

Further Characterization of NADPH Oxidase Activity of Human Polymorphonuclear Leukocytes

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ABSTRACT Mn^{2+} was shown to catalyze a nonenzymatic oxidation of NADPH in the presence of superoxide anion by means of an isotopic assay for measurement of the oxidation of NADPH to $NADP^+$. Human polymorphonuclear leukocyte granule NADPH oxidase activity was evaluated in the absence of Mn^{2+} and was found to be higher in granules from phagocytizing cells than in granules from resting cells. The drug phorbol myristate acetate, which affects the oxidative metabolism of the neutrophil like phagocytosis, was found to activate granule NADPH oxidase activity. Superoxide dismutase was shown to inhibit NADPH oxidase activity both in the presence and absence of added Mn^{2+} . The NADPH oxidase reaction in the absence of Mn^{2+} was optimal at pH 5.5, and was more linear with increasing time and protein concentration than in the presence of Mn^{2+} . No activity was measurable in granules isolated from resting cells until the level of NADPH added was above 0.25 mM. Activity was present in granules isolated from cells challenged with opsonized zymosan, even at 0.05 mM NADPH, and was higher than the activity found in granule fractions from resting cells at all levels of NADPH tested. The addition of as little as 0.1 μM NADH to the reaction mixture was found to inhibit granular NADPH oxidase activity, indicating a possible regulatory role for NADH. These results suggest that NADPH oxidase may be the enzyme that initiates the metabolic events accompanying phagocytosis.

INTRODUCTION

NADPH oxidase has been proposed as the initiating enzyme of the series of metabolic events that accompanies phagocytosis (1). These events include increases in oxygen consumption, hexose monophosphate shunt activity, and the production of both hydrogen peroxide

and superoxide anion (2). The activity of NADPH oxidase has been shown to increase upon phagocytosis (3-5), consistent with the enzyme being the initiator. However, the use of Mn^{2+} in the assay for the oxidase has been viewed somewhat skeptically. The concentration of Mn^{2+} in the human polymorphonuclear leukocyte (PMNL)¹ is not well established; thus; its physiological significance is unknown.

A recent publication reported that, in the presence of superoxide anion, Mn^{2+} stimulates a nonenzymatic oxidation of NADPH (6). It has been demonstrated that PMNLs generate superoxide (7) and could, therefore, serve as a source of superoxide for the Mn^{2+} -catalyzed reaction. These results have been confirmed by Patriarca et al. (8).

We have previously reported a sensitive isotopic assay system for measuring NADPH oxidase activity (4). In light of the recent information concerning Mn^{2+} , it seemed necessary to examine the effect of superoxide on the Mn^{2+} -catalyzed oxidation of NADPH in our assay system and to evaluate the characteristics of NADPH oxidase in the absence of Mn^{2+} . This communication reports the results of our investigation.

METHODS

Isolation of leukocytes. Human neutrophils were isolated from heparinized venous blood as described previously (4). The final cell pellet was resuspended in Dulbecco's phosphate-buffered saline (PBS) and a differential cell count was obtained with a hemocytometer. The cell suspensions, routinely containing more than 85% PMNL, were diluted in PBS to a cell concentration of 1.5×10^8 PMNL/ml.

Isolation of granules. Zymosan (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) was opsonized and prepared as previously

¹Abbreviations used in this paper: BSA, bovine serum albumin; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMNL, polymorphonuclear leukocytes; SOD, superoxide dismutase.

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described (4). Phorbol myristate acetate (PMA) (Consolidated Midland Corp., Chemical Div., Katonah, N. Y.) was stored frozen at a concentration of 2 mg/ml in dimethylsulfoxide. Immediately before use, PMA was diluted with PBS to a concentration of 3.0 $\mu\text{g}/\text{ml}$.

Isolated cells at a concentration of 1.5×10^8 PMNL/ml were incubated at a ratio of 1 vol cells to 2 vol opsonized zymosan or PBS. When cells were incubated with PMA, the drug was added to give a final concentration of 0.1 $\mu\text{g}/\text{ml}$. In these experiments, an equal quantity of dimethylsulfoxide was added to the resting and opsonized zymosan incubation mixes to exclude possible effects due to the small amount of solvent added with the PMA. Incubation of cells with PBS, opsonized zymosan, or PMA was carried out for 3 min at 37°C, after prior equilibration of the solutions to 37°C. Incubation was terminated by the addition of an equal volume of 0.68 M sucrose, followed by immediate immersion in an ice bath.

Homogenization was carried out as described previously (4), with the modification that every sample was homogenized for a total of 5 min. Cell breakage was estimated by phase microscopy and routinely found to be more than 90%. Homogenates were centrifuged at 500 *g* for 10 min in the cold to remove unbroken cells, nuclei, and cell debris. Supernates were recentrifuged at 27,000 *g* for 15 min at 4°C. The final pellets were then resuspended in a volume of 0.34 M sucrose equal to twice the original cell volume. The resuspended 27,000 *g* pellet is referred to as the granule fraction. If granule fractions were washed once with 0.34 M sucrose before assaying, no change in resulting NADPH oxidase activity was observed. The protein content of the granule fraction was routinely determined by the Lowry et al. method (9), and the fractions were adjusted to the same protein level before use.

Assay for NADPH oxidase. The isotopic assay for NADPH oxidase has been described in detail previously (4). The assay consists of a two-step incubation procedure in which NADP⁺ is first generated under optimal conditions for NADPH oxidase. The amount of NADP⁺ produced is then quantitated in a second incubation, in a system containing 0.1 μCi [¹⁴C]6-phosphogluconate (New England Nuclear Corp., Boston, Mass.) and 1 U 6-phosphogluconate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.). The ¹⁴CO₂ produced is trapped and counted as previously described (10).

Certain modifications have been made in this procedure. The first involves the elimination of Mn²⁺ from the initial incubation mix, based on the probability that it obscures the true enzymatic activity. A second modification is the substitution in the second incubation of isotope with a higher specific activity. Previously, [1-¹⁴C]6-phosphogluconate at a specific activity of 0.04 $\mu\text{Ci}/\mu\text{mol}$ was utilized. The present isotope has a 10-fold higher specific activity, 0.4 $\mu\text{Ci}/\mu\text{mol}$, achieved by reducing to one-tenth the amount of unlabeled 6-phosphogluconate used to dilute the isotope. The actual amount of isotope in an assay, 0.1 μCi , remained unchanged. This, in effect, increased the number of counts per minute obtained in an assay and was adopted because the elimination of Mn²⁺ from the assay system markedly lowered the measurable activity. Under these new conditions, levels of NADPH as high as 1.25 mM were found to have no effect on the activity of 6-phosphogluconate dehydrogenase.

In a series of experiments, the oxidation of NADPH was measured in the presence of the xanthine-xanthine oxidase system, used to generate superoxide. The assay medium for the first step was as follows: 0.1 M potassium phosphate buffer, pH 5.5; 0.5 mM MnCl₂; 0.17 mM NADPH;

0.25 mM xanthine (Sigma Chemical Co.); and 0.01 U xanthine oxidase (Sigma Chemical Co.); in a total volume of 1.0 ml. The concentrations of xanthine and xanthine oxidase were similar to those used by Patriarca et al. (8). In some instances, 100 μg superoxide dismutase (SOD) (Truett Laboratories, Dallas, Texas) or 100 μg bovine serum albumin (BSA) (Sigma Chemical Co.) were also included in the assay. SOD was inactivated by autoclaving at 260°F for 20 min. In one case, xanthine oxidase was boiled for 5 min and then substituted for the active enzyme at a final concentration of 0.01 U/ml. The second step of the incubation, in which the amount of NADP⁺ produced was quantitated, remained unchanged from that described above.

In some cases, NADPH oxidase activity was also determined polarographically as described by Hohn and Lehrer (3). Assay conditions were essentially the same as in the first step of the isotopic assay, with 0.17 mM NADPH and no manganese.

Assay for myeloperoxidase. Myeloperoxidase activity was determined with the *o*-dianisidine dye procedure (11). Activity was calculated with an extinction coefficient for *o*-dianisidine at 460 nm of 11.3 mM⁻¹cm⁻¹.

RESULTS

Initial experiments were concerned with the effect of a superoxide-generating system on the oxidation of NADPH. The results are summarized in Table I. At pH 5.5 and in the presence of Mn²⁺, the xanthine-xanthine oxidase system effectively oxidized NADPH to NADP. Optimal activity was dependent upon the presence of NADPH, Mn²⁺, and an active O₂⁻-generating system. SOD inhibited the oxidation, while autoclaved SOD did not, indicating that superoxide anion is involved in the reaction. If BSA was substituted for the SOD, no inhibition was observed. Several other proteins, including catalase, horseradish peroxidase, and glutathione reductase, were examined and none were inhibitory (data not shown). Thus, the effect of SOD seems to be spe-

TABLE I
Mn²⁺-Catalyzed Oxidation of NADPH by the Xanthine-Xanthine Oxidase System

Condition	Radioactivity
	<i>cpm</i>
Complete system	23,377 ± 3,574 (4)
– Mn ²⁺	2,010 ± 395 (5)
– NADPH	491 ± 36 (3)
– Xanthine	5,291 ± 793 (4)
– Xanthine oxidase	4,809 ± 1,329 (4)
+ Boiled xanthine oxidase	5,382 ± 73 (2)
+ SOD	6,643 ± 1,235 (4)
+ Autoclaved SOD	18,960 ± 4,996 (3)
+ BSA	23,931 ± 2,947 (2)

Oxidation of NADPH was determined as described under Methods. Numbers in parentheses are the number of experiments carried out. Values were determined in triplicate in each experiment. Each value reported represents the mean ± SE for the given number of experiments.

TABLE II
NADPH Oxidase Activity in Granules Isolated from Resting and Phagocytizing Cells

Condition	Radioactivity <i>cpm</i>
Spontaneous oxidation of NADPH	2,025±126 (15)
Granule fraction from resting cells	925±40 (8)
Granule fraction from phagocytizing cells	7,196±547 (14)

Assay was executed as described under Methods. Numbers in parentheses denote the number of experiments performed. Each value was determined in triplicate in each experiment. The values reported here represent the mean±SE for the given number of experiments.

cific. The xanthine oxidase preparation is devoid of significant NADPH oxidase activity, as indicated by the control, which omitted xanthine. These results suggest that the presence of Mn²⁺ in an assay system for NADPH oxidase may very well mask the true enzymatic activity by greatly stimulating a nonenzymatic oxidation of NADPH.

It was imperative, therefore, to reevaluate the NADPH oxidase found in the human PMNL in an assay system without Mn²⁺. Previously, in the presence of Mn²⁺, we had demonstrated that NADPH oxidase activity was higher in granules isolated from phagocytizing cells than in granules from resting cells (4). Table II shows the results of several experiments performed in the absence of Mn²⁺ measuring NADPH oxidase activity in isolated granules. Activity of the granule fraction isolated from resting cells never exceeded the level of spontaneous oxidation of NADPH; in fact, the presence of this granule fraction actually seemed to inhibit control activity. However, considerable activity was always present in granules from phagocytizing cells. Thus, the enzyme seems to be active in granules obtained from phagocytizing cells, but cannot be measured in granules from resting cells at the level of NADPH tested, i.e., 0.17 mM.

We have compared NADPH oxidase measurements obtained with our isotopic procedure to those acquired with the more conventional polarigraphic assay. Essentially no differences were apparent in NADPH oxidase activity found in granules from phagocytizing cells with either assay. From five separate experiments, a mean value of 3.22±0.65 nmol oxygen/min per mg protein was obtained polarigraphically. The mean value of 13 experiments performed isotopically was 3.24±0.63 nmol NADP⁺/min per mg protein. These results support the validity of the isotopic assay for measurement of NADPH oxidase activity.

The drug PMA has been shown to alter the oxidative metabolism of human PMNL similarly to phagocytosis

(12). Accordingly, we examined the effect of PMA on NADPH oxidase. Human neutrophils were incubated with either PBS (resting), opsonized zymosan (phagocytizing), or PMA. The granule fractions were subsequently isolated from each and were assayed for NADPH oxidase activity. The results (Table III) showed no activity in the granule fraction from resting cells above that of the control. The granule fractions from cells that had phagocytized zymosan or that had been stimulated by PMA both demonstrated NADPH oxidase activity. In one experiment, granules isolated from resting cells were assayed in the presence of PMA. No oxidase activity was observed under these conditions. Controls with PMA alone indicated no effect by the drug on the spontaneous oxidation of NADPH.

It was of interest to compare the effects of SOD on NADPH oxidase activity in the presence and absence of added Mn²⁺. Results are given in Table IV. As expected, in the presence of Mn²⁺, 100 µg SOD significantly inhibited the spontaneous oxidation of NADPH and NADPH oxidase activity exhibited by granules from resting and phagocytizing cells. In contrast, little inhibition was observed when either BSA or autoclaved SOD were substituted for the active dismutase. Similarly, SOD was also found to inhibit the auto-oxidation of NADPH and the activity of both granule fractions in the absence of added Mn²⁺. Under these conditions, BSA was slightly inhibitory, while autoclaved SOD caused a slight stimulation.

Other characteristics of the enzyme in granules from phagocytizing cells were investigated in the absence of Mn²⁺. The pH optimum was found to be 5.5, as shown in Fig. 1, which agrees with previous data obtained in the presence of Mn²⁺ (4). Fig. 2 shows the dependence of the enzymatic activity on time and on protein concentration. Except for an initial burst of activity in the first 2.5 min, the reaction was essentially linear with time. The same does not hold true for increasing protein concentration, since no linearity was observed above approximately 0.2 mg protein/ml. For this reason, the protein concen-

TABLE III
NADPH Oxidase Activity in Granules Isolated from Cells Incubated with Opsonized Zymosan or PMA

Condition	Exp. 1	Exp. 2	Exp. 3
	<i>cpm</i>		
Control	2,394	1,398	1,804
+Granule fraction from resting cells	777	—	1,087
+Granule fraction from cells which had phagocytized zymosan	6,898	6,502	7,871
+Granule fraction from cells exposed to PMA	4,925	5,151	3,286

Each value is the mean of closely agreeing triplicate determinations. Preparation of granule fractions and assay for NADPH oxidase activity are described in Methods.

TABLE IV
The Effect of SOD on NADPH Oxidase Activity in the Presence and Absence of Added Manganese Ion

Condition	Spontaneous oxidation	Granules from resting cells	Granules from phagocytizing cells
<i>cpm</i>			
Mn²⁺ present			
No addition	8,913 ± 15	17,581 ± 140	21,076 ± 861
+SOD	3,144 ± 116	4,763 ± 222	5,820 ± 149
+Autoclaved SOD	7,009 ± 163	15,614 ± 189	19,466 ± 380
+BSA	7,924 ± 231	15,715 ± 597	17,978 ± 361
Mn²⁺ absent			
No addition	2,595 ± 123	2,562 ± 2	6,694 ± 46
+SOD	1,852 ± 9	2,039 ± 43	2,482 ± 55
+Autoclaved SOD	3,029 ± 78	4,704 ± 15	8,693 ± 279
+BSA	2,444 ± 83	2,321 ± 39	4,841 ± 316

Assay was performed as described under Methods. Protein additions were made in 100- μ g quantities. Each value represents the mean \pm SE for triplicate determinations. The experiment shown is representative of two separate experiments.

tration of each granule fraction was determined, and the level was adjusted to within the range of linearity before use in each assay. No activity was measurable in the granule fraction from resting cells with either increasing time or increasing protein concentration.

The effects of NADPH concentration on the NADPH oxidase activity in granule fractions isolated from resting and phagocytizing cells are compared in Fig. 3. At every level of NADPH, phagocytizing activity was higher than resting activity. It is noteworthy that no activity was measurable in the granule fraction from

resting cells until the level of NADPH was above 0.25 mM. Thus, at low levels of NADPH, there was no detectable NADPH oxidase activity in the granule frac-

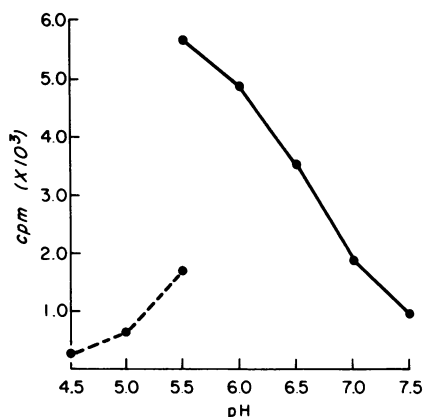


FIGURE 1 Effect of pH on NADPH oxidase activity. Granules were isolated from phagocytizing cells and assay was performed as described in Methods. Each point is the mean of triplicate determinations and has been corrected for spontaneous oxidation (determined with no granule fraction present). Protein concentration was 0.15 mg/ml and NADPH concentration was 0.17 mM. Dotted line, acetate buffer; solid line, phosphate buffer.

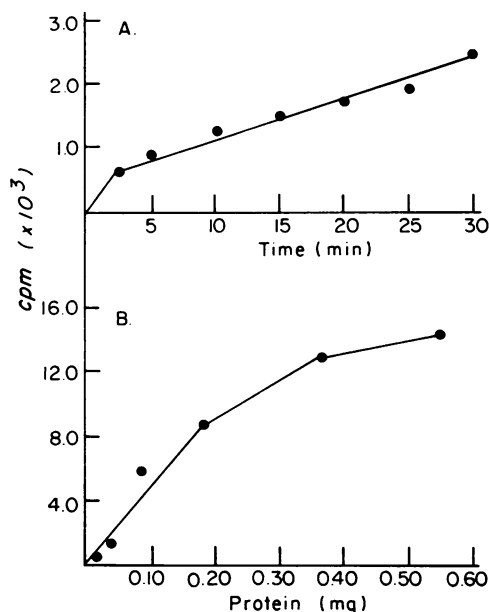


FIGURE 2 Effect of time and protein concentration on NADPH oxidase activity. Granules were isolated from phagocytizing cells as described in Methods. Each point has been corrected for nonenzymatic oxidation and represents the mean of triplicate determinations. Nonenzymatic oxidation was determined in the absence of added granule fraction. NADPH concentration was 0.17 mM. A. Effect of incubation time; protein concentration was 0.107 mg/ml. B. Effect of protein concentration; time of incubation was 30 min.

tion from resting cells, while significant activity was present in the granule fraction from phagocytizing cells.

We compared the myeloperoxidase activity in granules isolated from resting and phagocytizing cells. Assays were performed on five separate paired granule fractions from either source. The means for granule fractions from resting and phagocytizing cells were 0.830 ± 0.051 and 0.809 ± 0.039 $\mu\text{mol}/\text{min}$ per mg, respectively. The myeloperoxidase activity in both granule fractions was inhibited 75% by 0.67 mM potassium cyanide.

To investigate the possibility that the granule NADPH oxidase can also utilize NADH as substrate, we examined the effect of NADH on the production of NADP in our assay system. As shown in Fig. 4B, the presence of relatively low concentrations of NADH significantly inhibited the production of NADP by granules isolated from phagocytizing cells. Some inhibition was observed with concentrations as low as 0.1 μM NADH (data not shown). The inhibition could not be overcome by increasing the NADPH concentration (Fig. 4A). In this experiment, the presence of 0.05 mM NADH completely inhibited the NADPH oxidase activity of granules from phagocytizing cells, even when the level of NADPH was increased to 0.85 mM, a 17-fold increase over the NADH concentration. We observed slight day-to-day variation in the amount of inhibition by NADH, most likely due to variation among cells from different donors. In one experiment, NADH was oxidized to NAD by lactate dehydrogenase. In contrast to the reduced form, the oxidized preparation had no inhibitory effect on NADPH oxidase activity, suggesting that NADH and not a contaminant was the actual inhibitor. Because lactate dehydrogenase also oxidized NADPH in our assay system, it was necessary to in-

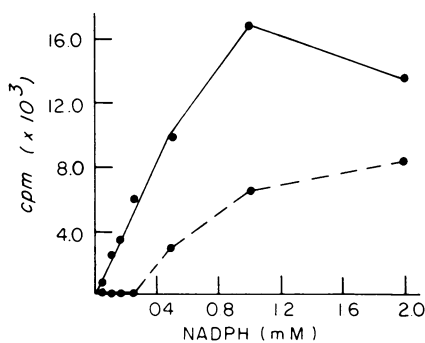


FIGURE 3 Effect of NADPH concentration on NADPH oxidase activity of resting and phagocytizing granule fractions. Protein concentration was 0.125 mg/ml and incubation time was 30 min. Each value is the mean of triplicate determinations and has been corrected for nonenzymatic oxidation (determined in the absence of added granules). Dotted line, granules from resting cells; solid line, granules from cells challenged with opsonized zymosan.

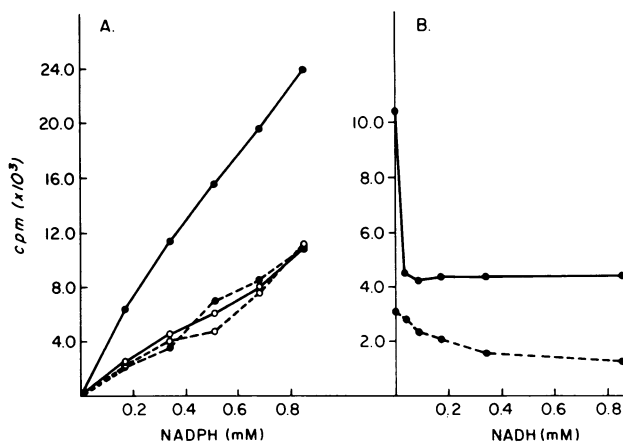


FIGURE 4 Effect of NADH on NADPH oxidase activity. Granules were isolated from phagocytizing cells and assays were performed as given in Methods. Each value represents the mean of triplicate determinations. A. Effect of varying NADPH concentration in the presence and absence of 0.05 mM NADH. Protein concentration was 0.20 mg. Dotted line, NADH added; solid line, NADH absent; closed circles, granules from phagocytizing cells; open circles, spontaneous oxidation of NADPH. B. Effect of varying NADH concentration, NADPH level was 0.17 mM. Protein concentration was 0.17 mg. Dotted line, spontaneous oxidation; solid line, granules from phagocytizing cells.

activate the lactate dehydrogenase-containing preparation by either boiling or acid treatment before use in the assay. These inactivated preparations had no effect on the spontaneous oxidation of NADPH. Other control experiments demonstrated that NADH had no effect on the 6-phosphogluconate dehydrogenase reaction used in the second incubation (data not shown).

DISCUSSION

Using our isotopic assay procedure, we have corroborated the observations of Curnutte et al. (6) and Patriarca et al. (8), that a superoxide-generating system such as xanthine-xanthine oxidase will catalyze the oxidation of NADPH in the presence of Mn^{2+} . The catalysis occurred at pH 5.5 and required both Mn^{2+} and the source of O_2^- (Table I).

Previously, we and others have found that granule fractions isolated from phagocytizing cells had higher NADPH oxidase activity than granule fractions isolated from resting cells (1, 3, 4, 13). This suggested that the oxidase could be the enzyme responsible for initiation of the respiratory burst. However, since assays were performed with Mn^{2+} present, it was possible that the increase in activity in phagocytizing cells was due solely to the increased production of superoxide by such cells (7), resulting in a stimulation of the Mn^{2+} -catalyzed nonenzymatic oxidation of NADPH. The present report demonstrates that phagocytizing activity was greater than resting activity, even in the absence of

Mn²⁺ (Table II). Similar results were described by Patriarca et al. (8), utilizing guinea pig cells, which contain much more NADPH oxidase activity (14), and an assay procedure that measured oxygen uptake (1).

There was no measurable activity in granules from resting cells below approximately 0.25 mM NADPH (Fig. 3). This may be compared with the estimated level of NADPH in the human PMNL of approximately 51 μ M (15). Even if this estimated value is in error by a factor of two or three, it is still evident that at the probable physiologic level of the pyridine nucleotide, NADPH oxidase seems to be inactive in resting cells and active in phagocytizing cells. These results support the theory that NADPH oxidase is the enzyme that initiates the series of metabolic events that accompanies phagocytosis.

When cells were incubated with the drug PMA and then fractionated, the NADPH oxidase activity of the granule fraction in the absence of Mn²⁺ was stimulated (Table III). The activity found in the PMA granule was notably different from the activity of the granule fraction from resting cells (which did not rise above the control level) and was quite similar to the activity found in granules from phagocytizing cells. The stimulation of NADPH oxidase by PMA parallels the effect of the drug on many of the events that normally accompany phagocytosis. PMA previously has been shown to stimulate degranulation (16), the reduction of nitroblue tetrazolium, oxygen consumption, and glucose oxidation (12). Data from this laboratory indicate that PMA also increases hydrogen peroxide formation, superoxide production, chemiluminescence, and iodination (17). Thus, a drug that affects neutrophils remarkably like phagocytosis also activates NADPH oxidase.

The mechanism of activation of NADPH oxidase in phagocytizing cells is still unknown. We found that PMA did not activate the oxidase in isolated granules and was only effective with intact cells. It would follow that the activation of NADPH oxidase during phagocytosis requires that the cell be intact, suggesting that the activation may occur at the level of the plasma membrane.

SOD was shown to inhibit NADPH oxidase activity both in the presence and absence of added Mn²⁺ (Table IV). This suggests that superoxide is involved even in the enzymatic oxidation of NADPH by isolated granules. These results agree with those reported by Patriarca et al. (8) in guinea pig cells and are also consistent with observations by Curnutte et al. (18), that NADPH-dependent superoxide production occurred in a 27,000 *g* granule fraction from human PMNL. An alternate interpretation of our results is that enough manganese ion is present in the granule fraction to cause, in combination with superoxide, the nonenzymatic oxidation of NADPH. No definite conclusions can be drawn until the concentration of Mn²⁺ in the granules is known.

We have clarified at least some of the problems of linearity reported previously (4). Linearity was seen over a narrow range of protein concentration (Fig. 2), while a complete lack of linearity was observed in the presence of Mn²⁺ (4). A linear relationship was obtained with increasing time of incubation after the first 2.5 min (Fig. 2). An initial burst of activity was seen previously with Mn²⁺ present; however, as with increasing protein, no linearity was observed. Thus, the elimination of Mn²⁺ has served to improve the linearity obtainable with increasing protein concentration and time.

Roberts and Quastel (19) have suggested that myeloperoxidase is responsible for the NADPH oxidase activity found in PMNL granules. We demonstrated that the myeloperoxidase activity in granule fractions isolated from resting and phagocytizing neutrophils was approximately the same, confirming results reported in a recent publication (20) with a different assay for myeloperoxidase activity. This is in contrast with results reported by Paul et al. (13), in which myeloperoxidase activity was greater in a 19,000 *g* pellet fraction isolated from phagocytizing cells than in one from resting cells. A possible explanation for the difference is that Paul's group studied guinea pig cells, while our studies and those of Patriarca utilized human cells. This hypothesis is further weakened by the presence of normal NADPH oxidase activity in the PMNL of patients deficient in myeloperoxidase (3, 18).

Controversy has arisen over whether an NADH or an NADPH oxidase is the enzyme that initiates the respiratory burst. The evidence presented here tends to support NADPH oxidase as the initiating enzyme. Further support is provided in the examination of the effect of NADH on NADPH oxidase activity. Our assay system is uniquely suited for such a study because of its specificity.

The presence of NADH in no way enhances NADPH oxidase activity and actually appears to cause a potent inhibition of the enzyme. These results are subject to two interpretations. The first is that NADH can be considered an inhibitor of NADPH oxidase. The second is that the enzyme can actually utilize both pyridine nucleotides as substrates, and prefers NADH. Comparative studies between NADH and NADPH oxidase have been made in this laboratory by a sensitive fluorometric procedure. In every cell fraction examined, NADPH oxidase was more active than NADH oxidase (21). These results do not support NADH as a better substrate for the oxidase. Further argument against this theory comes from the demonstration by Curnutte et al. (18) that granules from human cells produce more superoxide in the presence of NADPH than in the presence of NADH. From this evidence, it seems more likely that NADH simply inhibits granular NADPH oxidase activity and perhaps could modulate the enzyme *in vivo*.

Much of the evidence supporting either NADH or NADPH oxidase as the initiating enzyme has been obtained in the presence of Mn^{2+} , under suboptimal pH conditions, or with neutrophils from sources other than human. The evidence presented here attempts to overcome these problems to approach more physiologic conditions for measurement of the oxidases. We feel that our results are most consistent with theories propounding NADPH oxidase as the enzyme that initiates the metabolic events accompanying phagocytosis.

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