The Particulate Superoxide-Forming System from Human Neutrophils

PROPERTIES OF THE SYSTEM AND FURTHER EVIDENCE SUPPORTING ITS PARTICIPATION IN THE RESPIRATORY BURST

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ABSTRACT Studies were performed to characterize the previously reported particulate O_2^--forming system from human neutrophils. Of eight reducing agents examined, including glutathione, ascorbic acid, and intermediates of the glycolytic and hexose monphosphate shunt pathways, only the pyridine nucleotides could serve as electron donors. At 0.1 mM pyridine nucleotide, O_2^- production was relatively independent of pH. The K_m for NADH was approximately 0.7 mM regardless of pH, while with NADPH the K_m varied from 0.02 mM at pH 6.0 to 0.3 mM at pH 7.5. The molar ratio of NADPH oxidized to O_2^- produced was consistent with the reaction: $NADPH + 2\,O_2^- \rightarrow NADP^+ + H^+$; the product nucleotide was shown enzymatically to be NADP. O_2^- production was not inhibited by CN^-, N_3^-, EDTA, or 1,10-phenanthroline. Particulate O_2^- production accounted for 35% of the oxygen taken up during the respiratory burst by an equivalent number of intact neutrophils.

Greatly diminished O_2^- production was seen with particles prepared from cells obtained from three patients with chronic granulomatous disease, with 2.5 mM NADPH as electron donor. With 5.0 mM NADH similar observations were made with particles from two of the patients, but with this nucleotide, O_2^- production was only slightly reduced in the third case.

The evidence available suggests that this particulate O_2^--forming system is the one responsible for the respiratory burst in activated neutrophils. The relationship between this system and other O_2^--forming system found in human neutrophils is discussed.

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INTRODUCTION

When exposed to appropriate stimuli, neutrophils undergo a series of metabolic changes, collectively termed the "respiratory burst", the purpose of which appears to be the elaboration of bactericidal compounds by the partial reduction of oxygen (1). Recent evidence has suggested that the first reaction in the sequence responsible for the formation of these compounds is the one electron reduction of oxygen to produce superoxide (O_2^-) (2–7). Other bactericidal agents, including H_2O_2 (8–10) and possibly the hydroxyl radical (6, 11, 12) and singlet oxygen (12–14), appear to be formed at least in part by secondary reactions in which O_2^- serves as starting material.

Since its characterization by Sbarra and Karnovsky (15), the respiratory burst has been the object of numerous studies designed to elucidate its biochemistry. In particular, much effort has been directed toward the discovery of the enzyme responsible for the reduction of oxygen in this pathway. An enzyme (more accurately, an enzymatic activity) that has been discussed in these terms by several groups of investigators (16–19) is a NADPH oxidase located in the particulate fraction of neutrophil homogenates. This oxidase has been studied extensively by Rossi and his collaborators (17, 18), who characterized it as a manganese requiring enzyme with a pH optimum of 5.5.

It has recently become apparent that the manganese requirement is an artifact (20). The marked acceleration in oxygen consumption that occurs when the metal is added to the NADPH oxidase system was shown to be due to a nonenzymatic oxidation of NADPH by a free radical chain process involving manganese and O_2^-

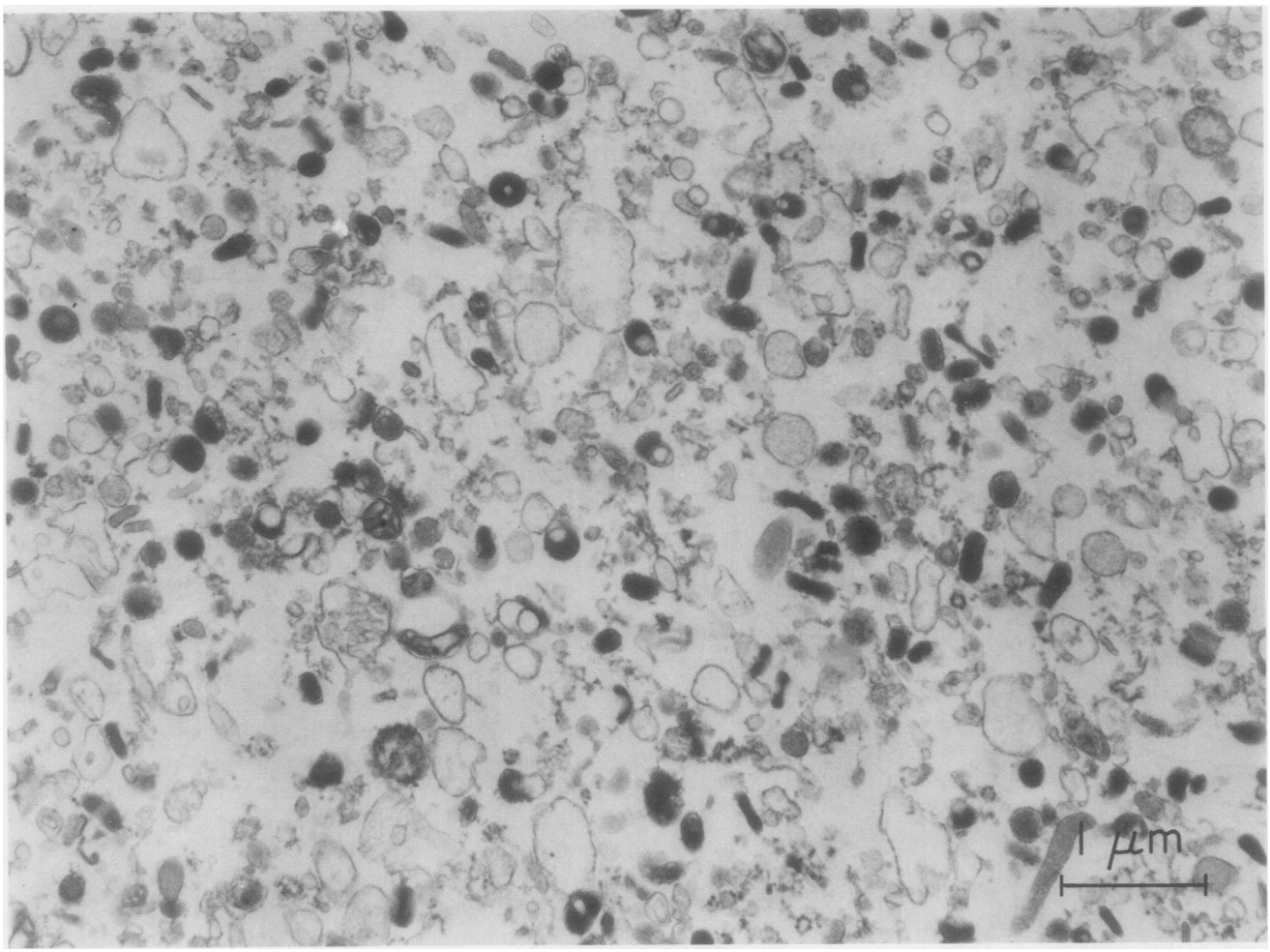


FIGURE 1 Electron micrographs of particles from resting (*left*) and zymosan-activated (*right*)

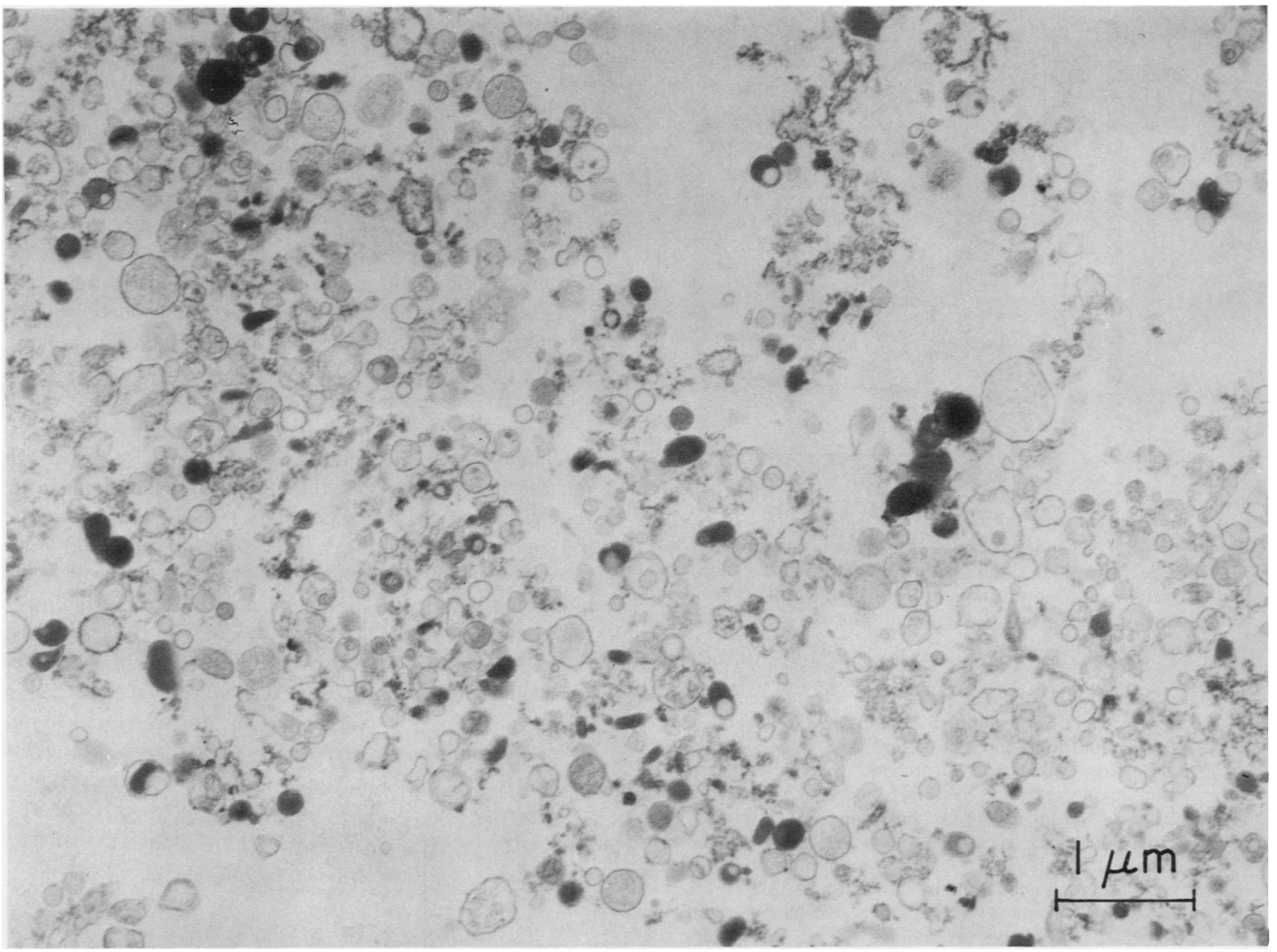
as propagating species. The omission of manganese from the system disclosed that the particulate NADPH oxidase prepared from zymosan-activated but not resting neutrophils catalyzed the formation of O_2^- at a substantial rate (21). Little O_2^- was generated by particles from neutrophils of patients with chronic granulomatous disease (CGD)[1] (21), a condition (or group of conditions) in which the respiratory burst does not take place on stimulation of the neutrophil (22). These findings indicated that the O_2^--forming system is probably involved in oxygen reduction during the respiratory burst and suggested that detailed study of this system would be of value in understanding neutrophil physiology. The further characterization of the particulate O_2^--forming system of human neutrophils is the subject of the present report.

[1] *Abbreviation used in this paper:* CGD, chronic granulomatous disease.

METHODS

Reduced pyridine nucleotides (preweighed vials), glycolytic and hexose monophosphate shunt intermediates, reduced glutathione, ascorbic acid, ferricytochrome *c* (Type VI), catalase (bovine liver), sodium isocitrate, and isocitrate dehydrogenase (Type IV) were obtained from Sigma Chemical Co., St. Louis, Mo. Superoxide dismutase was purchased from Truett Laboratories, Dallas, Tex. Zymosan was obtained from ICN Pharmaceuticals, Inc., Cleveland, Ohio. Other reagents were the best grade commercially available and were used without further purification.

Neutrophil particles. Human neutrophils were prepared as described previously. 27,000 *g* particles were obtained from these cells by the method of Hohn and Lehrer (19), washed once with 0.34 M sucrose adjusted to pH 7.4 with $NaHCO_3$, and suspended in the same medium, usually at a concentration of 1 mg particle protein/ml. For the experiments to be described below, only particles from zymosan-activated neutrophils were used. However, electron microscopy was performed on particles from both zymosan-activated and resting neutrophils. For electron microscopy, the particles were fixed for 18 h at 4°C in 2% glutaraldehyde



cells. The preparation of particles for electron microscopy is described in the text.

in 0.1 M cacodylate buffer (pH 7.5) containing 5% sucrose, stained with 2% OsO_4 in the same cacodylate/sucrose buffer, dehydrated, embedded in Epon 812 and prepared for microscopy by standard procedures. Electron micrographs (Fig. 1) revealed both preparations to be heterogeneous, each being composed of plasma membrane fragments, azurophil and specific granules, and occasional mitochondria.

Oxygen uptake. Oxygen uptake was determined manometrically by using Gilson all glass submersible differential respirometers (Gilson Medical Electronics, Inc., Middleton, Wis.) with siliconized 15-ml Warburg flasks as reaction vessels (2). Experiments with resting and zymosan-activated cells were performed simultaneously. Neutrophils (2×10^7 cells) in 0.5 ml phosphate-buffered saline (composition [mM]: NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5) were placed in the main compartment of each of two flasks. To the sidearm of one flask was added 6 mg opsonized (21) zymosan in 0.5 ml phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, while the sidearm of the other flask received 0.5 ml of the same buffer without opsonized zymosan. The flasks were then attached to the respirometers and placed in a Dubnoff shaker, the flasks themselves being immersed in the bath, which was maintained at 37°C. After 3 min the contents of the sidearms were tipped into the main compartments of the flasks, and the assemblies were incubated with shaking for 3 min. (These times and temperatures are identical to those used to activate neutrophils for the preparation of particles [21]). The assemblies were then placed in a shaking water bath maintained at 23°C for measurement of oxygen uptake at that temperature. 10 min was required for temperature equilibration within the assembly; changes in volume were not recorded during that period. Thereafter, readings were taken every 5 min for 40 min.

O_2^- production. O_2^- production was determined as previously described (ref. 21, method B) except that potassium instead of sodium phosphate buffer was used, the reference cuvet contained only 30 μg superoxide dismutase, and the quantity of cytochrome *c* in the reaction mixtures was 75 nmol. Doubling the cytochrome *c* concentration caused no change in the rate of dismutase-inhibitable reduction at either pH 5.5 or 7.5, indicating that the concentration used in the assay was sufficient to trap all the O_2^- generated in the reaction. The initial rate of O_2^- production as measured by this assay was proportional to the quantity of neutrophil particle protein up to a concentration of 0.1 mg/ml.

The stoichiometric relationship between O_2^- production and NADPH consumption was established as follows. O_2^--

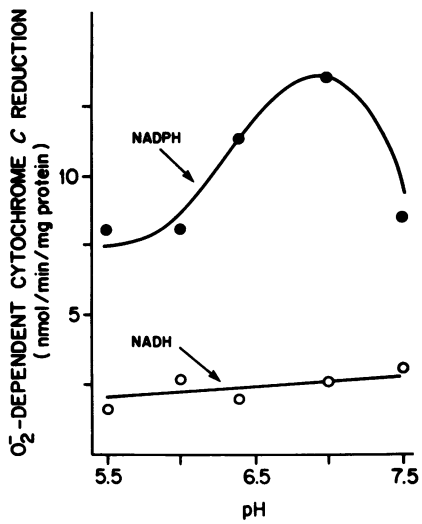


FIGURE 2 Dependence of O_2^- production on pH. The assays were performed as described in Methods. The concentration of pyridine nucleotide was 0.1 mM, and the concentration of particle protein was 0.1 mg/ml. The pH was as indicated. ●, NADPH, ○, NADH.

dependent cytochrome *c* reduction was determined using 0.1 mg/ml particle protein, 0.1 mM NADPH, and pH 7.4. NADPH consumption was determined under similar conditions, except that cytochrome *c* was omitted from the reaction mixtures, and NADPH and superoxide dismutase were omitted from the reference cuvet. NADPH disappearance was followed spectrophotometrically at 340 nm. For each measurement of NADPH consumption, two blank values were obtained: one by omitting NADPH from the sample cuvet and one by replacing active particles with particles heated for 1 min in boiling water. All values were expressed in terms of the quantity of substrate consumed (NADPH) or cytochrome *c* reduced (O_2^-) during the 3-min interval beginning 30 s after starting the reaction. Each figure for NADPH consumption was corrected by subtracting the sum of the corresponding blank values.

Miscellaneous methods. Protein was determined by the Lowry method (23). Spectra were taken on a Cary 118C recording spectrophotometer (Cary Instruments, Fairfield, N. J.). The Michaelis constants for the pyridine nucleotides were calculated using a nonlinear least squares curve fitting program (HYPER [24]), assigning a weight of 1 to each data point. Cells were counted by hand in a hemacytometer. Millimolar extinction coefficients used for the calculation of cytochrome *c* reduction and NADPH oxidation were, respectively, 18.5 (550 nm [25]) and 6.22 (340 nm [26]).

RESULTS

Properties of the system. Previous work indicated that the particulate O_2^--forming system from human neutrophils required a reduced pyridine nucleotide as electron donor. The reduced pyridine nucleotides, however, represent only one of several classes of biological reducing agents. Other classes include glycolytic intermediates, sulfhydryl compounds (e.g., glutathione), and ascorbic acid. To examine the substrate specificity of this system, we tested several such reducing agents for their ability to replace pyridine nucleotides as sources of electrons for O_2^- production. Assays were performed as described in Methods, with 0.1 mg/ml particle protein, 0.1 mM substrate, and pH 7.0. Under these conditions, O_2^--dependent cytochrome *c* reduction with NADPH and NADH were 5.3 and 1.5 nmol/min per mg protein, respectively. Rates with other substrates, including glutathione, L-ascorbic acid, L-lactic acid, DL-glyceraldehyde-3-phosphate, glucose-6-phosphate, and 6-phosphogluconic acid were all less than 0.05 nmol/min per mg protein.

The pH dependence of O_2^- production by this system is shown in Fig. 2. With both pyridine nucleotides O_2^- production took place over a wide range of hydrogen ion concentrations. With 0.1 mM NADPH as substrate, the activity at pH 5.5 was nearly 60% of activity at the pH optimum, which was in the vicinity of 7.0. With 0.1 mM NADH the reaction showed no clear pH optimum, the activity at pH 5.5 being only slightly lower than that at pH 7.0.

Determinations of K_m with the two pyridine nucleotide substrates revealed that the enzyme system had a greater affinity for NADPH than for NADH. Moreover, the affinity for NADPH varied with pH. This is shown in Fig. 3, in which the K_m is plotted against

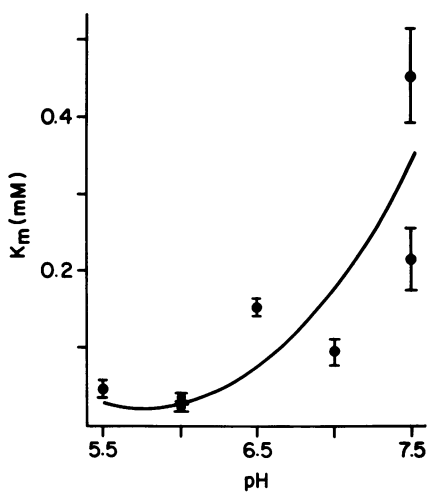


FIGURE 3 K_m for NADPH as a function of pH. The assays were performed as described in Methods, and the K_m calculated as described in the text. Each point represents the results obtained with a single preparation of particles, using eight concentrations of NADPH ranging from 0.02 to 0.5 mM. At the pH values where two separate measurements were made (pH 6.0 and pH 7.5), the cells used for the two measurements were obtained from different donors. The concentration of particle protein was 0.1 mg/ml, except for the experiment at pH 6.5 (80 μg/ml) and the experiment represented by the lower point at pH 6 (50 μg/ml). Error bars represent ±1 SE.

pH. There is a 10-fold fall in the K_m as the pH drops from 7.5 to 6.0, indicating stronger binding of NADPH to the enzyme under acidic conditions. In contrast, the values for the K_m of NADH measured under similar conditions (except that the concentrations of NADH ranged from 0.1 to 1.0 mM) were 1.0±0.17 and 0.59 ±0.07 SE mM at pH 6.0 and 7.0, respectively. The affinity of NADPH for the enzyme was substantially greater than that of NADH at both pH values tested.

The respiratory burst of intact neutrophils is characteristically insensitive to CN^- (15, 22), implying that the enzyme system responsible for the burst should be similarly cyanide-insensitive. The effects of CN^- and certain other inhibitors on the particulate O_2^--forming system were tested in an assay that contained 0.1 mM NADPH and 0.1 mg/ml particle protein at pH 7.0. O_2 production was not inhibited by the heme enzyme reagents CN^- (1 mM) [2] or N_3^- (0.5 mM), or the metal chelators Na_2EDTA (0.25 mM), or 1,10-phenanthroline (0.25 mM). The results with CN^- and N_3^- are in accord with previous observations in which whole cells were used (27).

NADPH consumption. It was possible to determine the stoichiometric relationship between pyridine nucleotide consumption and O_2^- production by measuring both processes spectrophotometrically under similar conditions. These experiments were conducted as described in Methods. In control experiments, the addition of superoxide dismutase or catalase to the reaction mixtures (30 μg to each cuvet) had little effect on the disappearance of NADPH, indicating that the consumption of reducing agent was not altered by the presence of O_2^- or H_2O_2. The results of three experiments, each conducted with a different particle preparation, showed that the ratio of NADPH consumed to O_2^- produced was 0.55±0.06 SE. This is in reasonable agreement with the value of 0.50 expected for the reaction:

$$2\,O_2 + NADPH \rightarrow 2\,O_2^- + NADP^+ + H^+$$

The compound formed from NADPH was identified as NADP by showing that it could be reduced back to the starting material by the isocitric acid-isocitrate dehydrogenase system. In the experiment a reaction mixture containing NADPH, neutrophil particles, isocitric acid, $MnCl_2$, and superoxide dismutase [3] was incubated

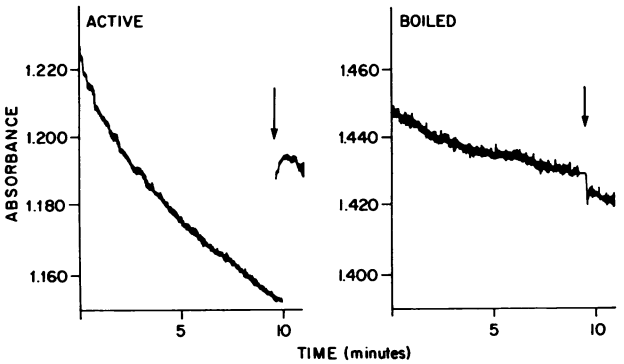


FIGURE 4 The identity of the product formed from NADPH. A reaction mixture that contained 0.1 mg particle protein, 0.1 μmol NADPH, 5 μmol sodium isocitrate, 0.5 μmol $MnCl_2$, 30 μg superoxide dismutase, and 65 μmol potassium phosphate buffer (pH 7.0) in a total volume of 1.0 ml was placed in a cuvet, and the oxidation of NADPH followed with time at 340 nm, reading against a water blank. The temperature of the incubation was 24°C. At the arrow, 0.01 ml (0.36 U) isocitrate dehydrogenase was added to the reaction mixture, and the reaction was followed for an additional minute, by which time the rise in absorbance at 340 nm associated with the isocitrate dehydrogenase reaction had reached completion. *Left:* active particles, *Right:* boiled particles.

at room temperature in the spectrophotometer, and NADPH disappearance was followed at 340 nm (Fig. 4). After 10 min isocitrate dehydrogenase was added, and the reaction was again followed spectrophotometrically until 30 s after the dehydrogenase-catalyzed reduction had reached completion. A similar experiment with boiled particles served as control. From the results of these experiments it is possible, after correcting for absorbance changes due to enzyme-independent processes (determined from the boiled particle control) and dilution by the added isocitrate dehydrogenase, to show that 84% of the NADPH oxidized by the neutrophil particles was regenerated by the isocitrate dehydrogenase reaction. This shows that the pyridine nucleotide product was NADP.

Oxygen uptake and O_2^- production. It was of interest to establish the extent to which O_2^- production by particles from activated neutrophils could account for the oxygen taken up by intact cells during the respiratory burst. To provide a basis for comparison, the oxygen uptake of neutrophils activated by the method used for preparing particles was measured at 23°C, the temperature at which O_2^- production by particles was determined. These experiments showed the rate of oxygen uptake by zymosan-activated neutrophils at 23°C to average 7.5 nmol/min per 10^7 cells. Oxygen uptake by resting cells was negligible.

An estimate of O_2^- production was obtained by averaging the results of the seven incubations reported in the present paper that were conducted at pH 7.0 with

[2] For this experiment, the reference cuvet contained 300 μg superoxide dismutase. This quantity of dismutase was necessary to overcome the inhibition resulting from the presence of CN^- in the cuvet.

[3] Manganese was present because of the metal ion requirement of isocitrate dehydrogenase (28); superoxide dismutase was present to prevent the occurrence of the Mn^{++}- and O_2^--requiring free radical chain oxidation of NADPH that has been described elsewhere (20).

TABLE I

O_2^- Production by Particles from CGD Neutrophils

Substrate	O_2^--Dependent cytochrome *c* reduction					
	Patient 1	Control	Patient 2	Control	Patient 3	Control
	nmol/min/mg					
NADPH						
pH 6.0	0.7	4.4	0	2.7	0.1	3.9
pH 7.0	0.5	*	0	4.9	0.4	10.2
NADH						
pH 6.0	1.9	2.1	0	1.2	0.6	4.5
pH 7.0	1.6	4.0	0	1.7	0.6	6.6

The assays were performed as described in Methods at the pH and with the substrates indicated. The concentrations of constituents were: NADPH, 2.5 mM; NADH, 5.0 mM; and particle protein, 0.1 mg/ml. In every case, control assays under the same conditions were performed on the same day using particles from normal granulocytes.

* Insufficient particles.

0.1 mM NADPH and 100 μg protein. From these values, the rate of O_2^- production by neutrophil particles was calculated to be 9.8±1.4 SE nmol/min per mg particle protein. When this figure is corrected for the fact that the NADPH concentration in neutrophils (0.35 mM in guinea pig cells [29]) is well above the K_m for the O_2^--forming enzyme, while the measurements of particle O_2^- formation were performed at an NADPH concentration approximately equal to the K_m, a value of 15.2 nmol O_2^-/min per mg particle protein is obtained. This value can be used to compare O_2^- production with oxygen uptake. Measurements of particle yield from homogenates prepared with known quantities of neutrophils showed that 0.17±0.03 SE mg particle protein were obtained from 10^7 cells. Thus, 2.6 nmol of O_2^-/min (corrected) was produced by particles equivalent to 10^7 cells. Comparison of this figure with the measured rate of oxygen uptake by intact cells shows that O_2^- production accounted for 35% of the oxygen taken up by activated granulocytes.

O_2^- production by particles from CGD neutrophils. With regard to the system responsible for the respiratory burst, the significance of the present study lies in the fact that O_2^- is not produced by particles obtained from CGD neutrophils. This finding strongly suggests that this particulate O_2^--forming system is identical with, or closely related to, the oxygen-consuming system which is activated when the neutrophil is exposed to appropriate stimuli. To understand the defect in the O_2^--forming system of CGD neutrophils more completely, we performed a series of further studies on the particles obtained from these neutrophils. The results (Table I) show the following: (*a*) both NADH-dependent and NADPH-dependent O_2^- production are diminished in CGD particles, suggesting that, at least in principle, either nucleotide may support the respiratory burst. There may be a second NADH-dependent O_2^--forming system in the particles, however, since the loss in NADH-dependent activity is not as complete as the loss in NADPH-dependent activity, when expressed as percent of control values; (*b*) the defect is expressed both at pH 7.0 and pH 6.0; (*c*) little activity was seen with either pyridine nucleotide despite the high levels used in these experiments, levels that for both nucleotides substantially exceed the K_m values.

DISCUSSION

Four lines of evidence support the idea that the particulate O_2^--forming system described above is the oxygen-consuming system responsible for the respiratory burst characteristic of activated neutrophils. The first two lines of evidence were reported elsewhere, and consist of the findings (*a*) that O_2^- production is catalyzed by particles from zymosan-activated but not resting neutrophils, and (*b*) that particles from zymosan-activated CGD neutrophils produce little if any O_2^- when incubated with NADPH (21). The lack of effect of cyanide on particle-catalyzed O_2^- production is another piece of evidence in support of this notion, since cyanide is known to have little effect on the respiratory burst in intact cells (15, 22, 27). Finally, calculations show that O_2^- production by particles from activated neutrophils can account for a large fraction of the increment in oxygen uptake that occurs during the respiratory burst. Since certain experiments suggest that the conditions used for measurements of O_2^- production may not have been ideal—it appears, for example, that certain chelatable metals present in the reaction mixture interfered with the assay (data not shown)—it seems reasonable to speculate that optimization of assay conditions could result in rates of O_2^- production that could

account for all the oxygen consumed in the respiratory burst.

The system described in the present paper, which in the guise of the manganese-dependent NADPH oxidase was first reported by Iyer and Quastel (16) and was later studied in detail by Rossi and his colleagues (17, 18) and by Hohn and Lehrer (19), is only one of three cell-free O_2^--forming systems that have been obtained from human neutrophils in the past 2 yr. The properties of the other two systems may be summarized as follows: (*a*) In one system described by Johnson and co-workers (6) and by Allen and his associates (13) and later characterized in this laboratory (29), nitro blue tetrazolium was employed to detect O_2^- production by whole neutrophil homogenates. The source of electrons in this system was a reduced pyridine nucleotide. Either NADH or NADPH could be used, but the K_m for the latter was two orders of magnitude smaller than the K_m for NADH. O_2^- production by this system was normal in homogenates of cells from CGD patients. (*b*) In the second system, the oxidation of epinephrine to adrenochrome was used to detect O_2^- production by whole neutrophil homogenates (30). Though O_2^- could be detected by this technique, its rate of production could not be quantitated owing to the nature of the reaction used to detect its formation, nor could a requirement for an electron donor be established. No further studies have been carried out with this system.

The existence of these three O_2^--forming systems presents a rather confusing picture, particularly since nothing is known of the relationships among the three. Rather than attempting to sort out these relationships, we have begun the study of that system which for the reasons set forth above appears to be of most immediate interest in terms of neutrophil function—namely, the particulate O_2^--forming system of activated neutrophils, which we believe is responsible for the respiratory burst.

The present observations confirm previous results showing that there is a defect in the particulate O_2^--forming system in CGD neutrophils. The defect was expressed under all incubation conditions employed. It is possible, however, that other conditions might have permitted O_2^- production by the particles to take place. Such correction would have indicated a kinetically abnormal O_2^--forming enzyme; however, our inability to demonstrate O_2^- production under the conditions employed clearly does not rule out such an enzyme as the cause of the CGD defect. Alternatively, the defect could represent absence of the O_2^--forming enzyme, or a lesion in the mechanism by which the enzyme is activated. Further study will be required to distinguish among these various alternatives.

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