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Edward P. Sloan
Dartmouth College

Dana R. Crawford
Dartmouth College

Donald L. Schneider
Dartmouth College

ISOLATION OF PLASMA MEMBRANE FROM HUMAN NEUTROPHILS AND DETERMINATION OF CYTOCHROME *b* AND QUINONE CONTENT*

By EDWARD P. SLOAN,‡ DANA R. CRAWFORD,§ and DONALD L. SCHNEIDER

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755

Neutrophils kill invading microbes with lethal concentrations of oxygen metabolites. Generation of these metabolites is accompanied by a cyanide-insensitive oxygen burst during which the rate of oxygen consumption increases up to 20-fold (1). The mechanism by which consumed oxygen is transformed into lethal concentrations of the oxygen metabolites, superoxide anion (O_2^-),[1] H_2O_2, and $\cdot OH$, is of current interest (2). On the basis of the preliminary studies, we have proposed that an electron-transport chain in the neutrophil is responsible for oxygen consumption (3). We suggested that the electron-transport chain may exist as two partial chains in resting neutrophils, one in the plasma membrane and the other in one of the cytoplasmic granule membranes. As phagocytosis occurs, membrane fusion and formation of the phagolysosome makes possible an association between the plasma and granule membranes. Such a combination may bring together partial chains and allow respiration to occur.

There are several candidates for components of such an electron transport chain: NAD(P)H dehydrogenase, cytochrome *b*, non-heme iron protein, and quinone. Although flavin-dependent NADH- and NADPH-oxidases have been recognized activities in leukocytes for nearly 20 years, it is as yet uncertain which is of primary importance in the respiratory burst and whether they act as dehydrogenases in the initial region of an electron transport chain or as functional oxidases independent of other oxido-reduction components (2). A novel *b*-type cytochrome has been found in horse (4) and rabbit neutrophils (5), and more recently in human (6, 7) and rat (3) neutrophils. The cytochrome has been shown to exist mainly in the plasma membrane purified from rat neutrophils. Quinone has also been found in the rat neutrophil (3), but its subcellular localization is as yet unclear. Quinone conceivably facilitates completion of the chain by acting, perhaps, as a mobile electron shuttle between the two partial chains.

This paper reports on the characteristics of plasma membrane isolated from human neutrophils that may be relevant to their microbicidal activity. It is shown that

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[1] *Abbreviations used in this paper:* E, extract (fraction); MLP, membrane (fraction); N, nuclear (fraction); NAβGase, glucosaminidase; O_2^-, superoxide anion; P, microsomal (fraction); S, soluble (fraction); TVS, 50 mM Tris-SO_4, pH 8; 200 mM NaCl; 1 mM EDTA.

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cytoplasmic granule membrane, not the plasma membrane, is the primary location of both cytochrome *b* and quinone.

Materials and Methods

Fresh blood was obtained from healthy donors or from donors who required therapeutic bleeding for treatment of hemachromatosis or polycythemia. Regardless of the source of the blood, the neutrophils obtained behaved similarly in every apparent way. Chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Preparation of Neutrophils. Leukocyte suspensions were prepared from whole blood after sedimentation of the erythrocytes in dextran as described (8), with slight modification of the procedure. The leukocyte-rich supernate was centrifuged at 800 *g* for 10 min to pellet the neutrophils. This pellet was resuspended in saline and exposed to 35 mM NaCl for 25 s to lyse any erythrocytes. Isotonicity was then immediately restored by addition of an appropriate volume of 615 mM NaCl. This suspension was centrifuged at 800 *g* for 5 min. The resultant pellet was resuspended in 50 ml of saline and centrifuged at 800 *g* for 5 min. Finally, the lysis and saline wash steps were repeated.

Preparation of Cell Fractions. Neutrophils were first homogenized and separated into nuclear (N)[1] and extract (E) fractions. This procedure involved exposing the neutrophils to 40 mM sucrose for 5 min, which is essentially the hypoosmotic procedure of DePierre and Karnovsky (9). The suspension was then homogenized 10 times in a Dounce tissue grinder with a tight-fitting A pestle. Isoosmolarity was restored by addition of an appropriate volume of chilled 2 M sucrose. Centrifugation at 800 *g* for 5 min separated the cell homogenate into a crude nuclear pellet and a partial extract. The above homogenization was repeated on the crude pellet with isotonicity being restored this time through addition of 1.45 M NaCl. After repeating the 800 g, 5-min spin, both supernates were combined as the cell E fraction.

The cell E fraction was then separated into membrane (MLP) and soluble (S) fractions by centrifugation at 35,000 rpm (g_{max} = 110,000) for 35 min in a Spinco 50 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The MLP fraction pellet was resuspended in TVS (50 mM Tris-SO_4, pH 8; 200 mM NaCl; 1 mM EDTA) and recentrifuged. The final membrane pellet was suspended in 75 mM NaCl and 125 mM sucrose.

Subfractionation of the MLP Fraction. A 25-ml linear sucrose gradient (20–50%; wt:wt) was layered above a 4-ml cushion of 60% sucrose. The MLP fraction was layered onto the gradient and immediately centrifuged in a Spinco SW27 rotor at 24,000 rpm (g_{max} = 110,000) for 3 h at 4°C. These conditions were sufficient to allow the organelles to attain their equilibrium density.

Fractions highest in 5′-AMPase and ouabain-sensitive, Na^+,K^+-ATPase activity were combined. The density range of these plasma membrane-enriched fractions was 1.08–1.12 gm/ml. The granules went farther into the gradient, for example, glucosaminidase equilibrated at densities of 1.16–1.18. After addition of two volumes of TVS, the combined sample was centrifuged in a Spinco 50 Ti rotor at 35,000 rpm (g_{max} = 110,000) for 35 min at 4°C. The pelleted plasma membranes were finally resuspended in a 75 mM NaCl, 125 mM sucrose solution.

Digitonin Shift Determination. A digitonin shift of the neutrophil plasma membrane was conducted as described (10). Digitonin was added to the membrane sample (0.15 mg digitonin/1 mg of membrane protein), mixed, and allowed to incubate for 5 min at room temperature. This treated plasma membrane was layered onto a 20–50% (wt:wt) sucrose gradient and centrifuged as described above.

b-Type Cytochrome Determination. To minimize the possibility of adsorbed hemoglobin interfering in these determinations, frozen neutrophils were washed with two volumes of TVS and centrifuged at 35,000 rpm (g_{max} = 110,000) for 35 min at 4°C in a Spinco 50 Ti rotor. After resuspension in TVS, the pelleted cells were homogenized in a Dounce tissue grinder with a tight-fitting A pestle and then recentrifuged. This second pellet was resuspended in 250 mM sucrose and centrifugation repeated a third time. The final total membrane preparation was suspended in 250 mM sucrose.

The total membrane preparation was analyzed in a solution containing 0.4% Triton X-100, 0.1% cholate, and 0.1 M potassium phosphate buffer at pH 7. The protein concentration was

2.5 mg/ml. Reduced vs. oxidized difference spectra were obtained with a Cary 118 spectrophotometer after addition of sodium dithionite and potassium ferricyanide, respectively. *b*-type cytochrome concentrations were calculated from the absorbance maximum at 558 nm using a millimolar extinction coefficient of 28.5 (11). Digitonin-shifted plasma membrane was analyzed as described above in a 0.1 M potassium phosphate solution. Plasma membrane protein concentration was 0.56 mg/ml.

Quinone Determination. Hexane-soluble quinones were extracted from either whole neutrophils or membrane preparations and quantitated by their ultraviolet absorption spectra as described (12).

Marker Enzyme Assays. 5′-Nucleotidase (5′-AMPase) activity was measured as described (13). Phosphate was analyzed by the ammonium molybdate-ascorbic acid method (14). Ouabain-sensitive, Na^+,K^+-ATPase activity was measured as described (15); 0.1 mM ouabain was used. Glucosaminidase (NAβGase) activity was measured using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate (16). Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate (17). Total ATPase activity was determined using Mg_2ATP as substrate with conditions similar to those of the ouabain-sensitive ATPase assay, except that ouabain was excluded. Protein was measured as described (18). Trichloroacetic acid precipitation of protein was performed twice on sucrose gradient fractions. The density of sucrose fractions was determined using a Fisher refractometer (Fisher Scientific Co., Pittsburgh, Pa.).

Results

Neutrophil Cell Preparation. In the preparation, 50–80% of the whole blood neutrophils were isolated. Each unit of blood (480 ml) yielded $\sim 5 \times 10^8$ cells and 100 mg of cell protein. Antibody to IgM was employed to detect the possible presence of lymphocytes. Using Ouchterlony plates, no cross-reaction was observed between the antibody and the neutrophil sample solubilized with Triton X-100. Large and distinct immunoprecipitation lines did appear when this same antibody was exposed to the discarded supernates from the neutrophil preparation. Therefore, lymphocyte contamination seems unlikely. With Wright's stain, microscopic analysis of the neutrophil preparation indicated that purity was at least 90%.

Neutrophil Characteristics. Enzyme levels of the neutrophils were consistent with data reported for 5′-nucleotidase (7), ouabain-sensitive ATPase (19), and alkaline phosphatase (7).

A novel *b*-type cytochrome with an alpha band at $A_{558\,nm}$, which has been previously reported in the human neutrophil (4–7), was observed using dithionite difference spectroscopy (Fig. 1, Table I). The neutrophil contains 60 pmol cytochrome/mg of protein. No other cytochromes were evident (Fig. 1); neutrophils are very deficient in mitochondria and contain extremely low levels of the mitochondrial marker enzyme, cytochrome oxidase.

Quinone was observed to be seven times more concentrated in the cell than the *b*-type cytochrome (Fig. 2, Table I). A quinone concentration of 430 pmol/mg of protein in the human neutrophil, a mitochondria-deficient cell, is consistent with the idea that quinone is a necessary component of the electron-transport system that produces lethal oxygen metabolites.

Neutrophil Plasma Membrane Enhancement. Enhancement of the plasma membrane is necessary for quantitative characterization. This procedure utilizing differential and gradient centrifugation resulted in purification of plasma membrane of suitable quality. The membrane fraction obtained by differential centrifugation contained only 17% of the cell protein, and >80% of the 5′-AMPase activity; thus, it was five times more pure than the cell homogenate. Twice in the purification procedure, a

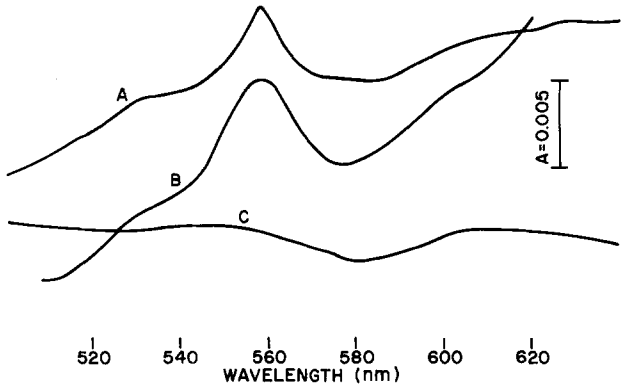

FIG. 1. *b*-Type cytochrome spectra of human neutrophils. Reduced vs. oxidized difference spectra were obtained after addition of sodium dithionite and potassium ferricyanide, respectively, to samples in matched cuvettes. (A) Plasma membrane protein equalled 0.56 mg/ml. (B) Neutrophil total membrane contained 0.5% Triton X-100 and 1.0% (wt:wt) cholate; protein equalled 2.5 mg/ml. (C) Reduced vs. oxidized buffer blanks. Peak absorbance occurred at $A_{558\ nm}$.

TABLE I

b-Type Cytochrome and Quinone Content of Human Neutrophil Fractions

Fraction	*b*-Type cytochrome	Quinone
	pmol/mg protein	
Total membrane fraction (NMLP)	60	430
Plasma membrane fraction	205 (3.4x)	740 (1.7x)

Cell fractions were prepared and analyzed as described in Materials and Methods. Total NMLP values were divided by total cell protein to include soluble proteins lost during preparation of the NMLP fraction. Millimolar extinction coefficients were assumed to be 28.5 for cytochrome *b* (11) and 12.1 for quinone (12).

buffered solution high in salt and EDTA was used to wash the membrane sample. These washes removed substantial amounts of protein, such that plasma membrane enrichment increased another 1.5 times.

Equilibrium centrifugation on a continuous sucrose gradient resolved the plasma membrane into a clear band which showed peak 5′-AMPase activity at a density of 1.12 gm/ml (Fig. 3). A modal density of 1.11 gm/ml has previously been reported for human neutrophil 5′-AMPase activity (21). Usually 50% of the 5′-AMPase activity was located in the density range of 1.08–1.12 gm/ml. Because only 25% of the membrane protein was present in these fractions, this procedure yielded a twofold enrichment in marker enzyme activity. The overall enrichment is therefore about fifteenfold.

Marker Enzymes. 5′-AMPase activity was used as a neutrophil plasma membrane marker. Data supportive of plasma membrane localization of 5′-AMPase include: (*a*) Up to 80% of the 5′-AMPase activity follows the initial MLP fraction with up to sevenfold enrichment in enzyme purity; (*b*) 5′-AMPase activity clearly occurs in a single peak at a low density. This points to localization in a low density organelle, such as the plasma membrane; (*c*) addition of digitonin to these 5′-AMPase-enriched fractions resulted in a shift of the 5′-AMPase activity to a greater density (Fig. 4). A

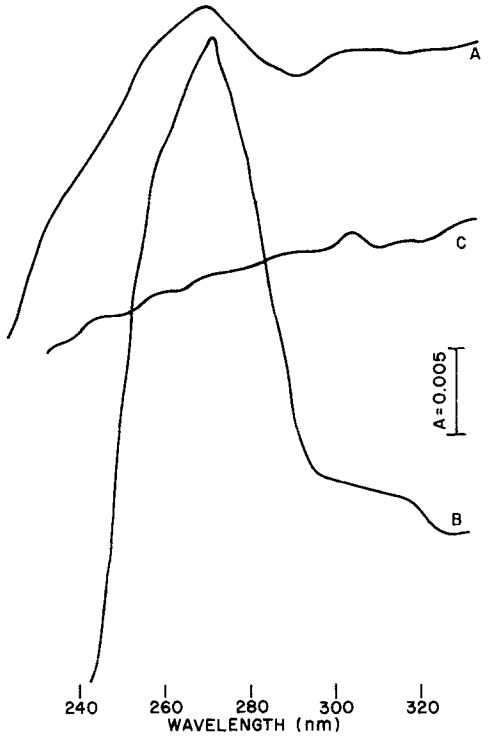

FIG. 2. Quinone spectra of human neutrophils. Quinones were solubilized in absolute ethanol and split between matched cuvettes. Reduced vs. oxidized difference spectra were obtained through addition of sodium borohydride to one sample. (A) Plasma membrane quinones extracted from 1.5 mg of membrane protein. (B) Neutrophil quinones extracted from 15.5 mg of cell protein. (C) Reduced vs. oxidized ethanol blanks. Peak absorbance occurred at $A_{270\ nm}$.

density shift of ~0.03 gm/ml was observed. A significant digitonin shift is indicative of a high cholesterol content and is a well-accepted hallmark of plasma membrane. In the case of rat liver, of the internal membranes and plasma membrane that occur in the microsomal fraction, only the plasma membrane exhibits a marked digitonin shift (10). The purified plasma membrane was enriched 14-fold in 5′-AMPase activity before digitonin shift of the membrane (Table II).

Ouabain-sensitive, Na^+,K^+-ATPase was also used as a plasma membrane marker. The observation that only 6% of the total ATPase was ouabain-sensitive is in good agreement with previous results (19). Because only 6% of the total ATPase activity of the neutrophil was ouabain-sensitive, it was difficult to quantify with confidence the absolute amounts of the ouabain-sensitive, Na^+,K^+-ATPase activity. The peak activities of both this enzyme and 5′-AMPase were located at the same density, and the enrichment for ouabain-sensitive was 10-fold (Table II). Furthermore, both activities were shifted by digitonin (Fig. 4), indicating localization in the same organelle. Such findings further support 5′-AMPase as a neutrophil plasma membrane marker.

Enrichment data for alkaline phosphatase in the digitonin-shifted plasma membrane fraction (Table II) suggest that 60% of the activity may be present in plasma membrane. Secondary localization is probably in the azurophilic granule. There is a

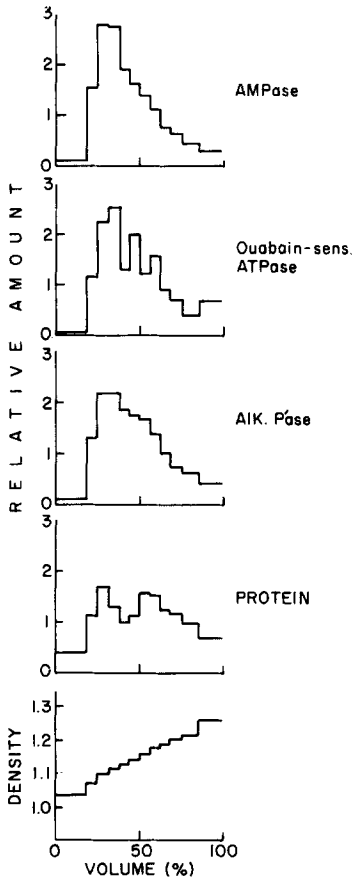

FIG. 3. Distribution patterns of enzymes after gradient centrifugation of neutrophil membranes (MLP fraction). The MLP fraction was prepared as described in Materials and Methods and layered on a linear 0–50% sucrose gradient. Plots of relative activity (RA versus percent volume are given, where RA is percent total activity divided by percent volume (20). These histograms represent the average of three repeated preparations used to isolate plasma membrane. 5′-AMPase, ouabain-sensitive Na^+,K^+-ATPase, and alkaline phosphatase all show maximal enrichment in the fractions with a density of 1.11–1.12 g/ml and presumed to be plasma membrane.

broad activity peak in the region of the plasma membrane (1.12 gm/ml), and a smaller amount of activity in the region of the granules (1.18–1.22 gm/ml) (Fig. 3).

Total ATPase enrichment data indicates that about one-third of neutrophil ATPase activity is in the plasma membrane fraction (Table II).

NAβGase activity was followed to determine the granule contamination of the plasma membrane fraction. NAβGase activity, which marks the azurophilic granule, clearly is not associated with the plasma membrane. This was shown by the negative enrichment for NAβGase activity in the pure plasma membrane fraction (Table II).

Protein yield was very consistent in each of the plasma membrane preparations. If these preparations of plasma membrane are pure, then plasma membrane contains ~8% of the total protein content of the neutrophil.

It was desirable to show that lymphocyte contamination was not present in the digitonin-shifted plasma membrane. Antibody against human IgM was diffused

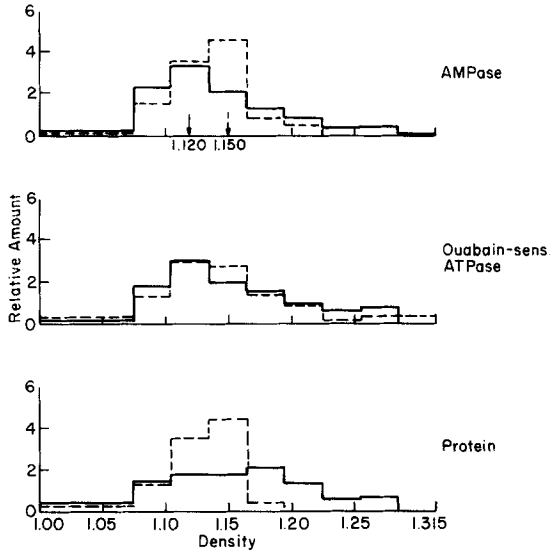

FIG. 4. Digitonin shift of purified plasma membrane. MLP fraction was incubated with digitonin as described in Materials and Methods, and layered on a linear 20–50% sucrose gradient. The histograms represent the average of three repeated preparations. (—) density distributions of untreated membranes; (---) density distributions of digitonin-treated membranes.

TABLE II

Marker Enzyme Data for Purified and Digitonin-shifted Plasma Membrane

Marker enzyme	Homogenate specific activity	Purified plasma membrane specific activity		Digitonin-shifted plasma membrane specific activity	
	mU/mg	*mU/mg*		*mU/mg*	
5′-Nucleotidase	0.80	11.3	(14.1-fold)	14.4	(18.0-fold)
Ouabain-sensitive Na^+,K^+-ATPase	0.62	6.21	(10.0-fold)	4.98	(8.0-fold)
Total ATPase	12.1	70.7	(5.8-fold)	62.0	(5.1-fold)
Alkaline phosphatase	3.02	36.2	(12.0-fold)	36.1	(12.0-fold)
NaβGase	38.7	18.6	(0.48-fold)	16.1	(0.42-fold)

Five marker enzymes were assayed as described in Materials and Methods. Homogenate values for the enzymes are an average of the three homogenates that were used to obtain purified and digitonin-shifted plasma membrane.

against purified plasma membrane in Ouchterlony plates. The antibody showed no reaction whatsoever with the purified plasma membrane.

Plasma Membrane Characteristics That Pertain to Microbicidal Activity. Digitonin-shifted plasma membrane was found to have 205 pmol of the *b*-type cytochrome per mg of membrane protein. This concentration is three times greater than that of the whole neutrophil (Fig. 1, Table I). This result indicates that only about one-quarter of the neutrophil's *b*-type cytochrome is located in the plasma membrane. The low NAβGase activity and the significant digitonin shift of the plasma membrane preparation indicate that contamination is low. It, thus, appears that cytochrome *b* may have a dual localization in plasma and in granule membranes.

Evidence that the majority of the cytochrome is associated with the granules was

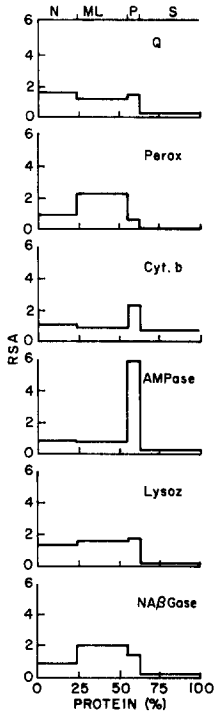

FIG. 5. Distribution patterns of enzymes, cytochrome *b*, and quinone after differential centrifugation of human neutrophil homogenate. Cells were collected and homogenized as described in Materials and Methods, and then the homogenate was separated into four fractions (N, ML, P or microsomes, and S) as described (20). Peroxidase and lysozyme activities were determined with *o*-dianisidine (22) and bacterial cell walls (23), respectively. RSA (relative specific activity) is calculated by dividing the percent activity in a fraction by the percent protein in that fraction. Recoveries were quinone 174%, cytochrome *b* 77%, peroxidase 87%, AMPase 110%, lysozyme 56%, and glucosaminidase 92%.

obtained by differential centrifugation of neutrophil homogenates (Fig. 5). The microsomal fraction (P) was enriched 5.8 times in 5′-nucleotidase and 2.3 times in cytochrome; most of the cytochrome was in the larger granule fractions (Fig. 5, N + MLP). Comparison with the distribution patterns of lysozyme, peroxidase and NAβGase suggests that granule cytochrome *b* and lysozyme are similar, both may be associated with the specific granules.

An alpha band absorbance peak was observed at $A_{558\ nm}$ for the *b*-type cytochrome of plasma membrane (Fig. 1). Although this peak is the same as that of the total cell membranes, the two spectra appear to be somewhat different. The more rounded appearance of the cell membrane cytochrome peak can probably be attributed to the slight increase in scattering which the greater particulate protein concentrations cause.

The quinone content of digitonin-shifted plasma membrane was measured to be 740 pmol/mg of protein, an amount two times greater than in the neutrophil homogenate (Fig. 2, Table I). Although there is a greater amount of quinone than *b* cytochrome in the cell, the quinone tends to be even less localized in the plasma membrane (12 vs. 24%). As with the *b*-type cytochrome, most of the quinone fractionated with the granules (Fig. 5).

The extracted quinones showed peak absorbance near $A_{270\ nm}$, which is the accepted

quinone absorbance peak (Fig. 2). The two spectra look similar although the amount of quinone which was present in the two samples was quite different.

Discussion

Current studies have emphasized the importance of NAD(P)H oxidases and cytochrome *b* in generation of lethal oxygen metabolites by neutrophils (2). Because the above oxido-reduction components appeared to be associated with the plasma membrane (2), it seemed as though phagocytosis and membrane fusion might not be required for generation of oxygen metabolites. This idea was supported by the observation that cytochalasin B inhibited the release of granule contents (24) without inhibiting the generation of oxygen metabolites. Although it seems possible that cytochalasin B does not inhibit membrane fusion, a subsequent report (25) using inhibitors of anion transport, which may block membrane fusion, further indicated that fusion is not required for production of oxygen metabolites. However, kinetic analyses show that oxidative events and fusion may occur coordinately (26). Therefore, it appears that the requirement for membrane fusion is an open question, and we suggest that the possible role of granule membranes in the generation of oxygen metabolites should still be evaluated. Here we report that the primary location of cytochrome *b* and of quinone may be the granule membrane. This suggestion rests on analyses of subcellular fractions obtained by differential centrifugation and of partially purified plasma membrane.

The procedure for the isolation of human neutrophil plasma membrane used 5′-AMPase and ouabain-sensitive ATPase as marker enzymes. Previous reports have questioned the existence of 5′-AMPase in the human neutrophil (27, 28). The discrepancy may be related to the fact that the 5′-AMPase activity in the neutrophil is significantly less than that in other tissues. However, this activity was measured using a colorometric assay; therefore, the measurable absolute levels of activity were not insignificant. Furthermore, a very recent report on purification of plasma membrane for human neutrophils also indicates that 5′-nucleotidase is a suitable marker enzyme (29).

Enrichment data show that the specific activity for 5′-AMPase in the gradient-purified plasma membrane is 14-fold greater than in the cell homogenate. Ouabain-sensitive ATPase was enriched 10-fold. These values indicate substantial enhancement of the plasma membrane, because most plasma membranes contain 5–10% of total cell protein. We obtained a 10-fold enrichment in 5′-AMPase activity when isolating rat neutrophil plasma membrane (3). A 20-fold enrichment of the same enzyme is usually attained when isolating rat liver plasma membrane (30). Finally, 17-fold enrichment was obtained for 5′-AMPase in plasma membrane isolated from fat cells of three species (31).

Enrichment of alkaline phosphatase activity indicated primary localization in the plasma membrane, which confirms previous data (32, 33). It is possible that nonmembranous alkaline phosphatase is associated with the azurophilic granules, because it has been suggested that the specific granules are devoid of this activity (34).

The total ATPase activity was only purified 4.4 times in the digitonin-shifted membrane fractions, which suggests that 30% of the ATPase activity of the neutrophil is localized in the plasma membrane. This result contrasts with a previous report (35), which indicated that 90% of the neutrophil ATPase activity is in the plasma

membrane. Because so much ATPase activity was found in the granule fraction, it seems worth considering that a granule-associated ATPase may function as a proton pump.

The neutrophil plasma membrane must have a relatively large amount of cholesterol, because it was shifted by digitonin more than that of most cells are shifted. This observation seems logical, because cholesterol adds to membrane fluidity and is essential for solute transport (36) and endocytosis (37).

Although 5′-AMPase activity was purified up to 14-fold, neither the *b*-type cytochrome nor the quinone in the neutrophil was equally enriched. In fact, the low enrichments of these electron carriers indicate that they are primarily located elsewhere in the cell. Differential centrifugation results suggest primary localization of these carriers to be in a granule-rich fraction of the neutrophil, perhaps the specific granule. In the case of the *b*-type cytochrome, this finding contradicts those of Segal and Jones (7), which suggests primary localization of human neutrophils in the plasma membrane. This lack of plasma membrane localization is also surprising because we achieved nearly parallel enrichment for 5′-AMPase and the *b*-type cytochrome in rat neutrophil preparations (3).

One possibility is that, in humans, there is no quinone or *b*-type cytochrome in the plasma membrane of resting neutrophils. It is possible that these carriers exist exclusively in the granules of virgin neutrophils—those which have not experienced phagocytosis or exocytosis. Our data might be explained by the fact that limited phagocytic activity may possibly have occurred before fractionation. In this regard, it may be very significant to note that rat peritoneal neutrophils were elicited by injection of caseinate (3).

Another interpretation is related to the possible existence of *b*-type cytochromes and quinones that are not functional components of the oxidase system. Perhaps, for example, there are nonfunctional quinone pools, a possibility which has precedent in mitochondria (38, 39). Such pool quinones would make identification of the functional quinone difficult.

A third possibility is that the respiratory burst depends on the presence of cytochrome *b* and quinone in both the granule membrane and in the plasma membrane. If this possibility is true, then our previous results with rat neutrophils are suspect inasmuch as all of the cytochrome *b* appeared to be located in the plasma membrane. The possibility of contamination by specific granule membranes in the rat preparation seems slight but was not rigorously ruled out (3).

Our suggestion that quinone is necessary for the oxygen burst in neutrophils is strengthened by the results observed after addition of exogenous quinones and quinone inhibitors.[2] One might point out that nonspecific resistance to bacterial infections is enhanced by treatment of mice with ubiquinone-8 (40). One can speculate that certain neutrophil diseases may be found to be caused by the absence or malfunction of quinone, as is the case with chronic granulomatous disease and the *b*-type cytochrome (41).

Purification of specific granules from virgin neutrophils and characterization of their membrane composition would provide useful information and may resolve some of the above uncertainties. Further, in view of the composition of other electron-

[2] Crawford, D. R., and D. L. Schneider. Evidence that a quinone may be required for the production of superoxide and hydrogen peroxide in neutrophils. *Biochem. Biophys. Res. Commun.* In press.

transport chains, it would seem appropriate to conduct studies aimed at determining the possible existence of non-heme iron proteins in this system. Reconstitution experiments involving the joining of purified plasma membrane and purified granules (both specific and azurophilic) might provide important insights. It should also be possible to analyze phagolysosomes, isolated from latex-treated neutrophils, for their *b*-type cytochrome and quinone contents.

Summary

Analyses of plasma membrane and other subcellular fractions indicate that the primary location of cytochrome *b* in human neutrophils is not the plasma membrane. The procedure developed for the purification of plasma membrane from fresh human neutrophils yielded a 14-fold enrichment in the marker enzyme 5′-nucleotidase and a 10-fold enrichment in ouabain-sensitive ATPase. On sucrose density gradients, the peak density of 5′-nucleotidase activity was 1.12 g/ml, and was shifted after digitonin addition to 1.15 g/ml. Protein in the plasma membrane equalled ~8% of the whole cell protein.

A *b*-type cytochrome was found to be present in the plasma membrane fraction at a concentration of 205 pmol/mg of protein, which is three times greater than that in the neutrophil overall. Although this cytochrome has been reported previously in the neutrophil, this is the first determination for purified plasma membrane and may indicate that *b*-type cytochrome has a dual localization in the human neutrophil. Differential centrifugation results suggest that the primary location is in the granules, probably specific granules.

Quinone content in the plasma membrane was found to be 740 pmol/mg of protein, a concentration two times greater than in the whole cell. Such a small enhancement of quinone indicates that quinone also is not primarily located in the plasma membrane.

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