

The Role of Superoxide Anion Generation in Phagocytic Bactericidal Activity

STUDIES WITH NORMAL AND CHRONIC GRANULOMATOUS DISEASE LEUKOCYTES

RICHARD B. JOHNSTON, JR., BERNARD B. KEELE, JR., HARA P. MISRA,
JOYCE E. LEHMEYER, LAWRENCE S. WEBB, ROBERT L. BAEHNER, and
K. V. RAJAGOPALAN

From the Departments of Pediatrics and Microbiology, Institute of Dental Research, and Comprehensive Cancer Center, the University of Alabama in Birmingham, Birmingham, Alabama 35294; the Department of Pediatrics, Division of Pediatric Hematology-Oncology, Indiana University School of Medicine and Riley Hospital for Children, Indianapolis, Indiana 46202; and the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT The capacity of human phagocytes to generate superoxide anion (O_2^-), a free radical of oxygen, and a possible role for this radical or its derivatives in the killing of phagocytized bacteria were explored using leukocytes from normal individuals and patients with chronic granulomatous disease (CGD). Superoxide dismutase, which removes O_2^- , consistently inhibited phagocytosis-associated nitroblue tetrazolium (NBT) reduction indicating the involvement of O_2^- in this process. Similarly, superoxide dismutase inhibited the luminescence that occurs with phagocytosis, implicating O_2^- in this phenomenon, perhaps through its spontaneous dismutation into singlet oxygen. Subcellular fractions from homogenates of both normal and CGD leukocytes generated O_2^- effectively in the presence of NADH as substrate. However, O_2^- generation by intact cells during phagocytosis was markedly diminished in nine patients with CGD. Leukocytes from mothers determined to be carriers of X-linked recessive CGD by intermediate phagocytic reduction of NBT elaborated O_2^- to an intermediate extent, further demon-

strating the interrelationship between NBT reduction and O_2^- generation in phagocytizing cells.

Activity of superoxide dismutase, the enzyme responsible for protecting the cell from the damaging effects of O_2^- , was approximately equal in homogenates of normal and CGD granulocytes. Polyacrylamide electrophoresis separated this activity into a minor band that appeared to be the manganese-containing superoxide dismutase associated with mitochondria and a more concentrated, cyanide-sensitive, cytosol form of the enzyme with electrophoretic mobility that corresponded to that of erythrocyte cuprozinc superoxide dismutase.

Superoxide dismutase inhibited the phagocytic killing of *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus viridans*. A similar inhibitory effect was noted with catalase which removes hydrogen peroxide. Neither enzyme inhibited the ingestion of bacteria. Peroxide and O_2^- are believed to interact to generate the potent oxidant, hydroxyl radical ($\cdot OH$). A requirement for $\cdot OH$ in the phagocytic bactericidal event might explain the apparent requirement for both O_2^- and H_2O_2 for such activity. In agreement with this possibility, benzoate and mannitol, scavengers of $\cdot OH$, inhibited phagocytic bactericidal activity. Generation of singlet oxygen from O_2^- and $\cdot OH$ also might explain these findings.

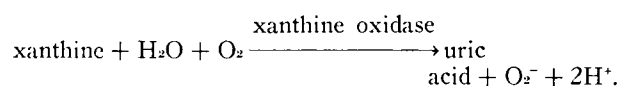
This work was presented in part at the 65th Annual Meeting of The American Society for Clinical Investigation, Atlantic City, N. J., 29 April 1973 (*J. Clin. Invest.* 52: 44a, 1973).

Received for publication 24 July 1974 and in revised form 18 January 1975.

It would seem clear from these and other studies that the granulocyte elaborates O_2^- as a concomitant of the respiratory burst that occurs with phagocytosis. To what extent the energy inherent in O_2^- is translated into microbial death through O_2^- itself, hydrogen peroxide, $\cdot OH$, singlet oxygen, or some other agent remains to be clearly defined.

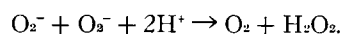
INTRODUCTION

Recognition that superoxide anion (O_2^-),¹ a free-radical form of oxygen, can be generated in biological systems has emerged primarily from study of the aerobic oxidation of xanthine by the enzyme xanthine oxidase (1), according to the reaction:



The radical can be detected by its capacity to reduce compounds such as ferricytochrome *c* (2) or nitroblue tetrazolium (NBT) (3, 4) or to oxidize epinephrine to adrenochrome (5). Auto-oxidative processes and enzymes capable of generating this highly reactive anion have been identified in various mammalian tissues, bacteria, and fungi (reviewed in reference one). However, it has not been clear whether or not O_2^- serves a useful biological purpose. On the contrary, its presence should pose a potential threat to those cells in which it is elaborated.

Greater understanding of the physiological control of O_2^- developed after the demonstration by McCord and Fridovich (6) of enzymic activity which catalyzes the dismutation of O_2^- in the reaction:



The enzyme, superoxide dismutase (SOD), is now known to be identical to "cuprein," the copper protein without known function previously isolated from mammalian erythrocytes, liver, brain, and other tissues (6). The presence of SOD in aerobic bacteria and its absence from strict anaerobes (7), and the capacity of increased intracellular concentrations of SOD to protect *Escherichia coli* (8) and *Saccharomyces cerevisiae* (9) against oxygen toxicity have led to the assumption that this enzyme serves to protect oxygen-metabolizing cells from the potentially detrimental effects of O_2^- .

¹Abbreviations used in this paper: BSA, bovine serum albumin; CGD, chronic granulomatous disease; KRP-D buffer, Krebs-Ringer phosphate buffer with dextrose but no albumin; KRP-DA, Krebs-Ringer phosphate buffer with dextrose and albumin; NBT, nitroblue tetrazolium; 1O_2 , singlet oxygen; O_2^- , superoxide anion; $\cdot OH$, hydroxyl radical; PBS, phosphate-buffered solution; SOD, superoxide dismutase.

Normal human neutrophils have the capacity to reduce NBT to formazan during phagocytosis; neutrophils from patients with chronic granulomatous disease (CGD), which can ingest bacteria normally but cannot kill them, lack this capacity (10). The ability of O_2^- to reduce NBT, as well as its potent reactivity and the apparent ubiquity of its production in biological systems, suggested to use the possibility that this free radical might be generated by leukocytes and involved in the killing of bacteria by these cells. Indeed, Babior, Kipnes, and Curnutte have demonstrated recently that normal leukocytes generate O_2^- during phagocytosis (11) and that CGD phagocytes do not (12). We describe here evidence to substantiate O_2^- production by the phagocyte during ingestion and involvement of this free radical in phagocytosis-associated NBT reduction and chemiluminescence and in the killing of ingested bacteria. A partial deficiency in O_2^- generation by leukocytes from carriers of X-linked CGD is also reported.

METHODS

Enzymes, $\cdot OH$ scavengers, and their controls. SOD was purified from bovine erythrocytes by the method of McCord and Fridovich (6). A portion of each preparation was autoclaved for 10 min at 124°C for use as the "heated SOD" control. Apoenzyme was prepared by dialysis for 40 h against acetate-EDTA buffer (6). The autoclaved enzyme no longer inhibited the reduction of cytochrome *c* in the aerobic xanthine-xanthine oxidase system (2); the apoenzyme lost approximately 90% of its original activity in this assay. Bovine liver catalase and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo. SOD contaminating the catalase was removed by filtration over Sephadex G-75 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) using 0.05 M potassium phosphate buffer with 0.1 M KCl, pH 7.8. The proteins were dissolved in saline (0.15 M NaCl) to a concentration of 1 mg/ml and sterilized by filtration (Millipore Corp., Bedford, Mass., 0.22 μm diameter pore size). Sodium benzoate, D-mannitol, and D-glucose (Fisher Chemical Co., Fair Lawn, N. J.) were made 0.2 M in water.

Source of leukocytes. Venous blood was obtained from healthy adult volunteers, patients with CGD, and mothers and maternal grandmothers of CGD patients. The diagnosis of CGD was established in each case by demonstration that the patient's phagocytes could ingest but not effectively kill *Staphylococcus aureus*, and in most cases *E. coli* also, and could not reduce NBT normally. The X-linked carrier state of CGD was determined in mothers and maternal grandmothers of three of the male patients by the finding of an intermediate value in the quantitative NBT assay (10) and a family history of CGD or possible CGD only in boys on the maternal side. The non-X-linked state was determined in five mothers of six patients by a normal result in the NBT assay on at least two occasions. The first diagnosis of CGD or suspected CGD in these families was made in the patients studied; five of the six were boys.

Preparation of leukocytes for functional assays. For determination of phagocytosis-associated NBT and cytochrome *c* reduction, chemiluminescence, bactericidal activity, and bacterial uptake, leukocytes were separated from heparinized blood by sedimentation of erythrocytes at room tempera-

ture for 20 min in an equal vol of 3% dextran (mol wt 250,000 or 500,000, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in saline. The leukocyte-rich plasma was centrifuged at 250 *g* for 10 min, and the cells were resuspended in 0.2% NaCl for 20 s to lyse erythrocytes; isotonicity was restored with an equal vol of 1.6% NaCl. Leukocytes were washed twice more and suspended in Hank's balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) containing 0.2% glucose, or in Krebs-Ringer phosphate buffer, pH 7.35, containing 0.2% glucose and 0.2% BSA (KRP-DA buffer). Final preparations routinely contained 80–90% granulocytes.

Preparation of granulocytes for homogenates. Peripheral blood was defibrinated with glass beads and diluted with 3 vol of 0.01 M phosphate-buffered saline, pH 7.4 (PBS). 3 vol of diluted blood were layered over 1 vol of a solution containing 6.3% Ficoll (Pharmacia Fine Chemicals Inc.) and 9.9% Hypaque (Winthrop Laboratories, New York), and the mixture was centrifuged at 300 *g* for 30 min at 20°C. The supernatant fluid containing lymphocytes and monocytes was removed. Sedimented erythrocytes and granulocytes were resuspended in PBS to approximately the original volume of defibrinated blood and mixed with an equal vol of 3% dextran in saline for sedimentation of erythrocytes. Remaining erythrocytes were removed by lysis with hypotonic saline, leaving preparations of 96–99% neutrophils, 1–3% lymphocytes, and no detectable platelets on examination of Wright's stained smears or counting-chamber preparations.

*Preparation of electron micrographs.*² The phagocytic bactericidal assay mixture, including latex particles, was prepared as described below. Latex spherules, 0.1 ml, were preincubated with horseradish peroxidase (Sigma Chemical Co.), 1 mg/ml, for 20 min at room temperature; 100 µg BSA, 0.1 ml serum, 5×10^8 *E. coli*, and 2.5×10^8 phagocytes were added, and the mixture was incubated on a rotating wheel at 37°C for 20 min. The reaction was stopped by adding chilled saline; the cells were centrifuged at 180 *g* for 5 min and washed gently in KRP buffer with dextrose but no albumin (KRP-D buffer), osmolarity adjusted to 290 mosmol with NaCl. The cells were suspended in 1-ml serum and centrifuged at 15,000 *g* for 1 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.). The cell button was resuspended in 2% glutaraldehyde in KRP-D buffer, final osmolarity 290 mosmol. After 1 h at 25°C, the cell pellet was removed, rinsed in buffer, diced into cubes, and reacted with diaminobenzidine for 1 h by the method of Graham and Karnovsky (13). After several buffer rinses, the specimen was fixed for 1 h at 4°C with 1% osmium tetroxide in KRP buffer, dehydrated in ethanol, and embedded in Araldite (Ciba Products Co., Summit, N. J.). Sections were stained with uranyl acetate or lead citrate, or both, and examined with a Philips 200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

Polyacrylamide electrophoresis of granulocyte homogenates. Purified granulocyte preparations were frozen and thawed three times, homogenized in 0.05 M potassium phosphate buffer, pH 7.8, with a motor-driven Teflon pestle in a Duall tube (Kontes Glass Co., Vineland, N. J.), cleared by centrifugation at 800 *g* for 15 min, and stored

at –70°C. The supernatant fluid was thawed within 40 h and tested for protein content (14); 0.2–0.4 mg protein in volumes up to 200 µl was placed on polyacrylamide for electrophoretic separation by the method of Davis (15). Gels were stained for SOD activity by the technique of Weisiger and Fridovich (16). Subcellular fractions from purified granulocytes homogenized in sucrose, obtained as described below, were also analyzed. Cyanide sensitivity of the electrophoresed enzyme was tested by adding 1 mM KCN to the gel with the photoreduction system.

Assays

Phagocytosis-associated NBT reduction. The NBT reduction accompanying phagocytosis was studied by a previously described technique (17) with the following modifications: Zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared by the method of Wardlaw and Pillemer (18) and suspended in saline to a concentration of 50 mg/ml. 1 vol of zymosan was incubated with 4 vol of normal human serum (processed and frozen at –70°C to preserve complement activity) for 30 min at 37°C. The opsonized zymosan was centrifuged and resuspended in saline to a concentration of 10 mg zymosan/ml. Duplicate reaction mixtures consisted of 0.1-ml opsonized zymosan particles, 0.2-ml (200 µg) SOD or its controls, 0.4 ml of 0.2% NBT in saline (17), and 2.5×10^8 phagocytes (granulocytes and monocytes) in 0.2-ml Hank's buffer. The particle:phagocyte ratio was approximately 50:1. Reaction time was 20 min at 37°C.

NBT reduction by granulocyte homogenates. The reduction of NBT by fractions from homogenates of human granulocytes was also studied. In one group of experiments approximately 10^8 cells were frozen and thawed three times and homogenized for 30 s in 0.05 M potassium phosphate buffer, pH 7.8, using an iced Duall glass tube and Teflon pestle. After homogenization no intact cells were seen on microscopic examination. A "particulate fraction" (primarily cell membranes and nuclear debris, as identified by electron microscopy) was pelleted by centrifugation at 650 *g* for 10 min; the supernatant fluid ("supernatant fraction") was removed for further processing and for study of its SOD activity (see below). The pellet was washed in a large volume of buffer then resuspended to 2 ml in phosphate buffer or in 0.154 M KCl containing 3.2×10^{-4} M KHCO_3 (alkaline isotonic KCl). Particulate fractions were subjected to sonication using a Branson W185 Sonifier with microtip (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.), power setting 55–60 W. The suspensions were placed in an iced bath and sonicated three times for 3 min each with 2-min intervals inbetween. The sonicates were cleared by centrifugation at 10,000 *g* for 15 min.

In a second group of experiments, 10^8 – 10^9 granulocytes were suspended in 2 ml of 0.34 M sucrose and homogenized as described above. The homogenate was centrifuged at 200 *g* for 10 min to remove any intact cells that remained. The pellet was washed once in 1-ml sucrose (200 *g* for 10 min), and the supernatant fluids were pooled and centrifuged at 1,000 *g* for 10 min. The resulting pellet contained nuclear debris and cell membranes by electron microscopy and was designated the "particulate fraction." This pellet was resuspended in 1-ml sucrose and centrifuged at 1,000 *g* for 10 min. Recentrifugation of the pooled supernates at 30,000 *g* for 20 min pelleted a "granular fraction" containing primarily lysosomal granules by electron microscopy. This pellet was washed in sucrose, and the pooled supernates were designated the "soluble fraction." The pellets were re-

² Electron micrographs were prepared by Dr. A. W. Atkinson, Jr. in the laboratory of Dr. Sidney P. Kent, Department of Pathology, University of Alabama in Birmingham.

suspended in 1-ml sucrose, and all fractions were sonicated four times in an iced bath for 1 min each with cooling inbetween. Particulate and granular fractions were cleared by centrifugation before use. Each of the fractions was studied for its ability to reduce NBT in a modification of the assay of Beauchamp and Fridovich (4), using 25°C incubation temperature, 2.5×10^{-4} M NBT, 0.05 M sodium carbonate buffer, pH 10.2, 1×10^{-4} M EDTA, and 5×10^{-5} M NADH or 2×10^{-3} to 5×10^{-8} M NADPH as substrate for the reaction, in a total vol of 3 ml. The reaction was initiated by addition of NBT, and the reduction of NBT was followed continuously by monitoring the increase in absorbance of light at 560 nm using a Gilford model 2,000 recording spectrophotometer (Gilford Instrument Company, Oberlin, Ohio). Involvement of O_2^- in the reaction was demonstrated by a decrease in the rate of NBT reduction when 15 μ g SOD was added before the addition of NBT. Duplicate assays of each cell preparation were run with and without 1 mM KCN and with and without NADH or NADPH. Certain preparations were studied at pH 7.0 or pH 7.4. Some of the starting leukocyte preparations were not sedimented through Ficoll-Hypaque and contained 85–95% granulocytes and the remainder lymphocytes. Results with these preparations and those obtained with homogenates of 96–99% granulocytes were almost identical and were therefore pooled.

Cytochrome *c* reduction. A modification of the assay of Babior et al. (11) was used. Phagocytes, 2.5×10^6 in 1 ml KRP-DA buffer, were preincubated for 5 min at 37°C in polypropylene tubes (Falcon Plastics Co., Division of BioQuests, Oxnard, Calif.) with KRP-DA buffer and 30 μ g SOD, as appropriate, to a total vol of 1.3 ml. $\frac{1}{10}$ ml each of ferricytochrome *c*, 1.2 mM (Type III, Sigma Chemical Co.), and opsonized zymosan, 10 mg/ml prepared as above, were added to begin the reaction. After incubation for 10 min the reaction was stopped by returning the tubes to an iced bath, and the contents of the tubes were promptly centrifuged at 4°C, 200 *g* for 10 min. Absorbance of the supernates at 550 nm was determined in a spectrophotometer, and the results with duplicate or triplicate tubes were averaged. Results were converted to nanomoles cytochrome *c* reduced using the extinction coefficient $E_{550\text{ nm}} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (19). Tubes containing only buffer and cytochrome *c* were incubated as above and served as blanks.

SOD activity. Leukocytes from patients with CGD and paired controls were lysed and homogenized as described above for the assay of NBT reduction. Supernatant fluids after the initial centrifugation at 650 *g* were cleared by centrifugation at 30,000 *g* and assayed in multiple dilutions for their ability to inhibit reduction of ferricytochrome *c* by O_2^- generated by the aerobic xanthine-xanthine oxidase system (6). In this assay a unit of SOD activity is defined as the amount of enzyme required to inhibit by 50% a standard rate of ferricytochrome *c* reduction (0.025 absorbance unit per minute) (6); our results are expressed as units of activity per milligram of cell protein.

Phagocytosis-associated chemiluminescence. Zymosan particles were prepared and opsonized as described above but resuspended in KRP-DA buffer to a concentration of 4-mg zymosan/ml. The 4-ml reaction mixture consisted of 1.0-ml opsonized zymosan suspension; 50 μ g SOD or BSA; 1.95 ml KRP-DA buffer; and 10^7 phagocytes in 1.0 ml KRP-DA. The leukocytes were kept at 4°C until added to begin the reaction; all of the other components were at ambient temperature. The reaction was carried out in siliconized liquid scintillation counting vials which had been stored

overnight in the dark and exposed only to red light during the experiment (20). Phagocytes were added at 1-min intervals, the contents of the vial were mixed by gentle swirling, and the vial was immediately placed in the chamber for the initial (time 0) 1-min reading, then remixed before each subsequent count. Luminescence measurements were made with a Beckman LS-150 ambient-temperature liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) in the tritium region, with only the front photomultiplier tube activated (20, 21). Empty vials were counted before the experiment, and those giving the lowest counts (1,200–2,000 per min) were selected for use.

Phagocytic bactericidal activity. A modification of the method of Quie, White, Holmes, and Good (22) was used, as previously described (23). The 1-ml reaction mixture consisted of 0.1 ml fresh human serum, approximately 2.5×10^6 or 5×10^6 bacteria in 0.3 ml KRP-DA buffer, 50–200 μ l of enzyme, benzoate or control solutions, and 2.5×10^6 phagocytes in KRP-DA buffer. In experiments in which latex particles were included, 0.1 ml latex spherules, 0.81 μ m diameter (23), were preincubated in the reaction tube with SOD, catalase, or their controls for 20 min at room temperature. The tubes were then chilled in an iced bath, and serum, bacteria, and phagocytes were added in that order. Samples were removed at timed intervals, the leukocytes were disrupted in water, and the diluted suspension was sampled to make agar pour plates for colony counting (23). To determine the “representative” experiments graphed in Figs. 2, 4, 6, 7, and 8, differences in colony counts at 60 and 120 min in the presence of the inhibitor (enzyme or scavenger) and its control were added for each experiment and then ranked. The representative experiment was selected from the middle one or two experiments of each set.

Phagocytosis. To determine the effect of enzymes and scavengers on the ingestion of particles in the assays described above, particle uptake was measured in each assay using the reaction mixture and conditions of that assay. For the assay of phagocytic NBT reduction, saline was substituted for NBT and duplicate tubes were prepared containing 200 μ g SOD or BSA. Smears made of the reaction mixture after incubation for 20 min were stained with Wright’s stain and coded. The number of zymosan particles in 200 phagocytes was determined by light microscopy for each of the four tubes without knowledge of the contents of the tube from which individual smears were made. To study uptake of zymosan in the chemiluminescence assay, smears were made after incubation for 14 min, and the number of particles ingested by 400 phagocytes was determined. The uptake of ^{125}I -labeled zymosan was also used to study phagocytosis in this system, as previously described (20).

The uptake of radiolabeled *E. coli* and *S. aureus* by phagocytes was studied in three to nine replicate tubes using the bactericidal assay system, except that the volume of each ingredient was doubled and the bacteria were cultivated overnight in 5-ml trypticase soy broth or minimal medium (24) to which 10 μ Ci [^{14}C]amino acid mixture (New England Nuclear, Boston, Mass.) had been added. After incubation for 8–40 min (as noted), the reaction was stopped with iced saline, and phagocytes containing radiolabeled bacteria were separated from uningested bacteria by centrifugation at 150 *g* for 5 min at 4°C. The cell pellet was washed and trapped on membrane filters, and radioactivity was determined in a liquid scintillation counter as detailed previously (25). Radioactivity of pelleted material in duplicate tubes in which the reaction was stopped immediately

after adding chilled phagocytes (time 0) or in duplicate tubes that lacked phagocytes was consistently less than 15% and usually less than 10% of that at the peak of bacterial uptake.

Phagocytic ingestion of bacteria was studied microscopically by making smears from duplicate assay tubes after 25 min incubation, staining them with Gram's stain, coding them, and determining the percentage of 400 phagocytic cells containing at least one bacterium and the number of bacteria per phagocyte, without knowledge of whether the original reaction tube contained SOD or its control, BSA.

RESULTS

Effect of SOD on phagocytic NBT reduction. The reduction of NBT by phagocytes ingesting opsonized zymosan particles was studied in the presence of SOD or an equal concentration (200 $\mu\text{g/ml}$) of BSA, heat-denatured SOD, or apoenzyme as controls. The extent of dye reduction achieved in the presence of SOD was consistently approximately two-thirds of that achieved in the presence of BSA or inactivated SOD (Table I). Ingestion of zymosan in the presence of active enzyme (2.33 particles/cell) was not significantly different from ingestion in the presence of BSA (2.37 particles/cell). We have previously reported that SOD did not inhibit ingestion of ^{125}I -labeled zymosan at this same particle to phagocyte ratio (20).

Effect of SOD on NBT reduction by leukocyte homogenates. In further attempts to determine if phagocytes have the capacity to generate O_2^- , homogenates of purified granulocytes in phosphate buffer were separated into a particulate fraction containing cell membranes and nuclei and a supernatant fraction containing the remaining cell constituents. These fractions were tested for their ability to reduce NBT in the presence or absence of SOD. The reaction was studied at pH 10.2 in order to maximize the one-electron reduction of oxygen to generate O_2^- (5, 26). As summarized in Table IIA, the rate of NBT reduction with either fraction was increased approximately threefold when NADH was added as substrate for the reaction. SOD inhibited the rate achieved with NADH by particulate and supernatant fractions from 10 normal control homogenates by 61% and 57%, respectively. Specific activity was greater in the particulate fraction both before and after the addition of SOD. Boiling this fraction eliminated its SOD-inhibitable NBT reducing activity. Although the mean rate of NBT reduction by both fractions from patients with CGD was approximately two-thirds the rate achieved with normal homogenate fractions, this difference was not significant ($P > 0.2$, Wilcoxon two-sample rank test, two-tailed [27]), and the extent of inhibition by SOD did not differ appreciably in the two groups. There was no inhibition of NBT reduction by heated SOD or in the presence of 1 mM KCN, which inactivates this enzyme (16).

TABLE I
Inhibition of Phagocytosis-Associated NBT Reduction by SOD

Protein added*	NBT reduction‡	Inhibition %
	OD_{615}	
BSA	0.230 ± 0.025 (7)	
SOD	0.158 ± 0.021 (7)	31 (25-46)§
SOD, heated	0.227 ± 0.021 (2)	
SOD, apoenzyme	0.228 ± 0.041 (3)	

* 200 $\mu\text{g/ml}$.

‡ Mean \pm SEM of averages of values for total dye reduction in duplicate tubes. The number of experiments is given in parentheses.

§ The mean and range of values are given for seven experiments comparing results with SOD and BSA.

At pH 7 or 7.4 the rates achieved by particulate fractions from three control homogenates in the presence of NADH were slower (mean 4.9 nmol/min per mg), but the inhibitory effect of SOD remained appreciable (37%). NADPH was much less effective as a substrate for the reaction. In three experiments at pH 10.2, NADPH concentrations as high as 40-fold that of NADH permitted only up to 60% as much NBT reduction as achieved with NADH. NBT reduction in the presence of NADH or NADPH was consistently increased slightly by 1 mM KCN. NADH oxidase activity of the particulate fraction from normal cells was demonstrable at pH 10.2 and pH 7.4, as determined by decrease in absorbance at 340 nm in the presence of 10^{-4} M NADH (10); SOD had no effect on this activity at either pH.

In additional experiments normal and CGD granulocytes were homogenized in sucrose, and particulate, granular, and soluble (cytosol) fractions separated by differential centrifugation were tested at pH 10.2 for O_2^- -generating activity. The mean total protein content of the three fractions and the mean number of cells homogenized were similar for control and CGD preparations (14.3 mg and 5.8×10^8 cells for controls; 19.1 mg and 6×10^8 cells for CGD homogenates). In the control preparations specific activity in the presence of NADH was again highest in the particulate fraction (Table IIB). Based upon the total protein content of each fraction, total activity was greatest in the cytosol, which accounted for almost two-thirds of the total activity found in the three fractions (data not shown). The decreased activity at pH 7.4 expected in an O_2^- -generating system (5, 26) was also found with all three fractions from control homogenates. Although SOD inhibited NBT reduction in the presence of NADH approximately equally in fractions from control and CGD homogenates, the rate of NBT reduction per milligram

TABLE II
Superoxide-Dependent NBT Reduction by Fractions from Normal and CGD Leukocyte Homogenates

Source of homogenates*	Subcellular fraction	Rate of NBT reduction			Inhibition by SOD†
		base line	+NADH	+NADH and SOD	
		nmol/min/mg‡			%
A. PO ₄ buffer					
Controls (10)	particulate	5.7±1.3	18.1±3.5	7.4±1.8	61
	supernate	3.4±0.1	11.3±1.6	4.8±1.3	57
CGD patients (6)	particulate	3.8±0.4	12.2±0.5	3.8±0.3	69
	supernate		7.8±0.8	3.1±0.5	60
B. Sucrose					
Controls (4)	particulate	18.6±7.5	38.7±4.8	16.4±0.8	58
	granular		21.7±4.3	8.5±1.8	61
	soluble	21.3±1.7	23.8±1.6	12.4±0.7	48
CGD patients (3)	particulate	10.8±0.7	19.2±3.3	8.7±1.6	55
	granular	9.0±0.1	18.5±4.0	8.3±1.7	55
	soluble	9.2±0.7	11.6±0.5	6.5±0.8	44

* Granulocytes were homogenized in phosphate buffer (A) or sucrose (B) and fractionated by differential centrifugation as described in Methods. The number of different homogenates tested is shown in parentheses.

† $\left(\frac{\text{result with NADH} - \text{result with NADH and SOD}}{\text{result with NADH}} \right) \times 100\%$.

‡ The mean ±SEM of the averages of two or three replicate determinations for each fraction are shown. The rate of increase in absorbance at 560 nm per mg of homogenate protein was determined as described in Methods; conversion to nanomoles NBT reduced was made using $E_{560 \text{ nm}} = 2.3 \text{ mM}^{-1} \text{ cm}^{-1}$, which was calculated from a standard curve obtained with known amounts of formazan generated by reduction of NBT with alkaline sodium ascorbate. The presence in the reaction mixture of $5 \times 10^{-6} \text{ M}$ NADH, or NADH and SOD, 5 µg/ml, is noted. All values listed here were determined in the absence of KCN. Each preparation was also tested in the presence of 1 mM KCN and NADH (without SOD); mean values were slightly higher than those shown. Preparation of particulate fractions from part A in alkaline isotonic KCl rather than phosphate buffer did not result in improved specific activity of the extracted material.

protein by particulate and cytosol fractions from patients was about half that of these fractions from normals (Table IIB). However, the difference was not significant ($P = 0.057$, Wilcoxon two-sample rank test, two-tail [27]).

Cytochrome c reduction by normal and CGD cells. The ability of O₂⁻ to reduce ferricytochrome *c* in the surrounding milieu was used to determine the capacity of phagocytizing leukocytes to generate this free radical (11). Cells from 23 controls, 9 patients with CGD,

TABLE III
Inhibition of Phagocytosis-Associated Ferricytochrome *c* Reduction by SOD

Subjects	No.	Ferricytochrome <i>c</i> reduction*		
		Resting	Phagocytizing	Phagocytizing and SOD
		nmol/min/2.5 × 10 ⁶ Phagocytes		
Controls	23	0.18±0.03	3.43±0.11	0.14±0.02
CGD patients	9	0.19±0.06	0.24±0.06	0.25±0.06
CGD carriers, XLR‡	5	0.13±0.02	1.69±0.29	0.15±0.04
CGD carriers, non-XLR‡	3	0.17±0.01	3.68±0.38	0.14±0.10

* Mean ±SEM of averages of two or three determinations.

‡ XLR stands for X-linked recessive (inheritance).

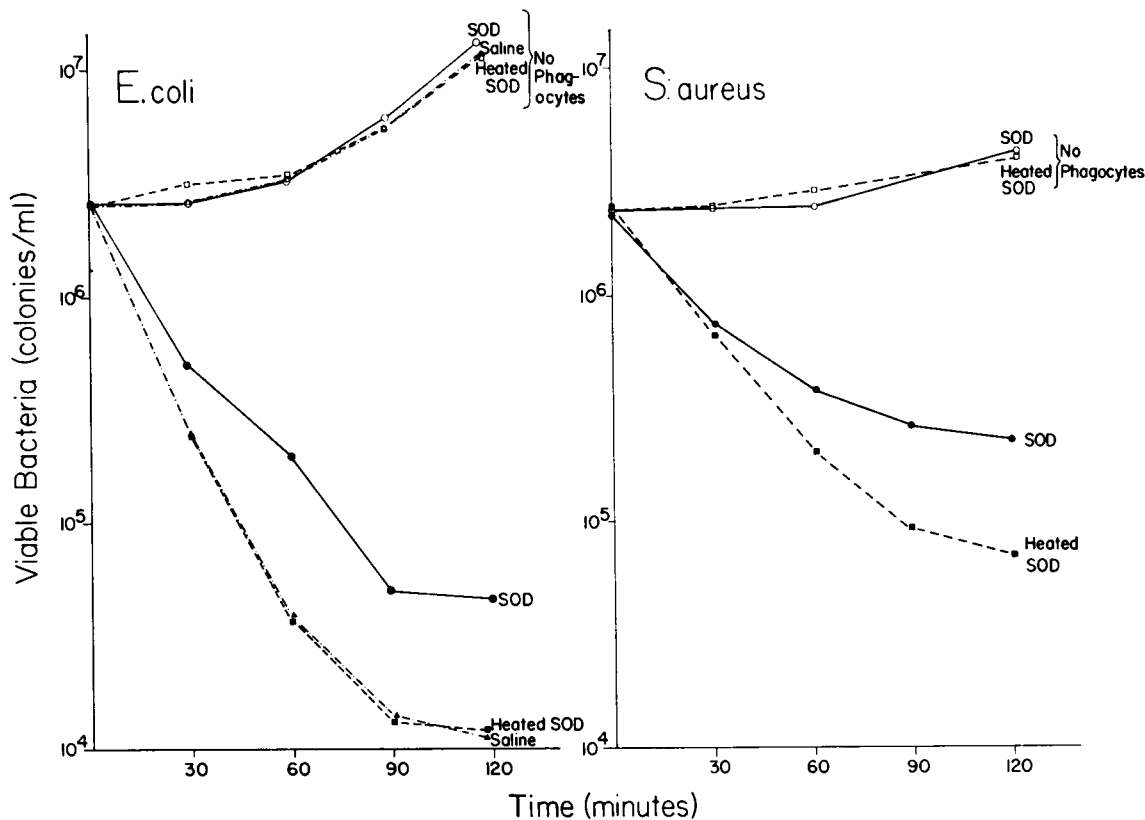


FIGURE 1 Inhibition of phagocytic bactericidal activity by SOD. The number of viable bacteria in the 1-ml reaction mixture, determined as colony-forming units, is plotted as a function of the time at which the mixture was sampled. In the experiment with *E. coli* the enzyme was present at a concentration of 100 $\mu\text{g}/\text{ml}$; with staphylococci the concentration was 200 $\mu\text{g}/\text{ml}$. Heated enzyme at equivalent concentrations and saline were used as controls. 2.5×10^6 phagocytes were used in all bactericidal assays. Selection of "representative" experiments is described in Methods.

and 8 mothers or grandmothers of children with CGD were studied. As summarized in Table III, normal control leukocytes reduced cytochrome *c* actively during phagocytosis, and this reduction was inhibited completely by SOD. However, there was no significant cytochrome *c* reduction by patients' leukocytes. All five carriers of the X-linked form of the disease, as determined by the quantitative NBT assay and a compatible family history, had values which fell between those of patients and controls: 23%, 54%, 56%, 59%, and 67% of the extent of cytochrome *c* reduction achieved by phagocytizing control cells in the same experiment. Cells from three of these carriers had activity equal to that of control cells if incubation was prolonged to 60 min from the usual 10 min; cells from the other two never achieved normal activity. Phagocytes from all three maternal carriers of the non-X-linked form of the disease, as determined by normal phagocytic reduction of NBT and compatible family history, had normal activity in this assay.

Effect of SOD on phagocytosis-associated chemiluminescence. During the process of phagocytosis, human leukocytes emit a burst of luminescence that can be quantitated in a liquid scintillation spectrometer (28). To obtain further evidence that O_2^- is generated by phagocytizing leukocytes and to determine if O_2^- generation plays a part in phagocytosis-associated chemiluminescence, we tested the capacity of SOD to inhibit this phenomenon. In the presence of BSA as control, luminescence rose rapidly from a background level of approximately 5×10^3 cpm at time 0 to a maximum of over 1.7×10^5 cpm at 13 min. When BSA was replaced by an equal concentration of SOD (50 $\mu\text{g}/\text{ml}$), the rate of luminescence generation fell from 2.3×10^4 cpm/min to 0.66×10^4 cpm/min, a reduction of 71%; and peak luminescence was reduced by 64%. In 11 experiments, SOD at a concentration of 100 $\mu\text{g}/\text{ml}$ inhibited peak luminescence by 65 to 76% (mean 70%) (20). Particle ingestion in the presence of the enzyme was not significantly different than that in the presence of BSA (2.81

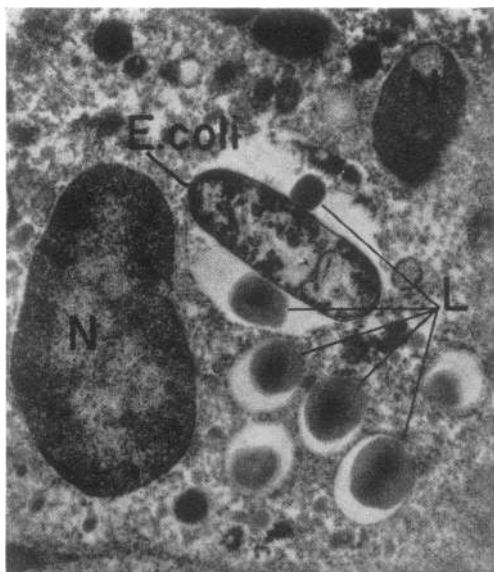


FIGURE 2 Electron micrograph of one granulocyte illustrating *E. coli* and latex particles (L) within the same phagocytic vacuole. The phagocytic bactericidal assay system was used, except that latex particles were preincubated with peroxidase in an attempt to mark the location of particles by surrounding deposition of electron-dense material (13). The nucleus (N) is shown. Magnification $\times 13,000$.

and 2.87 particles/phagocyte, respectively). In addition, SOD in a concentration of $100 \mu\text{g/ml}$ did not influence the phagocytosis of ^{125}I -labeled zymosan in this system, uningested labeled particles being separated from phago-

cytes with ingested particles by velocity sedimentation in an isokinetic gradient (20).

Effect of SOD on phagocytic bactericidal activity. The effect of SOD on the bactericidal capacity of human granulocytes was studied using strains of bacteria obtained from infected humans. In the presence of $50\text{--}250 \mu\text{g}$ SOD modest inhibition was achieved, as shown for two representative experiments in Fig. 1. However, the effect was reproducible in that inhibition was demonstrated in six consecutive experiments each with *E. coli* and staphylococci. Specifically, in 12 experiments in which colony counts were compared at 30, 60, and 120 min (and at 90 min in 5 experiments), the colony count in the presence of the enzyme preparation was greater than that with the control 35 of 41 times; of the six times the count was higher with the control, five were at the 30-min sampling when differences would be expected to be minimal. The probability that this might occur by chance is less than 1 in 10,000 (sign test, two-tailed [27]).

In an attempt to facilitate entry of SOD into the phagocytic vacuole, various concentrations of the enzyme or its controls (heat-denatured enzyme or BSA) were fixed to latex particles by preincubation in the reaction tube with 0.1 ml of the particles. Approximately 20% of the total enzyme activity in $100 \mu\text{g}$ of preparation incubated with the latex was associated with the particles when they were separated by centrifugation; 5% of the total activity remained after one wash with water. Electron micrographs made of this system after 20 min of phagocytosis indicated that particles and

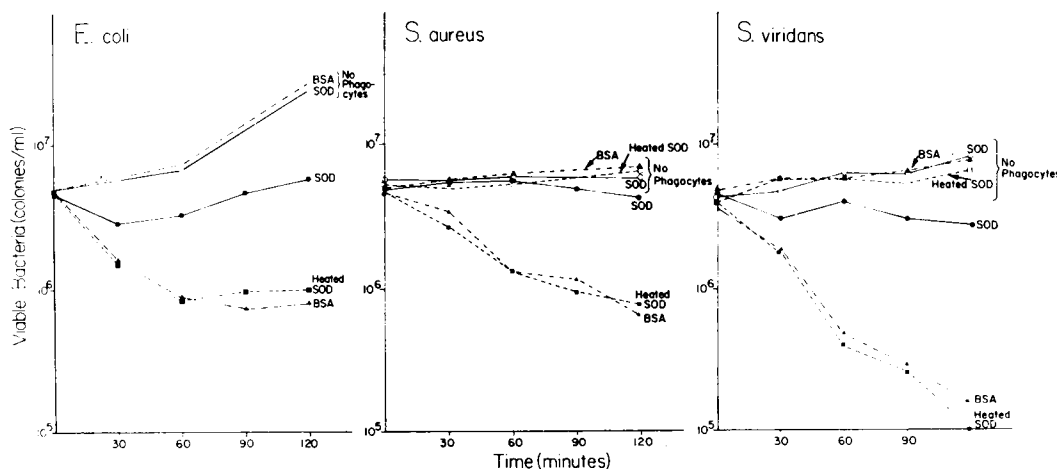


FIGURE 3 Inhibition of phagocytic bactericidal activity by SOD in the presence of latex particles. In the experiments shown here, $200 \mu\text{g}$ of either BSA, SOD, or heat-denatured SOD was present in an individual assay tube. Illustrated experiments are representative of 11 with *E. coli*, 5 with staphylococci, and 7 with streptococci done at various enzyme concentrations and bacteria: cell ratios. Seven additional confirmatory experiments showing a similar inhibitory effect were performed with staphylococci in a second laboratory (R.L.B.).

bacteria could commonly be found together within the same vacuole (Fig. 2).

In the presence of SOD and latex particles there was consistent, marked inhibition of the killing of *E. coli*, staphylococci, and streptococci by normal human leukocytes, as shown for three representative experiments in Fig. 3. In 23 experiments in which bacterial colony counts were compared at 30, 60, 90, and 120 min using inactivated SOD or BSA as controls, at each of 92 time points the colony count with SOD was higher than that with either control ($P < 0.0001$). Heat denaturation of the enzyme (or in experiments not shown, removal of its copper and zinc ions by dialysis against EDTA buffer) eliminated its ability to suppress bacterial killing. The enzyme had no effect on growth of the bacteria in the absence of phagocytes.

SOD did not inhibit the ingestion of *E. coli* or *S. aureus* whether or not latex particles were present, as determined by measuring the uptake of radiolabeled *E. coli* or staphylococci by phagocytes (Table IV). Counting the number of phagocytized *E. coli* on stained smears (without latex) gave similar results (1.80 bacteria/phagocyte with SOD; 1.91 bacteria/phagocyte with autoclaved enzyme). The rate of uptake of radiolabeled bacteria in the presence of SOD was not significantly different than that in the presence of an equal concentration of BSA (Fig. 4). The addition of 0.02 M sodium fluoride to inhibit ingestion reduced cell-associated radioactivity to base-line (time 0) counts.

Effect of catalase and ·OH scavengers on phagocytic bactericidal activity. The ability of catalase, which catalyzes removal of hydrogen peroxide, to inhibit phagocytic bactericidal activity was tested in the assay system used to demonstrate inhibitory activity by SOD. In

TABLE IV
Uptake of ¹⁴C-Labeled Bacteria by Phagocytes in the Presence of SOD or Control Preparations

Enzyme or control preparation	Latex	Phagocyte-associated radiolabeled bacteria	
		<i>E. coli</i> cpm*	<i>S. aureus</i> cpm*
SOD‡	absent	1,632 ± 126	2,352 ± 531
Saline	absent	1,702 ± 222	2,307 ± 268
BSA‡	absent		2,412 ± 244
SOD‡	present	1,134 ± 210	1,013 ± 79
SOD, heated‡	present	1,180 ± 160	1,049 ± 112
SOD, apoenzyme‡	present		1,079 ± 51

* Mean ±SD of values in counts per minute from 6–15 replicates in two or three experiments. Square root transformations of enzyme and control values were not significantly different ($P > 0.2$) by analysis of variance (27).

‡ 200 µg/ml.

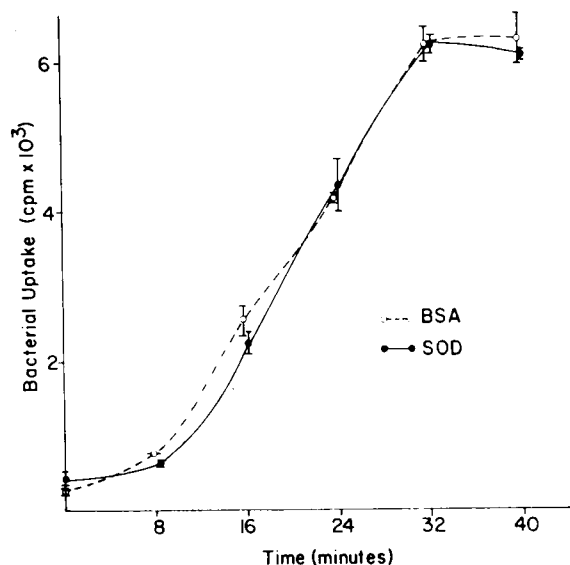


FIGURE 4 Uptake of radiolabeled *E. coli* by phagocytes in the presence of SOD, 200 µg/ml, or an equal concentration of BSA as control. The phagocytic bactericidal assay system was used, including latex particles, except that volumes of all ingredients were doubled. The reaction was stopped at 8-min intervals, and leukocytes were separated from un-ingested bacteria by low-speed centrifugation. Means of leukocyte-associated radioactivity in triplicate tubes are plotted as a function of the length of incubation. The bars represent the SE of each mean.

the absence of latex particles there was slight but consistent inhibition of the phagocytic killing of *E. coli* and staphylococci by catalase (Fig. 5). However, in the presence of latex, catalase inhibited the killing of all three bacterial strains as effectively as did SOD (Fig. 6). There was no enhancement of bacterial growth in the presence of the enzyme when leukocytes were omitted.

Inasmuch as bactericidal activity was markedly inhibited by enzymes that removed either O_2^- or H_2O_2 , we considered the possibility that optimal killing required the combined effect of both these agents. Interaction of O_2^- and H_2O_2 has been said to lead to production of hydroxyl radical ($\cdot OH$) (29). To test the possibility that $\cdot OH$ is generated by phagocytizing leukocytes and that this radical is required for the bactericidal event, the phagocytic bactericidal assay was performed in the presence of sodium benzoate and mannitol, scavengers of $\cdot OH$ (30–32). As shown in Fig. 7, there was modest but definite inhibition of phagocytic killing by benzoate concentrations that had no direct effect on the bacteria. At 50 of 52 time points sampled in 10 consecutive experiments, the colony count was higher in the presence of either 0.01 M or 0.02 M benzoate than the saline control ($P < 0.0001$). There was no apparent adherence of benzoate to latex particles,

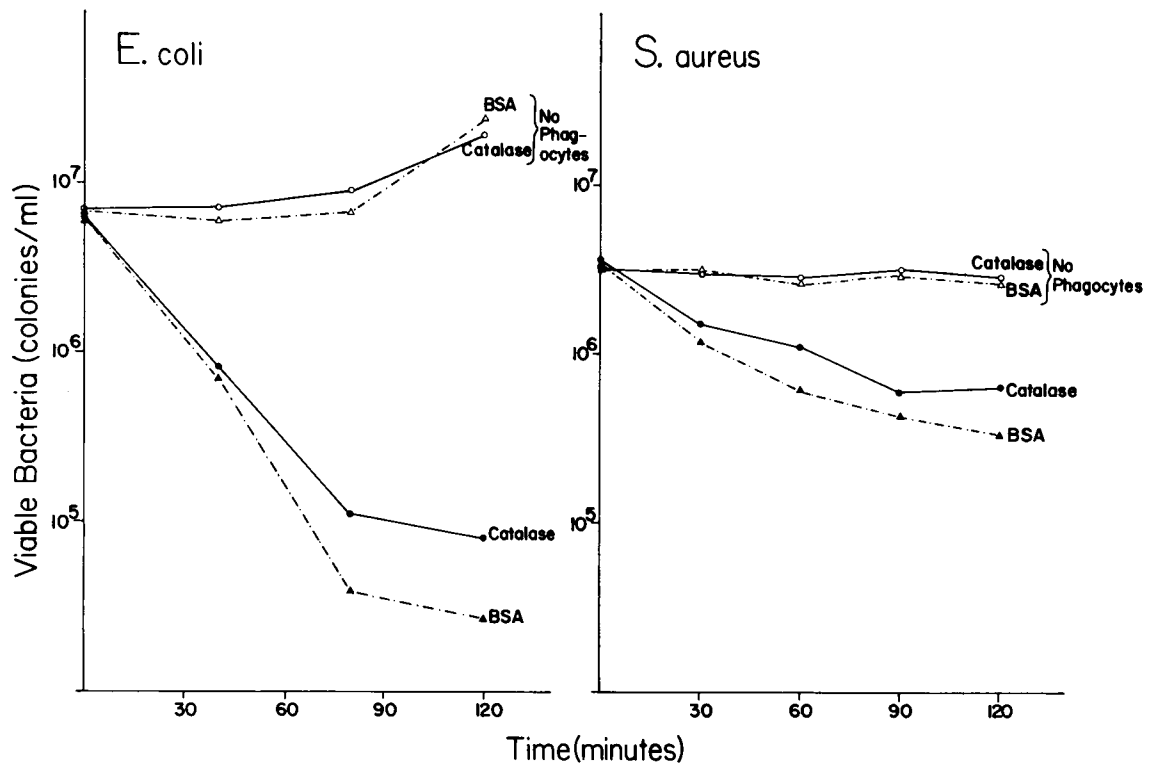


FIGURE 5 Inhibition of phagocytic bactericidal activity by catalase, 200 $\mu\text{g}/\text{ml}$, in the absence of latex particles. An equal concentration of BSA was used as control. These results are representative of the effect achieved in two experiments with *E. coli* and five with staphylococci. At 27 of 27 sampling times in these experiments the colony count was higher in the presence of catalase than in the presence of BSA ($P < 0.0001$, sign test, two-tailed [27]).

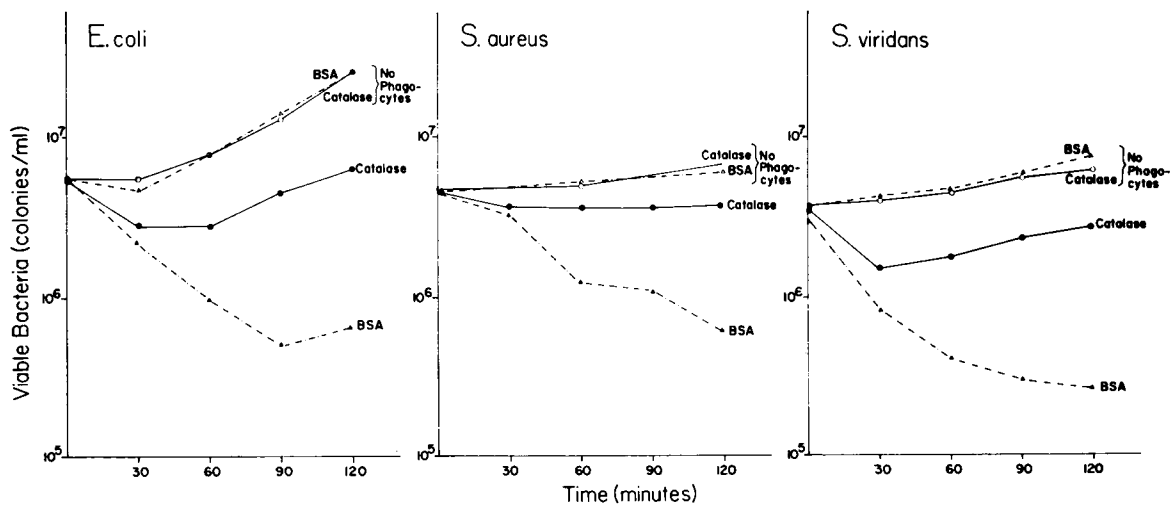


FIGURE 6 Inhibition of phagocytic bactericidal activity by catalase, 200 $\mu\text{g}/\text{ml}$, in the presence of latex particles. An equal concentration of BSA was used as control. The experiments plotted are representative of nine with *E. coli*, two with staphylococci, and four with streptococci done at various enzyme concentrations and bacteria: cell ratios. In these 15 experiments the colony count was higher with catalase than BSA at 59 of 60 sampling times ($P < 0.0001$).

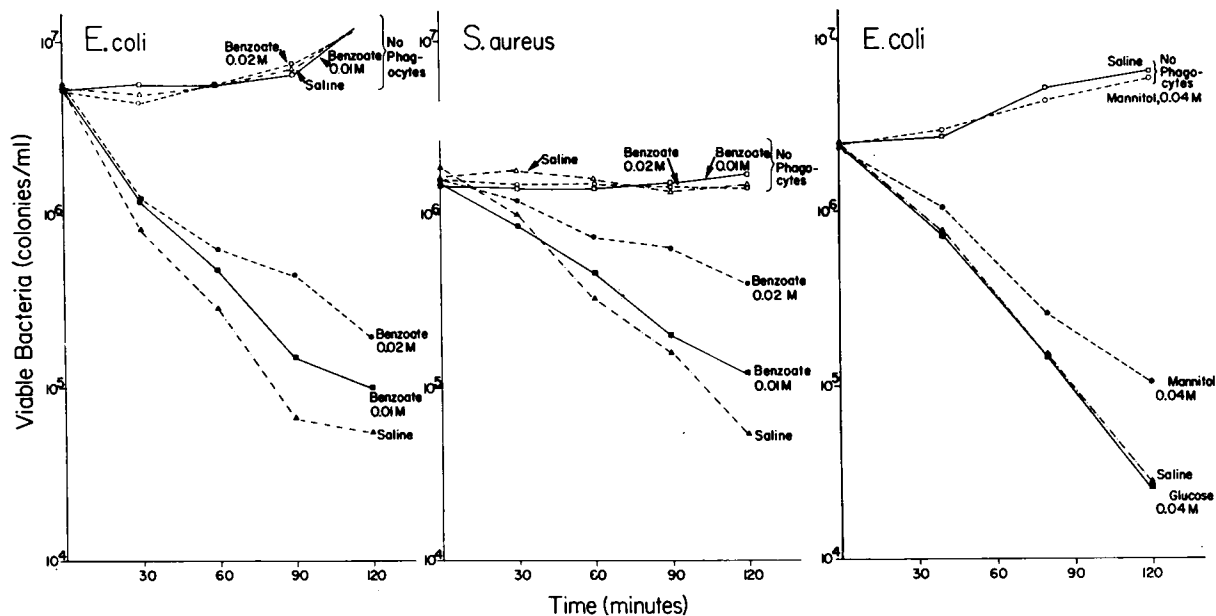


FIGURE 7 Inhibition of phagocytic bactericidal activity by sodium benzoate at a final concentration of 0.01 M or 0.02 M compared to 0.15 M NaCl, and by 0.04 M mannitol compared to 0.15 M NaCl and 0.04 M glucose. Latex particles were not present. The results with benzoate that are plotted are representative of those achieved in six experiments with *E. coli*, three with staphylococci, and one with streptococci. The presence of latex particles in several of these experiments had no influence on the inhibition achieved by benzoate. The results with mannitol shown are representative of those in six experiments with 0.03 M–0.05 M mannitol and *E. coli*. The osmolalities of the final reaction mixtures containing the saline, benzoate, mannitol, and glucose solutions were approximately equal.

and the addition of latex to the reaction mixture did not increase the extent of inhibition by benzoate.

Mannitol inhibited the phagocytic killing of *E. coli* slightly but consistently at concentrations of 0.03 M–0.05 M: at all 22 sampling points in six consecutive experiments colony counts were higher with mannitol than with glucose or saline ($P < 0.0001$). Mannitol had no direct effect on *E. coli* but inhibited slightly the growth of the two staphylococcal strains tested, preventing our use of this bacterium for accurate evaluation of mannitol effects on the neutrophil. We were unable to demonstrate consistent inhibition of phagocytic bactericidal activity by ethanol, a less reactive scavenger of $\cdot\text{OH}$ than benzoate (30, 31), in concentrations lower than those that inhibited ingestion slightly (0.08 M). There was no inhibition of the uptake of radiolabeled organisms by catalase, benzoate, or mannitol, as summarized in Table V.

Granulocyte SOD. Lysates of purified granulocytes were placed on polyacrylamide for electrophoretic separation. The location of SOD after electrophoresis was demonstrated by its capacity to inhibit the reduction of NBT by an O_2^- -producing system incorporated into the gel (4, 16). Two distinct achromatic bands were seen in all tested lysates from 12 normal individuals, 4

TABLE V
Uptake of ^{14}C -Labeled Bacteria by Phagocytes in the Presence of Catalase, $\cdot\text{OH}$ Scavengers, or their Controls

Catalase, scavenger, or control preparations	Latex	Phagocyte-associated radiolabeled bacteria	
		<i>E. coli</i>	<i>S. aureus</i>
		cpm*	cpm*
Catalase†	absent	1,553 ± 56	
BSA†	absent	1,225 ± 81	
Catalase†	present	878 ± 105	1,465 ± 704
BSA†	present	796 ± 175	1,387 ± 670
Benzoate, 0.01 M	absent	1,020 ± 165	
Benzoate, 0.02 M	absent	1,089 ± 185	
Saline	absent	966 ± 114	
Mannitol, 0.04 M	absent	2,312 ± 268	
Mannitol, 0.05 M	absent	2,372 ± 362	
Glucose, 0.05 M	absent	2,389 ± 473	

* Mean \pm SD of values from 6–12 replicates in two experiments. Square root transformations of catalase, benzoate, and mannitol values were not significantly different from those of their controls ($P > 0.2$) by analysis of variance (27).

† 200 $\mu\text{g}/\text{ml}$.

patients with chronic myelogenous leukemia, and 3 patients with CGD. A minor, slower band could be found slightly toward the cathode from electrophoresed manganese-containing SOD purified from *E. coli* (33). The major band corresponded in electrophoretic mobility to the most prominent of three closely associated bands from lysates of human erythrocytes; this band was slightly anodal to that of purified bovine SOD. The major granulocyte band (but not the minor one) and all three erythrocyte bands were removed by cyanide treatment of the lysates, a result expected with cupric enzyme (16, 34). Purified granulocytes were homogenized in 0.34 M sucrose, and subcellular fractions were separated by differential centrifugation. The supernatant fluid after centrifugation at 105,000 *g* (cytosol fraction) contained large amounts of the major, cyanide-sensitive SOD by this technique.

Supernatant fluids after centrifugation at 650 *g* of homogenates prepared in phosphate buffer were assayed for their ability to inhibit reduction of ferricytochrome *c* by O_2^- generated by the xanthine-xanthine oxidase system. Granulocyte homogenates from four normal controls had 2.4 ± 0.5 (mean \pm SEM) U of SOD activity per milligram protein; homogenates from four patients with CGD had 2.4 ± 0.2 U of activity per milligram protein. Treatment with 1 mM KCN reduced SOD activity in both normal and CGD homogenates by 74–100% (mean 87%); heating to 100°C for 15 min eliminated activity. Particulate fractions from these homogenates had negligible SOD activity.

DISCUSSION

Babior and colleagues have presented evidence that O_2^- is generated by normal granulocytes during phagocytosis (11); and Curnutte, Whitten, and Babior have shown defective O_2^- generation by phagocytes from two patients with CGD (12). We have confirmed defective O_2^- generation by phagocytizing leukocytes from three patients with the X-linked recessive form of CGD and six patients with the non-X-linked (probably autosomal recessive) form of this disease. The carrier state was detectable as an intermediate value in this assay in the three mothers and two maternal grandmothers whose cells reduced NBT to an intermediate extent during phagocytosis. CGD in these families occurred only in boys and in maternal relatives, consistent with X-linked transmission of the disease. The inhibition by SOD of the NBT reduction that accompanies phagocytosis further confirms the capacity of normal leukocytes to generate O_2^- . Moreover, this finding, in conjunction with the failure of NBT and cytochrome *c* reduction by CGD granulocytes and the occurrence of a partial defect in these two metabolic events in mothers and maternal

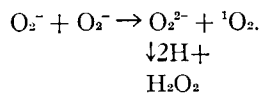
grandmothers of male patients, strongly implicates O_2^- in NBT reduction.

Subcellular fractions from granulocyte homogenates exhibited the capacity to generate O_2^- in the presence of NADH as substrate, the greatest activity per milligram protein being found in the fraction containing cell membranes and nuclear debris. Subcellular fractions from homogenates of CGD granulocytes had one-half to two-thirds the O_2^- -generating capacity that normal homogenates did, but this difference was not statistically significant and was slight compared to the marked deficiency of CGD cells in phagocytosis-associated O_2^- generation. Superoxide generation was not diminished by 1 mM cyanide treatment of either the normal or CGD homogenates. This cyanide-insensitive utilization of NADH as substrate for O_2^- formation and the demonstration of NADH oxidase activity in the particulate fraction suggest that O_2^- can be generated by NADH oxidase activity. Whether the O_2^- -generating activity in the membrane-rich particulate fraction is actually associated with the cell membrane must be determined by study of purer cell fractions. Approximately normal NBT reduction by homogenates of CGD leukocytes has been previously shown (35); our extension of these findings further interrelates O_2^- generation and NBT reduction. Demonstration of markedly deficient O_2^- generation by intact CGD cells but only a slight deficiency in O_2^- generation by CGD cell lysates again raises the possibility that the enzyme or enzymes controlling NBT reduction, and perhaps the phagocytosis-associated respiratory burst, are present within CGD leukocytes but not strategically placed or, for some other reason, not fully activated by ingestion.

Elaboration of luminescence by phagocytizing leukocytes appears to depend in large part on the generation of O_2^- during ingestion in that the rate of generation and the maximal extent of chemiluminescence achieved by phagocytizing granulocytes were significantly inhibited by SOD. That O_2^- is the actual luminescing agent has not been shown, however. Allen, Stjernholm, and Steele have suggested that singlet oxygen (1O_2) could be that agent (28). This electronically excited form of oxygen appears to participate in ordinary chemical reactions involving molecular oxygen (36, 37) where its presence has been monitored by its generation of luminescence (36–42). To our knowledge no direct proof that this species is generated by cells has been presented.

Several laboratories have reported that the aerobic xanthine-xanthine oxidase reaction, which generates O_2^- , can generate chemiluminescence (38–42). Stauff, Schmidkunz, and Hartmann (39) have attributed luminescence in this system to generation of singlet oxygen

from the basic O_2^- dismutation reaction:

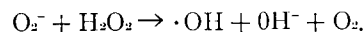


It would appear that SOD has no direct effect on 1O_2 once this agent has been formed (42). However, the enzyme-catalyzed dismutation does not give rise to 1O_2 (41, 42), which could explain the inhibition of chemiluminescence by SOD.

The involvement of O_2^- in the NBT reduction, cytochrome *c* reduction, and chemiluminescence exhibited by phagocytizing granulocytes strongly supports the concept that O_2^- is a significant by-product of metabolism in aerobic cells. The possibility that this phenomenon might serve some useful purpose would seem most likely for phagocytic cells. Results of the experiments reported here suggest that O_2^- generation is involved in the killing of ingested bacteria by such cells. SOD in the reaction mixture consistently diminished the killing of *E. coli*, staphylococci, and streptococci by phagocytes, compared to an equal concentration of heat-denatured enzyme or BSA. The effect was far more striking when latex particles were also present, presumably because the enzyme, shown to adhere to the particles, could be introduced more effectively into the phagocytic vacuole with the bacteria. Electron micrographs confirmed the coexistence of latex and bacteria within the same vacuole in this system. The enzyme did not inhibit ingestion of bacteria in the presence or absence of latex particles. The failure of SOD to inhibit phagocytosis of paraffin oil-*E. coli* lipopolysaccharide emulsions has also been shown (43).

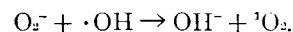
The inhibition of phagocytic bactericidal activity by SOD does not necessarily indicate that O_2^- is itself the lethal agent. In fact, results obtained in this system using catalase, which removes H_2O_2 , suggest otherwise. Evidence that peroxide is involved in the killing of phagocytized bacteria is well documented, as reviewed by Klebanoff and Hamon (44). The inhibition of phagocytic bactericidal activity by catalase reported here and previously by others (45, 46) supports that contention. However, inasmuch as the predicted effect of SOD would be to increase the rate of H_2O_2 formation from O_2^- , one might expect more H_2O_2 to be formed within the phagocytic vacuole in the presence of the enzyme and, therefore, bactericidal activity to be increased. That more H_2O_2 is formed under these conditions does in fact appear to be the case (47). One possible explanation for our findings might be the requirement for both H_2O_2 and O_2^- for optimal phagocytic bactericidal activity. Interaction of these agents has been shown to occur in vitro and to lead to production of the potent oxidizing agent, $\cdot OH$ (31, 32,

48) by the cycle of Haber and Weiss (29), as represented by the reaction:



The inhibition of phagocytic bactericidal activity by sodium benzoate and mannitol, scavengers of $\cdot OH$ but not of O_2^- or H_2O_2 (30, 32), is consistent with the possibility that $\cdot OH$ is formed during phagocytosis and is required for the bactericidal event. However, inasmuch as the activity of benzoate and mannitol upon phagocytes may not be limited to removal of $\cdot OH$, it must be stated that their antibacterial effect is compatible with but does not prove the importance of $\cdot OH$ in phagocytic killing.

A role for 1O_2 in the killing of phagocytized bacteria has been suggested by the finding that *Sarcina lutea* containing carotenoid, a scavenger of 1O_2 , were protected against phagocytic bactericidal activity compared to a colorless mutant (49). A second possible explanation for the apparent requirement for both O_2^- and H_2O_2 in the phagocytic bactericidal event, as well as for the relationship between O_2^- and chemiluminescence, might involve 1O_2 , as suggested by the experiments of Arneson. Using the aerobic xanthine-xanthine oxidase system to generate O_2^- , he noted that the addition of H_2O_2 greatly increased and the addition of catalase decreased chemiluminescence (40). He suggested that $\cdot OH$ was being generated in the Haber-Weiss reactions and that the reaction responsible for luminescence in this system would be:



Thus, SOD within the phagocytic vacuole could conceivably inhibit bactericidal activity (and luminescence) through its suppression of 1O_2 formation from $\cdot OH$ or from the spontaneous O_2^- dismutation reaction, as well as through suppression of formation of $\cdot OH$ itself.

It seems highly likely that protection of a cell from autogenous O_2^- and perhaps, thereby, other high-energy intermediates of oxidative metabolism, depends upon the presence of SOD within that cell. A copper- and zinc-containing form of this enzyme is located in the cytosol of eukaryotic cells which also possess smaller amounts of a manganese SOD in the matrix of their mitochondria (16). Aerobic bacteria contain an intracellular manganese enzyme (33) as well as a periplasmic iron enzyme (50). Evidence supporting a protective role for SOD includes the finding by McCord, Keele, and Fridovich of SOD activity in all eight species of aerobic bacteria analyzed, yet in none of the six species of strict anaerobes studied (7). Additional experiments have shown that increased concentrations of intracellular SOD could be induced in *E. coli* B or the eukaryote *Saccharomyces cerevisiae* by growth in 100%

oxygen; such preconditioning increased the resistance of these organisms to growth in 20 atm oxygen (8, 9). Cultivation in an aerated, iron-poor medium resulted in bacteria with high levels of the manganoenzyme but low levels of the iron enzyme and decreased resistance to death from exogenous O_2^- generated by a photochemical or enzymatic system (8). In experiments not reported here we have found decreased resistance to exogenous O_2^- in a mutant *E. coli* lacking the periplasmic enzyme.³

Beckman, Lundgren, and Tärnvik have found SOD with the electrophoretic mobility of cuprozinc enzyme in all human cells studied except peripheral blood granulocytes, which apparently contained only the mitochondrial enzyme (51). However, in our experiments, in agreement with other recent reports (52, 53), lysates of 96–99% pure neutrophils from normals and patients with CGD or chronic myelogenous leukemia have consistently shown two distinct bands on electrophoresis in polyacrylamide gels. A minor, slower band corresponds approximately in position to electrophoresed manganese-containing SOD, purified from *E. coli*; the major band corresponds in electrophoretic mobility to erythrocyte cupric enzyme and is removed by cyanide treatment, a result expected with cupric enzyme.⁴

The results of the studies described here, placed in the perspective of current understanding of granulocyte physiology, have led us to formulate the following conceptual model of the process of phagocytosis: Stimulated by our own and reported observations (54–57) we would hypothesize that the enzyme system responsible for the respiratory burst of phagocytosis provides the source of O_2^- and that this system is associated with the cell membrane. Inasmuch as the same membrane forms the boundaries of the phagocytic vacuole, the mechanism for converting molecular oxygen to superoxide, then to peroxide, would be located between the captured bacteria and the cytoplasm of the cell. Because O_2^- and H_2O_2 can diffuse even to the outside of the cell (11, 58–60), these agents would accumulate in the vacuole where they would be free to act on the captured micro-organisms or to interact to generate other oxidants such as $\cdot OH$ or 1O_2 . Within the vacuole

they would have interference from neither cytoplasmic catalase (61) nor, probably, cytoplasmic SOD; yet these enzymes would act to protect critical cellular components from the destructive effects of these agents. Lysosomal degranulation would introduce digestive hydrolytic enzymes, cationic proteins, and myeloperoxidase into the vacuole. The last might stimulate free-radical (57, 62) or 1O_2 (49, 63) formation from H_2O_2 , or H_2O_2 and hypohalite anions, respectively, as a means of enhancing the bactericidal effect of this agent (44, 64).

It would seem clear from these and other studies that the respiratory burst coincident with granulocyte phagocytosis is associated with significant production of O_2^- . Through this process the oxygen molecule is literally charged with new energy. The inhibition of phagocytic bactericidal activity, as well as chemiluminescence, by SOD suggests that this energy is involved in the killing of ingested bacteria. Exactly how this energy is translated into microbial death remains to be elucidated.

ACKNOWLEDGMENTS

We are grateful to Doctors A. W. Atkinson, Jr. and Sidney P. Kent for the electron microscopy, John A. Burdeshaw and Dr. Edwin A. Bradley for guidance in and performance of the statistical analyses, Doctors Irwin Fridovich and Dale Kessler for constructive comments, Doctors Blair Batson, Richard Hortman, Paul Bianco, Steven L. Shore, and Rebecca Buckley for referral of patients with CGD, and to Dr. Alexander R. Lawton for critical review of the manuscript. We are particularly appreciative of the diligence and industry of Shannon Skalley, Peggy Morris, Linda Guthrie, Susan Murrmann, and Jackie Broadley who performed many of the experiments reported here.

This work was supported by U. S. Public Health Service Grants AI-10286, CA-13148, DE-02670, RR-00032, AI-10892, and GM-00091 and a grant from the Riley Memorial Association.

REFERENCES

1. Fridovich, I. 1972. Superoxide radical and superoxide dismutase. *Accounts Chem. Res.* **5**: 321–326.
2. McCord, J. M., and I. Fridovich. 1968. The reduction of cytochrome *c* by milk xanthine oxidase. *J. Biol. Chem.* **243**: 5753–5760.
3. Rajagopalan, K. V., and P. Handler. 1964. Hepatic aldehyde oxidase. II. Differential inhibition of electron transfer to various electron acceptors. *J. Biol. Chem.* **239**: 2022–2026.
4. Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**: 276–287.
5. Misra, H. P., and I. Fridovich. 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247**: 3170–3175.

³ Miles, S. K., B. B. Keele, Jr., H. P. Misra, and R. B. Johnston, Jr. Manuscript in preparation.

⁴ DeChatelet, McCall, McPhail, and Johnston originally reported cyanide insensitivity of neutrophil homogenate SOD assayed by its interference with O_2^- -mediated autoxidation of adrenalin (52). It would appear that this assay is inappropriate for study of the cyanide sensitivity of homogenate SOD because such sensitivity can be demonstrated with the same homogenates when interference with reduction of cytochrome *c* or NBT, in fluid or gel systems, is used as the assay of enzyme activity (Johnston and DeChatelet, unpublished observations).

6. McCord, J. M., and I. Fridovich. 1969. Superoxide dismutase. An enzymic function for erythrocyte hemocuprein (hemocuprein). *J. Biol. Chem.* **244**: 6049-6055.
7. McCord, J. M., B. B. Keele, Jr., and I. Fridovich. 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 1024-1027.
8. Gregory, E. M., F. J. Yost, Jr., and I. Fridovich. 1973. Superoxide dismutases of *Escherichia coli*: intracellular localization and functions. *J. Bacteriol.* **115**: 987-991.
9. Gregory, E. M., S. A. Goscin, and I. Fridovich. 1974. Superoxide dismutase and oxygen toxicity in a eukaryote. *J. Bacteriol.* **117**: 456-460.
10. Baehner, R. L., and D. G. Nathan. 1968. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. *N. Engl. J. Med.* **278**: 971-976.
11. Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**: 741-744.
12. Curnutte, J. T., D. M. Whitten, and B. M. Babior. 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *N. Engl. J. Med.* **290**: 593-597.
13. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**: 291-302.
14. Ellman, G. L. 1962. The biuret reaction: changes in the ultraviolet absorption spectra and its application to the determination of peptide bonds. *Anal. Biochem.* **3**: 40-48.
15. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**: 404-427.
16. Weisiger, R. A., and I. Fridovich. 1973. Superoxide dismutase. Organelle specificity. *J. Biol. Chem.* **248**: 3582-3592.
17. Johnston, R. B., Jr., M. R. Klemperer, C. A. Alper, and F. S. Rosen. 1969. The enhancement of bacterial phagocytosis by serum. The role of complement components and two cofactors. *J. Exp. Med.* **129**: 1275-1290.
18. Wardlaw, A. C., and L. Pillemer. 1956. The properdin system and immunity. V. The bactericidal activity of the properdin system. *J. Exp. Med.* **103**: 553-575.
19. Massey, V. 1959. The microestimation of succinate and the extinction coefficient of cytochrome c. *Biochim. Biophys. Acta.* **34**: 255-256.
20. Webb, L. S., B. B. Keele, Jr., and R. B. Johnston, Jr. 1974. Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase. *Infect. Immun.* **9**: 1051-1056.
21. Stanley, P. E., and S. G. Williams. 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem.* **29**: 381-392.
22. Quie, P. G., J. G. White, B. Holmes, and R. A. Good. 1967. *In vitro* bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. *J. Clin. Invest.* **46**: 668-679.
23. Johnston, R. B., Jr., and R. L. Baehner. 1970. Improvement of leukocyte bactericidal activity in chronic granulomatous disease. *Blood J. Hematol.* **35**: 350-355.
24. Curtiss, R. 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. *J. Bacteriol.* **89**: 28-40.
25. Johnston, R. B., Jr., P. Anderson, and S. L. Newman. 1973. Opsonization and phagocytosis of *Hemophilus influenzae*, type b. In *Hemophilus Influenzae*. S. H. W. Sell and D. T. Karzon, editors. Vanderbilt University Press, Nashville. 99-112.
26. Fridovich, I. 1970. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J. Biol. Chem.* **245**: 4053-4057.
27. Snedecor, G. W., and W. G. Cochran. 1967. *Statistical Methods*. Iowa State University Press, Ames. 6th edition. 125-127, 130-131, 162-163.
28. Allen, R. C., R. L. Stjernholm, and R. H. Steele. 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochem. Biophys. Res. Commun.* **47**: 679-684.
29. Haber, F., and J. Weiss. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. R. Soc. Lond. A. Math. Phys. Sci.* **147**: 332-351.
30. Neta, P., and L. M. Dorfman. 1968. Pulse radiolysis studies. XIII. Rate constants for the reaction of hydroxyl radicals with aromatic compounds in aqueous solutions. *Adv. Chem. Ser.* **81**: 222-230.
31. Beauchamp, C., and I. Fridovich. 1970. A mechanism for the production of ethylene from methional. The generation of the hydroxyl radical by xanthine oxidase. *J. Biol. Chem.* **245**: 4641-4646.
32. Fong, K.-L., P. B. McCay, J. L. Poyer, B. B. Keele, and H. Misra. 1973. Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity. *J. Biol. Chem.* **248**: 7792-7797.
33. Keele, B. B., Jr., J. M. McCord, and I. Fridovich. 1970. Superoxide dismutase from *Escherichia coli* B. A new manganese-containing enzyme. *J. Biol. Chem.* **245**: 6176-6181.
34. Beauchamp, C. O., and I. Fridovich. 1973. Isozymes of superoxide dismutase from wheat germ. *Biochim. Biophys. Acta.* **317**: 50-64.
35. Nathan, D. G., R. L. Baehner, and D. K. Weaver. 1969. Failure of nitro blue tetrazolium reduction in the phagocytic vacuoles of leukocytes in chronic granulomatous disease. *J. Clin. Invest.* **48**: 1895-1904.
36. Khan, A. U. 1970. Singlet molecular oxygen from superoxide anion and sensitized fluorescence of organic molecules. *Science (Wash. D. C.)*. **168**: 476-477.
37. Khan, A. U., and M. Kasha. 1970. Chemiluminescence arising from simultaneous transitions in pairs of singlet oxygen molecules. *J. Am. Chem. Soc.* **92**: 3293-3300.
38. Greenlee, L., I. Fridovich, and P. Handler. 1962. Chemiluminescence induced by operation of iron-flavo-proteins. *Biochemistry*. **1**: 779-783.
39. Stauff, J., H. Schmidkunz, and G. Hartmann. 1963. Weak chemiluminescence of oxidation reactions. *Nature (Lond.)*. **198**: 281-282.
40. Arneson, R. M. 1970. Substrate-induced chemiluminescence of xanthine oxidase and aldehyde oxidase. *Arch. Biochem. Biophys.* **136**: 352-360.
41. Finazzi Agró, A., C. Giovagnoli, P. De Sole, L. Calabrese, G. Rotilio, and B. Mondoví. 1972. Erythrocyte and singlet oxygen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **21**: 183-185.

42. Goda, K., J. Chu, T. Kimura, and A. P. Schaap. 1973. Cytochrome c enhancement of singlet molecular oxygen production by the NADPH-dependent adrenoxin reductase-adrenoxin system: the role of singlet oxygen in damaging adrenal mitochondrial membranes. *Biochem. Biophys. Res. Commun.* **52**: 1300-1306.
43. Curnutte, J. T., and B. M. Babior. 1974. Biological defense mechanisms: the effect of bacteria and serum on superoxide production by granulocytes. *J. Clin. Invest.* **53**: 1662-1672.
44. Klebanoff, S. J., and C. B. Hamon. 1972. Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. *J. Reticuloendothel. Soc.* **12**: 170-196.
45. McRipley, R. J., and A. J. Sbarra. 1967. Role of the phagocyte in host-parasite interactions. XI. Relationship between stimulated oxidative metabolism and hydrogen peroxide formation, and intracellular killing. *J. Bacteriol.* **94**: 1417-1424.
46. Klebanoff, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. *J. Exp. Med.* **126**: 1063-1078 + plate 80.
47. Baehner, R. L., S. K. Murrmann, and R. B. Johnston, Jr. 1974. The role of superoxide anion and hydrogen peroxide in phagocytosis-associated oxidative metabolic reactions of human polymorphonuclear leukocytes. *Blood. J. Hematol.* **44**: 926 (Abstr.).
48. Goscin, S. A., and I. Fridovich. 1972. The role of superoxide radical in a nonenzymatic hydroxylation. *Arch. Biochem. Biophys.* **153**: 778-783.
49. Krinsky, N. I. 1974. Singlet excited oxygen as a mediator of the antibacterial action of leukocytes. *Science (Wash. D. C.)*. **186**: 363-365.
50. Yost, F. J., and I. Fridovich. 1973. An iron-containing superoxide dismutase from *Escherichia coli*. *J. Biol. Chem.* **248**: 4905-4908.
51. Beckman, G., E. Lundgren, and A. Tärnvik. 1973. Superoxide dismutase isoenzymes in different human tissues, their genetic control, and intracellular localization. *Hum. Hered.* **23**: 338-345.
52. DeChatelet, L. R., C. E. McCall, L. C. McPhail, and R. B. Johnston, Jr. 1974. Superoxide dismutase activity in leukocytes. *J. Clin. Invest.* **53**: 1197-1201.
53. Salin, M. L., and J. M. McCord. 1974. Superoxide dismutases in polymorphonuclear leukocytes. *J. Clin. Invest.* **54**: 1005-1009.
54. Karnovsky, M. L. 1975. Biochemical aspects of the functions of polymorphonuclear and mononuclear leukocytes. In *The Phagocytic Cell in Host Resistance*. J. A. Bellanti and D. H. Dayton, editors. Raven Press, New York. 25-43.
55. Graham, R. C., Jr., M. J. Karnovsky, A. W. Shafer, E. A. Glass, and M. L. Karnovsky. 1967. Metabolic and morphological observations on the effect of surface-active agents on leukocytes. *J. Cell. Biol.* **32**: 629-647.
56. Goetzl, E. J., and K. F. Austen. 1974. Stimulation of human neutrophil leukocyte aerobic glucose metabolism by purified chemotactic factors. *J. Clin. Invest.* **53**: 591-599.
57. Odajima, T. 1971. Myeloperoxidase of the leukocyte of normal blood. II. The oxidation-reduction reaction mechanism of the myeloperoxidase system. *Biochim. Biophys. Acta.* **235**: 52-60.
58. Paul, B., and A. J. Sbarra. 1968. The role of the phagocyte in host-parasite interactions. XIII. The direct quantitative estimation of H₂O₂ in phagocytizing cells. *Biochim. Biophys. Acta.* **156**: 168-178.
59. Baehner, R. L., D. G. Nathan, and W. B. Castle. 1971. Oxidant injury of caucasian glucose-6-phosphate dehydrogenase-deficient red blood cells by phagocytosing leukocytes during infection. *J. Clin. Invest.* **50**: 2466-2473.
60. Root, R. K., N. Oshino, and B. Chance. 1974. Determinants of H₂O₂ release by human granulocytes (PMN). *Clin. Res.* **22**: 402A (Abstr.).
61. Stossel, T. P., T. D. Pollard, R. J. Mason, and M. Vaughn. 1971. Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. *J. Clin. Invest.* **50**: 1745-1757.
62. Yamazaki, I., and L. H. Piette. 1963. The mechanism of aerobic oxidase reaction catalyzed by peroxidase. *Biochim. Biophys. Acta.* **77**: 47-64.
63. Allen, R. C., and R. H. Steele. 1973. Electronic excitation of phagocytes. Proceedings of the 166th National Meeting of the Am. Chem. Soc., Biol. Sect. 116. (Abstr.)
64. Klebanoff, S. J. 1974. Role of the superoxide anion in the myeloperoxidase-mediated antimicrobial system. *J. Biol. Chem.* **249**: 3724-3728.