Inhibition of Neutrophil Oxidative Metabolism by Lysosomotropic Weak Bases

By Barbara Styr and Mark S. Klemper

Maintenance of an acidic intralysosomal compartment may be relevant to multiple aspects of neutrophil function. The effect of lysosomal alkalization on the neutrophil respiratory burst was studied by measuring cytochrome c reduction in response to soluble stimuli in the presence of lysosomotropic weak bases. The weak bases chloroquine, ammonium chloride, methylamine, and clindamycin all raised the intralysosomal pH and inhibited neutrophil oxidative metabolism at concentrations ranging from 0.1 to 100 mmol/L. Inhibition was dose dependent for each base and correlated significantly with the degree of lysosomal alkalinization. Concentrations that did not alkalize the lysosome did not inhibit the respiratory burst. Inhibition by weak bases was seen when oxidative metabolism was stimulated by phorbol myristate acetate, calcium ionophore A23187, formyl-methionyl-leucyl-phenylalanine, opsonized zymosan, or sodium fluoride. Increasing the stimulus concentration (from 5 ng/mL to 5 μg/mL phorbol myristate acetate and from 0.5 to 1 μmol/L A23187) diminished or abolished inhibition by weak bases. Washing the cells after incubation with bases and before stimulation substantially reversed the inhibition. None of the bases impaired detection of superoxide in a cell-free xanthine-xanthine oxidase assay. Other indexes of oxidative metabolism, including oxygen consumption and hydrogen peroxide release, were also inhibited by weak bases. Analysis of particle NADPH oxidase activity from neutrophils stimulated in the presence of bases suggested that these cells assemble a subnormal amount of an enzyme complex with normal kinetic characteristics. Lysosomotropic weak bases alkalinized the neutrophil lysosome and produced inhibition of oxidative metabolism that was dose related, was not stimulus specific, and was largely reversed by washing the cells before stimulation. A possible explanation would be altered assembly of the enzyme complex involved in respiratory burst activation as a consequence of impaired intralysosomal pH.

Materials and Methods

The human neutrophil responds to a variety of stimuli, including phagocytosable particles and membrane-active soluble agents, with a complex sequence of events including degranulation and production of toxic oxygen metabolites. There is evidence to suggest that these two events including degranulation and production of toxic oxygen metabolites. There is evidence to suggest that these two events share a requirement for membrane fusion between the neutrophil granules and plasma membrane. In other cell systems, vesicular acidity is thought to be important to reactions between membranes, and we have previously demonstrated that weak bases that raise the pH of the acidic neutrophil lysosome are inhibitors of degranulation. To see whether this effect can be generalized to other fusion-requiring elements of the neutrophil activation sequence, we have now examined the effect of weak bases that alkalinize the intralysosomal pH on the neutrophil respiratory burst.

Materials and Methods

Hypaque (diatrizoate meglumine and diatrizoate sodium) was obtained from Winthrop Laboratories (New York), dextran (molecular weight, 200,000 to 300,000) from J.T. Baker Co (Phillipsburg, NJ), and phorbol myristate acetate (PMA) from L.C. Services (Woburn, Mass). Ficoll, 9-aminoacridine, cytochrome C, superoxide dismutase, formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe), cytochalasin B, calcium ionophore A23187, chloroquine, methylamine HCl, xanthine oxidase, catalse, superoxide, and zymosan were obtained from Sigma Chemical Co (St Louis). Clindamycin HCl was from the Upjohn Co (Kalamazoo, Mich) and horseradish peroxidase from Boehringer Mannheim (Indianapolis, Ind). Solutions of weak bases were made up at 100 times the desired concentration, and the pH was adjusted with concentrated HCl or NaOH to produce 0.1 pH or less unit change in the incubation buffer.

Isolation of neutrophils. Heparinized venous blood was obtained from healthy volunteer donors after informed consent was obtained from them. Neutrophils were isolated by Ficoll-Hypaque density gradient centrifugation and dextran sedimentation following the method of Boyum. Residual erythrocytes were removed with three cycles of hypotonic saline lysis, and cells were resuspended in Hanks' balanced salt solution (HBSS). The neutrophil concentration was 106/mL for superoxide assays and 108/mL for lysosomal pH monitoring. For assays of stimulation, the buffer contained 0.9 mmol/L calcium and 0.9 mmol/L magnesium.

Monitoring of intralysosomal pH. The method for following intralysosomal pH in intact neutrophils has been described previously. Briefly, fluorescence was monitored at 400-nm excitation and 456-nm emission in a solution (3 mL) of 0.05 μmol/L 9-aminoacridine maintained at 37 °C by a circulating water bath. Neutrophils (120 μL at 109/mL for a final concentration of 4 x 106/mL) were added, and fluorescence quenching was followed for four minutes as the indicator was taken up into the lysosomes. Weak bases were then added, and fluorescence quenching was followed for four minutes as the indicator was taken up into the lysosomes. Weak bases were then added, and displacement of the indicator from the cells, because of lysosomal alkalization, was monitored as increasing fluorescence. The degree of lysosomal alkalization was estimated from the percent reversal of initial fluorescence quenching, as correlated with previous observations of the transmembrane pH gradient in isolated lysosomes.

Measurement of neutrophil oxidative metabolism. Neutrophils (5 x 106) were preincubated with weak bases (10 μL at 100 times the desired final concentration) in a total volume of 0.9 mL for five minutes at 37 °C. Cytochrome c (0.1 mL at 0.001 mol/L) was added, followed by the stimulus, and the incubation was continued for an additional 20 minutes. When f-met-leu-phe or opsonized zymosan was used as the stimulus, cells were incubated with cytochalasin B (5 μg/mL) for an additional five minutes before...
stimulation. For all conditions, simultaneous controls contained 0.1 mg/mL superoxide dismutase (SOD).

Incubation was terminated by centrifuging the samples for ten minutes at 1,200 rpm and 4 °C. Absorbance of supernatants (diluted 1 in 4 in 0.1 mol/L phosphate buffer, pH 7.2) was scanned from 335 to 555 nm in a Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio), either immediately or after storage at −70 °C. The optical density (OD) was compared in duplicate samples with and without SOD, and superoxide production was calculated as the SOD-inhibitable reduction of cytochrome c using an extinction coefficient of 21.1/mmol/L·cm.

The effect of weak bases was calculated as the decrease in cytochrome c reduction by neutrophils incubated with weak bases relative to control cells from the same donor processed identically except for the absence of the base. Results are expressed as percent inhibition (mean ± SEM of two to three determinations).

Reversibility. To document whether the effect of weak bases was reversible, neutrophils were preincubated with 1 mmol/L chloroquine or 50 mmol/L NH₄Cl for ten minutes at 37 °C, centrifuged for ten minutes at 1,200 rpm at 4 °C, washed once with HBSS, and resuspended in HBSS with divalent cations. Superoxide production was then measured as previously outlined.

Xanthine-xanthine oxidase assay. Superoxide scavenging by weak bases was determined by using a cell-free enzyme system to generate superoxide. Reaction mixtures contained 33 μmol/L cytochrome c, 100 μmol/L xanthine, and 0.26 U/mL xanthine oxidase in 0.05 mol/L phosphate buffer (pH 7.5) as well as 67 μg/mL SOD and weak bases where indicated. OD₅₅₀ was read after five minutes of incubation at room temperature. SOD-inhibitable cytochrome c reduction was determined for duplicate samples with and without the base, and the percent inhibition by the base was calculated.

Preparation of particulate fraction containing NADPH oxidase activity. To prepare particles containing NADPH oxidase activity from stimulated cells, neutrophils were suspended in 2 mL HBSS at approximately 1 to 1.5 × 10⁶/mL. Zymosan (45 mg in 5 mL 1 N NaOH) was boiled for ten minutes, washed twice, opsonized in AB serum for 30 minutes at 37 °C, washed twice, and suspended in 2 mL HBSS with 2 mmol/L NaNO₂. Neutrophils and zymosan were incubated for three minutes separately at 37 °C. The two suspensions were combined, and incubation was continued for another seven minutes, then terminated by the addition of 4 mL ice-cold HBSS and centrifugation for 12 minutes at 1,200 rpm and 4 °C. The cells were resuspended in 0.34 mol/L sucrose containing 5 × 10⁻⁴ mol/L phenyl methyl sulfon fluoride and sonicated in an ice bath with an ultrasonic probe (Branson Sonic Power Co, Danbury, Conn) for one minute at a setting of 1.5. Undisrupted cells and zymosan were removed by centrifugation at 1,400 rpm for ten minutes at 4 °C. The supernatant was then ultracentrifuged at 4 °C and 25,000 rpm for 30 minutes. The pellet from this ultracentrifugation was suspended in 5 mL 0.34 mol/L sucrose and stored at −70 °C until used. Protein was determined by the method of Bradford.

NADPH oxidase assay. NADPH oxidase activity in the particulate fraction from stimulated neutrophils was determined by measuring the reduction of cytochrome c by monitoring the OD at a wavelength of 550 nm at room temperature. The reaction was initiated by adding 0.2 mL particles to a total of 3 mL HBSS containing 0.6 mg/mL cytochrome C, 0.02% Triton X-100 (Sigma Chemical Co, St Louis) and varying concentrations of NADPH. The OD was read every 10 seconds for three minutes, and the rate of cytochrome c reduction was taken as the initial linear rate of change after subtracting out control values from identical reaction mixtures to which SOD (0.1 mg/mL) was added.

Determination of kinetic constants for NADPH oxidase. The kinetic constants Kₘ (substrate concentration at which the rate is half maximal) and Vₘₐₓ (reaction rate when enzyme is saturated) for NADPH oxidase were determined by graphic methods from rates of cytochrome c reduction at NADPH concentrations ranging from 0.01 to 2 mmol/L. A Hewlett-Packard 9845A computer was used to generate Lineweaver-Burk plots using least-squares fitting of lines to the data, and Kₘ and Vₘₐₓ were calculated from the slope and intercept. The effect of lysosomotropic bases on these values was assessed by adding 1 mmol/L chloroquine or 50 mmol/L NH₄Cl at three different time points: before activation of neutrophils with zymosan, seven minutes after activation (with 37 °C incubation continued for an additional five minutes after the addition of bases), and after isolation of particles (bases added to the reaction mixture for the NADPH oxidase assay).

Oxygen consumption. Oxygen consumption was monitored with a Yellow Springs oxygen electrode (Yellow Springs Instrument Co, Yellow Springs, Ohio). Neutrophils were suspended in HBSS at 5 × 10⁶/mL and loaded with chloroquine (1 mmol/L) or ammonium chloride (50 mmol/L) for five minutes at 37 °C. Cytochalasin B (5 μg/mL) was added in a second five-minute, 37 °C incubation for experiments using f-met-leu-phe as the stimulus. The remainder of the experiment was carried out at room temperature. One milliliter of cells was added with constant stirring to 1 mL of buffer containing the same concentration of base in the electrode chamber. After the electrode baseline was recorded for three minutes, the stimulus (10 μL of 10⁻¹ mol/L f-met-leu-phe or 500 ng/mL PMA) was added and the change in pO₂ followed for ten minutes. This value was converted to an estimate of the quantity of oxygen consumed on the basis of the calculated oxygen content of water in equilibrium with air at room temperature.

Hydrogen peroxide production. Neutrophil production of hydrogen peroxide was measured fluorometrically by the scopoletin reduction assay. Neutrophils (10⁶/mL) were incubated for five minutes at 37 °C in HBSS containing 4 μmol/L scopoletin and 20 mmol/L horseradish peroxidase with or without 50 mmol/L NH₄Cl. Cytochalasin B (final concentration of 5 μg/mL from stock kept at 1 mg/mL in dimethyl sulfoxide [DMSO]) was added for a further five-minute incubation. Then f-met-leu-phe (final concentration of 10⁻¹ mol/L) was added. Lag time was estimated from the continuous chart recording, and the initial rate of H₂O₂ production was determined from the reduction in scopoletin fluorescence (excitation wavelength, 350; emission wavelength, 460). Standards of a known H₂O₂ concentration with and without NH₄Cl were used to convert the results to nanomoles of H₂O₂ produced.

RESULTS

Measurement of lysosomal alkalization. Release of the indicator 9-aminoacridine from neutrophils by weak bases is shown in Fig 1, which compares the concentration response to ammonium chloride and other bases. The rise in intralysosomal pH can be extrapolated from reversal of quenching, as previously reported. Thus, 20% reversal of quenching corresponds approximately to 0.5, and 30% to 1.5-pH-unit alkalinization. Chloroquine, ammonium chloride, methylamine, and clindamycin all produced substantial alkalization of the lysosome at concentrations ranging from .01 to 100 mmol/L.

Inhibition of superoxide production. To observe either augmentation or inhibition of the respiratory burst, our initial experiments were designed to produce approximately half-maximal stimulation of neutrophils. This level of stimulation was observed using 5 ng/mL PMA, with minimal superoxide production at 0.5 ng and maximum stimulation at
Fig 1. Alkalinization of the neutrophil lysosome by weak bases as monitored by the reversal of 9-aminoacridine fluorescence quenching. Each point represents the mean of two to three determinations.

50 ng and above. The mean superoxide production by normal cells after each of the stimuli used is listed in Table 1.

Figure 2 shows the effect of the four weak bases on superoxide production stimulated by 5 ng/mL PMA. At concentrations that did not alkalinize the lysosome, none of these agents significantly affected oxidative metabolism. At concentrations producing more than 0.5-pH-unit alkalinization, all four bases were inhibitory. Clindamycin inhibition, which we have discussed in detail elsewhere, is here compared with that produced by the other bases. Chloroquine and clindamycin showed somewhat more inhibition relative to their alkalinizing capacity than ammonium chloride and methylamine.

Effect of stimulus concentration. To determine whether the inhibitory effect of weak bases could be overcome, we repeated these experiments using 5 μg/mL PMA for maximal stimulation. As shown in Fig 3, inhibition by 1 mmol/L chloroquine was reduced in this setting. At the highest concentrations of ammonium chloride and methylamine used, inhibition was abolished by raising the stimulus to maximal levels.

Table 1. Relationship Between Neutrophil Response to Different Stimuli and Inhibition of Superoxide Production by 1 mmol/L Chloroquine

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cytochrome c Reduction by Control Cells (nmol)</th>
<th>Percent Inhibition by 1 mmol/L Chloroquine</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA, 5 μg/mL</td>
<td>67.0 ± 5.1</td>
<td>45.5 ± 10.5</td>
</tr>
<tr>
<td>PMA, 5 ng/mL</td>
<td>44.4 ± 6.3</td>
<td>69.5 ± 13.9</td>
</tr>
<tr>
<td>NaF, 15 mmol/L</td>
<td>42.1 ± 3.5</td>
<td>96.1 ± 1.5</td>
</tr>
<tr>
<td>Opsonized zymosan, 2 mg/mL</td>
<td>33.7 ± 3.7</td>
<td>91.4 ± 7.2</td>
</tr>
<tr>
<td>f-met-leu-phe 0.1 μmol/L</td>
<td>30.8 ± 6.9</td>
<td>87.4 ± 6.4</td>
</tr>
<tr>
<td>Opsonized zymosan, 0.5 mg/mL</td>
<td>19.3 ± 4.8</td>
<td>89.0 ± 7.1</td>
</tr>
<tr>
<td>A23187, 1 μmol/L</td>
<td>13.4 ± 2.5</td>
<td>58.9 ± 18.8</td>
</tr>
<tr>
<td>A23187, 0.5 μmol/L</td>
<td>11.2 ± 2.0</td>
<td>90.6 ± 0.3</td>
</tr>
<tr>
<td>f-met-leu-phe, 0.01 μmol/L</td>
<td>4.2 ± 2.1</td>
<td>58.4 ± 8.4</td>
</tr>
</tbody>
</table>

Assessment of stimulus specificity. To determine whether inhibition by weak bases was stimulus specific, we repeated superoxide assays using other stimuli. Figure 4 shows the effects on superoxide production stimulated by the calcium ionophore A23187 (0.5 μmol/L). Chloroquine, ammonium chloride, and methylamine all produced even more marked inhibition than in the experiments using PMA. A modest reduction in inhibition was produced by increasing the stimulus to 1 μmol/L. As shown in Fig 5, with 0.1 μmol/L f-met-leu-phe as the stimulus in the presence of 5 μg/mL cytochalasin B, results were similar to those seen with 0.5 μmol/L A23187. Zymosan-induced superoxide production was inhibited by chloroquine (Table 1) and ammonium chloride (73.9% ± 7.0% inhibition by 50 mmol/L ammonium chloride with 2 mg/mL zymosan stimulus). Chloroquine also inhibited superoxide production induced by 15 mmol/L fluoride (18.9% ± 21.7% inhibition at 0.01 mmol/L chloroquine, 29.7% ± 11.9% at 0.1 mmol/L, and 96.1% ± 1.5% at 1 mmol/L).

Correlation between lysosomal alkalinization and inhibition of superoxide production. The relationship between lysosomal alkalinization (as percent reversal of quenching) and inhibition of superoxide production by the various bases tested is illustrated in Fig 6 for cells stimulated by 5 ng/mL.
PMA (as in Fig. 2) and by 1 μmol/L A23187. Similar relationships were seen with other stimuli, with correlation coefficients of $r = 0.904$ ($P < 0.001$) for 0.1 μmol/L f-met-leu-phe and 0.752 ($P < 0.05$) for 0.5 μmol/L A23187. To see whether these correlations were affected by the slight, non-significant increase in superoxide production seen with low concentrations of some bases, correlations were also computed omitting negative values for percent inhibition. This procedure reduced $r$ to 0.548 for cells stimulated with 5 ng/mL PMA but did not significantly weaken any of the other correlations cited.

Although increased concentrations of PMA or A23187 reduced the inhibitory effect of bases on superoxide production, the relationship of greater stimulation to lesser inhibition did not hold when different stimuli were compared. Table 1 shows the amount of superoxide production by control cells (no base) in response to a variety of stimuli. As shown, the ability of chloroquine to inhibit superoxide production was not related to the total amount of superoxide produced.

Reversal of weak base effects. Reversibility of inhibition was assessed by incubating cells with ammonium chloride or chloroquine, washing away the base, and then stimulating the cells with 5 ng/mL PMA. Preliminary experiments had indicated that base-mediated decreases in fluorescence quenching were abolished by this procedure so that the pH gradient across the lysosomal membrane was presumably reestablished at or near its original value before the neutrophils were stimulated. Although slight inhibition was seen after washing, most of the weak base effect on superoxide production was reversed by removal of the bases. Thus, cells treated with 1 μmol/L chloroquine and washed displayed 10.7% ± 8.7% inhibition, compared with 69.5% ± 13.9% with base present throughout the assay; corresponding values for 50 mmol/L ammonium chloride were 15.7% ± 3.0% for washed cells and 46.3% ± 2.7% for cells with base present during stimulation. At these concentrations of bases we did not see functional enhancement, as reported by Phillips et al. for iodinating activity of neutrophils washed after treatment with isotonic ammonium chloride.

Effect of osmolarity and ionic strength. To assess the contribution of hyperosmolarity and increased ionic strength to the weak base effects, sucrose or NaCl was added to the (already isotonic) reaction mixture. The addition of NaCl up to 50 mmol/L and sucrose up to 100 mol/L had little or no effect on superoxide production stimulated by 5 ng/mL PMA. Moderate inhibition was seen when these concentrations were doubled: 33.8% ± 4.6% with 200 mmol/L sucrose and 31.9% ± 19.8% with 100 mmol/L NaCl. Thus, only extreme hyperosmolarity produced inhibition comparable to that seen for lower concentrations of weak bases.

Superoxide scavenging. The xanthine-xanthine oxidase assay was used to detect scavenging of superoxide by weak bases. There was no significant inhibition of cytochrome c reduction in this system by any of the weak bases tested at the highest concentrations used. Indeed, values higher than the control were obtained in some experiments (39.4% ± 24.8% greater than the control in the presence of 1 mmol/L chloroquine and 22.3% ± 15.2% greater than the control in the presence of 50 mmol/L NH4Cl). To evaluate whether this represented genuinely enhanced superoxide detection in the presence of chloroquine and NH4Cl, assays were repeated using a different lot of xanthine oxidase (0.53 U/mL) and 10 μmol/L cytochrome c, with OD measure-
ments every 10 seconds to permit calculation of an initial linear rate of cytochrome c reduction. The results showed –3.4% ± 8.0% inhibition by 50 mmol/L NH4Cl and 8.2% ± 7.8% inhibition by 1 mmol/L chloroquine. Thus, there was no consistent augmentation of cytochrome c reduction, and the data taken together indicate no significant effect on the detection of superoxide in the presence of any of the bases used.

**NADPH oxidase activity in particulate fractions.** Table 2 shows the effect of ammonium chloride and chloroquine on the NADPH oxidase activity of particles from zymosan-stimulated neutrophils. Both bases produced marked inhibition of activity when added to cells before stimulation, but not when added to intact cells after stimulation or to particle preparations before the assay of NADPH oxidase activity. Relative potency of the two bases was similar to their inhibitory effect on zymosan-stimulated superoxide production by intact cells in suspension.

Kinetic constants for NADPH oxidase, determined using NADPH concentrations from 0.01 to 2 mmol/L, are shown in Table 3. Cells without added base had a mean Km of 0.09 ± 0.02 mmol/L and a Vmax of 42.8 ± 13.6 nmol cytochrome c reduced per minute per mgm protein (n = 7; no significant difference between controls for bases added before and after stimulation). The addition of bases did not affect the Km but profoundly reduced the Vmax if and only if they were added before the cells were stimulated. This suggests that bases did not alter the activity of the NADPH oxidase complex after it was formed and that the presence of bases during cell stimulation led to the production of a reduced quantity of enzyme with normal substrate affinity.

In several experiments, very high concentrations of NADPH (usually more than 20 times the Km) produced decreased activity and deviation from the Michaelis-Menten kinetics used to derive the kinetic constants. These data were reanalyzed by fitting the equation $V = (V_{max} \times S)/ (K_m + S + S^2/K_c)$ for substrate inhibition to determine Km, Vmax, and the inhibition constant Kc. The results (Table 4) yielded Kc values comparable to those obtained assuming Michaelis-Menten kinetics at lower substrate concentrations and suggested significant substrate inhibition only at NADPH concentrations far higher than the Km. The influence of ammonium chloride on Vmax was unchanged using this procedure.

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**Table 2. Effect of Bases Added at Various Times on NADPH Oxidase Activity**

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<th>Time of Addition</th>
<th>Chloroquine (1 mmol/L)</th>
<th>NH4Cl (50 mmol/L)</th>
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<tr>
<td>Before activation</td>
<td>17.3 ± 11.6</td>
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<td>After activation, before disruption</td>
<td>91.1 ± 16.9</td>
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<td>After disruption</td>
<td>89.0 ± 9.1</td>
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All values represent the mean ± SEM of two or more experiments. Rates are cytochrome c reduction expressed as a percentage of simultaneous control cells processed identically except for the absence of added base, adjusted for equal protein content, and assayed in the presence of 0.1 mmol/L NADPH.

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**Table 3. The Effect of Weak Bases on Kinetic Constants for NADPH Oxidase**

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<td>Km (mmol/L NADPH)</td>
<td>0.07 ± 0.03</td>
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<td>Vmax (% of control)</td>
<td>100</td>
<td>15.4 ± 1.10</td>
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**Table 4. Kinetic Constants for Substrate Inhibition of NADPH Oxidase Activity**

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Each value represents the mean ± SEM of two to seven experiments. No values were obtained for chloroquine-pretreated cells because the total activity was very low and no definite substrate inhibition was demonstrated.

**DISCUSSION**

When neutrophils are activated by phagocytic stimuli or soluble agents, a respiratory burst is initiated, with resulting production of reactive oxygen metabolites that are toxic to ingested microorganisms. This process involves an increase in oxygen consumption,17 production of the superoxide anion,18 and its dismutation to hydrogen peroxide.19 These metabolites can then react further to produce hydroxyl radicals18 and active halogenated species such as chlorinating agents19,20 that are thought to be the principal microbialid reaction products.

Using superoxide production as an indicator of respiratory burst activity, we have demonstrated that permeant weak bases inhibit oxygen consumption,114.14 suggest significant substrate inhibition only at NADPH concentrations far higher than the Km. The influence of ammonium chloride on Vmax was unchanged using this procedure.

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**Effect of weak bases on neutrophil oxygen consumption.** Oxygen consumption was measured to determine whether the entire sequence of oxidative metabolism was affected by weak bases. The estimated consumption by control cells was 38 ± 14 nmol/5 x 10⁶ cells after stimulation by f-met-leu-phe and 22 ± 5 nmol/5 x 10⁶ cells after stimulation by PMA. As Table 5 indicates, oxygen consumption was decreased in the presence of bases as with superoxide production, chloroquine was a more potent inhibitor than ammonium chloride on a molar basis, and less inhibition was seen in PMA-stimulated cells than in f-met-leu-phe–stimulated cells.

**Effect on hydrogen peroxide release.** Cells treated with ammonium chloride released less hydrogen peroxide after stimulation with f-met-leu-phe (0.1 μmol/L) than control cells, as shown in Fig 7. The effect on the initial rate of release was most striking, again suggesting that the immediate response of the cell to stimulation is affected rather than a subsequent secondary event in oxidative metabolism.

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bases, which raise the intralysosomal pH in neutrophils as well as other phagocytic cells, inhibit neutrophil oxidative metabolism. This inhibition was dose dependent, was decreased by increasing the concentration of some stimuli, and could be observed using five different stimuli. Washing away the bases largely reversed their inhibitory effect.

Assessment of other components of the respiratory burst showed that oxygen consumption and hydrogen peroxide release were also inhibited. Similar patterns of inhibition were seen when cells were disrupted for analysis of NADPH oxidase activity after activation by a particulate stimulus (opsonized zymosan) and even though oxygen consumption and NADPH oxidase activity were assayed at room temperature after the cells were incubated at 37 °C with weak bases. Kinetic analysis of the NADPH oxidase suggested the formation of reduced amounts of active enzyme having normal substrate affinity.

There are several possible mechanisms for this inhibition. The simplest would be scavenging of superoxide by weak bases, with resulting failure to detect the presence of the superoxide anion by measuring cytochrome c reduction. In such a situation we would be measuring not a true inhibition of cellular function but an impairment of our ability to detect the products of stimulated cells. The xanthine-xanthine oxidase assay was used to test this possibility. Since none of the weak bases produced any impairment of superoxide detection in the cell-free system, scavenging of the superoxide anion cannot explain our results. The impairment of oxygen consumption and \( \text{H}_2\text{O}_2 \) production also argues against this possibility, as does the fact that particles from amine-loaded cells had diminished NADPH oxidase activity although most of the base was washed away during preparation of the particles.

Another explanation on the basis of artifact would be that very large amounts of superoxide are produced and dismutated to hydrogen peroxide, which in turn reoxidizes the reduced cytochrome c. This could not explain the reduction of oxygen uptake and hydrogen peroxide release. Thus, our findings appear to reflect a genuine depression of cellular activity.

A second hypothesis would be that the addition of weak bases to a balanced salt solution produces a rise in osmolarity and/or ionic strength sufficient to inhibit neutrophil function. Although we were able to inhibit superoxide production with extremely high concentrations of sucrose or NaCl, we saw remarkably little inhibition using up to 50 mmol/L NaCl or 100 mmol/L sucrose. It is unlikely, therefore, that these factors make a major contribution to the inhibition observed using weak bases, although they may play a role at the highest methylamine concentrations studied.

A third possibility is that weak bases interfere with binding of stimuli to membrane receptors. In this case inhibition might be expected to be stimulus specific. We observed inhibition of superoxide production in response to four different stimuli that are presumed to activate the respiratory burst by different mechanisms. In particular, inhibition was seen with an ionophore stimulus (A23187) and a halide stimulus (fluoride anion) as well as with receptor-mediated activation (zymosan, f-met-leu-phe). The fact that the phenomenon is so general argues against the likelihood that inhibition results purely from interference with stimulus-receptor binding.

A fourth possibility is suggested by recent work of Thomas and co-workers. These authors propose that when the neutrophil respiratory burst is activated in the presence of permeant amines, hypochlorous acid produced as a result of the respiratory burst reacts with the amines to form toxic nitrogen-chlorine derivatives. These compounds then diffuse into the cell where they produce oxidative damage to cell components and inhibition of further cellular activity. Clark and Borregaard and Ohno and Gallin have proposed that direct inactivation of neutrophil products by oxygen metabolites and similar processes might likewise be augmented by the presence of amines. Such a sequence of events could explain many of our results, although we have not seen increased cell death when neutrophils are activated in the presence of permeant amines (also unpublished observations, 1983) and the reversal of inhibition by high concentrations of PMA would be somewhat surprising in this context.

If this were the explanation, we would also expect to see inhibition of NADPH oxidase activity in particles from cells treated with bases after stimulation, but we observed no such effect. An additional argument against this hypothesis is the immediate inhibition of hydrogen peroxide release we observed, which would not be expected if cell damage occurs.

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<th>Table 5. Effect of Bases on Neutrophil Oxygen Consumption</th>
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<td><strong>Base</strong></td>
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<tr>
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<td>Chloroquine</td>
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<td>( \text{NH}_4\text{Cl} )</td>
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*nmol/min/10⁶ control cells with 60% inhibition of \( \text{H}_2\text{O}_2 \) production for a period of 5 minutes was 2.31 \pm 0.22 nmol/10⁶ cells with 40.0% \pm 0.22% inhibition by \( \text{NH}_4\text{Cl} \).
only after the backdiffusion of the initial products of oxidative metabolism.

We suggest that the inhibition of oxidative metabolism, which correlates with lysosomal alkalinization by weak bases, is at least in part a direct result of that alkalinization. This hypothesis is in accordance with the significant correlation between release of 9-aminacridine quenching and inhibition of superoxide production. It also explains the substantial reversal of inhibition when alkalinization is reversed by washing away the base.

By what mechanism does a disturbance of lysosomal pH regulation lead to suppression of the respiratory burst? One possibility is that these results reflect a general alteration of membrane fusion processes. The existence of an acid compartment and transmembrane pH gradient has been shown to be either a prerequisite or a concomitant factor in many processes involving fusion. For example, the Semliki Forest virus can be induced to fuse with cell membranes by low pH treatment. Low pH promotes fusion of small unilamellar vesicles in synthetic lipid-membrane systems. Rapid acidification of endocytic vesicles takes place not only in the neutrophil phagosome but also in mouse fibroblasts and single-cell organisms. Permeant weak bases such as methylamine and ammonium chloride, which should alkalinize the lysosome and/or the intermediary endocytic vesicle, have been reported to inhibit phagosome-lysosome fusion and to block various steps in receptor-mediated endocytosis including receptor clustering into coated pits and delivery of internalized ligand to the lysosome. Another method of acidicizing intracellular vesicles using the ionophore monensin has likewise been reported to block delivery of endosome contents to the macrophage lysosome. In addition to phagosome-lysosome fusion, our previous results suggest that lysosomotropic weak bases inhibit lysosome-plasma membrane fusion and thus impair neutrophil degranulation. Although others have reported more variable effects of weak bases on neutrophil function, the relationship between pH change and functional effects was not explicitly examined.

Reduction of the pH gradient across the lysosomal membrane may therefore explain inhibition of neutrophil oxidative metabolism by weak bases to the extent that the respiratory burst depends upon lysosome/plasma membrane fusion. Activation of the enzyme complex responsible for production of reactive oxygen metabolites may involve assembly of a variety of components: those that have been proposed include a flavoprotein, a b-type cytochrome, ubiquinone, and an unidentified chromophore. The assembly process may involve the fusion of plasma membrane with subcellular membranes and the transfer of components from one membrane location to another. For example, flavin adenine dinucleotide reportedly is present in plasma membrane and specific granules of resting cells but is increased in phagolysosomes relative to the plasma membrane. Transfer of ubiquinone from granules to phagolysosome has been proposed as an important step in the respiratory burst. There is evidence that activation of the NADPH oxidase involved in neutrophil superoxide generation may require translocation of cytochrome b from granule membranes to the plasma membrane, including a report that abnormal localization of cytochrome b in the plasma membrane may be associated with a supranormal respiratory burst. Thus, by impairing membrane fusion, lysosomal alkalinization could interfere with assembly of the active enzyme complex. This is not an all-or-none effect, as demonstrated by the failure to abolish superoxide production completely and the lessening of inhibition at high-stimulus concentrations. This in turn would be consistent with the report that granule-depleted cytoplasts are capable of oxidative metabolism, which implies that the requirement for granule-plasma membrane interaction may not be absolute. That is, the elements necessary for enzyme assembly may all be present in plasma membrane, as suggested by analysis of cytoplasts, but augmented by further supplies from granule fusion. A recent report that PMA produces less cytochrome b translocation relative to superoxide release than either A23187 or f-met-leu-phe/ cytochlasin B would then be compatible with our finding that cells stimulated by high concentrations of PMA show relatively less inhibition by bases than cells activated by other stimuli. The ability to overcome inhibition at very high PMA concentrations is consistent also with the reported graded dose-response of oxidative metabolism to PMA and may reflect recruitment of additional receptor sites or of alternative pathways of enzyme activation. Failure to reverse the chloroquine effect completely at high-PMA concentrations suggests that this particular base may have direct effects on receptor interactions in addition to the pH-dependent effects that it shares with other bases, and other studies have been undertaken to elucidate this.

Measurement of NADPH oxidase activity in particular preparations from stimulated neutrophils did suggest that lysosomotropic weak bases interfere with the assembly of the active enzyme complex, but without altering its substrate affinity. We speculate that this may reflect alterations in the intracellular calcium flux that occurs during neutrophil activation by diverse stimuli. In support of this hypothesis, recent data from this laboratory indicate that alkalinizing weak bases can modify the rise in cytosolic calcium associated with activation. Studies are in progress to delineate the step in the activation sequence at which weak base inhibition of neutrophil function occurs and to further elucidate the mechanism of inhibition.

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Inhibition of neutrophil oxidative metabolism by lysosomotropic weak bases

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