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Effect of Cyanide on NADPH Oxidation by Granules From Human Polymorphonuclear Leukocytes

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Cyanide has been shown to stimulate both oxygen uptake and hexose monophosphate shunt activity in phagocytizing human polymorphonuclear leukocytes. It also stimulates the oxidation of NADPH by a particulate fraction derived from phagocytizing cells. This stimulation of NADPH oxidase is not observed in the presence of exogenous Mn^{2+}. Studies with purified enzymes have shown that CN^- also stimulates NADPH oxidation by horseradish peroxidase or lactoperoxidase, suggesting that the respiratory burst might be initiated by activation of a peroxidase-like enzyme in the human polymorphonuclear leukocyte. Based on studies of others, however, it does not appear as though the enzyme is identical to myeloperoxidase. The mechanism of the CN^- stimulation appears to involve an oxidatic chain reaction, since it stimulates markedly NADPH oxidation in the presence of an artificial superoxide-generating system.

DURING THE PROCESS OF PHAGOCYTOSIS by human polymorphonuclear leukocytes (PMN), the cell exhibits a "respiratory burst" that encompasses an increased consumption of oxygen, increased oxidation of glucose by way of the hexose monophosphate shunt, and increased production of both hydrogen peroxide and superoxide anion.[1] These metabolic alterations are not inhibited by cyanide and are thought to be crucial to the ability of the cell to kill bacterial parasites.[2] Although the enzymatic basis of the respiratory burst is controversial,[3,4] there is general agreement that activation of some oxidative type of enzyme is the likely initiating event.[5] One enzyme which has been proposed as a candidate in this sequence of events is NADPH oxidase.[6]

We recently reported a sensitive isotopic assay for this enzyme and described some of the characteristics of the enzyme in human PMN.[7] It was seen to be particulate in nature, had an acid pH optimum, was markedly stimulated by Mn^{2+} ions, and appeared to be insensitive to CN^-.

Recent investigations have questioned the use of Mn^{2+} in the assay procedure by demonstrating that this metal ion can catalyze a nonenzymatic oxidation of NADPH in the presence of superoxide generated from any source.[8,9] Accordingly, we have initiated a series of experiments to characterize NADPH oxidase in the absence of exogenous Mn^{2+}. Under these conditions, the enzyme was not simply insensitive to CN^-, it was markedly stimulated by the compound. This unexpected phenomenon forms the basis of the present report.

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MATERIALS AND METHODS

Enzymes employed. Lactoperoxidase (62 units/mg protein), horseradish peroxidase (190 units/mg), xanthine oxidase (1 unit/mg), and catalase (14,400 units/mg) were all products of Sigma Chemical, St. Louis, Mo. No catalytic activity (as determined by a decrease in absorbance at 240 nm due to destruction of H_2O_2) could be detected in 100 μg of either peroxidase, nor was there measurable superoxide dismutase (SOD) activity as determined by the assay of McCord and Fridovich.[10] A similar amount of catalase was free of detectable SOD activity and exhibited no peroxidative activity using o-dianisidine as substrate, in either the presence or absence of cyanide. The xanthine oxidase also had no peroxidatic activity as judged by the o-dianisidine assay.

Isolation of leukocytes. Human neutrophils were isolated from heparinized venous blood as previously described.[11] The final cell pellet was resuspended in Dulbecco's phosphate-buffered saline (PBS) and a differential cell count was obtained using a hemocytometer. The cell suspensions, routinely containing greater than 85% PMN, were diluted with PBS to give the desired final cell concentrations (described in the individual experiments).

Isolation of granules. Isolated cells at a concentration of 1.5×10^8 PMN/ml were incubated at a ratio of one volume of cells to two volumes of opsonized zymosan (designated phagocytizing cells) or PBS (designated resting cells). Zymosan (ICN Nutritional Biochemical Division, International Chemical and Nuclear, Cleveland) was opsonized and suspended in PBS as previously described.[7] Incubation of the cells with either PBS or opsonized zymosan was carried out for 3 min at 37° C after equilibration of the component solutions to 37°C. The incubation was terminated by the addition of an equal volume of cold 0.68 *M* sucrose followed by immediate immersion in an ice bath.

Homogenization was carried out as previously described[7] 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 greater than 90% with both the resting and phagocytizing cell samples. Homogenates were centrifuged at 500 *g* for 10 min in the cold to remove unbroken cells, nuclei, cell debris, and zymosan particles. The supernatants were carefully removed and centrifuged 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 subsequently referred to as the "granule fraction" for sake of simplicity. The composition of this fraction has not been characterized, although it is undoubtedly heterogeneous in nature, containing fragments of plasma membrane in addition to the true granules of the cell. The protein content of the various granule fractions was determined by the Lowry procedure,[12] and the fractions were adjusted to the same protein concentration before use in the assay for NADPH oxidase activity.

Assay for NADPH oxidase. The isotopic assay for NADPH oxidase has been described in detail previously.[7] The assay consists of a two-step incubation in which $NADP^+$ is first generated by the granule fraction under conditions which are optimal for NADPH oxidase activity. Controls are routinely included in which no granule fraction is added to measure the spontaneous oxidation rate of NADPH which occurs during the course of the incubation. The reaction is stopped with acid, neutralized, and the amount of $NADP^+$ produced is quantitated in a second incubation using a system containing 1-^{14}C-6-phosphogluconic acid (New England Nuclear, Boston) and 6-phosphogluconic acid dehydrogenase (Sigma Chemical). The $^{14}CO_2$ produced in the course of this second incubation is trapped in a center well containing hydroxide of hyamine (New England Nuclear) and counted as previously described.[7]

Certain modifications have been made in the procedure as originally described. Recent work has demonstrated that Mn^{2+}, in the presence of superoxide anion, can act as an amplification mechanism by stimulating a nonenzymatic oxidation of NADPH.[8,9] We have confirmed this observation employing the isotopic assay system described here and the xanthine-xanthine oxidase system for generating superoxide anions and have characterized the assay system in the absence of added manganese.[13] Accordingly, we have now eliminated Mn^{2+} from the initial incubation mixture, based on the probability that it obscures the true enzymatic activity. The second modification involves increasing the specific activity of the isotope used in the second incubation. Previously 1-^{14}C-6-phosphogluconate with a specific activity of 0.04 μCi/μmole has been utilized. The isotope employed in the present communication has a 10-fold higher specific activity (0.4 μCi/μmole), achieved by reducing to one-tenth the

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amount of unlabeled 6-phosphogluconate used to dilute the isotope. This procedure effectively increased the sensitivity of the assay and has been adopted because the elimination of Mn^{2+} from the assay system markedly lowered the measurable activity.

One experiment was performed in which 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; 0.17 m*M* NADPH; 0.25 m*M* xanthine; and 0.01 unit xanthine oxidase in a total volume of 1.0 ml. KCN was added, where indicated, to a final concentration of 2.0 m*M* and Mn^{2+} to a final concentration of 0.5 m*M*. The second step of the incubation in which the amount of NADP produced was quantitated remained unchanged from that described above.

Assays on intact cells. Oxygen consumption by intact cells was monitored continuously by means of a Clark oxygen electrode. For these assays, 2×10^7 cells were incubated in a total volume of 2.4 ml of PBS containing 7.5 m*M* glucose and, where indicated, 2.0 m*M* KCN. Phagocytosis was initiated, where indicated, by the addition of 0.2 ml of a standard suspension of latex particles (0.8 μm diameter; approximately 25 particles/cell).[14] The order of addition was varied so that in some cases CN^- was added to a suspension of resting cells before the addition of latex and in other cases the CN^- was added once phagocytosis was already initiated. The measurement of oxygen consumption was made over a 5-min time interval when the slope was at a maximum.

Hexose monophosphate shunt activity was determined by measuring the oxidation of 1-^{14}C-glucose to $^{14}CO_2$ over a 15-min time interval as previously described.[15]

RESULTS

Table 1 illustrates the effect of 2.0 m*M* KCN on the oxygen consumption of human PMN. The addition of CN^- to resting cells results in a consistent inhibition of the basal level of oxygen consumption. The same concentration of CN^-, however, markedly stimulates the oxygen consumption of phagocytizing cells.

This stimulation by CN^- is apparent if the compound is added to a suspension of cells already undergoing phagocytosis by latex particles, but is even more pronounced if the CN^- is preincubated with the cells for 5 min before the addition of latex particles to initiate phagocytosis. In four paired experiments, the prior addition of CN^- always resulted in a significantly greater rate of oxygen consumption.

The effect of cyanide on NADPH oxidation by granule fractions from human PMN is illustrated in Table 2. In the absence of cyanide, the granule fraction from resting cells shows no significant NADPH oxidase activity above the control, while the granule fraction from phagocytizing cells shows about a twofold increase in NADPH oxidation. The addition of 2.0 m*M* potassium cyanide results in a stimulation of the spontaneous oxidation and a marked stimulation of NADPH oxidation by the granule fraction from phagocytizing

Table 1. Effect of Cyanide on Oxygen Consumption by Human PMN

Description	Oxygen Consumption ($\mu l\ O_2/10^8$ cells/min)
Resting cells (16)	0.43 ± 0.17
+ CN^- (7)	0.09 ± 0.07
+ latex (9)	1.91 ± 0.41
+ latex + CN^- (4)	3.63 ± 0.72
+ CN^- + latex (7)	4.85 ± 0.56

Cyanide was added, where indicated, to a final concentration of 2.0 mM. Numbers in parentheses represent number of individual experiments; values represent mean ± SEM for the given number of experiments.

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Table 2. Effect of Cyanide on NADPH Oxidation by Granule Fractions of Human PMN

Description	0.17 mM NADPH		0.85 mM NADPH	
	−KCN	+KCN	−KCN	+KCN
Spontaneous oxidation	826 ± 75 (6)	2025 ± 126 (15)	2173 (2)	6,007 ± 464 (7)
Granule fraction from resting cells	919 ± 102 (3)	925 ± 40 (8)	3545 (2)	13,571 ± 1493 (7)
Granule fraction from phagocytizing cells	1565 ± 66 (5)	7196 ± 547 (17)	4032 (2)	19,299 ± 2617 (7)

CN^- was added, where indicated, to a final concentration of 2.0 mM. Numbers in parentheses denote number of individual experiments; each value was determined in triplicate in each experiment. Values given are mean ± SEM in counts per minute.

cells, but does not alter the degree of oxidation exhibited by the granule fraction from resting cells. In the presence of cyanide the granule fraction from resting cells appears to inhibit the spontaneous oxidation. These results have been obtained with 0.17 m*M* NADPH in the absence of added Mn^{2+} ions. At higher levels of substrate (i.e., 0.85 m*M*), activity above the spontaneous is observed with the granule fraction from either resting or phagocytizing cells, although the latter shows substantially greater activity. In this case, the addition of 2.0 m*M* CN^- markedly stimulates NADPH oxidation by both resting and phagocytizing granule fractions, in contrast to the results seen at lower concentrations of NADPH.

The stimulation of NADPH oxidation by cyanide is not observed if the reaction is run in the presence of 0.5 m*M* Mn^{2+}, the usual conditions for the assay.[4,6,7] Figure 1 contrasts the effect of CN^- on the reaction in the presence and absence of Mn^{2+} ions. As described by others,[8,9] the presence of Mn^{2+} markedly increases the rate of NADPH oxidation under all conditions studied. However, the stimulation of activity by CN^- is no longer observed with granules from either resting or phagocytizing cells when Mn^{2+} is included in the incubation mixture (Table 3). There is, however, a moderate stimulation of the spontaneous oxidation under these conditions.

Figure 2 illustrates the effect of CN^- concentration on the NADPH oxidase activity of isolated granules obtained from phagocytizing PMN. For comparative purposes, the effect of CN^- concentration on hexose monophosphate shunt activity of intact phagocytizing PMN is also illustrated. Both activities are stimulated by CN^- and the maximal stimulation is observed in each case at a concentration of KCN between 2.0 and 5.0 m*M*. The hexose monophosphate shunt activity appears to be more sensitive to low levels of CN^- than the

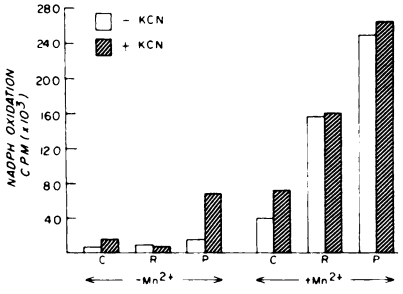


Fig. 1. Effect of CN^- on NADPH oxidase activity of human PMN granules. Mn^{2+} was present, where indicated, at a final concentration of 0.5 mM, CN^- at a final concentration of 1.0 mM. NADPH concentration was 0.17 mM in all cases. C, control value (spontaneous oxidation); R, granules prepared from resting cells; P, granules prepared from phagocytizing cells. Values illustrated are means of closely agreeing triplicate determinations; the data are from a single experiment that is representative of two such experiments.

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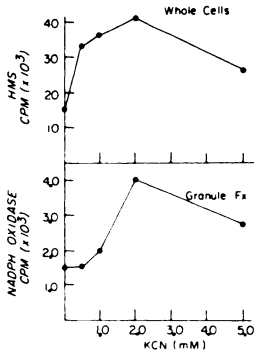


Fig. 2. Effect of CN^- concentration on hexose monophosphate shunt activity of intact phagocytizing PMN and on NADPH oxidase activity of the isolated granule fraction obtained from phagocytizing human PMN. The two assays were run in parallel; each value represents the mean of closely agreeing triplicate determinations.

oxidase activity itself; this may be due to procedural differences in the two assays.

To determine if CN^- might be involved in an oxidatic chain reaction, as has previously been proposed for manganese,[8] we have investigated the effect of CN^- on the superoxide catalyzed oxidation of NADPH using xanthine-xanthine oxidase as the source of superoxide. The results in Table 4 indicate that CN^- does, indeed, amplify the oxidation of NADPH to an extent very similar to that exhibited by Mn^{2+}. The effect of CN^- in this system differs from that observed using granule fractions of PMN in that the effects of CN^- and Mn^{2+} are additive in the model system, while CN^- is without effect in the presence of Mn^{2+} in the leukocytic assay. Other investigators have not observed this stimulation by CN^- in the xanthine-xanthine oxidase system.[8] It is possible

Table 3. Lack of Effect of Cyanide on NADPH Oxidation in the Presence of Manganese

Description	−KCN	+KCN
Spontaneous oxidation	8,001 ± 328	12,472 ± 431
Granule fraction from resting cells	16,781 ± 4028	17,659 ± 4071
Granule fraction from phagocytizing cells	30,570 ± 1309	31,171 ± 951

Assays utilized 0.5 mM $MnCl_2$ and 0.17 mM NADPH. Values are expressed as mean ± SEM in counts per minute for four separate experiments. Each value was determined in triplicate in each experiment.

Table 4. Oxidation of NADPH by the Xanthine–Xanthine Oxidase System for Generating Superoxide

Description	Exp. 1	Exp. 2
Complete system	2,446	2,162
−Xanthine	—	1,749
−Xanthine oxidase	1,962	1,738
$+Mn^{2+}$ (0.5 mM)	16,372	16,638
$+CN^-$ (2.0 mM)	19,038	19,578
$+Mn^{2+}$ (0.5 mM) + CN^- (2.0 mM)	35,882	35,210

Incubations were performed in 0.1 M phosphate buffer, pH 5.5. Incubations were for 30 min at 37°C. Two separate experiments are illustrated; each value represents the mean (in counts per minute) of closely agreeing triplicate determinations.

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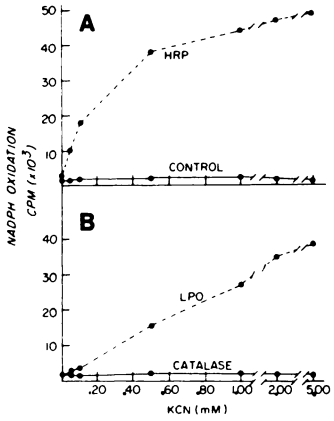


Fig. 3. Effect of CN^- concentration on the NADPH oxidase activity of purified peroxidases. Two separate experiments are illustrated. (A) Solid line, control (spontaneous oxidation observed in the absence of enzyme); dashed line, horseradish peroxidase (0.10 mg) added. (B) Solid line, catalase (0.10 mg) added; dashed line, lactoperoxidase (0.10 mg) added. Each point represents the mean of triplicate determinations.

to cause a maximal stimulation by Mn^{2+} so that the effect of CN^- is no longer observed (data not shown); this effect may partially explain the discrepancy.

In an attempt to clarify further the stimulatory effect of CN^- on the oxidation of NADPH, we turned to a model system in which the leukocytic granule fraction was replaced by 0.10 mg of either purified horseradish peroxidase or lactoperoxidase (Sigma Chemical). Two separate experiments in which the CN^- concentration was varied from 0.0 to 5.0 m*M* are depicted in Fig. 3. Low concentrations of CN^- markedly increased the oxidation of NADPH by the purified peroxidases; horseradish peroxidase was much more sensitive to CN^- than was lactoperoxidase. The degree of stimulation was dose dependent and appeared to reach a near-maximal value at a cyanide concentration of 2.0 m*M*, at least in the case of horseradish peroxidase. Control experiments demonstrated that cyanide had little effect on the spontaneous oxidation of NADPH over the concentration range studied; similarly, catalase could not replace the peroxidases in the model system.

The ability of a number of purified enzymes to oxidize NADPH was studied in the presence and absence of CN^- (Table 5). Of the enzymes examined, only

Table 5. Effect of Cyanide on NADPH Oxidation by Purified Enzyme Preparations

Description	−KCN	+KCN
Spontaneous oxidation	1246	1345
Lactoperoxidase	1561	14,766
Horseradish peroxidase	3104	42,698
Glucose oxidase	2008	1717
Xanthine oxidase	3723	2968
D-amino acid oxidase	1811	1375
Glucose-6-phosphate dehydrogenase	1624	1644
6-phosphogluconate dehydrogenase	1367	1699
Lactic dehydrogenase	1681	1558
Glutathione reductase	18,922	11,183
Catalase	1375	1842
Superoxide dismutase	1056	1007

Incubations were for 30 min at 37°C in 0.1 *M* phosphate buffer, pH 5.5. NADPH concentration was 0.17 mM and enzyme concentration was 0.10 mg/ml in all cases; CN^- was added, where indicated, to a final concentration of 2.0 mM. Values represent the mean (in counts per minute) of closely agreeing triplicate determinations.

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the peroxidases exhibited a CN^- stimulated oxidation of reduced pyridine nucleotide. Glutathione reductase showed considerable activity, but this was inhibited by CN^-.

The concentrations of CN^- employed were sufficient to cause nearly complete inhibition of the peroxidative activity of horseradish peroxidase as determined by the o-dianisidine assay procedure.[16] A concentration of 0.067 m*M* CN^- caused 80% inhibition of peroxidase activity, while a concentration of 0.67 m*M* CN^- completely abolished the activity. The peroxidative activity of lactoperoxidase in this assay system was somewhat more resistant to CN^-; 0.67 m*M* KCN resulted in 41% inhibition of activity. The concentration employed in the present experiment (i.e., 2.0 m*M*) resulted in 60% inhibition of activity. Complete inhibition of this enzyme was achieved only with a concentration of 10 m*M* KCN. Myeloperoxidase was 75% inhibited by 0.67 m*M* CN^- and completely inhibited by 2.0 m*M*.

DISCUSSION

The respiratory burst which accompanies phagocytosis by human PMN is often referred to in the literature as being "insensitive to cyanide."[1,2,5] The data in the present communication clearly indicate that both oxygen consumption and hexose monophosphate shunt activity are markedly stimulated by cyanide in intact phagocytizing PMN (Table 1, Fig. 2). Careful examination of the literature reveals that this is not a new observation; Reed and Tepperman have reported that CN^- stimulates the postphagocytic oxygen consumption in rat PMN,[17] and Klebanoff and Hamon have presented data illustrating the same effect in normal human PMN.[18] A similar effect of CN^- on the postphagocytic hexose monophosphate shunt activity has been reported.[19]. The significance of these observations has generally gone unnoticed and investigators have emphasized the fact that CN^- does not inhibit the respiratory burst without actually recognizing the stimulatory effect.

Of particular interest is the observation that NADPH oxidation by a granule fraction from human PMN is also stimulated by CN^- (Table 2, Figs. 1 and 2). A number of investigators have reported that the activity of NADPH oxidase is increased in granules obtained from phagocytizing cells as opposed to resting cells,[4,6,20] but the enzyme is generally referred to as "cyanide insensitive" and no mention is made of increased pyridine nucleotide oxidation in the presence of cyanide. This failure is not unexpected, however, since these workers have invariably performed the assay in the presence of exogenous Mn^{2+}, and the stimulation by cyanide is not observed under these conditions (Fig. 1, Table 3). Karnovsky et al.[21] have made the statement that the NADH oxidase from guinea pig PMN is somewhat stimulated by cyanide; this observation appears as a single sentence in the paper and no data are presented. Because of the absolute specificity for NADPH of the assay system used in the present communication, we have not determined whether oxidation of NADH is also stimulated by CN^- in human PMN.

A stimulatory effect of CN^- on the activity of oxidative enzymes is not without precedent in the literature. Cyanide has been shown to enhance the rate of reduction of D-amino acid oxidase by β-chloroalanine[22] and to increase

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the activity of the L-amino acid oxidase reaction.[23] Nicholls[24] has reported that CN^- increases the rate of dihydroxyfumaric acid oxidation catalyzed by horseradish peroxidase and has proposed that CN^- may act by promoting an oxidatic chain reaction. This concept is very similar to the role which has been postulated for Mn^{2+} in the oxidation of NADPH by human PMN[9] and is supported by the experiments which demonstrate that CN^- increases the oxidation of NADPH using xanthine–xanthine oxidase to generate superoxide (Table 4). Such a chain reaction would probably utilize the superoxide produced by the initiating oxidase; detailed steps of the possible chain reactions have already been published.[8,9] It is also possible that the chain reaction might be propagated through interaction of both superoxide and hydrogen peroxide, perhaps by generation of the hydroxyl radical.[25] We have no evidence that this species is actually involved in the reaction. However, if the chain reaction does involve interaction of superoxide with hydrogen peroxide, then the stimulation by CN^- might be indirect in that it could inhibit an enzyme which utilized hydrogen peroxide, and thus increase the concentration of this reactant.

The fact that the effects of Mn^{2+} and CN^- are additive in the model system (Table 4) but not in the leukocytic granule system (Table 3) suggests that the two systems are not entirely comparable. We have no ready explanation for this discrepancy.

The literature cited above led us to investigate the oxidation of NADPH by a model system (either horseradish peroxidase or lactoperoxidase); the ability of such enzymes to oxidize reduced pyridine nucleotides has been well documented.[26,27] Cyanide markedly stimulated NADPH oxidation by both enzymes at levels which completely abolished peroxidative activity of horseradish peroxidase and substantially inhibited similar activity of lactoperoxidase. Control experiments (Table 5) showed that this effect was relatively specific for peroxidative type enzymes.

Roberts and Quastel in 1964[28] first postulated that myeloperoxidase might be responsible for initiation of the respiratory burst, but this was considered unlikely in view of the fact that CN^- completely inhibited myeloperoxidase activity but had no effect on the respiratory burst. Takanaka and O'Brien[29] recently demonstrated the fallacy of this argument by showing that although CN^- markedly inhibited the peroxidative activity of guinea pig myeloperoxidase, it did not significantly affect the NADPH oxidase activity of the enzyme under the same conditions. Their failure to observe appreciable stimulation by cyanide of the myeloperoxidase-mediated oxidation of NADPH was probably due to the fact that the assay was run in the presence of 0.50 m*M* Mn^{2+}. Since their preparation of myeloperoxidase was only partially purified, the possibility that it was significantly contaminated with another oxidative enzyme could not be eliminated and would provide an alternate explanation for their results.

A second major objection which has been raised against the possible involvement of myeloperoxidase in the initiation of the respiratory burst lies in the observation that leukocytes deficient in the enzyme have normal or increased oxidative metabolism.[17,30,31] This argument appears reasonably compelling since demonstration of the deficiency state has been based on lack of peroxidative activity and, in at least one instance, on lack of immunologic reactivity.[32]

The present results are consistent with the hypothesis that a peroxidaselike

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enzyme is responsible for initiation of the respiratory burst in human PMN. Based on the experiments cited above, it is highly unlikely that myeloperoxidase itself is the initiating enzyme, although the crucial oxidase does share certain characteristics with this enzyme. We cannot, however, exclude the possibility that the effect of CN^- on intact leukocytes is mediated through factors affecting the primary oxidase by altering regulatory factors (substrate, competing enzymes, etc.) rather than the (per)oxidase itself.

Finally, these results raise the disquieting possibility that the use of CN^- in measuring the oxidation of pyridine nucleotides might result in an artifactual nonenzymatic amplification of the true activity, as previously demonstrated for manganese.[8] Further experiments are required to explore this possibility.

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