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Research Article

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NAD(P)H Oxidase Activity in Human Neutrophils Stimulated by Phorbol Myristate Acetate

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ABSTRACT Phorbol myristate acetate activated in normal human neutrophils a single enzymatic entity that was dormant in unstimulated cells, optimally active at pH 7.0, and capable of oxidizing either NADH or NADPH, producing $NAD(P)^+$ and superoxide (O_2^-). Comparative fluorometric and spectrophotometric measurements supported the stoichiometry $NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + 2O_2^- + H^+$. The seemingly considerable $NAD(P)^+$ production at pH 5.5 and 6.0 was due largely to nonenzymatic oxidation of NAD(P)H by chain reactions initiated by HO_2^- (perhydroxyl radical), the conjugate acid of O_2^-. This artifact, responsible for earlier erroneous assignments of an acid pH optimum for NAD(P)H oxidase, was prevented by including superoxide dismutase in fluorometric assays. NAD(P)H oxidase was more active towards NADPH ($K_m = 0.15 \pm 0.03$ mM) than NADH ($K_m = 0.68 \pm 0.2$ mM). No suggestion that oxidase activity was allosterically regulated by NAD(P)H was seen. Phorbol myristate acetate-induced O_2^- production was noted to be modulated by pH in intact neutrophils, suggesting that NAD(P)H oxidase is localized in the plasma membrane where its activity may be subject to (auto)regulation by local H^+ concentrations.

INTRODUCTION

After exposure to various stimuli, normal neutrophils mount a metabolic response, the "respiratory burst," that underlies their ability to kill microorganisms by oxidative or peroxidative chemical attack (1, 2). Among the prominent components of this response are enhanced O_2 consumption (3) and production of O_2^- and H_2O_2 (4–6).

It is generally agreed that the respiratory burst results from activation of an oxidase catalysing the following reaction: $NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + 2O_2^- + H^+$. This reaction has been analysed by sensitive fluorometric procedures that measure $NAD(P)^+$ (7) or by spectrophotometric assays that quantitate O_2^-. The latter use ferricytochrome *c* (8) or other superoxide "traps" (9), gaining specificity from appropriate use of superoxide dismutase. Despite recent progress, controversies persist as to the nature of the primary oxidase(s) and physiologic substrate(s), as well as to the molecular basis of oxidase activation.

We studied the metabolic activity of human neutrophils exposed to phorbol myristate acetate (PMA).[1] This amphipathic lipid, best known for its tumor-promoting activity in mice, activates the respiratory burst of normal human neutrophils (10, 11), but not those from subjects with chronic granulomatous disease.

This report describes selected properties of NAD(P)H oxidase, including its unitary nature, pH optimum, apparent K_m and V_{max} for NAD(P)H, and the stoichiometry of NAD(P)H oxidation and O_2^- formation. Our data also identify and remedy methodologic artifacts that have compromised some previous attempts to characterize NAD(P)H oxidase activity fluorometrically.

METHODS

Leukocytes. Neutrophils (PMN) were obtained from heparinized venous blood by previously described techniques of Hypaque-Ficoll centrifugation, dextran sedimentation, and hypotonic lysis (12). Our preparations (92–97% neutrophils,

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[1] *Abbreviations used in this paper*: BS-H, a balanced salt solution buffered with Hepes; BS-M, a balanced salt solution buffered with Mes; GRF, "granule-rich fraction", a heterogeneous subcellular particulate fraction from neutrophils; Mes, 2-*N*-morpholinoethane sulfonic acid; PMA, phorbol myristate acetate; PMN, neutrophil; PMN-eq, neutrophil equivalents; SOD, superoxide dismutase; V_{max}, maximum rate of oxidation.

3–7% eosinophils, 1–3% other leukocytes) were 95–99% viable by trypan blue exclusion. In most experiments, PMN were suspended in a solution that contained 130 mM NaCl, 10 mM Hepes pH 7.35–7.4 (Sigma Chemical Co., St. Louis, Mo.), 5.6 mM glucose, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.035% bovine serum albumin (Sigma Chemical Co.) (BS-H). Some experiments were conducted in BS-M, a buffer identical to BS-H except for the substitution of 10 mM Mes (2-*N*-morpholinoethane sulfonic acid, Sigma Chemical Co.) for Hepes. BS-M was used at pH 5.0, 5.5, and 6.0; and BS-H was used at pH 6.0, 6.5, 7.0, 7.5, and 8.0.

Activation. PMA (12-*O*-tetradecanoyl-phorbol-13-acetate, Consolidated Midland Corp., Brewster, N. Y.) was dissolved at 1 mg/ml in dimethyl sulfoxide. This stock solution maintained full biological activity for several months when stored at 25°C in the dark (data not shown).

Unless otherwise specified, PMN, 5×10^7/ml in BS-H, were put into plastic centrifuge tubes (Falcon No. 2070, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and brought to 37°C. PMA stock solution was added to provide a final concentration of 1 μg/ml of PMA. "Resting" (control) cells were exposed to 1% (vol/vol) dimethyl sulfoxide. After 10 min at 37°C, we added 2–5 vol of cold 0.34 M sucrose and centrifuged the cells for 10 min at 200 *g* at 4°C. The cells were resuspended in cold 0.34 M sucrose at 1×10^8 PMN/ml and homogenized in melting ice for 5 min (~95% cell breakage) with a motor-driven Potter-Elvjhem glass-teflon homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.).

After dilution with cold sucrose to 10 ml, the homogenate was centrifuged at 200 *g* for 10 min at 4°C to sediment unbroken cells and coarse cellular fragments. The opalescent supernate was centrifuged at 27,000 *g* for 20 min to deposit a "granule-rich fraction" (GRF), previously shown to contain vesicles, membrane profiles, and abundant cytoplasmic granules (12). The clear supernate was discarded. The GRF was resuspended by gentle hand homogenation in 0.34 M sucrose at 1×10^8 PMN equivalents (PMN-eq)/ml. In one experiment, GRF was prepared from neutrophils activated by a 3-min exposure to opsonized zymosan, as previously described (12).

Although most experiments were performed on freshly isolated GRF, a few experiments (noted in the text) employed GRF that had been frozen immediately after their preparation and stored at −70°C. After slow thawing in an ice bath, these manifested fully intact enzyme activity even after storage periods >6 mo, as reported for the soluble NAD(P)H oxidase preparation of Gabig and Babior (13).

Fluorescence assay. This procedure was based on the general method of Lowry et al. (14), initially using conditions approximating those adopted by Iverson et al. (7). Incubations were conducted at room temperature (22–25°C) in 1.5-ml conical polypropylene microsample tubes (Kew Scientific, Colombus, Ohio). We added 50 μl of GRF to 100 μl of 0.2 M sodium phosphate buffer at the pH specified in the text. In some experiments, described in the text, 0.2 M Tris (2-amino-2-hydroxymethyl-1,3-propanediol, Sigma Chemical Co.) or 0.2 M Mes replaced the phosphate buffer.

Reactions were started by adding 50 μl of NADPH or NADH, prepared in 5 mM sodium phosphate buffer, pH 8.0, with 8 mM sodium azide. Incubations were terminated after 20 min, unless otherwise specified, by adding 100 μl of 0.6 N HCl to destroy rapidly residual NAD(P)H, without affecting the $NAD(P)^+$ (14). After 10 min, the tubes were centrifuged for 2 min at 8,000 *g* with an Eppendorf model 3200 centrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.), and 100-μl aliquots of the supernatant solution were removed and added to 150 μl of 10 N NaOH in 10 × 75-mm borosilicate glass tubes (diSPo culture tubes, Scientific Products Div., American Hospital Supply Corp., McGaw Park, Ill.). This was mixed vigorously and allowed to stand for at least 60 min at room temperature to allow the intensely fluorescent alkali reaction products of $NAD(P)^+$ to form. Finally, we added 1.6 ml of distilled water, mixed again, and measured the fluorescence with an Aminco fluorocolorimeter (American Instrument Co., Travenol Laboratories, Silver Spring, Md.).

Standard curves, prepared by incorporating 1–50 nmol of $NAD(P)^+$ in 0.2 ml of assay buffer and sucrose, were linear. Alkali-treated GRF displayed minimal intrinsic fluorescence in the absence of $NAD(P)^+$ and did not quench the alkali-induced fluorescence of $NAD(P)^+$. We screened any reactants employed in our assays to detect any that quenched or otherwise affected $NAD(P)^+$ fluorescence. All bottles of NAD(P)H were screened to eliminate any with high background fluorescence (rare). NADH and NADPH were relatively stable, even at pH 5.5, for the brief incubation periods used in our studies. As determined spectrophotometrically at 340 nm, ~8% of NAD(P)H was destroyed in 30 min at pH 5.5, without yielding any alkali-fluorescent product. Stability was substantially greater at or above pH 7.0.

In several experiments, we incubated GRF with mixtures of NADPH and NADH, and differentiated the resultant $NADP^+$ and NAD^+ with glucose 6-phosphate dehydrogenase and glucose 6-phosphate (Sigma Chemical Co.) as described by Lowry et al. (14). Under our conditions, ~93% of reagent $NADP^+$ or of $NADP^+$ arising from the oxidation of NADPH by GRF was reduced by glucose 6-phosphate dehydrogenase and its substrate. Reagent NAD^+ and NAD^+ resulting from the action of GRF on NADH were not reduced by glucose 6-phosphate dehydrogenase under identical conditions.

Spectrophotometric assays. Assays were run at room temperature in 1.5-ml polypropylene centrifuge tubes (Kew Scientific) that contained 50 μl of each of the following three stock solutions: 0.2 M sodium phosphate with 8 mM sodium azide, pH 7.0; 2 mM ferricytochrome *c* (Sigma Chemical Co.) in distilled water; and 2 mM NADH or NADPH in 5 mM sodium phosphate buffer, pH 8.0. When appropriate, other NAD(P)H concentrations replaced the standard stock solution. Incubations were started by adding 50 μl of GRF or 0.34 M sucrose (control). Increased concentration of ferricytochrome *c* did not increase trapping efficiency.

After 10 min, ferricytochrome *c* reduction was stopped by adding 25 μg of superoxide dismutase (SOD; Miles Laboratories, Elkhart, Ind., or Sigma Chemical Co.) and 0.8 ml of ice-cold water. The tubes were immediately centrifuged (90 s, 8,000 *g*), and the optical densities of the clarified supernates were measured at 550 nm in a Gilford model 222A spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Controls lacking enzymes and/or substrate or containing 10 μg of SOD from the outset of the enzymatic reaction were used. Superoxide production, equated to SOD-inhibitable ferricytochrome *c* reduction, was calculated with a millimolar extinction coefficient of 18.5 $mM^{-1}\ cm^{-1}$ at 550 nm for reduced (ferro)cytochrome *c*–oxidized (ferri)cytochrome *c* (15).

Effect of pH on O_2^- production by intact cells. PMN were prepared at 2×10^7 cells/ml in BS-H. Just before use, aliquots (0.5 ml) were taken, centrifuged, resuspended in 37°C Hepes-free BS-H (pH 7.35) at 1×10^7 cells/ml, and exposed to 1 μg/ml PMA for 3 min. Immediately thereafter, 100-μl aliquots were removed and transferred to cuvettes containing 0.9 ml of BS-H or BS-M with 50 μM ferricytochrome *c*. Other cuvettes contained, in addition, either 2 mM NaN_3, 50 μg/ml SOD, or both. Changes in optical density at 550 nm were recorded at 23°C on a Gilford model 222A spectrophotometer, and the differences between cuvettes containing and lacking SOD were used to calculate O_2^- production. We obtained identical results whether or not azide was present in these tests (data not shown). In other studies, PMN were prepared and ex-

posed to PMA as described, and 250-μl aliquots were transferred to the water-jacketed vessels (1.15-ml capacity), containing 0.9 ml of BS-H or BS-M, of a Gilson K-1C Oxygraph with Clark-YSI electrodes (Gilson Medical Electronics, Inc., Middleton, Wis.). O_2 consumption, measured for 5 min, was linear throughout this period.

We used two systems to determine how pH affected O_2^- detection by SOD-inhibitable ferricytochrome *c* reduction. Xanthine oxidase catalyses the two-electron oxidation of xanthine to uric acid, producing both O_2^- and H_2O_2. Total electron flux was derived spectrophotometrically from the rate of uric acid production (16). O_2^- production was measured by including 5×10^{-5} M ferricytochrome *c* ±50 μg/ml of SOD in the reaction mixtures and following its absorbance at 550 nm. Typical reaction mixtures contained, in a final volume of 1 ml, 0.1 mM xanthine, 0.1–0.2 IU of xanthine oxidase, and 0.7 ml of BS-H or BS-M buffer. When indicated, 2 mM NaN_3, 50 μg/ml SOD, or both of these agents were also added.

In the other system, we prepared a 3.9 mM solution of tetramethylammonium superoxide in anhydrous dimethyl sulfoxide, performing all manipulations in a nitrogen atmosphere. The compound was synthesized by the method of McElroy and Hashman (17), and its purity (86%) was checked by the procedure of Seyb and Kleinberg (18). The solution was transferred anaerobically to a gas-tight Hamilton 5-ml syringe (Hamilton Co., Reno, Nev.) and delivered by a Sage model 341 syringe pump (Orion Research Inc., Cambridge, Mass.) into 12 × 75-mm tubes containing 475 μl of BS-H or BS-M and 25 μl of 10 mM ferricytochrome *c*. The contents were continuously mixed by a teflon-coated magnetic stirring bar. After addition of 44 μl of $(CH_4)_4NO_2$ solution over 30 s, the volume was brought to 5.0 ml with BS-H or BS-M, and the optical density at 550 nm was compared with similarly treated controls that had received 44 μl of dimethyl sulfoxide.

We also used the xanthine:xanthine oxidase system to probe secondary oxidation of NAD(P)H by O_2^-. In such assays, 1 IU of enzyme activity was defined as the oxidation of 1 μmol xanthine/min (16), and NAD(P)$^+$ formation was determined fluorometrically, as previously described.

RESULTS

Initial fluorometric experiments. Our earliest fluorometric measurements of NAD(P)H oxidase activity were performed under conditions later found to be suboptimal (pH 5.5, no added SOD, 2 mM KCN present), and will not be described in detail. In essence, they revealed substantial NAD(P)$^+$ production by GRF from PMA-stimulated normal neutrophils, but little or no NAD(P)$^+$ production by GRF from unstimulated normal neutrophils or PMA-stimulated neutrophils from a patient with X-linked chronic granulomatous disease. Although NAD(P)$^+$ production was reasonably linear with GRF concentration and incubation time, NAD(P)$^+$ production did not reach a plateau with increasing substrate concentrations, and anomalous, nonhyperbolic reaction kinetics were simulated (data not shown).

Identification of artifacts. We determined that two important artifacts had compromised the accuracy of the aforementioned measurements. Despite our presumption that optimal NAD(P)H oxidation occurred at pH 5.5, we noted a substantially higher pH optimum when we tested this assumption experimentally (Fig. 1, left). GRF prepared from PMA-activated and zymosan-activated normal neutrophils showed similar pH profiles, whereas GRF from resting neutrophils differed considerably. Meager in amount, NADP$^+$ production by resting GRF was maximal at pH 5, the lowest pH we tested, and diminished as pH increased. A similar pH response was noted for NAD$^+$ production (data not shown).

Not only was NADP$^+$ production by PMA-activated

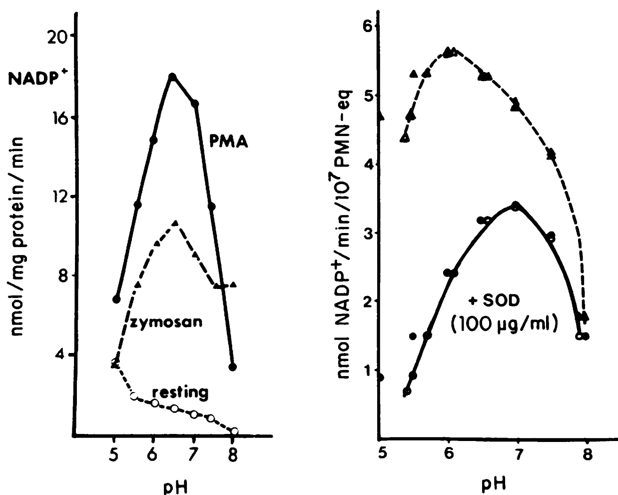


FIGURE 1 Effect of pH and SOD on NADPH oxidation by PMN fractions. Left: GRF was prepared from resting, PMA-stimulated, and zymosan-treated PMN. Assay conditions included 20 min incubation, 2 mM NADPH, 2 mM NaN_3, 0.1 M phosphate buffer, and 90–96 μg of each GRF. SOD was not present. NADP$^+$ was measured fluorometrically. Right: GRF from PMA-stimulated neutrophils was tested in the aforementioned manner ±100 μg/ml of SOD. Open symbols denote the actual pH of the final incubation mixture, filled symbols show the pH of its buffer component.

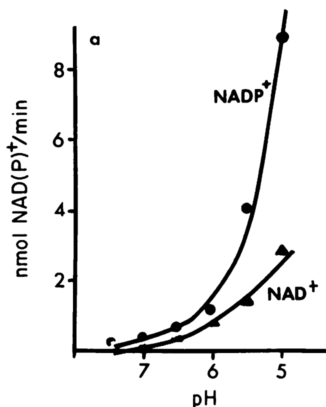


FIGURE 2 Effect of pH on oxidation of NAD(P)H by xanthine plus xanthine oxidase. Incubation mixtures contained 1 mM NAD(P)H, 0.1 mM xanthine, either 0.1 M Hepes (pH 6.0–7.5) or 0.1 M Mes (pH 5.0, 5.5), and xanthine oxidase sufficient to produce 1.4 nmol of urate/min at each pH tested. $NAD(P)^+$ production was measured fluorometrically.

GRF suboptimal at pH 5.5, but its formation was inhibited ~80% if SOD was present in the initial incubation mixture at that pH (Fig. 1, right). When SOD was present, maximal $NADP^+$ production was noted at pH 7.0.

Nonenzymatic effects of $O_2^{\cdot}$. The striking, pH-dependent inhibition of $NADP^+$ production by SOD, shown in Fig. 1, led us to examine another $O_2^{\cdot}$ generating system: xanthine plus xanthine oxidase. When NAD(P)H was exposed to this system, pH-dependent $NAD(P)^+$ production was readily demonstrated (Fig. 2). Other experiments (data not shown) established the following: (*a*) $NAD(P)^+$ production required both xanthine oxidase and xanthine, and was abrogated if SOD (50 μg/ml) was also added; (*b*) SOD minimally affected urate production by xanthine oxidase; and (*c*) $NAD(P)^+$ production was a relatively linear function of NAD(P)H concentration when ≤2 mM NAD(P)H was used.

When nonenzymatic (i.e., SOD-inhibitable) $NADP^+$ production in the GRF system (Fig. 1) and in the xanthine:xanthine oxidase system (Fig. 2) were compared, virtually identical pH dependencies of these reactions were apparent (Fig. 3). Indeed, when graphed as a logarithmic function of pH (Fig. 3, right), nonenzymatic $NADP^+$ production by either system was found to increase ~4.8-fold for each 1 U decline in pH between 7.5 and 5.0.

We concluded that fluorometric assays performed without SOD were unreliable, especially with high (>0.5 mM) NAD(P)H substrate concentrations or at lower pH levels. Under such circumstances, nonenzymatic secondary oxidation of NAD(P)H by enzymatically generated $O_2^{\cdot}$ obscured the primary oxidase reaction. Unless otherwise noted, our subsequent experiments were performed at pH 7 and in the presence of SOD. Linearity of $NAD(P)^+$ production with incubation time and granule concentration under these conditions are shown in Fig. 4. We observed no $NAD(P)^+$ production when resting GRF preparations were tested under these conditions.

Spectrophotometric assay. We measured NAD(P)H oxidase activity at pH 7.0, using ferricytochrome *c* as an $O_2^{\cdot}$ trap and SOD to render the reaction specific for $O_2^{\cdot}$. As shown in Fig. 5, considerable ferricytochrome *c* reduction occurred when a PMA-stimulated GRF was exposed to 0.5 mM NADPH, and most of this reduction was inhibited by the addition of SOD to the reaction mixture. Relatively little ferricytochrome *c* reduction occurred when a resting GRF was similarly tested.

pH optimum of intact cells. As NAD(P)H oxidase is thought by many to be localized to the plasma membrane, its activity might be influenced by pH changes in the neutrophil's external milieu. We tested this hypothesis by stimulating neutrophils maximally with PMA under uniform conditions in an unbuffered balanced salt solution and then transferring the activated cells to appropriately buffered solutions containing ferricytochrome with or without SOD.

Fig. 6 compares the effect of pH on $O_2^{\cdot}$ production by intact cells (solid line) with the pH profile of NADPH oxidase (dotted line). Is the remarkable similarity of the profiles evidence that both reactions are modulated equivalently by hydrogen ion concentrations, or are the data with intact PMN merely the result of inefficient

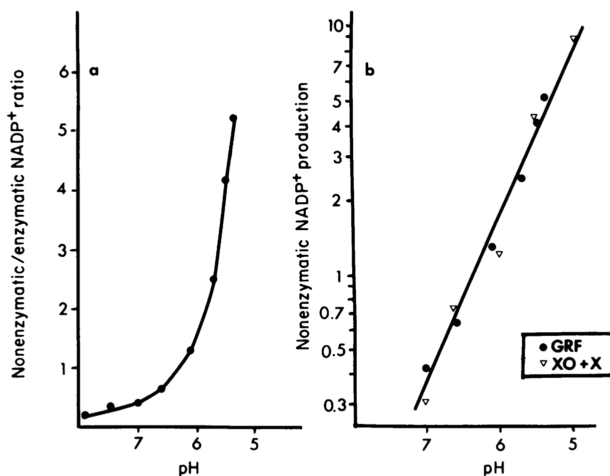


FIGURE 3 Effect of pH on the nonenzymatic oxidation of NADPH by $O_2^{\cdot}$. (a) From the data shown in Fig. 1, right, we derived a ratio—(T − E)/E—quantifying the nonenzymatic amplification of NADPH oxidation by $O_2^{\cdot}$ produced enzymatically by neutrophil GRF. T equals $NADP^+$ production measured in the absence of SOD and E equals $NADP^+$ produced in the presence of SOD. (b) The data from Fig. 1, right (●), and Fig. 2 (▽) are redrawn on semilogarithmic coordinates. It is apparent that whether $O_2^{\cdot}$ is generated by NAD(P)H oxidase (●) or xanthine oxidase (▽), its subsequent nonenzymatic oxidation of NADPH is governed equivalently by pH.

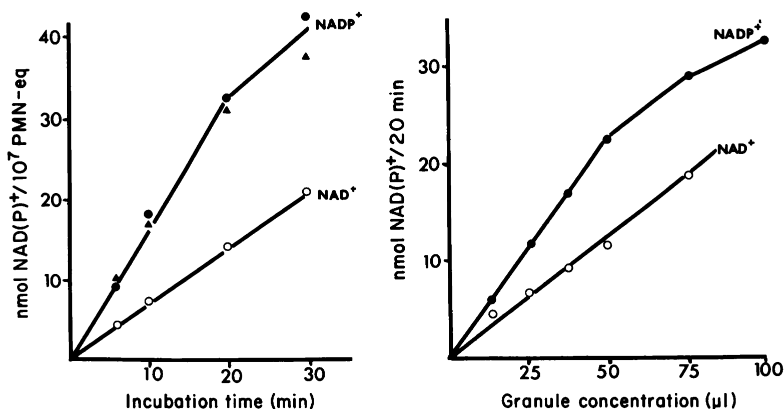


FIGURE 4 Linearity of NAD(P)H oxidation with time and granule concentration in the improved fluorometric assay method. Left: GRF (50 μl) from 5 × 10^6 PMA-stimulated neutrophils was added to a solution containing, in a final volume of 0.2 ml, 0.1 M phosphate buffer (pH 7.0), 0.2 mM NADPH or 1 mM NADH, 2 mM NaN_3 and 50 μg/ml SOD. $NADP^+$ (●), NAD^+ (○), and $NADP^+$, with phosphate buffer replaced by 0.1 M Tris buffer pH 7.0, all other constituents unaltered (▲). Right: GRF was incubated for 20 min in 0.2 ml of a solution containing 0.2 mM NADPH or 1.0 mM NADH and the aforementioned constituents.

O_2^--trapping by the ferricytochrome system at or below pH 6? We examined these alternatives in several ways. When ferricytochrome *c* was used to trap O_2^- generated by xanthine oxidase, it captured 25.0% of the total electron flow at pH 7, 17.5% at pH 6, and 12.2% at pH 5. Total electron flow was inferred from the rate of uric acid production, and O_2^- as SOD-inhibitable ferricytochrome *c* reduction. When tetramethylammonium superoxide was infused, ferricytochrome *c* trapped 38.0% at pH 8, 25.1% at pH 7.5, 19.3% at pH 7, and 11.5% at pH 6. Thus, even if interpreted in light of the reduced trapping efficiency of ferricytochrome *c* at pH 6, Fig. 6 still suggests diminished production of O_2^- by intact cells at that pH. PMA-stimulated intact PMN, handled analogously to the cells shown in Fig. 6, revealed the following rates of O_2 consumption at 23°C (nanomoles O_2 per 10^7 PMN per minute): 25 at pH 7.5, 23 at pH 7.0, 10.1 at pH 6.5, 9.4 at pH 6.0, 3.3 at pH 5.5,

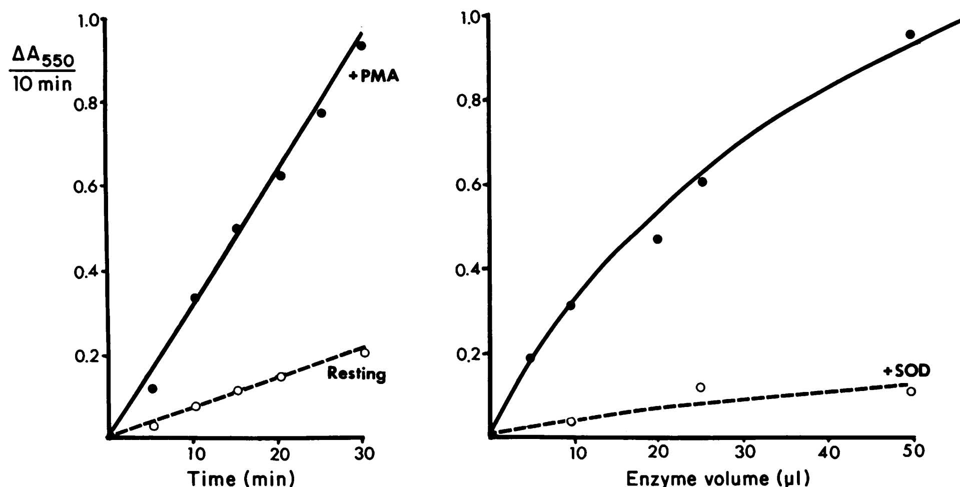


FIGURE 5 Spectrophotometric measurement of O_2^- produced by GRF. Left: mixtures containing 50 μl of GRF (equivalent to 5 × 10^6 PMN), 0.1 M phosphate buffer at pH 7.0, 0.5 mM NADPH, 2 mM NaN_3 and ±SOD (25 μg) in a final volume of 0.2 ml were incubated for 10 min at 23°C and processed as described in the text. Filled circles represent ΔA 550 nm (relative to a GRF-free control) in the absence of SOD and open circles represent ΔA 550 nm in the presence of SOD. Right: varying quantities of GRF (5–50 μl) were added to an incubation mixture, otherwise as described above, and the ΔA 550 nm was determined after 10 min.

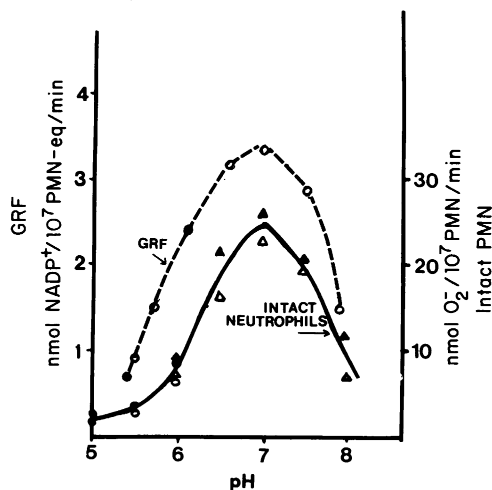


FIGURE 6 Effect of pH on O_2^- production by intact neutrophils. The solid line shows O_2^- generated by intact PMA-stimulated PMN in BS-H (triangles) or BS-M (circles). Solid symbols denote the presence of 2 mM NaN_3 and open symbols, its absence. The dashed line showing the effect of pH on $NADP^+$ production by GRF is taken from Fig. 1, right.

and 1.5 at pH 5.0. As these responses closely resemble the data for O_2^- production shown in Fig. 6, they offer independent support that oxidative metabolism was modulated by pH.

We compared the fluorometric and spectrophotometric methods for measuring NAD(P)H oxidase on a GRF obtained from PMA-stimulated neutrophils. $NAD(P)^+$ production was measured fluorometrically at pH 7 in the presence of SOD and 2 mM NaN_3. O_2^- was measured spectrophotometrically as SOD-inhibitable ferricytochrome *c* reduction under comparable conditions with or without SOD as described in Methods. As indicated by Fig. 7, we observed 2 nmol of O_2^-/nmol of NAD(P)H oxidized, confirming the postulated stoichiometry of NAD(P)H oxidase to be $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$. In this experiment we noted, as we did throughout, that NADPH was favored over NADH as the preferred electron donor for NAD(P)H oxidase.

K_m and V_{max} of the oxidase. We performed several experiments under optimal conditions, i.e., at neutral pH in the presence of SOD. A representative study, using frozen GRF from a single donor, is illustrated in Fig. 8. This shows both an Eadie-Hofstee reciprocal plot (used to determine K_m and V_{max}) and a velocity: substrate concentration plot (inset). For clarity of display, NADH and NADPH data are plotted on different scales. NADPH oxidase activity showed a K_m of 0.15 mM NADPH and a V_{max} of 2.22 nmol $NADP^+/10^7$ PMN-eq per min. NADH oxidase activity showed a K_m of 0.55 mM NADH and a V_{max} of 1.34 nmol $NAD^+/10^7$ PMN-eq per minute. The lower K_m and higher V_{max} for NADPH, relative to NADH, were regularly noted in our measurements, as shown in Table I.

Before defining optimal enzyme conditions and performing the experiments listed in Table I, we determined the apparent K_m for NAD(P)H at pH 5.5 or 7.0 in the absence of SOD in 11 experiments. In these, the apparent K_m (mean±SD) for NADPH was 0.09 ±0.02 mM, n = 4, at pH 5.5, and 0.13±0.03 mM, n = 3, at pH 7.0. In contrast, the K_m for NADH was 0.85 mM, n = 1, at pH 5.5 and 0.56±0.18 mM, n = 3, at pH 7.0. The values are surprisingly close to those obtained under the more appropriate incubation conditions.

Unitary nature of NAD(P)H oxidase. Were NADH and NADPH oxidation effected by a single NAD(P)H oxidase or by discrete NADH and NADPH oxidases? We tested this by examining GRF preparations with the substrates individually or in combination, using glucose 6-phosphate dehydrogenase and glucose 6-phosphate to distinguish $NADP^+$ from NAD^+. Assays with GRF were run in Tris buffer to avoid inhibition of glucose 6-phosphate dehydrogenase by phosphate. The results are shown in Table II. We selected concentrations of NAD(P)H that approximated twice the mean K_m we had determined for each nucleotide.

Note that NADPH inhibited NADH oxidation by 43.7 and 51.4% in experiments 1 and 2, whereas NADH inhibited NADPH oxidation by only 3.8 and 15.0% in these experiments. The total rate of $NAD(P)^+$ production was diminished 26.8 and 34.2% from the simple

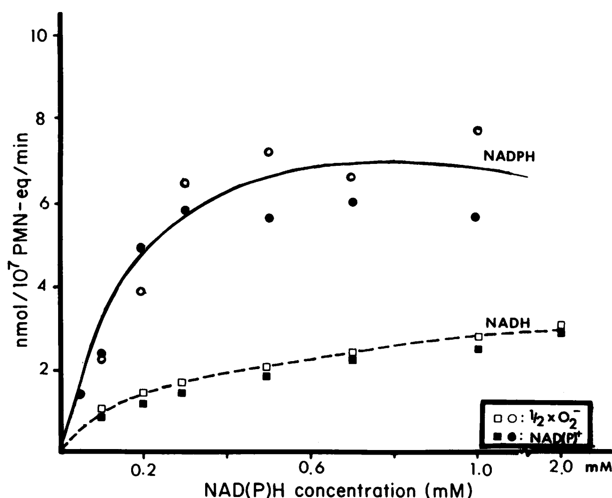


FIGURE 7 NAD(P)H oxidase activity of PMA-treated neutrophils. GRF, prepared from PMA-stimulated neutrophils, was tested for $NAD(P)^+$ production and O_2^- generation by the described fluorometric and spectrophotometric assays. Incubation times were 20 min (fluorometric assay) or 10 min (spectrophotometric assay). Superoxide generation (SOD-inhibitable ferricytochrome *c* reduction) is graphed at half-scale, to illustrate better the stoichiometric relationship $NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + 2O_2^- + H^+$.

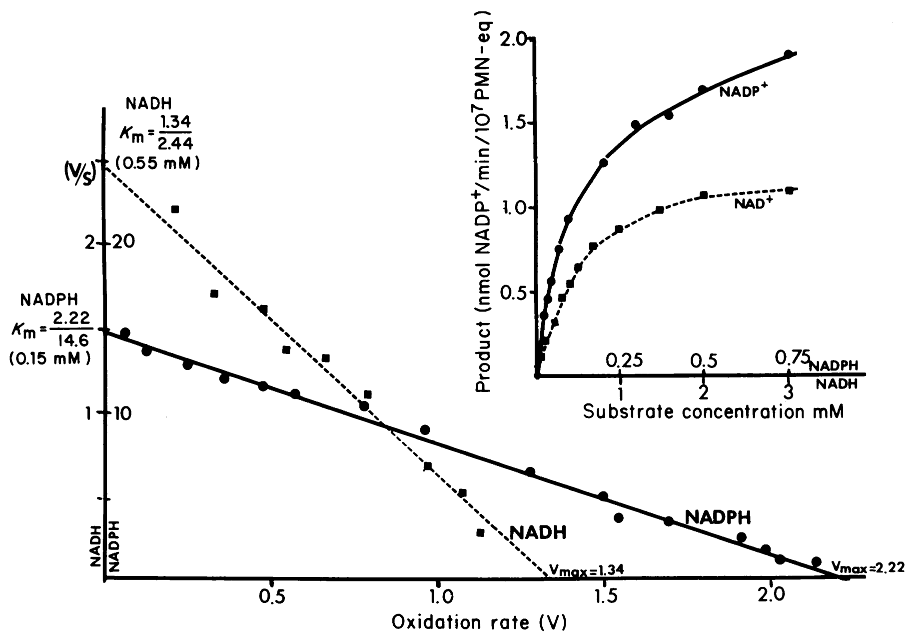


FIGURE 8 Eadie-Hofstee plot showing apparent K_m and V_{max} for NAD(P)H oxidase activity. Incubations were performed on successive days under standard conditions (pH 7.0 phosphate buffer, 50 μg/ml SOD, 2 mM NaN_3) on GRF, obtained from a single subject, that had been frozen at −70°C. The inset shows $NAD(P)^+$ production as a function of substrate concentration. Note that different scales are used for NADPH and NADH on the inset and on the ordinate of this representative reciprocal plot.

additive rates that would be expected if discrete NADH and NADPH oxidases were present.

We noted comparable results in two additional experiments, performed with 0.1 mM NADPH and 1.0 mM NADH (data not shown). This pattern of reciprocal inhibition indicates that PMA-activated GRF contains a single NAD(P)H oxidase that can utilize either substrate. The more effective inhibition of NADH oxidation by NADPH than vice versa is consistent with the lower K_m for NADPH noted in Table I.

DISCUSSION

The respiratory burst, a complex metabolic response whereby neutrophils generate O_2^-, is initiated by activation of an enzyme that oxidizes reduced phosphopyridine nucleotides. The primary oxidase involved in this reaction has been reported to use NADH (19) or NADPH (12, 20) preferentially. It has been assigned a pH optimum of 5.5–6.0 (7, 21–23) or near neutrality (20, 24). It has been localized to the plasma membrane (25–27), or to other cellular organelles (7, 24). Suggestions that its activation involves allosteric phenomena have been made (21), but not confirmed.

Although numerous innovative approaches have been applied in attempted characterizations of the "primary oxidase," two general methods—one fluorometric and the other spectrophotometric—have been most widely used. Both approaches were used in the present study. We began by applying a two-step procedure, devised by Auclair et al. (28–30) and Iverson et al. (7), that involves incubating GRF with NAD(P)H under specified conditions, followed by the measurement of $NAD(P)^+$ fluorometrically (14).

TABLE I
Kinetic Features of NAD(P)H Oxidase

Substrate	K_m	V_{max}	
	mM	*nmol/min/10⁷ PMN-eq*	*nmol/min/mg protein*
NADPH	0.15	2.2	9.2
	0.14	2.1	10.0
	0.07	1.4	11.7
	0.23	2.8	13.3
Mean±SEM	0.15±0.03	2.1±0.29	11.1±0.91
NADH	0.55	1.3	5.6
	0.68	1.6	8.4
	0.26	0.6	5.3
	1.23	1.6	13.3
Mean±SEM	0.68±0.20	1.3±0.22	8.2±1.8

GRF was prepared from PMA-stimulated neutrophils and $NAD(P)^+$ production was measured fluorometrically under the optimal conditions described in the text.

TABLE II

Competitive Effects of NADH and NADPH on NAD(P)H Oxidase

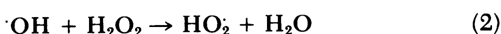
Experiment	Substrate		Product		
	NADPH	NADH	$NAD(P)^+$	$NADP^+$	NAD^+
			nmol/10^7 PMN-eq/min		
1	+	0	2.63	2.63	—
	0	+	3.59	—	3.59
	+	+	4.55	2.53	2.02
2	+	0	3.66	3.66	—
	0	+	4.07	—	4.07
	+	+	5.09	3.11	1.98

Experiments were performed with two different frozen (−70°C) PMA-activated GRF preparations. NADH concentration was 1.2 mM, NADPH concentration was 0.3 mM. (+) signifies the presence of a reactant, (0) shows its absence. The efficiency of $NADP^+$ conversion by glucose 6-phosphate dehydrogenase and glucose 6-phosphate was 93.4% in both experiments. In contrast to our usual assay conditions, these incubations were performed in Tris buffer (see text) for 10 (GRF step) and 20 min (glucose 6-phosphate dehydrogenase step), respectively. Each value is the mean of four closely agreeing sample values. Note the reciprocal inhibition of NADPH oxidation by NADH and of NADH oxidation by NADPH.

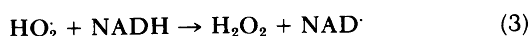
We found that two major and distinct processes caused NADPH to be oxidized to $NAD(P)^+$ in step 1. One of these was a direct enzymatic effect of NAD(P)H oxidase. However, a nonenzymatic process arose from reactions between NAD(P)H and O_2^- (produced by the oxidase) or its conjugate acid, $HO_2^\cdot$. Such a nonenzymatic reaction occurred whether O_2^- was produced by subcellular GRF fractions from neutrophils or by xanthine oxidase plus xanthine. Artifactual oxidation of NADPH by oxygen metabolites at acid pH has previously been described in related systems (31, 32). NADPH was more susceptible to $O_2^-/HO_2^\cdot$-initiated oxidation than was NADH, and both were protected by SOD. The effect, strongly pH dependent, increased dramatically as the pH approached 5.0 (see Figs. 2 & 3).

Nadezhdin and Dunford (33) recently reported that $HO_2^\cdot$, generated by flash photolysis of H_2O_2, oxidized NADH to NAD^+ via the intermediate production of $NAD^\cdot$, a potent reducing agent capable of univalently reducing O_2 (34). Their data suggested that the following reactions occurred:

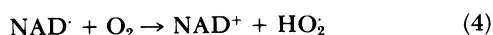
$$H_2O_2 \xrightarrow{h\nu} 2\,{}^\cdot OH \quad (1)$$



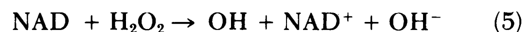
$${}^\cdot OH + H_2O_2 \rightarrow HO_2^\cdot + H_2O \quad (2)$$



$$HO_2^\cdot + NADH \rightarrow H_2O_2 + NAD^\cdot \quad (3)$$



$$NAD^\cdot + O_2 \rightarrow NAD^+ + HO_2^\cdot \quad (4)$$



$$NAD + H_2O_2 \rightarrow OH + NAD^+ + OH^- \quad (5)$$

Reactions (3) and (4) have the capacity to propagate or sustain a free radical chain reaction initiated by $HO_2^\cdot$ (hydroperoxyl radical), the protonated form of O_2^-. As the pK_a of $HO_2^\cdot$ is 4.9±0.1 (35), the striking increase in NAD(P)H oxidation noted in Fig. 3 as the pH approaches this pK_a would be expected if $HO_2^\cdot$ were the active oxidant species.

Inclusion of SOD in our assays abrogated the O_2^--initiated, non-enzymatic oxidation of NAD(P)H, lent improved specificity to the fluorometric procedure, and allowed us to demonstrate that the true pH optimum for NAD(P)H oxidase was 7.0. This value agrees with recent reports by Gabig and Babior (13) and Tauber and Goetzl (24), who used spectrophotometric methods.

Other, more minor methodologic alterations also improved the sensitivity of our fluorometric procedure relative to its antecedents. By diminishing the total reaction volume to 0.2 ml while adding 0.1–0.2 mg of GRF, we achieved an excellent ratio of enzymatic/nonspecific or spontaneous oxidation of substrate. Our inclusion of NaN_3 rather than NaCN in the reaction was intended to maintain a myeloperoxidase inhibitor in the assay, and was dictated by the report that cyanide ions greatly augment nonenzymatic oxidation of NADPH by O_2^- (36). In practice, however, we obtained equivalent results in our fluorometric assay whether or not azide was included.

DeChatelet et al. (21) suggested that initiation of the respiratory burst is associated with allosteric transformation of a reduced pyridine nucleotide oxidase, allowing its expression at physiologic substrate concentrations. Unfortunately, these experiments were conducted under suboptimal experimental conditions. Our data show that under conditions minimizing nonenzymatic substrate oxidation, the enzyme of resting cells is wholly inactive. Furthermore, we detected no evidence suggesting sigmoidal kinetics or allosteric regulation of active NAD(P)H oxidase by its substrates.

Auclair and associates (28–30, 38) also used fluorometric procedures to study oxidation of NAD(P)H by subcellular fractions from resting and phagocytic neutrophils. Their early experiments were performed under conditions (pH 5.5, 0.5 mM Mn^{++}) favoring spontaneous generation of O_2^- and cannot be readily interpreted. Unfortunately, even their more recent studies use resting neutrophils and were carried out at pH 5.0.

We believe that our data concerning the stoichiometry of NAD(P)H oxidase—$NAD(P)H + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$—are straightforward. Babior et al. (20) used zymosan-activated GRF and spectrophotometric assays of NADPH oxidation and O_2^- production, and reported similar stoichiometry. The reciprocal inhibition of NADPH oxidation by NADH and vice versa appears to afford adequate assurance that a single oxi-

dase, capable of oxidizing either NADPH or NADH, is present.

We believe that the substantial inhibition of O_2^- production seen in PMA-stimulated neutrophils exposed to low external pH can be explained most simply by postulating a membrane localization for the oxidase to render it susceptible to the external milieu. Proton production is a prominent, if little recognized component of the respiratory burst (37), and the neutrophil membrane must be highly permeable to these metabolically generated hydrogen ions. Although detailed consideration of the relationships between ionic and proton fluxes and metabolic activation would exceed the scope of this discussion, we hope to deal with these questions in subsequent reports.

Our values for the respective K_m of NADPH and NADH (Table I) compare reasonably well with values reported by Babior et al. for particulate (20) or detergent-solubilized preparations (13) from zymosan-treated neutrophils. Our determination of the K_m for NADPH (0.15 ± 0.03 mM) is consistent with that reported by Tauber and Goetzl (22.7 μM) for a deoxycholate-solubilized preparation from PMA-treated neutrophils (24). Our specific activity (11.1 ± 0.91 nmol/min per mg protein) for NADPH appears to be somewhat lower than theirs (21 ± 3.6 nmol/min per mg protein, mean$\pm$SEM), presumably reflecting partial purification of their preparation. It should be noted that our values were obtained by the fluorometric procedures described in the text, whereas the other values cited above were obtained by spectrophotometric procedures.

The concentration of both NADH and NADPH in resting human neutrophils was reported by Bachner et al. (39) to be 2.4 nmol/10^8 cells. Assuming their even distribution in total cell water, this corresponds to ~0.1 mM NAD(P)H. Whether one accepts the K_m from our experiments or the slightly dissimilar ones advanced by Babior et al. (13, 20) or Tauber and Goetzl (24), it follows that oxidation of NADPH should substantially exceed oxidation of NADH at their physiologic concentrations.

The improved fluorometric procedures described in this report allow many of the discrepant literature reports to be resolved. It is clear that both NADH and NADPH afford excellent in vitro substrates for a single enzyme, NAD(P)H oxidase. This fact, combined with the myriad of technical subtleties affecting the various oxidase assay procedures, has fueled the longstanding conflict between proponents of primary roles of separate NADH and NADPH oxidases.

We hope that application of the sensitive and specific fluorometric procedures described here will assist efforts to resolve the many important unanswered questions concerning the activation and regulation of NAD(P)H oxidase in states of normal and disordered neutrophil function.

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