us to demonstrate that human neutrophils contain an intracellular granule pool of AlkPase activity which becomes associated with the cell surface after cell stimulation. Recognition of the existence of this organelle as well as its unusual behavior during cell stimulation is important for a better understanding of neutrophil biology.

The number and types of neutrophil granules have generated much debate over the years (e.g., Brederoo et al., 1983). We propose that the small Alk compartment described in this study be added to the list of neutrophil granule types. The precise role of this organelle is not known at present; however, the rapid and dramatic alterations observed during cell stimulation suggest that this secretory compartment plays an important role in neutrophil function. Perhaps this compartment should be considered as a secretory granule. It is interesting to speculate further on the Alk compartment. As indicated the neutrophil AlkPase is a glycosyl-phosphatidylinositol-linked protein. Furthermore, AlkPase is absent in neutrophils from individuals with paroxysmal nocturnal hemoglobinuria (Burroughs et al., 1988); this is a clinical condition in which abnormal cells lack glycosyl-phosphatidylinositol-linked proteins. The neutrophil contains other glycosyl-phosphatidylinositol-linked proteins which are important in pathophysiological responses mounted by these cells. Two such proteins are Fc- $\gamma$  receptor class III (FcRIII) and complement decay-accelerating factor. Furthermore, paroxysmal nocturnal hemoglobinuria neutrophils lack normal levels of these proteins (Selvaraj et al., 1988). It has also been shown that there are intracellular stores of decay-accelerating factor (Berger and Medof, 1987) and FcRIII (Jost et al., 1990), and that after treatment with fMLP there is increased expression of these proteins on the cell surface (Berger and Medof, 1987; Huizinga, et al., 1988). The site(s) of the intracellular stores for these proteins is not well characterized. It may be that these proteins colocalize with AlkPase and that the Alk compartment is a unique organelle for the intracellular storage of glycosyl-phosphatidylinositol-linked proteins in human neutrophils before their expression on the cell surface. We are presently conducting experiments to test this hypothesis.

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