Neutrophil Plasma Membranes
I. High-Yield Purification of Human Neutrophil Plasma Membrane Vesicles by Nitrogen Cavitation and Differential Centrifugation

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ABSTRACT Neutrophil chemotaxis, phagocytosis, and oxygen-dependent microbicidal activity are initiated by interactions of stimuli with the plasma membrane. However, difficulties in neutrophil plasma membrane isolation have precluded studies on the precise structure or function of this cellular component. In this paper, a method is described for the isolation of representative human neutrophil plasma membrane vesicles, using nitrogen cavitation for cell disruption and a combination of differential centrifugation and equilibrium ultracentrifugation in Dextran gradients for membrane fractionation. Multiple biochemical markers and galactose oxidase-tritiated sodium borohydride surface labeling were employed to follow the yield, purity, and distribution of plasma membranes, nuclei, lysosomes, endoplasmic reticulum, mitochondria, and cytosol. According to these markers, neutrophil plasma membranes were exposed to minimal lysosomal hydrolytic enzymes and could be isolated free of other subcellular organelles. In contrast, disruption of neutrophils by mechanical homogenization resulted in >20% lysosomal rupture and significant plasma membrane proteolysis. Electron microscopy demonstrated that plasma membranes isolated after nitrogen cavitation appeared to be sealed vesicles with striking homogeneity.

Participation of polymorphonuclear leukocytes (PMN) in host defense against infection is dependent on the ability of this cell to respond to its chemical and physical environment. Although the list of "factors" that stimulate or inhibit PMN function is enormous and ever increasing, the cell's repertoire of responses is essentially limited to three general processes: (*a*) locomotion in a random or directed fashion (chemotaxis), (*b*) phagocytosis and killing of ingested material, and (*c*) secretion of lysosomal constituents. A fourth process, namely protein synthesis, may contribute to the above responses as well as lead to the appearance of new proteins (i.e., leukocytic pyrogen [1] or serum amyloid A protein [2]). Each of these major PMN responses is thought to be initiated by an interaction of the appropriate activating factor with the neutrophil plasma membrane. Despite the central role of the plasma membrane in neutrophil function, little information is available on the structural organization and biochemical nature of this cellular component. For example, the structures and dispositions of chemotactic-factor, Fc, and C3b receptors within the membrane are unknown, as are their vicinal relationships. Moreover, the relative contributions of different enzymatic processes of the plasma membrane and intracellular organelles to cell stimulation have been difficult to assess.

The major difficulty in these studies has been in the isolation of purified plasma membranes. Previous studies on PMN plasma membranes have relied on neutrophil homogenization by mechanical shear force. Use of mechanical homogenization is an empirical procedure and depends on the investigators notion of how a plasma membrane should fragment, i.e., large clearances and a few gentle strokes to obtain large plasma membrane fragments. However, even gentle homogenization can contribute to >25% lysosomal disruption and extensive nuclear disruption and can strip off >10% of the outer mitochondrial membrane (3). For cells rich in lysosomes, such as

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PMN, the release of lysosomal hydrolytic enzymes results in serious problems during plasma membrane purification as to the quality of the isolated membranes. Nuclear disruption can lead to poor and possibly unrepresentative yields of plasma membranes because nuclear basic proteins promote adherence of negatively charged surfaces, including nuclear and plasma membranes or lysosomes (4).

In this report, we present a purification scheme for neutrophil plasma membranes. Cell disruption by nitrogen cavitation minimizes lysosomal and nuclear rupture and allows for the purification in high yield of plasma membranes as sealed vesicles.

MATERIALS AND METHODS

Isolation of Neutrophils

Heparinized venous blood (20 U/ml) was obtained from healthy adult volunteers and neutrophils were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) density centrifugation followed by Dextran sedimentation (5). Residual erythrocytes were lysed with three cycles of hypotonic saline and the neutrophils washed twice in Hanks' balanced salt solution and finally suspended at 1×10^7/ml in modified Hanks' balanced salt solution (pH 7.4) containing 2.5 mM $MgCl_2$ but without calcium (disruption buffer).

Membrane Fractionation Procedures

Neutrophils in disruption buffer were placed in the cell disruption bomb (Artisan Industries, Waltham, Mass.) at 4°C as previously described (6). Preliminary studies were performed to define the optimum equilibration pressure and duration of equilibration to achieve a high degree of cell disruption, membrane recovery, and minimal nuclear and lysosome fragmentation. To achieve these goals, cells were equilibrated at 350 psi (pounds per square inch) for 20 min at 4°C. After release from the cavitation bomb, the suspension was collected into an EDTA (trisodium salt) solution so that the final concentration was 2.5 mM EDTA. An aliquot of the disrupted cells was removed to determine nuclear recovery, percent disrupted cells, total protein, enzymes, and DNA. To sediment nuclei and undisrupted cells, the remaining suspension was centrifuged at 1.5×10^4 g_{min} at 4°C and designated pellet 1. The supernate was centrifuged at 4×10^5 g_{min} at 4°C, and the recovered pellet containing lysosomes and mitochondria was designated pellet 2. The remaining supernate was then centrifuged at 6×10^7 g_{min} in a Sorvall ultracentrifuge (Du Pont Co., Sorvall Biomedical Div., Wilmington, Del.) and the pellet containing endoplasmic reticulum and plasma membrane vesicles (pellet 3) was isolated. The supernate containing the disrupted cells cytosol was designated supernate 3. A flow diagram of the above procedure is shown in Fig. 1.

Separation of Plasma Membrane from Endoplasmic Reticulum

Because endoplasmic reticulum cosedimented with the plasma membrane fraction in pellet 3, separation of these two membrane fractions was achieved by centrifugation in polymeric Dextran gradients. Pellet 3 was resuspended and centrifuged at 6×10^7 g_{min} in 10 mM HEPES (pH 7.5) and then in 1 mM HEPES (pH 7.5) to remove soluble cytoplasmic proteins trapped in the plasma membrane vesicles. The final membrane pellet was resuspended in 1 mM HEPES (pH 8.2) plus 0.1 mM $MgCl_2$. Dextran (average mol wt 175,000; Sigma Chemical Co., St. Louis, Mo.) in 1 mM HEPES (pH 8.2) and 0.1 mM $MgCl_2$ was used to prepare the gradients, which consisted of a 3-ml cushion of density 1.09 and 1 ml of continuous gradient with a density range of 1.0–1.09. The membrane material was loaded on the top of the gradient and centrifuged at 10^8 g_{min} at 4°C. Plasma membrane vesicles, which sediment within the continuous gradient were isolated by aspiration, as was the endoplasmic reticulum, which pellets through the Dextran cushion. Each fraction was washed in disruption buffer and assayed for the presence of appropriate markers.

Isolation of Plasma Membranes from Homogenized Neutrophils

In some experiments we compared plasma membrane fractions prepared from cells disrupted by nitrogen cavitation to membranes obtained from cells disrupted by mechanical shear with a Teflon pestle homogenizer as previously described (7). Neutrophils from 150 ml of blood were suspended in 2 ml of a 0.34-M sucrose solution or 2 ml of disruption buffer and homogenized with 45-s bursts (at 1,900 rpm) in a homogenizer (Wheaton Scientific Div., Wheaton Industries, Millville, N.J.). Phase microscopy was used to monitor cell disruption and the homogenization continued until 60–80% of the cells appeared to be ruptured. The homogenate was then centrifuged at 1.5×10^4 g_{min} to remove nuclei and unbroken cells (pellet 1) and the supernate processed as for the nitrogen-cavitated neutrophils to obtain pellets 2 and 3 and supernate 3.

Fate of Surface and Cytoplasmic Markers after Cell Disruption

The isolation of plasma membranes requires quantitative monitoring of the fate of various organelle markers. Phase microscopy and hemocytometer counts were used to determine recovery of nuclei after nitrogen cavitation. The distribution of protein among the pellets and supernates was determined by the method of Lowry et al. (8), with bovine serum albumin as standard.

The following enzyme markers were used to follow subcellular organelles in the fractionation scheme. Lactic dehydrogenase (LDH) activity was assayed by the method of Bergmeyer (9) to monitor percent disruption and the fate of the cytosol. The lysosomal enzymes lysozyme, β-glucuronidase, and myeloperoxidase were assayed to determine the fate of primary and specific granules and to quantitate lysosomal disruption. Lysozyme was measured by the rate of lysis of *Micrococcus lysodeikticus* according to a turbidometric method (10). β-glucuronidase by measuring the release of phenolphthalein from its β-glucuronate after a 6-h incubation at 37°C (pH 4.5) (11), and myeloperoxidase by using *o*-tolidine as substrate in the presence of H_2O_2, with horseradish peroxidase standards (12). Glucose-6-phosphatase activity was measured according to the method of Swanson (13) to follow endoplasmic reticulum and monoamine oxidase according to the method of Wurtman and Axelrod (14) to monitor mitochondria distribution. Because monoamine oxidase is a marker of the outer mitochondrial membrane and intermembrane space, this determination also follows "stripping off" of the outer membrane during disruption. Ouabain-sensitive Na^+,K^+-ATPase activity was determined as described by Wallach and Ullrey (15), and 5′-nucleotidase activity as described by Widnell (16) to monitor plasma membrane recovery and distribution. DNA was measured according to the method of Puzas et al. (17) to determine nuclear recovery and nuclear disruption.

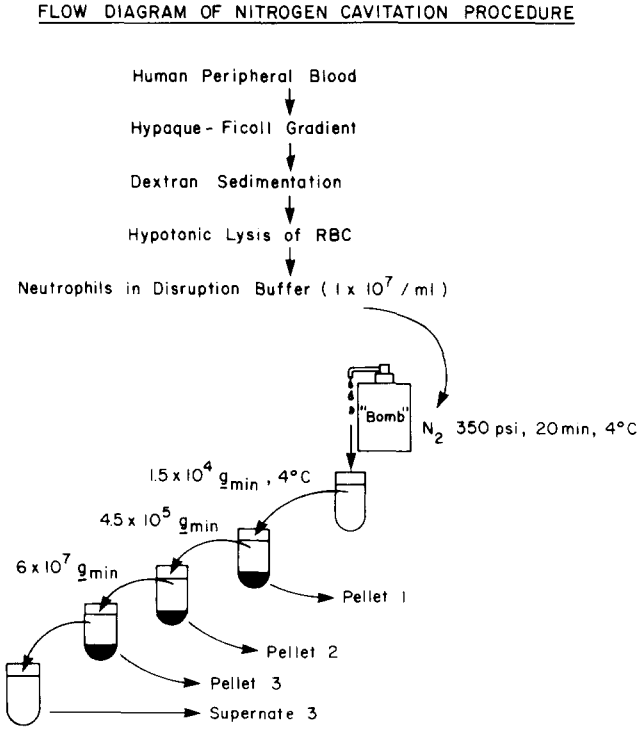

FIGURE 1 Schematic representation of procedure for neutrophil isolation, disruption by nitrogen cavitation, and subcellular fractionation by differential centrifugation.

Surface Labeling of Neutrophil Glycoproteins with Galactose Oxidase and Tritiated Borohydride

The galactose oxidase–tritiated sodium borohydride method (18) was used to surface-label neutrophil glycoproteins and follow their distribution after nitrogen cavitation and subsequent subcellular fractionation. 1 ml of neutrophils (2×10^7) in phosphate-buffered saline (PBS) was incubated with 5 U of galactose oxidase, 87 U/mg, (Worthington Biochemical Co., Freehold, N. J.) for 30 min at 30°C. A second 1-ml aliquot of cells was incubated under identical conditions but without galactose oxidase. The cells were then washed twice in PBS and resuspended in 1 ml of PBS. Enzyme-treated and control cells were then incubated with 0.5 mCi tritiated sodium borohydride (NaB^3H_4, 100 mCi/mmol, New England Nuclear, Boston, Mass.) for 30 min at room temperature and then washed three times in cold PBS. After washing, the cells were resuspended in disruption buffer (1×10^7/ml) and enzyme-treated and control suspensions were added to separate 18-ml aliquots of neutrophils in disruption buffer. Both samples were then nitrogen cavitated and fractionated as described above. A 0.5-ml aliquot of the cavitated-cell suspension or supernate 3 was mixed with 0.5 ml of bovine serum albumin (BSA, 10 mg/ml in disruption buffer) and precipitated with 1.0 ml of 10% ice-cold TCA. Pellets 1, 2, and 3 were resuspended in 1.0 ml of the BSA solution and precipitated with an equal volume of TCA. TCA precipitates were isolated by centrifugation, mixed with 10 ml of scintillation fluid (Aquasol, New England Nuclear) and counted in a liquid scintillation counter (model LS7000, Beckman Instruments Inc., Irvine, Calif.). Enzyme-specific surface labeling was determined by subtracting the radioactivity in untreated cell suspensions from galactose oxidase–treated neutrophils.

Electron Microscopy

As an adjunct method for following subcellular organelles during the fractionation procedure, electron microscopy was employed. Neutrophils were disrupted by nitrogen cavitation and pellets 1, 2, and 3 were isolated and resuspended in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 3°C (19). The samples were then centrifuged (1.5×10^4 g_{min}, 4.5×10^5 g_{min}, and 6×10^7 g_{min} at 4°C for pellets 1, 2, and 3, respectively), and subsequent procedures were carried out without disturbing the pellets. Postfixation was done with 1% osmium tetroxide for 1 h with subsequent dehydration in graded alcohols. Ultrathin sections of Epon-embedded material were mounted on uncoated grids, stained with 0.5% alcohol solution of uranyl acetate for 5 min and counterstained with 0.2% lead citrate. Sections were examined with a Hitachi HU-11E electron microscope (75 kV, objective aperture 50 μm).

SDS-PAGE of Plasma Membranes

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was employed to determine the molecular weight distribution of plasma membrane proteins and to investigate whether plasma membranes obtained by nitrogen cavitation or homogenization contained similar membrane proteins. Neutrophils were disrupted by nitrogen cavitation or homogenization and the plasma membrane fractions isolated as described above. Membrane protein was solubilized in 1% SDS and 10^{-2} M dithiothreitol (DTT) by heating at 100°C for 5 min. Electrophoresis was performed in 10% polyacrylamide slab gels with bromphenol blue as tracking dye. ~100 μg of membrane fraction protein was loaded into each lane and the electrophoresis was carried out at room temperature at 150 V. Molecular weight standards (chymotrypsinogen B, ovalbumin, bovine serum albumin) were run in adjacent lanes. Gels were stained with Coomassie blue and decolorized overnight in 7.5% acetic acid.

RESULTS

Membrane Isolation

To maximize neutrophil plasma membrane yield and minimize disruption of subcellular organelles, the nitrogen equilibration pressure was varied. By phase microscopy, equilibration pressures between 200–300 psi for 20 min at 4°C resulted in disruption of <70% of the neutrophils. Nitrogen equilibration pressures exceeding 400 psi for 20 min at 4°C resulted in virtually 100% cell disruption; however, many nuclear fragments were observed. For this reason, 350 psi was chosen as the optimal N_2 equilibration pressure. Under these conditions (350 psi, 20 min, 4°C) 85.6% of neutrophils were disrupted and, by phase microscopy, we could visually account for 94.3% of nuclei after cavitation. However, unlike other cells, the neutrophil nucleus is polylobed and visualization of a 2- or 3-lobed structure was microscopically scored as a recovered nucleus. By DNA determination and electron microscopy, nuclear material was also recoverable from pellet 2 (see below).

The presence of $MgCl_2$ in the disruption media has been shown to influence nuclear integrity when lymphocytes are disrupted by nitrogen cavitation (20). When 0.1 mM $MgCl_2$ was added to the disruption buffer and neutrophils cavitated at 350 psi for 20 min at 4°C, 99% of the cells were disrupted, however, only 40.4% of intact nuclei were recovered. Increasing the $MgCl_2$ concentration to 1 mM resulted in disruption of 96% of neutrophils and recovery of 47.6% intact nuclei. At 2.5 mM $MgCl_2$, 85.6% of neutrophils were disrupted, with microscopically determined nuclear recovery being 94.3%. Therefore, all subsequent experiments were performed in disruption buffer containing 2.5 mM $MgCl_2$.

Distribution of Protein

As shown in Table I, protein was determined on the subcellular fractions. In six separate experiments the majority of protein (62.6%) was recovered from the soluble fraction, supernate 3. Whereas the membrane fraction (pellet 3) contained 2.5% of the total protein, this represented a contribution from both plasma membranes (77%) and endoplasmic reticulum (23%) (see Table III). In a typical preparation, cavitation of 21.0×10^7 PMN yielded 9.26 mg of total protein distributed as follows: pellet 1, 1.56 mg (16.8%); pellet 2, .88 mg (9.4%); pellet 3, .25 mg (2.7%); and supernate 3, 5.90 mg (63.7%).

Distribution of Biochemical Markers

As shown in Table II, nitrogen cavitation provided a high yield of plasma membranes with minimal subcellular organelle disruption. LDH was used as a marker of the cytosol. Pellet 1 retained 14.6% of the LDH activity, which agreed closely with our light microscope observation that 14.4% of neutrophils were not disrupted under the cavitation conditions. Similarly, virtually all the remaining LDH activity was found in the nonsedimentable supernate (81.2%, supernate 3).

DNA was determined as a measure of nuclear recovery and integrity. Whereas 94.3% of nuclei could be visually accounted for in pellet 1, only 64.6% of the DNA was contained in this pellet. The remainder was found in the lysosomal fraction, pellet 2 (25.5%). Examination of pellet 2 by light and electron microscopy showed that single nuclear lobes with intact nuclear

TABLE I

Protein Recovery from Nitrogen-cavitated Neutrophils

Fraction	Protein recovery
	%
Cavitated sample	100
Pellet 1 (nuclei and undisrupted cells)	15.2
Pellet 2 (lysosomes)	10.2
Pellet 3 (plasma membrane)	2.5
Supernate 3 (cytosol)	62.6
Total recovery	90.5

Distribution of protein in subcellular fractions from nitrogen-cavitated human neutrophils. Mean values from six separate experiments.

membranes were present in this fraction. Nuclear integrity was further documented by the small amount of DNA (0.6%) found in supernate 3. Thus, although nuclei are mostly recovered intact in pellet 1, some nuclear lobes with lower sedimentation velocity purified with the lysosomal fraction. There was virtually no complete nuclear disruption with liberation of DNA into the supernate. These observations underscore the necessity of following biochemical as well as morphologic markers of subcellular organelles in fractionation procedures.

The distribution of lysosomal enzyme markers was of particular interest. Pellet 1 contained an average of 27.0% of the activity for lysozyme, β-glucuronidase, and myeloperoxidase. Although ~15% of this activity could be accounted for by undisrupted cells, the additional 12% activity in this dense fraction was initially puzzling. Because we were aware that even slight loss of nuclear integrity could result in release of basic nuclear proteins and the adherence of negatively charged surfaces such as lysosomes to these nuclei (3), we suspected that some intact lysosomes might adhere to the nuclei and thus sediment with this fraction. Electron microscopy confirmed this suspicion. Of further interest was the very low degree of lysosomal enzyme contamination in the plasma membrane fraction or supernate 3. Unlike the results of homogenization, a mean of 8.4% of lysosomal enzymes appeared in the supernate attesting to the small degree of lysosomal disruption. This is of particular importance for the biochemical characterization of plasma membranes as discussed below.

Mitochondria are sparse in neutrophils and activity of the mitochondrial outer membrane marker, monoamine oxidase was low (one-twentieth of that in thymocytes on a cell-equivalent basis; Mikkelsen, unpublished results). Nevertheless, two points deserve mentioning. First, mitochondria were essentially absent from the plasma membrane fraction (pellet 3), which again is important for interpretation of data on plasma membrane biochemical composition. Also, the disruption procedure did not strip the outer membrane from these organelles, because <1.0% of the monoamine oxidase activity was in the supernate. This important consideration has been confirmed in other cells disrupted by nitrogen cavitation (20).

Because endoplasmic reticulum (ER) sediments with the plasma membrane fraction when lymphocytes are cavitated, it was not surprising to observe that almost 50% of the glucose-6-phosphatase activity was found in pellet 3. However, using equilibrium ultracentrifugation, ER could be separated from plasma membranes on Dextran gradients, because at appropriate divalent cation concentrations and pH, ER are more dense than plasma membranes. When pellet 3 was centrifuged at 10^8 g_{min} over a continuous Dextran gradient with a 3-ml cushion of density 1.09 and 1 ml of continuous gradient with a density range of 1.0–1.09, plasma membrane sedimented within the gradient and ER formed a pellet (Fig. 2). 100% of the glucose-6-phosphatase activity was recovered in the pellet, whereas all the plasma membrane marker (see below) was found within the gradient fraction (Table III).

The plasma membrane marker, ouabain-sensitive Na^+,K^+-ATPase activity, was restricted to pellets 1 and 3. Although 18.2% of the ouabain-sensitive Na^+,K^+-ATPase activity was found in pellet 1, all but 3% of this activity could be attributed to undisrupted cells. Judging by electron microscopy, we suspect that this remaining small percentage represents plasma membrane vesicles adhering to nuclei, similar to the lysosomal data. Virtually all (80.7%) of the remaining ouabain-sensitive Na^+,K^+-ATPase activity was contained in the plasma membrane fraction, pellet 3. It is important to note that the demonstration of Na^+,K^+-ATPase activity required the presence of 0.01% deoxycholate. No ouabain-sensitive ATPase activity

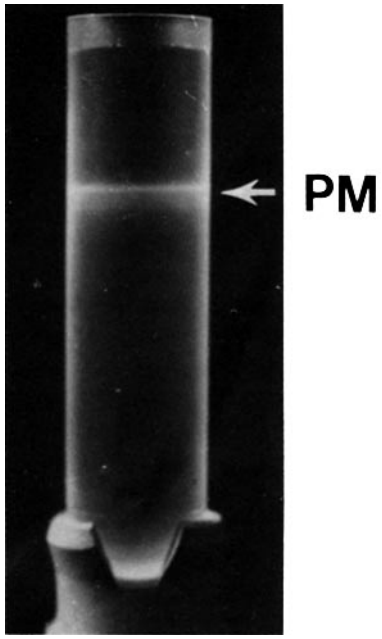

FIGURE 2 Separation of plasma membranes and endoplasmic reticulum on Dextran gradients by equilibrium ultracentrifugation. Plasma membranes (*PM*) sediment within the continuous portion of the gradient (density, 1.00–1.09), whereas endoplasmic reticulum pellets through Dextran cushion (density, 1.09).

TABLE II

Distribution of DNA and Marker Enzymes among Subcellular Fractions from Nitrogen-cavitated Human Neutrophils

Fraction	LDH	DNA	Lysozyme	β-Glucuronidase	Myeloperoxidase	Glucose-6-phosphatase	Monamine oxidase	5′-Nucleotidase	Oubain-sensitive Na^+,K^+-ATPase
					%				
Cavitated sample	100	100	100	100	100	100	100	100	100
Pellet 1	14.6	64.6	30.1	29.5	21.5	21.0	68.0	18.0	18.2
Pellet 2	1.8	25.5	53.7	46.3	72.3	20.0	21.5	6.0	0
Pellet 3	0.8	3.3	2.1	1.1	2.1	60.0	7.0	59.0	80.7
Supernate 3	81.2	0.6	12.7	10.0	2.7	0	0.7	13.0	0
Total recovery	98.4	94.0	98.6	86.9	98.6	101.0	97.2	96.0	98.9

Results are the mean of 18 separate fractionations. See text for assay details.

TABLE III

Dextran Gradient Separation of Plasma Membranes and ER

Fraction	Protein	Glucose-6-phosphatase*			Oubain-sensitive Na^+,K^+-ATPase‡		
		Total activity	Specific activity	Distribution	Total activity	Specific activity	Distribution
	μg			%			%
Pellet 3	54.0	1,350.4	25.0	100	157.0	2.9	100
Gradient fraction	39.5	16.7	0.4	1.2	161.0	4.1	102.5
Gradient pellet	8.0	1,171.2	146.4	86.7	0.0	0.0	0
Recovery	47.5	1,187.9	—	87.9	161.0	—	102.5

* Total activity expressed as micromoles of P_i liberated per hour from glucose-6-phosphate and specific activity expressed as micromoles of P_i per hour per microgram.

‡ Total activity expressed as nanomoles of P_i liberated per hour from ATP and specific activity expressed as nanomoles of P_i per hour per milligram.

TABLE IV

Distribution of ^3H among Subcellular Fractions after Galactose Oxidase and NaB^3H_4 Treatment of Human Neutrophils

Fraction	Total activity	Distribution	Protein	Specific activity
	cpm	%	mg	cpm/mg protein
Cavitated sample	4.56×10^4	100.0	8.20	5.58×10^3
Pellet 1	$.88 \times 10^4$	19.2	1.40	6.28×10^3
Pellet 2	$.36 \times 10^4$	7.8	.80	4.50×10^3
Pellet 3	1.52×10^4	33.2	.19	80.00×10^3
Supernate	$.64 \times 10^4$	13.9	5.04	1.26×10^3
Recovery	3.28×10^4	74.1	7.43	

18.0×10^7 cells were fractionated.

from either undisrupted cells (pellet 1) or plasma membrane vesicles (pellet 3) was measurable in the absence of detergent, implying that the vesicles and cells were impermeable to the labeled ATPase substrate in their native state. 5′-Nucleotidase activity had a similar distribution to ouabain-sensitive Na^+,K^+-ATPase activity. However, as has been shown with muscle (O'Neill and Mikkelsen, unpublished observations) and liver cells, the 5′-nucleotidase activity was only partially membrane bound and a fraction of this enzyme activity appeared in the soluble cytosolic supernate (supernate 3).

Fate of Surface-Labeled Glycoproteins after Nitrogen Cavitation

As shown in Table IV, 47% of the recovered label was found in the plasma membrane fraction pellet 3. Thus almost 50% of the recovered tritiated glycoproteins were concentrated in 2.3% of the recovered protein. The specific activity of the plasma membrane fraction was >10-fold greater than any other subcellular fraction. An additional 26.8% of recovered label was found in the fraction containing nuclei and unbroken cells (pellet 1) and 19.5% of recovered label was contained in the soluble cytoplasmic protein fraction (supernate 3). The recovery of labeled glycoproteins in the cytosolic fraction may have been the result of entrance of galactose oxidase into the cells by pinocytosis or of release of tritiated glycoproteins from the plasma membranes during disruption or fractionation.

Electron Microscopy

Each pellet was minute and could be embedded in three to four blocks of Epon. The blocks were then serially cut and examined in their entirety. Electron micrographs of representative sections from pellets 1, 2, and 3 are shown in Figs. 3, 4, and 5. The nuclei seen in Fig. 3 appeared as polylobed structures with intact nuclear membranes The chromatin distribution was unchanged from that observed in nuclei contained within undisrupted whole cells. Indeed, as mentioned above, there were some undisrupted PMN in pellet 1 whose electron microscopic morphology was unaltered from preparations of isolated PMN not exposed to nitrogen cavitation. In occasional sections, lysosomes were seen that appeared to adhere to the

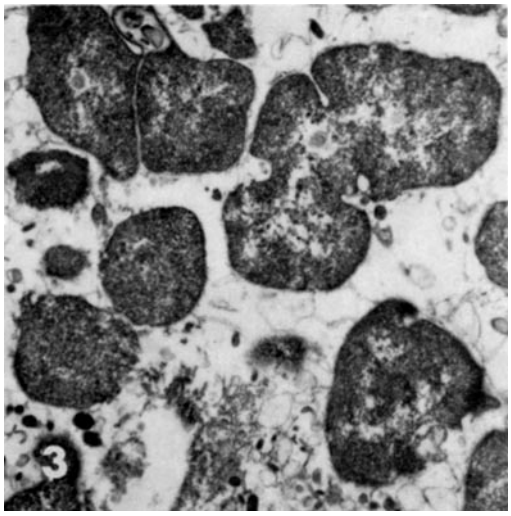

FIGURE 3 Pellet 1. Several nuclei are seen. × 3,000.

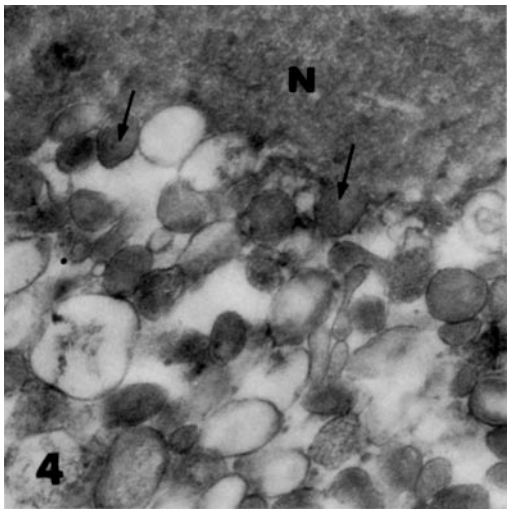

FIGURE 4 Pellet 2. Numerous lysosomes are visible, some of them (arrows) close to a nuclear fragment (*N*). × 35,000.

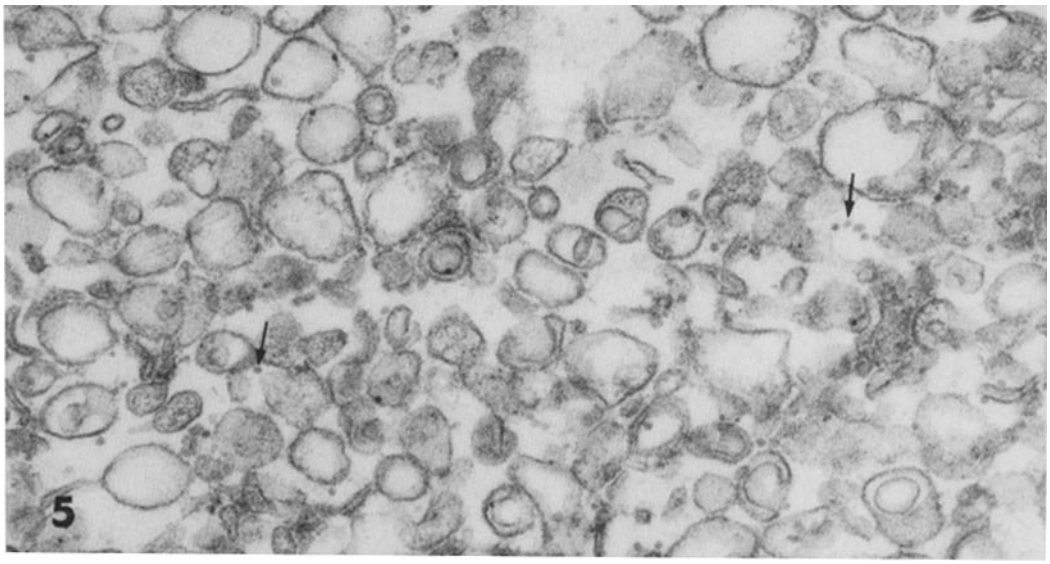

FIGURE 5 Pellet 3. Sealed membrane vesicles of variable size and a few glycogen granules (arrows) are shown. × 44,000.

nuclear outer membrane. This finding was in agreement with our observation that ~10% of the lysosomal enzymes not accountable for from undisrupted cells was present in pellet 1.

Fig. 4 shows an electron micrograph of pellet 2. Typical lysosomes with their electron-dense matrices were the major constituent of this fraction. No attempt was made to further separate the lysosomal fraction into primary and specific granules. As previously noted, single nuclear lobes were also present in this fraction. Lysosomes appeared to adhere to these nuclear lobes. In addition, some vesicles with empty matrices were observed in pellet 2. The origin of these structures was not clear because no ouabain-sensitive Na^+,K^+-ATPase activity was present in this fraction. Similarly, although >20% of the mitochondrial marker, monoamine oxidase, was detected in pellet 2, no clearly identifiable mitochondria were seen in the electron micrographs.

A representative section from pellet 3 is shown in Fig. 5. These plasma membrane vesicles appeared to be sealed and had typical trilamellar membrane structure. Although there was considerable variation in size, probably resulting from transecting the vesicles at various points along their polar axis, the average vesicle measured 1,200–1,800 Å. There was a complete absence of vesicles with electron-dense matrices (lysosomes) in this fraction. In addition to plasma membrane vesicles, multiple small (<100 Å) electron-dense granules were seen in pellet 3. These have the appearance of glycogen granules. Because of their size and the lack of lysosomal enzymes in this fraction, it is unlikely that these represent what has been termed azurophil variants (21).

SDS-PAGE of Neutrophil Plasma Membranes

Using sedimentability at 1×10^8 g_{min} as an operational definition of protein solubilization, >90% of neutrophil membrane proteins were nonsedimentable after treatment with 1% SDS and 10^{-2} M DTT for 5 min at 100°C. There were no differences in the solubilization of plasma membranes obtained by nitrogen cavitation or homogenization.

A representative gel of solubilized membranes obtained by homogenization (lane *A*) and nitrogen cavitation (lane *B*) is shown in Fig. 6. From membranes prepared by nitrogen cavitation, multiple protein bands were observed that were distributed throughout the entire molecular weight spectrum. Gels were highly reproducible from plasma membranes obtained by nitrogen cavitation. In comparison, plasma membranes prepared from homogenized neutrophils had a different gel electrophoresis pattern. The very high molecular weight bands (>100,000) observed in the membranes obtained by nitrogen cavitation were either markedly reduced or absent and there was a prominent low molecular weight protein smear. Additional high molecular weight proteins (≃56,000 and 62,000) were also absent from homogenized neutrophil plasma membranes. Even when homogenization was performed in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (10^{-4} M), there was a diminution of the high molecular weight species. After homogenization and differential centrifugation, the fraction comparable to supernate 3 from the

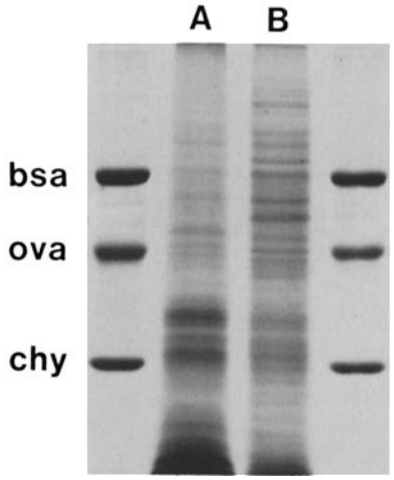

FIGURE 6 SDS-PAGE of neutrophil plasma membranes obtained by homogenization (lane *A*) and nitrogen cavitation (lane *B*). Membranes (100 μg of protein) were solubilized in 1% SDS and 10^{-2} M DTT and run in 10% polyacrylamide gels at 150 V. Standards: bovine serum albumin (*bsa*, 68,000 daltons), ovalbumin (*ova*, 45,000 daltons) and chymotrypsinogen B (*chy*, 25,000 daltons) were run in the adjacent lanes.

nitrogen-cavitated material contained 22% of the total lysosomal enzyme activity.

DISCUSSION

To investigate the role of the neutrophil plasma membrane in cellular function, a major goal of our studies has been to obtain a preparation, without other cellular organelles, of neutrophil plasma membranes that retain structural and functional integrity. The procedure we have chosen, namely nitrogen cavitation, has been extensively used to isolate and characterize plasma membranes from erythrocytes (22), lymphocytes (20), and fibroblasts (23) and also to obtain a preparation enriched for plasma membranes from rabbit peritoneal exudate neutrophils (24).

A critical facet of cell fractionation is the identification of subcellular organelles. Although electron microscopy is helpful, it is not sufficiently quantitative and most investigators employ biochemical markers that, presumably, unambiguously identify a specific cell component. There is little controversy regarding appropriate markers for the nucleus (DNA), cytosol (LDH), lysosomes (e.g., lysozyme), ER (glucose-6-phosphatase), and mitochondria (monoamine oxidase). For the plasma membrane, 5′-nucleotidase is apparently restricted to plasma membranes of guinea pig neutrophils (25) but can be cytochemically identified in primary granules of rabbit neutrophils (26). Although there have been reports of the absence of this enzyme in human neutrophils (27), we detected 5′-nucleotidase activity in both plasma membranes and the cytosol. Thus we believe that this is not the best marker for human neutrophil plasma membranes. Cation-dependent ATPases are widely distributed in biologic membranes and both Na^+,K^+-ATPase and Mg^+-ATPase have been found in rabbit (28) and human neutrophils (29). Because ouabain-inhibitable Na^+,K^+-ATPase is apparently restricted to a fraction enriched for rabbit neutrophil plasma membranes, we chose this as our marker and confirmed its localization to human neutrophil plasma membranes.

A significant problem that confronts all investigations involving plasma membrane isolation is membrane "sidedness." In the erythrocyte, osmotic changes can result in inside-out plasma membrane vesicles that can confuse interpretation of membrane function and structural vicinal relationships (30). It is noteworthy that we were unable to demonstrate ouabain-sensitive Na^+,K^+-ATPase activity in plasma membrane vesicles or whole neutrophils unless conditions were appropriate for substrate (ATP) entry into the plasma membrane interior (i.e., deoxycholate). Similar results have been reported by Becker et al. for a nonhomogeneous plasma membrane preparation. Not only does this suggest that the vesicles are right side out but also suggests that the membranes are not permeable to large molecules.

The use of organelle-specific labeling techniques has been widely used to follow subcellular organelles during fractionation schemes. For studies of plasma membranes, lactoperoxidase-catalyzed iodination has been the most commonly used method. However, as has been recently pointed out (31), neutrophils present particular problems for labeling by peroxide-dependent halogenization reactions because they contain lysosomal myeloperoxidase, membrane-associated oxidases, and concentrate extracellular iodine. Because of these intrinsic problems with iodination, we used labeling of surface glycoproteins with tritiated sodium borohydride after galactose oxidation treatment as previously reported. Using this method, 33.2% of the total label was incorporated into the plasma membrane fraction with a 15- to 60-fold increase in specific activity of plasma membranes compared to other subcellular fractions.

In summary, we have isolated an essentially homogeneous preparation of neutrophil plasma membranes in high yield that are only minimally exposed to lysosomal hydrolytic enzymes and retain structural and biochemical integrity. These membrane vesicles appear to be sealed and with a right-side-out orientation. This preparation should be useful in the further characterization of neutrophil membrane receptor structure and vicinal relationships and will permit studies of the events related to neutrophil "activation" in the normal and abnormal neutrophil.

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