Subcellular Localization of the *b*-Cytochrome Component of the Human Neutrophil Microbicidal Oxidase: Translocation during Activation

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ABSTRACT We describe a new method for subcellular fractionation of human neutrophils. Neutrophils were disrupted by nitrogen cavitation and the nuclei removed by centrifugation. The postnuclear supernatant was applied on top of a discontinuous Percoll density gradient. Centrifugation for 15 min at 48,000 *g* resulted in complete separation of plasma membranes, azurophil granules, and specific granules. As determined by ultrastructure and the distribution of biochemical markers of these organelles, ~90% of the *b*-cytochrome in unstimulated cells was recovered from the band containing the specific granules and was shown to be in or tightly associated with the membrane. During stimulation of intact neutrophils with phorbol myristate acetate or the ionophore A23187, we observed translocation of 40–75% of the *b*-cytochrome to the plasma membrane. The extent of this translocation closely paralleled release of the specific granule marker, vitamin B_{12}-binding protein. These data indicate that the *b*-cytochrome is in the membrane of the specific granules of unstimulated neutrophils and that stimulus-induced fusion of these granules with the plasma membrane results in a translocation of the cytochrome. Our observations provide a basis for the assembly of the microbicidal oxidase of the human neutrophil.

Neutrophils generate toxic oxygen derivatives such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·) when stimulated by a variety of particulate and soluble stimuli (1–4). These oxygen species are generated through the cyanide-insensitive reduction of oxygen (5, 6) by electrons ultimately originating from oxidation of glucose in the hexose monophosphate shunt (6, 7). They are essential, either alone or in combination with myeloperoxidase and a halide, for optimal function of the neutrophil in the killing of bacteria and fungi (8–11), for lysis of antibody-coated target cells (12–14), and for inactivation of chemotactic factors (15) and of protease inhibitors (16, 17).

The biochemical basis for this reduction of oxygen, known as the respiratory burst, is still a matter of debate. One candidate is an "enzyme" functioning as an NADPH-oxidase (18–20). This enzyme activity can be measured in plasma membrane preparations from activated normal neutrophils, but is not detectable in plasma membranes from resting neutrophils or from activated neutrophils from patients with chronic granulomatous disease (CGD) (21–23). Several lines of evidence support the view that the NADPH-oxidase may consist of an electron transport chain, one link of which is a *b*-type cytochrome (23–26). This cytochrome is present in substantial amounts in normal neutrophils and is reduced when intact neutrophils from normals, but not from CGD patients, are stimulated under anaerobic conditions (27–29). Moreover, the cytochrome is absent from the neutrophils of patients with the most common form of CGD, the classical X-linked type (30).

The subcellular localization of this cytochrome is not yet agreed upon. An association with cytoplasmic granules has been indicated by Shinagawa et al. (24) in rabbit neutrophils and by Sloan et al. (31) in human neutrophils. In contrast, Segal et al. (32) have argued for a plasma membrane localization in human neutrophils based on the observation that all *b*-cytochrome is reduced when dithionite is added to intact

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cells, whereas the intragranular heme protein, myeloperoxidase, is only reduced by dithionite following disruption of the cells. However, subcellular fractionation indicated the presence of cytochrome *b* both in the plasma membrane and in more dense fractions (33). Millard et al. (34) reported a plasma membrane localization of the *b*-cytochrome in rat peritoneal leukocytes, and Gabig et al. (23) found a plasma membrane localization in phorbol myristate acetate (PMA)-stimulated human neutrophils.

Our preliminary studies indicate that the traditional methods of subcellular fractionation, which make use of shear force to disrupt cells followed by either density gradient centrifugation or differential centrifugation, are unsatisfactory because artifacts are introduced by centrifugation on hypertonic sucrose gradients and because separation of the two main types of granules is impossible following differential centrifugation. We circumvented these problems as follows: the cells were disrupted by nitrogen cavitation. This technique has been shown by Klempner et al. (35) to induce minimal granule damage and proteolysis. Centrifugation of the postnuclear supernatant on discontinuous Percoll gradients results in a fast and efficient separation of granules and plasma membrane vesicles.

MATERIALS AND METHODS

Isolation of Neutrophils: 450 ml of blood was withdrawn from healthy donors who gave informed consent. The blood was anticoagulated with 25 mM sodium citrate and mixed with an equal volume of 3% Dextran T-500 (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.9% NaCl to ease the sedimentation of erythrocytes. After 45 min at room temperature, the leukocyte-rich supernatant was siphoned off and the cells were pelleted in plastic tubes by centrifuging at 200 *g* for 10 min. The cell pellets were resuspended in 0.9% NaCl. Mononuclear cells were separated from polymorphonuclear cells and residual erythrocytes by centrifugation through Ficoll-Hypaque (Ficoll; Pharmacia Fine Chemicals) (Hypaque, Winthrop Laboratories, New York, NY) as described by Boyum (36). The resulting granulocyte-erythrocyte pellets were resuspended in ice-cold distilled H_2O for 30 s to lyse the erythrocytes. Isotonicity was restored with an equal volume of 1.8% NaCl. The granulocytes were pelleted at 200 *g* for 6 min; the lysis of erythrocytes was repeated with 0.2% NaCl instead of distilled H_2O. After an additional wash in ice-cold saline, the cells were resuspended either in ice-cold relaxation buffer minus EGTA-100 mM KCl, 3 mM NaCl, 1 mM ATP(Na_2) (Sigma Chemical Co., St. Louis, MO), 3.5 mM $MgCl_2$, 10 mM PIPES, pH 7.3, if cavitation was to follow, or in Krebs Ringer-phosphate-119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.75 mM $CaCl_2$, 15 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, containing 5.5 mM glucose, if the cells were to be stimulated before cavitation. More than 97% of the cells were polymorphonuclear leukocytes and more than 98% excluded trypan blue.

Disruption of Cells: Purified neutrophils, 0.5–1.5 × 10^9 in 20 ml of ice-cold relaxation buffer minus EGTA, were pressurized with N_2 for 20 min at 350 psi with constant stirring in a nitrogen bomb (Parr Instrument Company, Moline, IL) at 4°C (35). The cavitate was then collected dropwise into EGTA, pH 7.4, sufficient for a final concentration of 1.25 mM.

Subcellular Fractionation: Nuclei and unbroken cells were pelleted (P_1) by centrifugation of the cavitate at 500 *g* for 10 min at 4°C. The supernatant (S_1) was decanted and loaded onto gradients precooled to 4°C.

Density Centrifugation on Percoll Gradients: The tonicity of Percoll (Pharmacia Fine Chemicals) was adjusted by adding one-tenth the final volume of a 10 times concentrated relaxation buffer (1,000 mM KCl, 30 mM NaCl, 35 mM $MgCl_2$, 10 mM ATP[$Na]_2$, 12.5 mM EGTA, 100 mM PIPES, pH 6.8). For continuous Percoll gradients, the Percoll was adjusted to density 1.08 g/ml, and 30 ml was centrifuged for 10 min at 20,000 rpm (48,000 *g*) in an SS34 rotor in a Sorvall RC-5B centrifuge (DuPont Co., Wilmington, DE) at 4°C. Thereafter, 8–10 ml of sample, S_1, was applied on top of this preformed gradient and centrifuged for an additional 35 min at 20,000 rpm. For discontinuous Percoll gradients, 14 ml of Percoll, density 1.120 g/ml, was layered under 14 ml of Percoll, density 1.050 g/ml, through a spinal needle. 8–10 ml of sample, S_1, was then applied on top and centrifugation was carried out at 4°C for 15 min at 20,000 rpm in an SS34 rotor. The density of the gradient was estimated from the bands of calibration beads of known density (Pharmacia Fine Chemicals) in gradients run in parallel.

Continuous sucrose gradients were made from sucrose solutions of density 1.112 g/ml and 1.250 g/ml by a gradient mixer. The volume of gradients was 30 ml, and 8 ml of the sample, S_1, was carefully layered on top. The gradients were then centrifuged for 210 min at 25,000 rpm (100,000 *g*) in an SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA).

Fractions of ~1 ml were collected at 4°C by aspiration from the bottom of the gradients through a 50-μl disposable glass pipet attached to a polyethylene tube which was connected to a peristaltic pump. Since the resolution into three distinct and well-separated bands was excellent on the discontinuous Percoll gradients, these bands, referred to as α, β, and γ in order of decreasing density, could alternatively be collected by hand through a pasteur pipette.

Percoll was removed from pooled fractions by spinning at 35,000 rpm (180,000 *g*) for 120 min in an SW41 rotor (Beckman Instruments, Inc.). To prevent trapping of biological material in the Percoll pellet, it is essential that the density of the pooled fractions in the centrifuge tubes is at least as high as that of the biological material. Therefore, Percoll in relaxation buffer, density 1.122, was added to fill and balance the centrifuge tubes. This is particularly important in the preparation of plasma membranes since these fractions near the top of the gradient contain the least amount of Percoll. After centrifugation, the sedimentable biological material was layered directly on a hard-packed pellet of Percoll from which it was easily separated by aspiration.

Disruption of Granules: To separate granule membranes from granule contents, the granules from the β-band were resuspended in 1.5 ml of relaxation buffer and were subjected to freezing and thawing seven times (37). To deplete the membranes of adsorbed proteins, 6 ml of extraction buffer (1 M KCl, 3.5 M urea, 50 mM glycine, 10 mM Na_2HPO_4/NaH_2PO_4, pH 6.8) was added and the sample incubated for 45 min at 4°C (38). Membranes were then pelleted by centrifugation, 45,000 rpm (220,000 *g*) for 90 min at 4°C in an SW50.1 rotor (Beckman Instruments, Inc.). The supernatant was aspirated and the membrane pellet resuspended in relaxation buffer.

Stimulation of Neutrophils: For each experiment, the isolated intact neutrophils from one donor were suspended in 20 ml of Krebs-Ringer phosphate buffer containing 5.5 mM glucose. Half of the cells were incubated with stimulus, either PMA (Sigma Chemical Co.) 5 μg/ml for 20 min, or A23187 (Eli Lilly and Co., Indianapolis, IN), 1 μM for 10 min, at 37°C in a water bath with shaking (90 strokes/min). The incubation was terminated by adding 10 ml of ice-cold Krebs-Ringer phosphate buffer. The cells were then pelleted by centrifugation at 200 *g* for 6 min at 4°C. The supernatant, S_0, was removed for enzyme and spectral analyses and the cell pellet resuspended in 20 ml of ice-cold relaxation buffer minus EGTA and cavitated as described above. The other half of the cells, the control, was treated in exactly the same way except that only solvent, dimethyl sulfoxide (7 mM) for PMA, ethanol (18 mM) for A23187, was added.

Spectroscopy: Absorption spectra from 400 to 600 nm were measured and recorded in the turbid sample compartment of a Perkin-Elmer 576 ST spectrophotometer (Coleman Instruments Division, Oak Brook, IL). Samples of 3 ml were divided into two plastic cuvettes and one, the sample, was reduced by adding solid dithionite (1–2 mg). The spectral scans were repeated following addition of 0.2% Triton X-100 (Fisher Scientific Co., Fair Lawn, NJ) from a stock of 10% to both sample and reference cuvette. Cytochrome *b* was quantitated using an absorption coefficient of the 559-nm peak of 21.6 mM^{-1} cm^{-1} (39). Myeloperoxidase was quantitated using an absorption coefficient for the 472-nm peak of 75 mM^{-1} cm^{-1} (40).

Enzyme Assays: Alkaline phosphatase (EC 3.1.3.1) was assayed with p-nitrophenyl phosphate (Sigma Chemical Co.) 1 mg/ml as substrate in a 1 mM $MgCl_2$, 50 mM sodium barbital buffer, pH 10.5 (41). 50-μl samples were assayed in duplicate. Samples were incubated for 30 min at 37°C in a total volume of 1 ml and the reaction was terminated by addition of 1 ml of ice-cold barbital buffer. Sodium hydroxide, which normally terminates this assay, was found to induce flocculence in Percoll-containing samples. The absorbance at 410 nm was read immediately after the assay was terminated, and the enzyme activity was calculated using an absorption coefficient for p-nitrophenol of 18.6 mM^{-1} cm^{-1} (42). For samples containing Percoll, an identical assay with omission of p-nitrophenyl phosphate was always run in parallel to estimate the light scattering at 410 nm induced by the presence of Percoll. These values were subtracted from the enzyme assay values. 1 U of enzyme liberates 1 μmol product per minute.

β-glucuronidase (EC 3.2.1.31) was assayed in duplicates as described (43) by liberation of phenolphthalein from 1 mM phenolphthalein β-monoglucuronic acid (Sigma Chemical Co.) in 100 mM sodium acetate buffer, pH 4.4, at 37°C for 4 h. The assay was terminated by adding 120 mM glycine, pH 10.5. The activity was calculated using an absorption coefficient for phenolphthalein of 33 mM^{-1} cm^{-1} at 550 nm (44). 1 U of enzyme liberates 1 μmol of substrate per minute. Samples containing Percoll became very turbid during incubation at the low pH but clarified immediately upon addition of the glycine buffer. However, it was found that the enzyme activity in samples to which Percoll

was added was inhibited in proportion to the content of Percoll. We observed a 40% inhibition when Percoll was added to samples to give the same content as that in the α-peak.

Cytochrome *c* oxidase was assayed as described by Cooperstein and Lazarow (45). Cytochrome *c* was purchased from Sigma Chemical Co.

Lysozyme (EC 3.2.1.17) was assayed kinetically by following the decrease in turbidity measured at 450 nm of 0.2 mg/ml *Micrococcus lysodeikticus* (Sigma Chemical Co.) in a 67 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.2, at room temperature (46). Egg white lysozyme (Sigma Chemical Co.) was used as standard.

Vitamin B_{12}-binding protein was measured in duplicates on 25-, 50-, and 100-μl samples essentially as described by Gottlieb et al. (47). To 1.0 ml of saline was added 500 μl ^{57}Co-vitamin B_{12} (New England Nuclear, Boston, MA), 4 ng/ml, sp act 10^5 cpm/ng. The sample was then added and, after mixing, 1 ml of albumin-coated charcoal was added. The samples were centrifuged for 15 min at 1,000 *g* at room temperature and 1 ml of the supernatant was aspirated and counted in a Packard auto-gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) to determine the amount of bound ^{57}Co-B_{12}.

Protein was determined as described by Lowry (48) using bovine serum albumin (Sigma Chemical Co.) as a standard. Addition of Percoll to a concentration comparable to that of the samples had no effect on color development in the standards. DNA was measured as described by Giles and Myers (49) with standard obtained from Sigma Chemical Co.

Electron Microscopy: Electron microscopy was done on samples fixed for 12 hours in relaxation buffer containing 4% formalin, 1% glutaraldehyde. After rinsing, the samples were fixed in 1% osmium tetroxide for 1 h and stained in 0.1% uranyl acetate. After dehydrating by incubating in ethanol, 70%, 95%, and 100%, the samples were covered with propylene oxide, infiltrated in Epon 812 propylene oxide, 1:1, and imbedded in Epon 812. Sections of 400 Å were cut and stained with uranyl acetate and viewed on a Zeiss electron microscope.

RESULTS

The neutrophils were disrupted by nitrogen cavitation, and fractionation of the cavitated sample was accomplished by an initial centrifugation, 500 *g* for 10 min, to pellet nuclei and undisrupted cells, P_1, followed by centrifugation of the supernatant S_1, on Percoll density gradients. Table I shows the distribution after the first centrifugation of markers for nuclei (DNA), azurophil granules (myeloperoxidase, β-glucuronidase [50–52]), specific granules (vitamin B_{12}-binding protein [53, 54]), and plasma membranes (alkaline phosphatase [31, 33, 34, 50, 52, 54]). 5′-AMP-ase was abandoned as a plasma membrane marker since it has been shown to be absent from pure human neutrophils (55, 56). Ouabain-inhibitable-Na^+/K^+-ATP-ase activity, although specific for plasma membranes of human polymorphonuclear leukocytes (31, 35), was also abandoned since only ~6% of the total activity was ouabain-sensitive even in the presence of 0.01% deoxycholate (31, 35, 57). Relaxation buffer, a high-potassium, low-sodium, calcium-free buffer containing MgATP, was designed to mimic cytoplasmic conditions in the neutrophil, based in part upon conditions shown to promote cytoplasmic relaxation in non-muscle contractile systems (58). We found that the use of this buffer resulted in less adherence of granules to nuclei during the first centrifugation and in better separation of organelles during density centrifugation than when Hanks' buffer was used (35) (data not shown).

To determine the densities of the granules and of the plasma membranes, S_1 was centrifuged on a continuous Percoll gradient (see Materials and Methods). This resulted in three visible bands, α, β, and γ, at densities 1.137 g/ml, 1.100 g/ml, and 1.028 g/ml, respectively (mean of three experiments). However, since the gradient formed in this way is steep in the high-density end near the bottom of the centrifuge tube, the spatial separation of the α and β bands was not satisfactory. Therefore, discontinuous Percoll gradients, designed on the basis of the densities of the α and β bands given above, were made as described in Materials and Methods. The sample, S_1, was applied on top of this gradient. As much as 10 ml of material (3.5 mg/ml protein) can safely be applied on a 28-ml gradient. Centrifugation at 48,000 *g* for 15 min at 4°C resulted in the formation of a gradient with a density profile as shown in Fig. 1. The low viscosity of Percoll permitted the organelles to equilibrate at their isopycnic densities, as indicated by the formation of sharp bands (Figs. 2 and 3*a*) at the same densities (Fig. 1) as obtained on the continuous gradient. The mean density of the α band was 1.135 g/ml (range 1.124–1.150 g/ml, $n = 7$); the mean density of the β band was 1.084

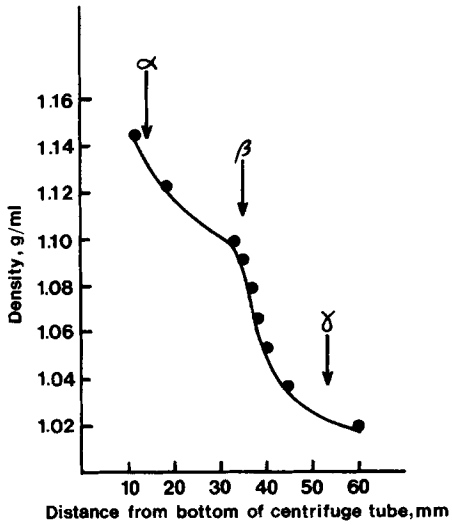

FIGURE 1 Density profile of Percoll gradient. Discontinuous Percoll gradient, initial density 1.050/1.120 g/ml after centrifugation at 48,000 *g* for 15 min. Arrows indicate visually determined positions of the three observed bands (α, β, and γ bands) from neutrophil homogenates (postnuclear supernatants) run in parallel. Dots indicate visually determined positions of the density beads.

TABLE I

Distribution of Markers among Subcellular Fractions from Nitrogen-cavitated Human Neutrophils

	Protein (4)	DNA (2)	Lysozyme (3)	β-Glucuronidase (7)	Vitamin B_{12}-binding protein (7)	Myeloperoxidase (7)	Alkaline phosphatase (5)	Cytochrome *b* (8)
				%				
Cavitate	100	100	100	100	100	100	100	100
P_1	16.4	82.7	13.4	17.4	15.7	18.9	17.6	15.0
	[11–28]	[80–85]	[10–19]	[8–30]	[11–27]	[15–24]	[10–26]	[9–23]
S_1	84.0	12.6	85.5	86.7	89.7	93.7	80.2	86.8
	[75–90]	[11–14]	[81–92]	[67–93]	[70–91]	[86–96]	[74–91]	[81–92]

Results are mean of the number of experiments indicated in parentheses, range indicated in brackets.

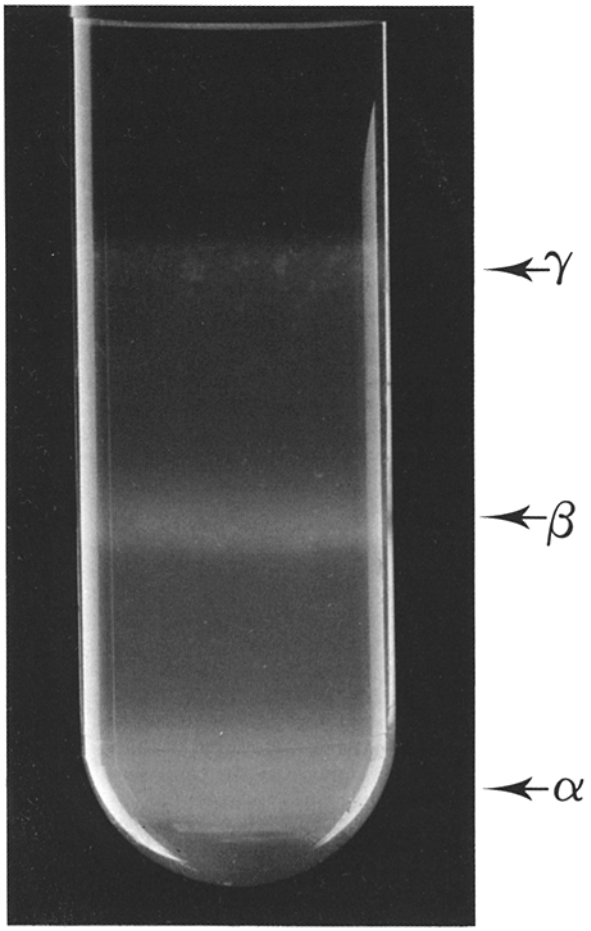

FIGURE 2 Photo of postnuclear cavitate, S_1 after centrifugation on discontinuous Percoll gradients (see Materials and Methods). The α (lowest), β (middle), and γ (highest) bands are readily visible. Magnification, 1.5-fold.

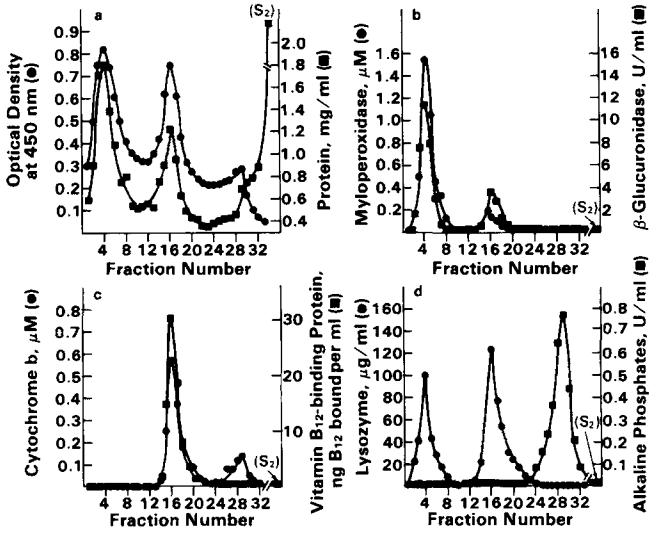

FIGURE 3 Distribution of marker enzymes on discontinuous Percoll gradients. 8 ml of postnuclear supernatant S_1 (protein concentration 3.02 mg/ml) was layered on each of two discontinuous Percoll gradients. Fractions ~1 ml each were collected by aspiration from the bottom of each gradient until only the supernatant, S_2, was left. The fractions from the two gradients were combined and assays performed on these. Spectra, for determination of myeloperoxidase and cytochrome *b*, were obtained in the absence and presence of 0.2% Triton X-100 on samples diluted sixfold with relaxation buffer. Enzyme assays were done in sixfold diluted samples in the presence of 0.2% Triton X-100. S_2 indicates assay on undiluted supernatant from the gradient.

g/ml (range 1.066–1.096, $n = 7$); the mean density of the γ band was 1.026 g/ml (range 1.022–1.029, $n = 7$).

Analysis of the gradients collected in ~1-ml fractions (Fig. 3, *a–d*) identified the α band as azurophil granules by their content of myeloperoxidase and β-glucuronidase with no contamination by markers of specific granules or plasma membranes. The β band was identified as specific granules by the content of vitamin B_{12}-binding protein, with no contamination by plasma membranes, but with some contamination by azurophil granules, as indicated by the presence of 10% of the myeloperoxidase in this peak. Of the β-glucuronidase recovered, ~25% was in the β band; however, this gives an artificially high estimate of the contamination of this band with azurophil granules since the high Percoll concentrations in the fractions containing most of the β-glucuronidase (the α band) markedly inhibited the enzyme assay (see Materials and Methods). The γ band was identified as plasma membranes by the distribution of alkaline phosphatase. The recoveries from gradients of the marker enzymes were as follows: myeloperoxidase 78% (range 71–91%), vitamin B_{12}-binding protein 90% (82–100%), alkaline phosphatase 80% (73–85%), and β-glucuronidase 54% (52–56%). The reason for the apparently lower recovery of β-glucuronidase is given above. Cytochrome *c* oxidase activity was too low to identify the position of mitochondria which are known to be scarce in neutrophils (59).

It is clearly observed from Fig. 3 that the markers for the azurophil granules, specific granules, and plasma membranes closely follow the peaks of light scattering measured as optical density at 450 nm. Therefore, routinely the fractions containing each type of organelle were pooled based on the optical density peaks. The Percoll was removed by centrifuging (see Materials and Methods). The recovery of the marker enzymes was essentially 100% after spinning out the Percoll (data not shown). A suspension of granules from the α band was intensely chartreuse colored in accordance with the azurophils' content of myeloperoxidase. The granules from the β band were beige and the plasma membranes were slightly whitish and opalescent in suspension. Electron micrographs of each fraction (Fig. 4) show the α and β bands to consist of granules bound by a single membrane, each fairly uniform in size and shape, the β granules slightly more electron-dense than the α granules. The γ band was found to contain closed vesicles bound by a single membrane, heterogeneous in size and shape, and empty but for residual filamentous material.

The major peak of cytochrome *b* migrates with the vitamin B_{12}-binding protein, a specific granule marker (Fig. 3*c*). None was found in the azurophil granules and only minimal amounts were in the plasma membrane (mean 11.7% of the total, range 6.9–17.4% in four experiments). The recovery of the *b*-cytochrome from the gradients was 86% (range 75–109%).

Absorption spectra, from 400 to 600 nm, of oxidized versus dithionite-reduced samples of α, β, and γ bands in the presence and absence of 0.2% Triton X-100 (Fig. 5) showed that all of the *b*-cytochrome in the β peak was fully reduced by dithionite in the absence of Triton X-100, but that only 4.5% (mean of eight experiments, range 2.6–6.3%) of the myelo-

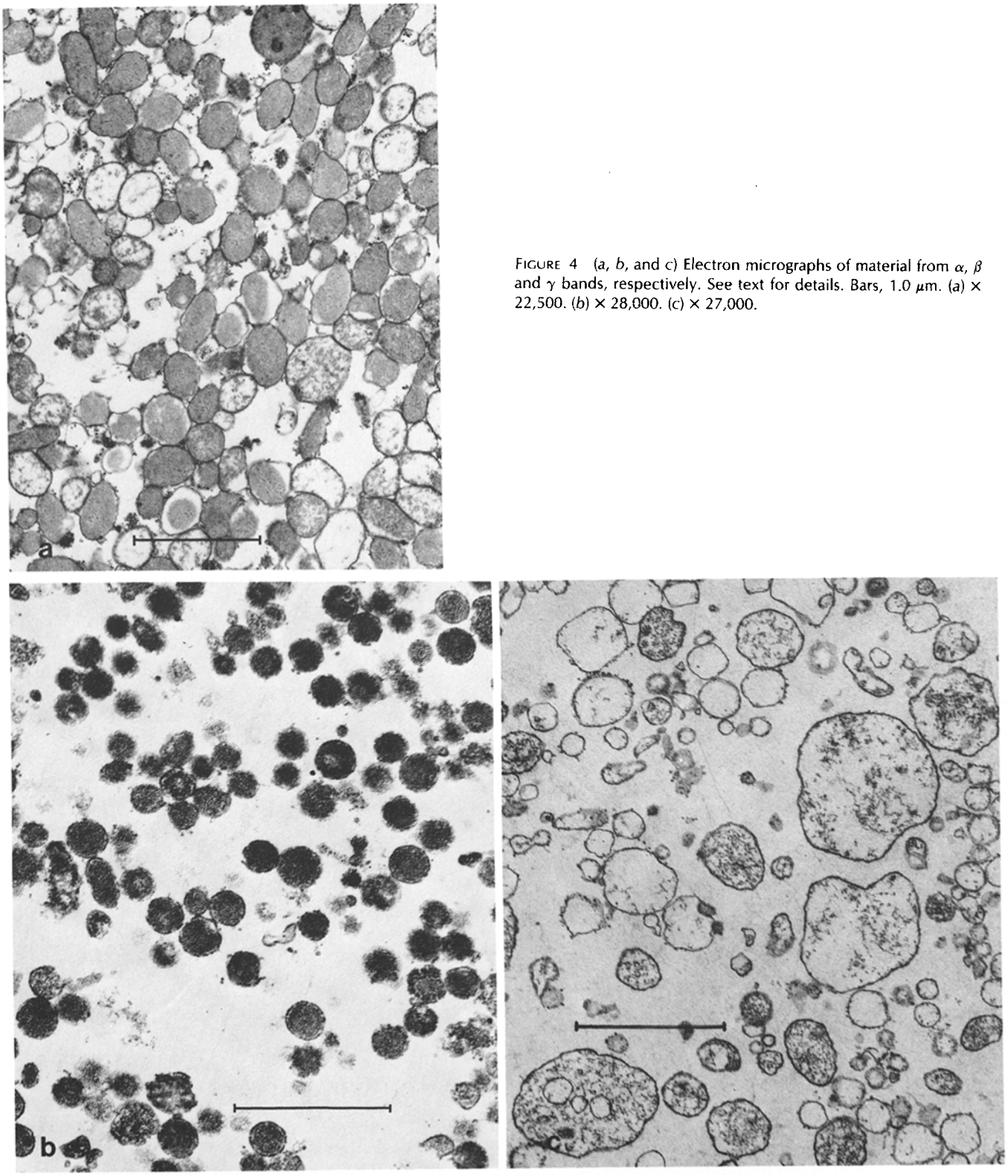

FIGURE 4 (*a*, *b*, and *c*) Electron micrographs of material from α, β and γ bands, respectively. See text for details. Bars, 1.0 μm. (*a*) × 22,500. (*b*) × 28,000. (*c*) × 27,000.

peroxidase in the α band was reduced in the absence of detergent. Spectra of the γ band confirmed that only little cytochrome *b* is present and is fully reduced in the absence of detergent. The resistance of myeloperoxidase in the azurophil granules to dithionite reduction has not been previously observed when subcellular fractionation was carried out on sucrose gradients (33). We also found that in azurophil granules, isolated from a sucrose gradient, the myeloperoxidase was indeed susceptible to dithionite reduction in the absence of Triton X-100 (Fig. 5*d*), thus indicating that granules isolated by our method maintain greater structural integrity.

The immediate susceptibility of the *b*-cytochrome in the β granules to reduction by dithionite indicates, although does not prove, a granule membrane as opposed to an intragranular

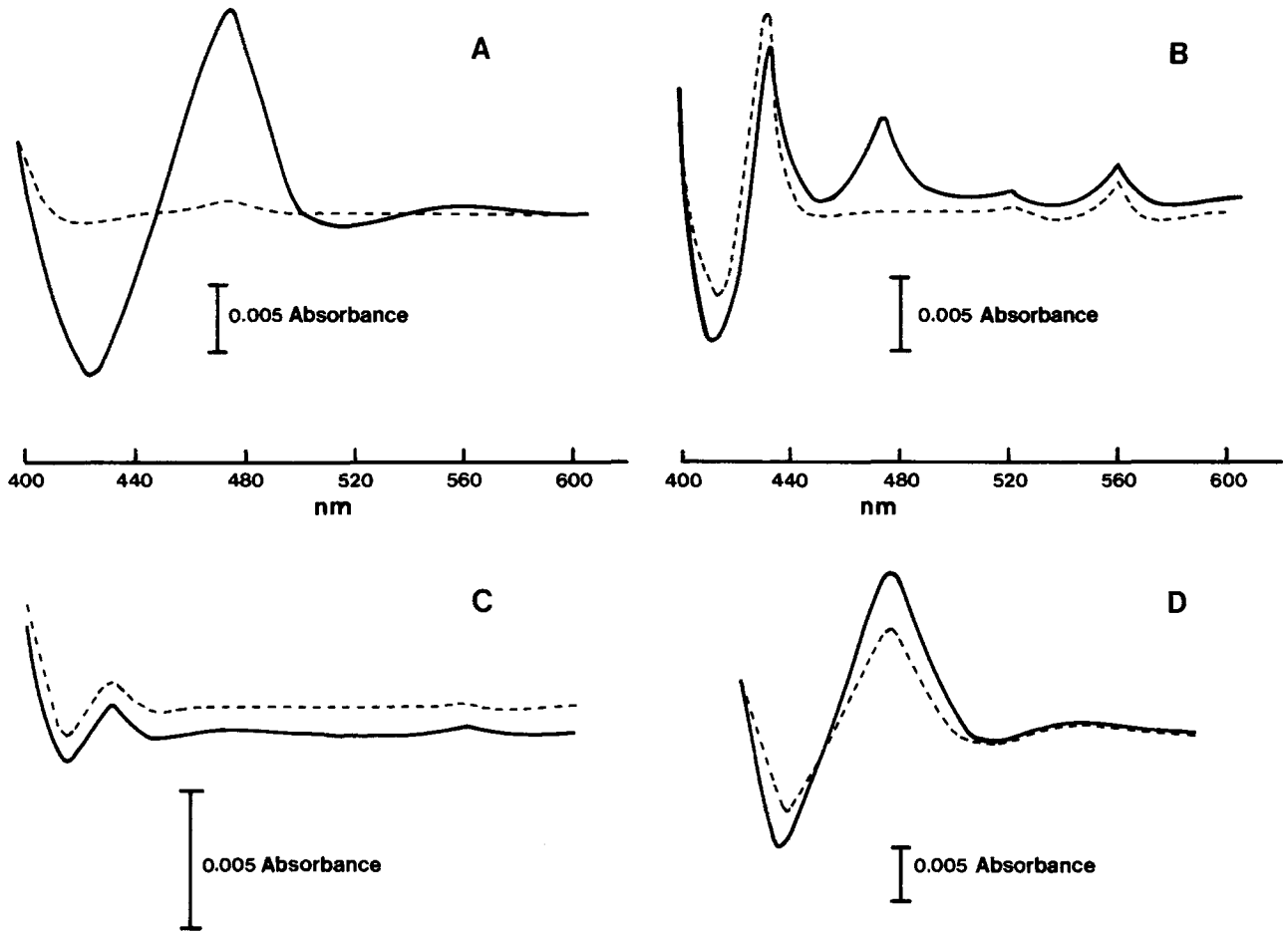

FIGURE 5 Difference spectra of dithionite reduced minus oxidized samples in the absence (broken lines) or presence (solid lines) of 0.2% Triton X-100. *A*, *B* and *C* are spectra of sixfold diluted samples of the α, β, and γ bands, respectively, from a discontinuous Percoll gradient after the Percoll has been removed and the biological material from each band has been resuspended in 2 ml of relaxation buffer. *D* is a spectrum of the fraction containing peak amounts of myeloperoxidase obtained from a continuous sucrose gradient (see Materials and Methods). The fraction, 1 ml, was diluted five times with relaxation buffer and a difference spectrum was obtained under absence and presence of 0.2% Triton X-100. Height of bars indicates 0.005 absorbance.

localization of this cytochrome. To investigate this, β granules were lysed by freezing and thawing seven times followed by incubation in a high ionic strength buffer (see Materials and Methods) to dissociate adsorbed protein from the membranes. The membranes, pelleted by centrifugation (see Materials and Methods), were visibly reddish. All of the vitamin B_{12}-binding protein was found in the supernatant (Table II), indicating complete lysis of the granules, a conclusion that was further supported by electron microscopy, Fig. 6. Spectral analysis of the pellet and supernatant showed that all the *b*-cytochrome was present in the pelleted membranes, whereas all myeloperoxidase from contaminating azurophil granules was in the supernatant. The same distribution was found when the neutrophils had been treated with 5 mM diisopropylfluophosphate for 5 min prior to cavitation to inhibit serin proteases (data not shown).

Since the *b*-cytochrome is located in or tightly associated with the membrane of the β granules, it might be expected to be translocated to the plasma membrane during stimulation of the cells as a result of fusion of granules with the plasma membrane. To investigate this possibility, cells were stimulated with either PMA or the ionophore A23187, both of which are known to induce degranulation primarily of specific

TABLE II
Localization of Cytochrome b within β-Granules

	Cytochrome *b*	Vitamin B_{12}-binding protein	Myeloperoxidase
	(nmol)	*(ng B_{12}-bound)*	*(nmol)*
β-granules	1.114	121.9	0.45
Supernatant of disrupted β-granules	0	93.6	0.39
Pellet of disrupted β-granules	1.06	0	0.01

β-granules, isolated from a discontinuous Percoll gradient, were resuspended in 1.5-ml relaxation buffer. A sample was taken for determination of cytochrome *b*, myeloperoxidase and vitamin B_{12}-binding protein in the presence of 0.2% Triton X-100. The granules were lysed by freezing and thawing and incubated in extraction buffer and centrifuged as described in Materials and Methods. The supernatant and the pellet, resuspended in relaxation buffer, were analyzed for cytochrome *b*, vitamin B_{12}-binding protein and myeloperoxidase in the presence of 0.2% Triton X-100.

granules and activation of the respiratory burst (60–63). Stimulation resulted in a decrease in the height of the β peak and an increase in the γ peak (as determined by the optical density at 450 nm) but no change in the position of the peaks on the

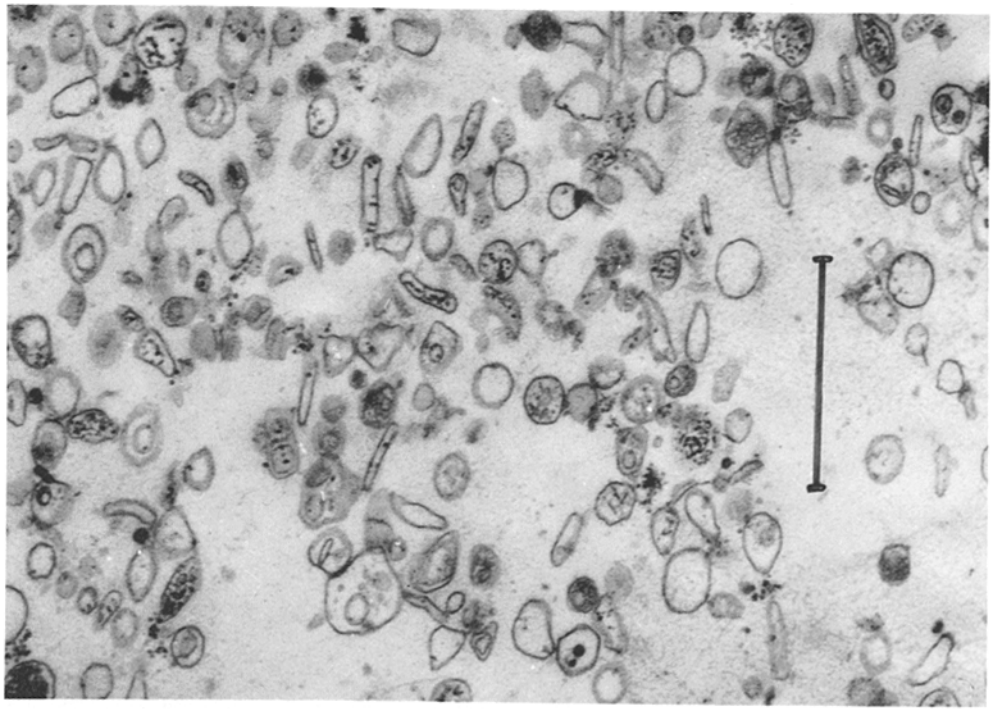

FIGURE 6 Electron micrograph of membranes from lysed and extracted β-granules (see Materials and Methods and legend to Fig. 2). Bar, 1.0 μm. × 30,000.

gradient (data not shown). Stimulation by either PMA or A23187 resulted in liberation of vitamin B_{12}-binding protein from the cells and in a corresponding translocation of the *b*-cytochrome from β granules to the plasma membranes (Fig. 7 and Table III). These data indicate fusion of the cytochrome *b*-containing granule membranes with the plasma membranes. Electron microscopy (Fig. 8) shows that the γ membranes from activated cells are not just a mixture of intact granules and plasma membranes but look like empty vesicles. Recovery of granular enzymes extracellularly during activation has been shown to be an unreliable quantitative measure of degranulation since the enzymes liberated are subject to damage by products of the respiratory burst (64). Therefore, quantitation of enzymes in the remaining granules is a better measure of degranulation during activation. However, especially for activation experiments, an artifact in comparing total amounts of enzyme left in activated cells versus control cells may be induced because activated cells stick to surfaces which may affect their recovery during fractionation. Therefore, in order to correlate the liberation of vitamin B_{12}-binding protein from the β band with disappearance of cytochrome *b* from the β band during activation, the ratio of cytochrome *b* to vitamin B_{12}-binding protein in the β band was calculated in each experiment for activated and control cells. These figures were almost identical for control and activated cells in each experiment, but some interexperimental variation in the determination of vitamin B_{12}-binding protein occurred. To eliminate the effect of this variation, the ratio

$$\frac{\left(\frac{[\text{cytochrome } b \text{ in } \beta \text{ band}]}{[\text{vitamin } B_{12}\text{-binding protein in } \beta \text{ band}]}\right)\text{ activated cells}}{\left(\frac{[\text{cytochrome } b \text{ in } \beta \text{ band}]}{[\text{vitamin } B_{12}\text{-binding protein in } \beta \text{ band}]}\right)\text{ control cells}}$$

from activated cells to control cells was calculated. The fact that this ratio was close to 1.00 shows that the ratio of cytochrome *b* to vitamin B_{12}-binding protein in the β granule was the same in resting cells as in cells that had degranulated up to 85% of their specific granules, providing further evidence that the *b*-cytochrome is in the specific granules.

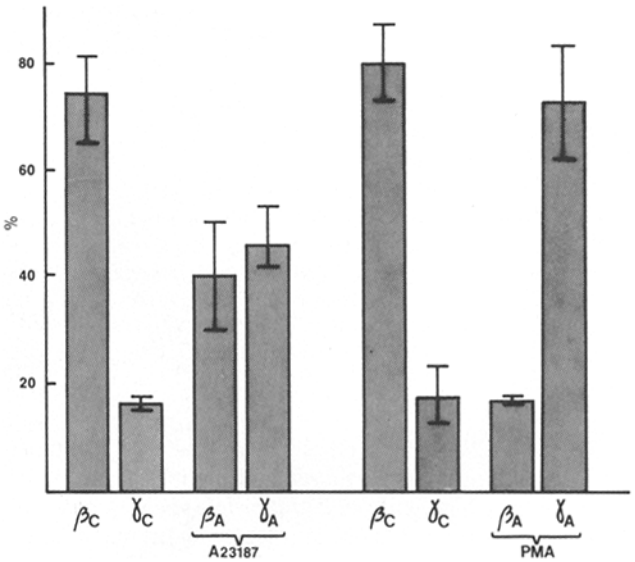

FIGURE 7 Translocation of cytochrome *b* from the β band (specific granules) to the γ band (plasma membranes) upon activation. Two sets of experiments are described in which for each experiment 0.8–1.4 × 10^9 purified neutrophils were divided. One-half of the cells was stimulated by either 1 μM A23187 for 10 min or by 5 μg/ml PMA for 20 min at 37°C. The other half of the cells was treated as a control (see Materials and Methods). Release of cytochrome *b*, β-glucuronidase and vitamin B_{12}-binding protein into the supernatant, S_0, was measured after pelleting the cells. The pelleted cells were cavitated and fractionated on discontinuous Percoll gradients as described in Materials and Methods. The percentage of loaded *b*-cytochrome in the β and γ bands is given. The values are corrected for recovery of alkaline phosphatase in the γ band and B_{12}-binding protein in the β bands. Bars are mean and range of three experiments with A23187 and two experiments with PMA.

TABLE III

Translocation of Cytochrome b during Activation

	Liberation of vitamin B_{12}-binding protein to medium, mean (range)	Liberation of β-glucuronidase to medium, mean (range)	Disappearance of vitamin B_{12}-binding protein from β-band mean (range)	*b*-Cytochrome in γ-band mean (range)	[(Cytochrome *b* in β band/Vit. B_{12}-binding protein) Activated]/[(Cytochrome *b* in β band/Vit. B_{12}-binding protein) Control] mean (range)
	(%)	(%)	(%)	(%)	
A23187 1 μM (3)	33.5 (29–36)	5.0 (4.9–7)	46 (42–50)	45.7 (42–53)	0.95 (0.76–1.07)
Control (3)	2.6 (0–6)	4.7 (4.0–6)		16.4 (14.9–17.4)	
PMA 5 μg/ml (2)	48.0 (37–59)	33.5 (25–42)	85 (84–86)	72.4 (62–82.8)	0.88 (0.76–1.00)
Control (2)	5.2	6.5 (5–8)		17.5 (12.6–23)	

The data were obtained from the experiments described in Fig. 7. Disappearance of vitamin B_{12}-binding protein was determined as ratio of total vitamin B_{12}-binding protein in the β-band of activated cells versus control cells.

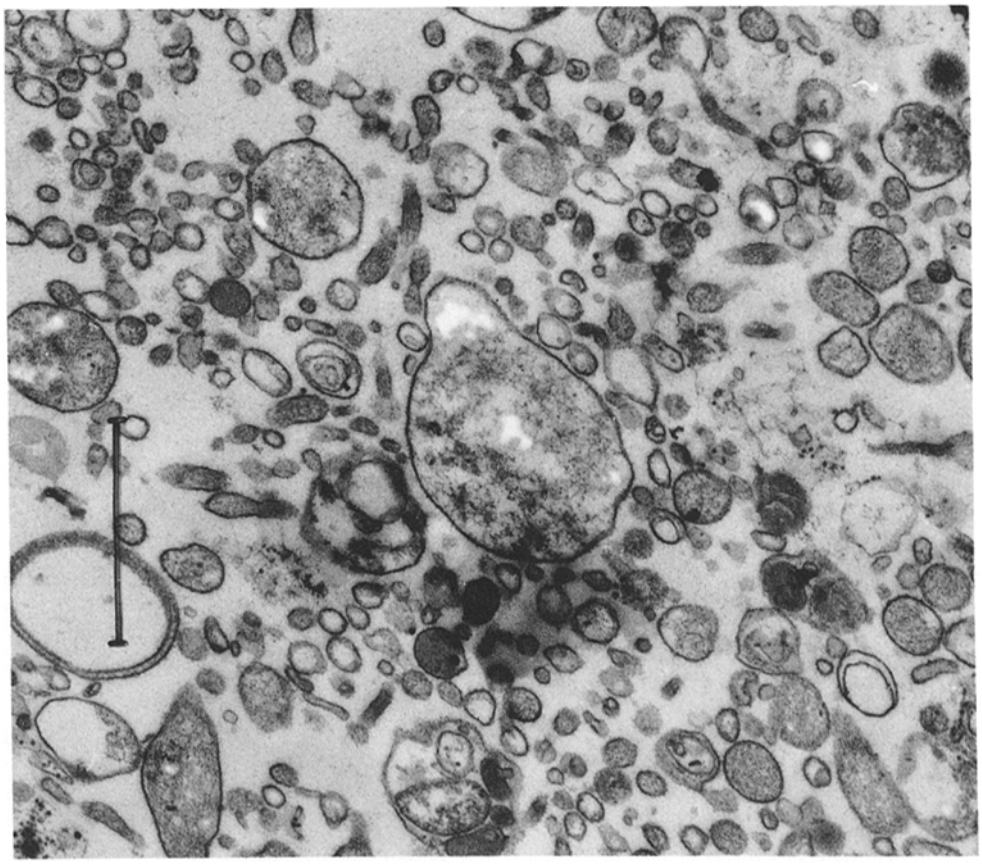

FIGURE 8 Electron micrograph of material from the γ band from cells that have been activated with PMA 5 μg/ml for 20 min (see Materials and Methods). Bar, 1.0 μm. × 30,000.

DISCUSSION

The method described here for subcellular fractionation of human neutrophils by nitrogen cavitation and discontinuous Percoll density gradient centrifugation is rapid, simple, and reproducible. Furthermore, it offers several distinct advantages over traditional methods. First, the use of relaxation buffer circumvents the problem with aggregation of organelles that has forced others to use high concentrations of heparin in the subcellular fractionation of human neutrophils (52). Second, the method appears to result in less damage to the organelles than techniques based on hypertonic sucrose gradients. The indication for this is the difference between Percoll- and sucrose-sedimented azurophil granules on addition of dithionite. The reducing agent readily enters the granules isolated on sucrose gradients and reacts with intragranular myeloperoxidase, whereas reduction of this enzyme within granules isolated on Percoll gradients is detected only in the presence of Triton X-100, suggesting major differences in granule membrane integrity. Third, the densities reported here are significantly less than those reported for the same organelles isolated on sucrose density gradients (52). Such differences between organelles isolated from Percoll and sucrose gradients have been reported from other types of cells (65). This is not unexpected, considering the high osmolarity of the sucrose, which is likely to cause the organelles to shrink. Also, for the plasma membranes, which re-seal into vesicles containing the medium in which the cells are disrupted, the

use of relaxation buffer as opposed to a sucrose-heparin buffer does not artificially increase the density of this structure. In a previous attempt to use colloidal silica for subcellular fractionation of human neutrophils, Olsson (66) reported a higher density for the granules than we find, but in his preparation granules were heavily coated with silica beads, thus increasing their density.

Using our method, we have achieved a clear-cut separation of azurophil granules, specific granules, and plasma membrane vesicles. Each of these populations of organelles was easily detected in the gradients by visual examination, optical density at 450 nm, and assay of marker enzymes.

A major stimulus for developing this method was the desire to determine the localization within the neutrophil of the *b*-cytochrome. We have demonstrated that in the resting neutrophils ~90% of the *b*-cytochrome is associated with the membranes of the specific granules. It is, of course, an open question whether the cytochrome that is present in the plasma membrane, ~10%, is a result of partial degranulation at some step(s) on the route of the neutrophil from the bone marrow to the test tube. This issue will very likely be clarified when an antibody against the *b*-cytochrome becomes available, permitting identification of *b*-cytochrome containing organelles in intact cells, including bone marrow preparations.

In previous reports on the subcellular localization of the *b*-cytochrome, fractionation by differential centrifugation has indicated a granule localization (24, 31). However, when sucrose gradients have been used, most of the *b*-cytochrome in unstimulated cells was found in the plasma membrane (33, 34). The demonstration here, that the cytochrome is in the membrane of the granules in unstimulated cells but translocates to the plasma membrane upon stimulation of the cell, shows the results of the experiments based on sucrose gradients to be artifactual. A further argument for a plasma membrane localization has been the ability of dithionite, when added to intact resting neutrophils, to reduce cytochrome *b* but not myeloperoxidase (32). However, we found that myeloperoxidase within isolated azurophil granules was protected from reduction by dithionite unless a detergent was added. Thus, our data suggest that in intact neutrophils, dithionite penetrates the plasma membrane but does not penetrate the membrane of the azurophil granule, leaving myeloperoxidase unaffected.

The demonstration here that the *b*-cytochrome is in the membrane of granules with the same density as the specific granules does not prove that these granules are the site of the cytochrome since gelatinase-containing (vitamin B_{12}-binding protein-free) granules with the same density as the specific granules have recently been described (54). These gelatinase-containing granules were reported to fuse much more readily than the specific granules with the plasma membrane, particularly in response to stimulation by ionophore A23187 (54). We found that following either PMA or A23187 stimulation, the extent of translocation of cytochrome *b* to the plasma membrane closely parallels release of vitamin B_{12}-binding protein and that a constant ratio of *b*-cytochrome to vitamin B_{12}-binding protein was found in the remaining intact granules of the β band after stimulation by both agents. This close association suggests that cytochrome *b* is in the membrane of the granules that contain vitamin B_{12}-binding protein, i.e., the specific granules. Definite proof of this may await the development of an anticytochrome *b* antibody, which will permit positive morphologic identification of the *b*-cytochrome containing granules.

During formation of phagolysosomes, granules fuse with the plasma membrane at the site of the developing phagosome (67). Thus, our demonstration of the translocation of the *b*-cytochrome to the plasma membrane in response to stimulation provides a mechanism by which the oxidase responsible for H_2O_2-generation is brought into close contact with the targets enclosed in the phagosome. In these experiments, only soluble stimuli have been used to induce fusion of granules with the plasma membrane, but *b*-cytochrome has also been detected in IgG latex containing phagolysosomes isolated by flotation on sucrose (32, 68). Work is in progress to isolate phagolysosomes induced by different particulate stimuli.

We propose that fusion of granule membrane with plasma membrane is an essential step in activation of the neutrophil respiratory burst. The similarity of the kinetics of granule fusion with phagosomes and the respiratory burst (69) is compatible with this concept. If, as accumulating evidence suggests (26, 28, 34, 70), the respiratory burst machinery is a multicomponent system which transfers electrons from pyridine nucleotides to oxygen, membrane fusion would be an ideal mechanism for bringing components from separate sites in the cell together during stimulation and would also provide a safe control to ensure that the oxidase is not activated unless the cell is challenged. Some of these components are very likely in the plasma membrane and others, such as the *b*-cytochrome, are in granule membranes. Definitive proof of this hypothesis will require the identification and localization of all components of the system. Nonetheless, the concept is fully supported by the findings we have reported here and has the appeal of explaining a number of other observations such as the lack of oxidase activity in resting cells (18–20), the rapid appearance of such activity upon stimulation (26, 32, 69), and the apparent plasma membrane site of the oxidase isolated from stimulated neutrophils (23).

We gratefully acknowledge James Fontaine and Dr. Christian Haudenschild for the electron micrographs, Dr. D. L. Taylor for the use of his nitrogen bomb, Dr. Mark Klempner for demonstrating his cavitation technique, and Lisa Houck for the figures.

This work was supported by U.S. Public Health Service grants HL19717 and AI19962 to Robert A. Clark, HL15335 and AM31056 to Elizabeth R. Simons, by a National Research Service Award grant HL06280 to Jeanne M. Heiple, and by grants from Knud Højgaard's Fund and the North Atlantic Treaty Organization to Niels Borregaard.

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Received for publication 6 December 1982, and in revised form 17 March 1983.

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