Manganese-Dependent NADPH Oxidation by Granulocyte Particles

THE ROLE OF SUPEROXIDE AND THE NONPHYSIOLOGICAL NATURE OF THE MANGANESE REQUIREMENT

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ABSTRACT Recent work has indicated that superoxide is involved in the manganese-stimulated oxidation of NADPH by crude granule preparations of guinea pig neutrophils. The characteristics of a model manganese-requiring NADPH-oxidizing system that employs a defined O_2^- generator have now been compared to the original neutrophil-granule system. With respect to pH dependence, cyanide sensitivity, and reduced pyridine nucleotide specificity, the properties of the two systems are very similar. Additional information has been obtained concerning cation specificity and the kinetics of the metal-catalyzed NADPH oxidation. From the similarities between the properties of the model and neutrophil particle systems, we postulate that the manganese-dependent NADPH oxidation observed in the presence of neutrophil granules represents in large part a nonenzymatic free radical chain involving the oxidation of NADPH to NADP, with O_2^- as both the chain initiator and one of the propagating species. In this reaction, the neutrophil particles serve only as a source of O_2^-. Further, the same changes in kinetics (decrease in apparent K_m for NADPH) observed previously when granules from phagocytizing rather than resting cells were employed could be mimicked by varying the rate of O_2^- generation by the model system. We conclude from these results that it is unnecessary to invoke a manganese-requiring enzyme as a component of the phagocytically stimulated respiratory system of the neutrophil.

INTRODUCTION

When granulocytes are activated by phagocytosis or other appropriate stimuli, characteristic changes occur in the oxidative metabolism of the cells that play a key role in their bactericidal activity. Included among these changes are a cyanide-insensitive increase in oxygen uptake (the "respiratory burst"), a rise in both superoxide (O_2^-) and hydrogen peroxide formation, and a 10-fold increase in glucose oxidation via the hexose monophosphate shunt (1–4).

Despite extensive study, the identity of the enzyme responsible for the increase in oxygen uptake by the activated granulocyte has not yet been established. Among the candidates is a particulate NADPH oxidase discovered by Iyer and Quastel (5) and studied extensively by Rossi and his collaborators (6, 7). Certain properties of this enzyme have been interpreted to favor its participation in the respiratory burst, including its insensitivity to cyanide and a pH optimum (5.0–5.5) that corresponds to the pH within the phagocytic vesicle (8). Most important in relating this enzyme to metabolic changes during phagocytosis, a difference in K_m has been reported between enzyme isolated from resting and activated granulocytes. This difference has been interpreted to indicate an increased affinity of enzyme for substrate due to phagocytosis and has been proposed as the biochemical mechanism for the activation of the cell (7). One puzzling feature that has been difficult to explain in physiological terms, however, is the requirement of the enzyme for manganese, and questions regarding the role of this enzyme in granulocyte metabolism have been raised on the basis of this requirement.

In connection with our interest in the role of O_2^- in granulocyte metabolism, we began to examine the possibility that the manganese-dependent NADPH oxidase is an O_2^--forming enzyme. Early experiments showed that oxygen uptake catalyzed by this oxidase

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was greatly inhibited by superoxide dismutase. This finding suggested that O_2^- was involved in an important way in the NADPH oxidase reaction. On the basis of this observation an investigation was undertaken which revealed that an oxidation of NADPH which displayed all the salient features of the granulocyte oxidase-catalyzed reaction took place when granulocyte particles were replaced by a model O_2^--generating system. In addition, an explanation was provided for the manganese requirement in the NADPH oxidase reaction. This investigation is the subject of the present report, which amplifies and extends work reported independently from our own (9) and another (10) laboratory. Thus, we now present detailed comparisons between the model system and the crude granule system, data on various cations, and especially information concerning the manner in which the rate and extent of O_2^- production affects the stoichiometry and kinetics of NADPH oxidation.

METHODS

Superoxide dismutase (3,000 U/mg) obtained from Truett Laboratories, Dallas, Tex., was reconstituted with water to a concentration of 3 mg/ml. NADPH and NADH (both chemically reduced), isocitric acid, purine, bovine serum albumin, horse heart cytochrome *c* (grade VI), bovine liver catalase, isocitric dehydrogenase (type IV), and milk xanthine oxidase (grade I) were obtained from Sigma Chemical Co., St. Louis, Mo. Milk xanthine oxidase purified to homogeneity by the criterion of disk gel electrophoresis was the generous gift of Prof. I. Fridovich. Pooled guinea pig serum was obtained from Grand Island Biological Co., Grand Island, N. Y. Dextran 70 (6% in 0.9% saline) was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

Bacillus subtilis were grown overnight at 37°C in nutrient broth. The bacteria were then washed twice in 0.9% saline, autoclaved 15 min at 121°C, washed once again with saline, and suspended in calcium-free Krebs-Ringer phosphate buffer containing 135 mM glucose. Opsonization was performed by adding guinea pig serum to the bacterial suspension (final concentration, 30% [vol/vol]) and incubating at 37°C for 5 min. The opsonized bacteria were washed once with calcium-free Krebs-Ringer phosphate buffer containing glucose, then suspended in the same buffer to an absorbance at 620 nm of 4.0. Guinea pigs (500–1,000 g, of both sexes) purchased from Charles River Breeding Laboratories, Wilmington, Mass., were maintained on chow pellets and water ad libitum.

$RhCl_3 \cdot 3H_2O$ and $CeCl_3$ were purchased from Alfa Inorganics, Beverly, Mass. Other reagents were the highest quality commercially available and were used without further purification.

Preparation of granulocytes. Granulocytes were obtained from guinea pigs by the method of DePierre and Karnovsky (11). This method involved the intraperitoneal injection of 30 ml of 12% (vol/vol) sodium caseinate in 0.9% saline to elicit the granulocytes. The cells were washed and suspended in calcium-free Krebs-Ringer phosphate buffer, pH 7.4, according to Patriarca et al. (7). Phagocytosis was induced with preopsonized autoclaved *B. subtilis* as described by Patriarca et al. (7).

Assays of NADPH oxidase activity. Assays of the NADPH oxidase activity of granulocyte particles were conducted by the method of Patriarca et al. (7), with oxygen uptake monitored with a Yellow Springs model 53 oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Spectrophotometric determinations of NADPH oxidase activity were also performed according to Patriarca et al. (7), with an assay volume of 1 ml. These determinations were carried out at 23°C in a Cary 118C recording spectrophotometer (Cary Instruments, Monrovia, Calif.). Protein concentration was determined by the Lowry method (12).

Measurement of O_2^- production by xanthine oxidase. The rate of O_2^- production by xanthine oxidase was determined spectrophotometrically by a modification of the cytochrome *c* assay described by McCord and Fridovich (13). Two identical cuvettes, each containing 65 μmol of Na-K phosphate buffer, pH 5.5 (prepared by adjusting 0.13 M NaH_2PO_4 to pH 5.5 with 0.13 M K_2HPO_4), 125 μmol sucrose, 0.5 μmol purine (in some cases, 5.0 μmol of purine was used), and 0.16 μmol cytochrome *c* in a volume of 1.0 ml were prepared. To the reference cuvette was added 20 μl of superoxide dismutase, while 20 μl of water was added to the sample cuvette. The reaction was started by the addition to each cuvette of the quantity of xanthine oxidase under study, and the change in absorbance at 550 nm was measured at 23°C in a Cary 118C recording spectrophotometer. Absorbance changes were converted to rates of O_2^--dependent cytochrome *c* reduction using a value of 15.5 for $\Delta\epsilon_{mM}$ (ferrocytochrome *c* minus ferricytochrome *c*) at 550 nm (14).

NADPH oxidation in the presence of xanthine oxidase. Spectrophotometric and oximetric measurements of the rates of NADPH oxidation by xanthine oxidase were performed by modifications of the methods described by Patriarca et al. (7), with xanthine oxidase plus purine replacing granulocyte particles. Superoxide dismutase at the concentrations indicated was used to assess the fraction of NADPH oxidation that was dependent on O_2^-. Constituents of the reaction mixtures are indicated in the legends to the tables and figures.

RESULTS

Dependence on O_2^- of manganese-requiring NADPH oxidation by granulocyte particles. During the course of a study on the relationship between O_2^- production and the particulate NADPH oxidase of guinea pig granulocytes, we examined the effect of superoxide dismutase on the activity of this enzyme system. These experiments revealed that superoxide dismutase was a powerful inhibitor of the NADPH oxidase reaction (9), a finding in agreement with that recently reported by Rossi's group (10). Prior attempts to demonstrate O_2^- production with this manganese-containing system had failed, yet the above observation strongly suggested that O_2^- was in fact being generated by the granulocyte particles and was somehow involved in the oxidation of NADPH. This in turn raised the possibility that the granulocyte particles were not essential per se, but served only as a source of O_2^-. Further considerations suggested that the O_2^- produced by these particles might be interacting with manganese, inasmuch as a prece-

TABLE I

*NADPH Oxidation in the Presence of Mn^{++} by the Xanthine Oxidase-Purine O_2^--Producing System**

Conditions	NADPH oxidized‡	Oxygen consumed§
	nmol/min	
Complete mixture	71.4±1.6 (SD)	113.0±11.3 (SD)
Plus superoxide dismutase	12.1±3.4	0±0
Plus inactivated superoxide dismutase‖	74.5±6.2	101.5±16.3
Plus albumin	66.1±4.7	124.5±10.6
Plus catalase	158.6±27.9	111.0±2.8
Omit Mn^{++}	5.5±0.8	14.0±2.8
Omit xanthine oxidase	1.5±0.9	0±0
Boiled xanthine oxidase¶	1.5±0.8	0±0
Omit purine	7.2±1.8	19.0±0
Omit NADPH	0±0	0±0
Complete mixture plus 2 mM CN^-	50.7±4.5	89.5±16.3
Replace NADPH with NADH	41.2±19.6	72.0±8.5

* Note that conditions for the two series of measurements are quite different, making stoichiometric comparisons of NADPH and oxygen consumed inappropriate.

‡ NADPH oxidation was measured spectrophotometrically at 340 nm on a Cary 118C recording spectrophotometer. The reaction was performed in a 1-cm quartz cuvette at 23°C. The reaction mixtures contained Na-K phosphate buffer (65 μmol), purine, (0.5 μmol), $MnCl_2$ (0.5 μmol), and pyridine nucleotide (0.16 μmol) in a total volume of 1.0 ml. When indicated, 120 μg of superoxide dismutase, inactivated dismutase, albumin, or catalase was present. For the CN^- control, 2 μmol of NaCN was present. The reference cuvette was identical to the reaction cuvette except that water replaced the pyridine nucleotide. The reaction was started by adding 0.1 U of xanthine oxidase (except where omitted), an amount which generated 13 μmol O_2^-/min (see Methods for this determination). Absorbance changes were converted to rates of NADPH oxidation by using a value of 6.22 for ϵ_{mM} at 340 nm. Rates in the table represent the mean of three experiments.

§ Oxygen consumption was measured on a Yellow Springs model 53 oxygen electrode at 37°C. A reaction mixture containing Na-K phosphate buffer, pH 5.5 (130 μmol), sucrose (250 μmol), $MnCl_2$ (1.0 μmol), and purine (10 μmol) in a volume of 1.96 ml was placed in the reaction vessel and warmed to 37°C. When indicated, 360 μg of superoxide dismutase, inactivated dismutase, albumin, or catalase was also present. Reduced pyridine nucleotide was then added (20 μl containing 5 μmol NADPH) and the spontaneous rate of oxidation measured. After 10% of the oxygen was consumed, 20 μl of xanthine oxidase (0.015 U) was added. The rate of oxygen consumption was calculated by subtracting from the total rate the rate of spontaneous NADPH oxidation and the rate of oxygen consumption by the xanthine oxidase-purine reaction. The rates reported represent the mean of two experiments.

‖ Inactivated by autoclaving for 15 min at 121°C.

¶ Inactivated by boiling for 1 min.

dent for the interaction of manganese with O_2^- exists in the form of the manganese-requiring class of superoxide dismutases (15), and this metal is generally regarded as a requirement for the NADPH oxidase reaction. In principle, then, the oxidation of NADPH could be envisioned not as a direct enzymatic oxidation of NADPH by a granulocyte oxidase, but rather as a manganese-catalyzed oxidation of NADPH by O_2^-.

If granulocyte particles served only as a source of O_2^-, then it should be possible to replace them with another source of O_2^- and still see rapid oxidation of NADPH. In the present experiments, the xanthine oxidase-purine system was used as a source of O_2^-. Table I shows that when this system was incubated with NADPH at pH 5.5 in the presence of Mn^{++}, rapid oxidation of the nucleotide took place. The addition of the active superoxide dismutase diminished the rate of oxidation to less than 20% of control, but albumin and dismutase inactivated by autoclaving were without effect. These observations have been reported previously by ourselves (9), and very similar data have been given by others (10). Little oxidation was seen

TABLE II

The Effect of Various Cations on Superoxide-Dependent NADPH Oxidation

Cation (as chloride)	O_2^--dependent NADPH oxidation	
	Acetate buffer	Phosphate buffer
	nmol/min	
Mn^{++}	23.2±2.8 (SD)	61.1±18.5 (SD)
Ce^{+++}	9.9±2.5	3.2±2.2
Co^{++}	3.6±0.1	
Cu^{++}	0.0	
Zn^{++}	2.2±0.1	
Fe^{++}	1.0±0.9	
Fe^{+++}	0.0	
Rh^{+++}		2.6±2.9
Cr^{+++}		2.3±2.3
Ni^{++}		2.7±1.9
None	4.4±1.2	

NADPH oxidation was measured spectrophotometrically as described in Table I, except that both reaction and reference cuvettes contained NADPH. In order to measure superoxide-dependent NADPH oxidation, the reference cuvette contained superoxide dismutase (120 μg). Each cation was present at a concentration of 0.5 mM. Because of solubility problems, some of the cations were assayed in 65 mM sodium acetate/acetic acid buffer, pH 5.5, instead of 65 mM Na-K phosphate buffer, pH 5.5 The mean values for two experiments are reported.

when xanthine oxidase, purine,[1] Mn^{++}, or NADPH was omitted, or when active xanthine oxidase was replaced by enzyme inactivated by boiling. These findings indicate that, like the granulocyte oxidase, the xanthine oxidase-purine system carries out a manganese-dependent oxidation of NADPH in which O_2^- is involved. Results similar to those presented in Table I were obtained with xanthine oxidase purified to homogeneity (see Methods) so that NADPH oxidation cannot be ascribed to a contaminant present in the partially purified commercial preparation.

In the presence of catalase, NADPH oxidation was stimulated. This effect was observed as a doubling in the rate of disappearance of NADPH itself, as measured spectrophotometrically (Table I, left-hand column). No net increase in oxygen uptake was observed, however (Table I, right-hand column), because the rise in oxygen consumption associated with the increase in NADPH oxidation was offset by the catalase-mediated regeneration of oxygen from the H_2O_2 formed in the reaction. The increase in NADPH oxidation in the presence of catalase is not understood, but may be a manifestation of the well-known peroxidatic activity of this enzyme (7).

The product of oxidation was identified enzymatically as NADP in the following manner. A reaction mixture (1.0 ml total volume) was prepared as described in Table I (NADPH oxidation assay), and NADPH oxidation was permitted to take place until 39.5% of the nucleotide had been oxidized, as indicated by a fall in absorbance of 340 nm from an initial value of 1.00 to a final value of 0.605 *A*. 40 μl of superoxide dismutase were then added to the reaction mixture to retard the further oxidation of NADPH. After the addition of superoxide dismutase, 10 μl (5 μmol) of sodium isocitrate and 10 μl (0.36 U) of isocitric dehydrogenase were added to the reaction mixture, and the change in absorbance at 340 nm was followed with time. On completion of the isocitrate dehydrogenase reaction, A_{340} was 0.975, a value only 2.5% smaller than the initial value. Correction for the dilution that accompanied the additions at the end of the oxidation reaction shows that this figure corresponds to the recovery of 103% of the NADPH originally present in the reaction mixture. Thus, the product of oxidation of NADPH in this system was NADP.

Studies of cyanide sensitivity and pyridine nucleotide specificity revealed further similarities between the granulocyte NADPH oxidase and the oxidation of NADPH by the xanthine oxidase-purine system. Neither is particularly sensitive to cyanide, the activity of both systems (7) falling only slightly in the presence of 2 mM CN^- (Table I). Both catalyze NADH oxidation at about 60% the rate seen with

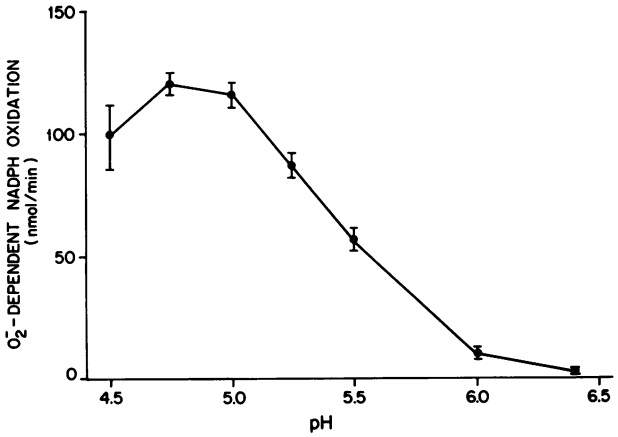

FIGURE 1 Effect of pH on superoxide-dependent NADPH oxidation in the presence of Mn^{++} by the purine-xanthine oxidase system. Superoxide-dependent NADPH oxidation was measured spectrophotometrically as described in Table II. Assays were conducted in 65 mM Na-K phosphate buffers at the pH's indicated. Data points and error bars represent the mean±SE of four experiments.

[1] The activity seen in the absence of purine may represent the direct oxidation of NADPH by xanthine oxidase (16), an enzyme of unusually broad specificity. The oxidizing agent for this reaction would be oxygen, not O_2^-, a point whose significance is explained in the discussion.

NADPH (Table I; reference 7). As with NADPH oxidation, NADH oxidation by the xanthine oxidase-purine system is sensitive to superoxide dismutase, insensitive to CN^-, and requires Mn^{++} (data not shown).

Properties of the xanthine oxidase-purine system. The correlations thus far observed between NADPH oxidation as catalyze by the granulocyte oxidase on one hand, and by the xanthine oxidase-purine system on the other, suggested that further investigation might reveal additional similarities. A systematic evaluation of the xanthine oxidase-purine system was therefore undertaken to document the extent to which its properties are congruent with those of the granulocyte oxidase.

One of the characteristic properties of the granulocyte NADPH oxidase is its metal ion requirement. The activity of the oxidase is greatly stimulated by manganese, but not by any of several other cations tested, including Mg^{++}, Ca^{++}, Fe^{+++}, Cu^{++}, and Zn^{++} (18). The cation specificity of the xanthine oxidase-purine system is shown in Table II. Apart from Mn^{++} and Ce^{+++}, the latter an ion not previously studied with the granulocyte oxidase, none of the cations tested caused the rate of O_2^--dependent oxidation of NADPH to increase above control levels. With Ce^{+++}, the increase in the NADPH oxidation rate was 40% that seen with Mn^{++}.

Iron represents a special case. Although the *O_2^--dependent* oxidation of NADPH was not affected by iron, both Fe^{++} and Fe^{+++} greatly accelerated the oxidation of NADPH by an *O_2^--independent* process. Rates of NADPH oxidation observed under the conditions in Table II were as follows: with Fe^{++}, 0.8 nmol/min in the absence of xanthine oxidase and 30.9 in its presence; with Fe^{+++}, 3.7 and 28.9 in the absence and presence of xanthine oxidase, respectively. These results

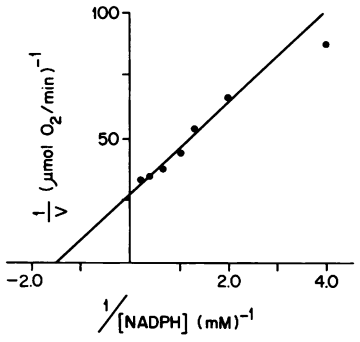

FIGURE 2 Double reciprocal plot of the rate of NADPH oxidation as a function of NADPH concentration. The assay was performed on the oxygen electrode at 37°C, and the rate of oxygen consumption was calculated as described in Table I except that 0.06 U of xanthine oxidase was used. Each reaction mixture also contained 2 μmol KCN and NADPH at the concentrations indicated.

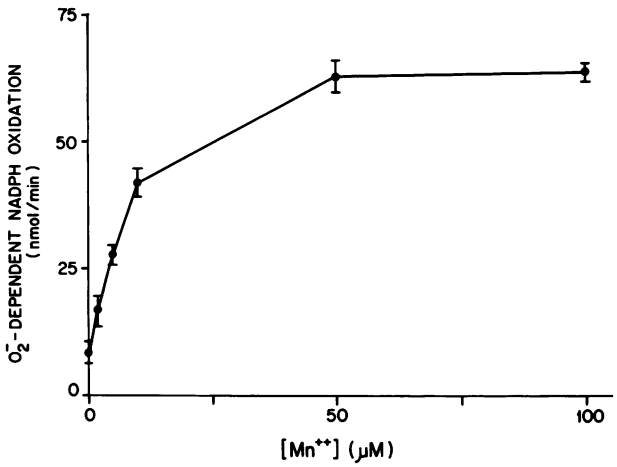

FIGURE 3 The effect of Mn^{++} concentration on superoxide-dependent NADPH oxidation. O_2^--dependent oxidation was measured spectrophotometrically as described in Table II. Assays were conducted at the Mn^{++} concentrations indicated. Data points and error bars represent the mean±SE for three experiments.

are at variance with those reported by Hohn and Lehrer for the human granulocyte NADPH oxidase (18). However, their experiments with iron appear to have been carried out in phosphate buffer under conditions which in our hands led to the precipitation of iron as the insoluble phosphate salt. Our results were obtained by using acetate buffer, in which both Fe^{++} and Fe^{+++} are soluble. It is possible that these circumstances explain the differences between their findings and ours.

The granulocyte oxidase-catalyzed reaction shows a characteristic dependence on pH, optimum activity occurring between pH 5.0 and 5.5, with very little activity at pH 7.0 (10, 18, 19). In Fig. 1, the pH dependence of the xanthine oxidase-purine-catalyzed oxidation of NADPH is shown. The curve of activity is strikingly similar to previously published curves showing the pH dependence of the granulocyte oxidase. Since the pH optimum of xanthine oxidase is 8.3 (20), the low pH optimum for NADPH oxidation reflects the intrinsic properties of the reaction, not those of the O_2^- source.

The granulocyte oxidase displays saturation kinetics with respect to NADPH. Saturation kinetics were also observed in the xanthine oxidase-purine-mediated reaction. Fig. 2 shows a representative reciprocal plot of reaction velocity against concentration for NADPH. Under the conditions employed, the Michaelis constant for NADPH, determined by nonlinear least-squares fitting of the points shown to the Michaelis-Menten equation, was 0.61±0.05 mM (mean±SD).

We also observed saturation kinetics with manganese in the xanthine oxidase-purine system (Fig. 3). The

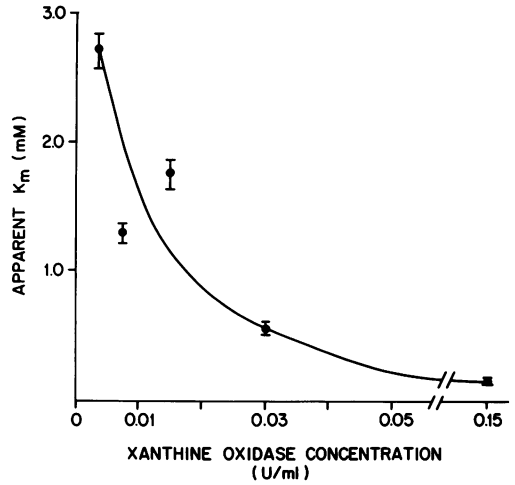

FIGURE 4 Change in K_m of the O_2^-/Mn^{++} oxidation of NADPH as a function of xanthine oxidase concentration (i.e., of the rate of O_2^- production). K_m's for the NADPH oxidation reaction were determined at various concentrations of xanthine oxidase by the method described in Fig. 3. Xanthine oxidase was diluted in 2.3 M $(NH_4)_2SO_4$ containing 0.2% sodium salicylate, the medium in which the commercial xanthine oxidase was supplied. 2 mM KCN was also present in all reaction mixtures. Because of the rapid rate of oxygen consumption at the highest concentration of xanthine oxidase (0.15 U/ml), it was necessary to determine the K_m at this concentration spectrophotometrically at 23°C. The method described in Table I was used and the following concentrations of NADPH were employed (all micromolar): 40, 60, 80, 120, 160, 180, and 240. The reaction and reference cuvettes each contained 2 μmol KCN (final volume, 1.0 ml).

points in Fig. 3 did not fall on a straight line in a reciprocal plot, indicating that the kinetics with manganese did not follow Michaelis-Menten behavior. Similar results have been obtained by Hohn and Lehrer (18) and by DeChatelet et al. (19), using the human granulocyte oxidase. Wide differences in the manganese concentration at half maximal velocity observed among the three studies (the present study and the two cited) may reflect differences in reactant concentrations in the various experiments.

Previous studies with the NADPH oxidase from guinea pig granulocytes have shown that phagocytosis is accompanied by a shift in the K_m for NADPH, which would be an important control mechanism in cellular metabolism during phagocytosis. The K_m determined with oxidase isolated from resting cells was ten times greater than the K_m with oxidase from phagocytizing cells, a finding that was interpreted to indicate an increase in the affinity of the enzyme for NADPH upon initiation of phagocytosis. This change in K_m has been proposed as the biochemical basis for the respiratory burst. Fig. 4 shows that this property of the granulocyte oxidase is also reproduced by the xanthine oxidase-purine system. An increase in the rate of O_2^- formation in the system was accompanied by a fall in the apparent K_m for NADPH. The range over which the K_m varied (2.7–0.15 mM) is in reasonable agreement with the range obtained with the granulocyte oxidase (4.0–0.4 mM) (7).

Stoichiometric relationship between O_2^- production and NADPH oxidation. To further characterize the xanthine oxidase-purine catalyzed reaction, we examined the rate of oxidation of NADPH as a function of the rate of O_2^- production. When the stoichiometric relationship between O_2^- production and NADPH oxidation was analyzed (Fig. 5), it became apparent that at all levels of O_2^- production, the NADPH oxidation rate exceeded that of O_2^- production by the xanthine oxidase-purine system. The ratio of NADPH oxidized to O_2^- produced was not constant, but varied as rates of O_2^- production changed. At the lowest levels of O_2^- production, more than 20 mol of NADPH was oxidized for each mole of O_2^- formed by the xanthine oxidase-purine system. As the rate of O_2^- production increased, this ratio declined, until at the highest O_2^- production rates examined, only 2.3 mol of NADPH was oxidized per mole of O_2^- formed. Thus, the quantity of NADPH oxidized is not related in a simple manner to the quantity of O_2^- produced but represents the latter value amplified by a factor that varies with the rate of O_2^- formation in the system under study.

DISCUSSION

Although manganese-dependent oxidation of NADPH by the xanthine oxidase-purine system displays certain properties generally thought to be characteristic of

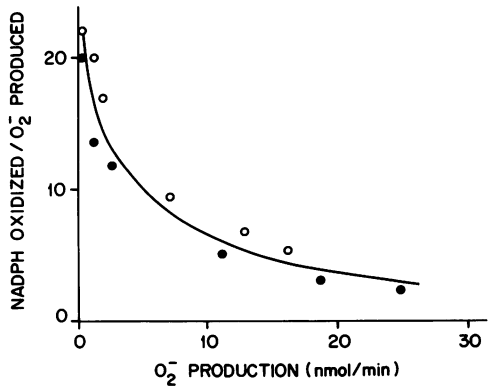

FIGURE 5 Superoxide-dependent NADPH oxidation at various rates of superoxide production. O_2^--dependent NADPH oxidation was measured as described in Table II except that the concentration of xanthine oxidase was varied to provide different rates of O_2^- production. The rate of O_2^- production was determined as described in Methods. Open circles represent one experiment while closed circles represent a second.

enzyme-catalyzed reaction, it is unlikely that the reaction involves the interaction of either the metal or the pyridine nucleotide with xanthine oxidase. This is indicated by several lines of evidence. In the first place, a requirement for a metal ion apart from those normally bound to xanthine oxidase (namely, iron and molybdenum [21, 22]) has not been demonstrated for any previously described reaction catalyzed by this enzyme. Secondly, the pH optimum for the manganese-dependent oxidation of NADPH is nearly three units away from the normal pH optimum for xanthine oxidase (20). Thirdly, the results obtained with superoxide dismutase show that O_2^- utilization is an essential feature of the xanthine oxidase-purine catalyzed oxidation of NADPH, indicating that O_2^- is serving in this reaction as an oxidizing agent. The usual oxidant employed by xanthine oxidase, however, is oxygen, O_2^- being a product—not substrate—of xanthine oxidase-catalyzed oxidations. Finally, the unusual stoichiometric relationship shown in Fig. 5 constitutes very strong evidence against the direct participation of xanthine oxidase in the O_2^--mediated oxidation of NADPH. If xanthine oxidase were involved directly in this reaction, catalyzing the oxidation of NADPH at the expense of the O_2^- formed by the same enzyme during the oxidation of purine, a maximum of $\frac{1}{2}$ mol of NADP would be formed for each mole of O_2^- released into the solution (assuming a one-electron reduction of O_2^- to H_2O_2).

$$\text{oxygen} + \text{purine} \xrightarrow[\text{xanthine oxidase}]{} O_2^- + \text{products}$$

$$2O_2^- + \text{NADPH} + 3H^+ \xrightarrow[\text{xanthine oxidase}]{} 2H_2O_2 + NADP^+$$

$$2O_2^- \searrow \text{side reactions}$$

Even if O_2^- were reduced to water, there is no way that a mechanism involving a xanthine oxidase-catalyzed reaction between O_2^- and NADPH can explain the formation of over 20 mol of NADP per mole of O_2^- released into the solution.

Largely on the basis of the stoichiometry shown in Fig. 5, we have concluded that the manganese-dependent oxidation of NADPH in the presence of xanthine oxidase and purine is best formulated as a free radical chain reaction. A similar mechanism was also proposed by Patriarca et al. (10), and both mechanisms resemble that offered by Bielski and Chan to explain the lactic dehydrogenase-catalyzed oxidation of NADPH by O_2^- (23). The chain is initiated by the enzyme-catalyzed formation of O_2^- a portion of which is instantly protonated at the pH of the reaction mixture to form the hydroperoxyl radical (24). The propagation steps of the reaction sequence may be postulated to involve first a one-electron oxidation of Mn^{++} by the hydroperoxyl radical to generate Mn^{+++} and hydrogen peroxide. Mn^{+++} is then presumed to form a complex with NADPH which can dissociate either to the starting materials or to Mn^{++} plus an NADP·radical. Once formed, the NADP·radical gives its free electron to an oxygen molecule, yielding NADP and another hydroperoxyl radical, which can then initiate another cycle of the propagation sequence.

$$O_2 + H^+ + e^- \longrightarrow HO_2\cdot \qquad \text{(Initiation)}$$

$$\left.\begin{aligned} &HO_2\cdot + Mn^{++} + H^+ \longrightarrow H_2O_2 + Mn^{+++} \\ &Mn^{+++} + \text{NADPH} \rightarrow Mn^{+++}\cdot\text{NADPH} \rightarrow Mn^{++} + \text{NADP} + H^+ \\ &\text{NADP}\cdot + O_2 + H^+ \longrightarrow \text{NADP}^+ + HO_2\cdot \end{aligned}\right\} \text{(Propagation)}$$

These propagation steps, in contrast to the initiation step, are nonenzymatic. This free radical chain process, then, can account for the large amount of NADPH oxidation that is O_2^--dependent, as well as for the increase in the rate of oxygen uptake that occurs when an O_2^--generating system is added to an acidic solution containing NADPH and manganese.

This mechanism can also explain the *variable amplification* that characterizes the stoichiometry of the reaction. At the pH at which NADPH oxidase is usually determined (7), O_2^- undergoes an unusually rapid spontaneous dismutation:

$$O_2^- + HO_2\cdot + H^+ \longrightarrow O_2 + H_2O_2$$

It would be expected that as rates of O_2^- production increase, a larger fraction of the O_2^- undergoes the bimolecular dismutation reaction, resulting in a relative increase in the amount of O_2^- diverted from participation in NADPH oxidation. As a consquence of this shift in the relative rates of the competing O_2^--consuming reactions, there is a nonlinear relationship between the rate of O_2^- production and the rate of NADPH oxidation. An additional factor that might contribute to the decrease in amplification at high O_2^- production rates would be the accompanying rise in the steady-state concentration of O_2^- and $HO_2\cdot$, which, by participating in termination reactions, could reduce the length of the free radical chain. Hypothetical examples of such termination reactions might include the following:

$$\text{NADP}\cdot + HO_2\cdot \longrightarrow \text{NADPH} + O_2$$

$$\text{NADP}\cdot + HO_2\cdot + H^+ \longrightarrow \text{NADP}^+ + H_2O_2$$

In the second reaction of the propagation sequence, the formation of a complex between NADPH and Mn^{+++} is indicated. The existence of such a complex is one way to rationalize both the saturation kinetics seen in the xanthine oxidase-purine-mediated reaction and the fall in the apparent K_m at higher rates of O_2^- production. We wish to emphasize, however, that this complex is purely hypothetical and is introduced only to provide a chemically acceptable explanation for the kinetic data. We have no direct evidence that it exists.

The pH optimum for the xanthine oxidase-purine-mediated oxidation of NADPH may be a manifestation of the fact that the hydroperoxyl radical is a more powerful oxidizing agent than the superoxide anion. The half-cell potentials for the one-electron reduction of $HO_2\cdot$ and O_2^- are, respectively, 1.7 and 1.0 V (25). The half-cell potential of the $Mn^{++} \rightarrow Mn^{+++} + e^-$ couple is such that Mn^{++} can be oxidized by $HO_2\cdot$ but not O_2^-. The pH dependence of the reaction can thus be explained by the increase in concentration of the more potent oxidizing agent as the pH decreases.

The finding of variable amplification in the manganese-containing system raises questions regarding the quantitative interpretation of previous NADPH oxidase studies (6, 7, 18, 19). Varying quantities of granules with different activities would exhibit different degrees of amplification and NADPH oxidation depending upon the rates of O_2^- generation by the granules. Additional complications arise because radical chain reactions are very sensitive to radical scavengers such as sulfhydryl compounds, and the occurrence of variable amounts of such scavengers in the crude granule fraction could affect the amplification of NADPH oxidation in an unpredictable fashion.

On the basis of our findings, we have concluded that the bulk of the oxidation that takes place when NADPH is incubated with granulocyte particles in the presence of Mn^{++} occurs via the nonenzymatic O_2^--dependent free radical chain reaction described above. The evidence for this conclusion may be summarized by the statement that we have demonstrated, in a system devoid of granulocyte components, a reaction that duplicates in virtually every particular the reaction catalyzed by the manganese-dependent particulate NADPH oxidase of granulocytes. Though the granulocyte-free system does contain an enzyme, its function appears to be the production of the O_2^- required to initiate the reaction. We further conclude, therefore, that the likely role of the granulocyte particles in the manganese-dependent NADPH oxidase reaction is to serve as a source of O_2^-. This formulation implies that granulocyte particles must catalyze the reduction of oxygen to O_2^- with NADPH as the source of electrons, since NADPH is the only reductant customarily present in the NADPH oxidase assay mixture. We believe, however, that the granulocyte particle-catalyzed oxidation of NADPH accounts for only a small fraction of the NADPH oxidation that takes place in the NADPH oxidase reaction as it is usually carried out; most of the oxidation occurs nonenzymatically.

Extending this line of reasoning to the intact granulocyte, we would argue that manganese has nothing to do with the events of the respiratory burst. We suggest instead that among the enzymes activated during the respiratory burst is a pyridine nucleotide oxidase that is responsible for the O_2^- production by stimulated granulocytes and that does not require manganese for its activity. Such an enzyme, if isolated from the cell and incubated with a reduced pyridine nucleotide in the presence of Mn^{++}, would show behavior similar to that reported in this paper. The production of O_2^- would be difficult to observe, because O_2^- would be rapidly consumed by the radical chain reaction outlined above. The omission of manganese, however, should permit the demonstration of O_2^- production in that system. We have recently carried out such an experiment, using the particulate NADPH oxidase preparation of Hohn and Lehrer (18), and have found that O_2^- is produced under these conditions at a respectable rate (26). These observations provided additional evidence in support of the conclusions set forth above.

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