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Research Article

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Superoxide Generation by Digitonin-Stimulated Guinea Pig Granulocytes

A BASIS FOR A CONTINUOUS ASSAY FOR MONITORING SUPEROXIDE PRODUCTION AND FOR THE STUDY OF THE ACTIVATION OF THE GENERATING SYSTEM

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ABSTRACT Stimulation of guinea pig granulocytes by digitonin results in superoxide (O_2^-) generation. A continuous assay shows that there is a lag between the addition of digitonin and the onset of O_2^- production. The rate of activation of the O_2^- generating system is dependent upon the concentration of digitonin and the temperature. The final linear rate of O_2^- production is affected by the concentration of digitonin, temperature, pH, and the presence of exogenous reduced pyridine nucleotides. Thus, factors which alter either the activation process or the activity of the O_2^- generating system can affect O_2^- production by stimulated granulocytes.

INTRODUCTION

Superoxide (O_2^-)¹, the product of the one-electron reduction of oxygen, is produced during the phagocytosis of particles or bacteria by neutrophils (1, 2), monocytes (3), macrophages (4), and eosinophils (5). The enzyme system responsible for O_2^- production is felt to be a reduced pyridine nucleotide oxidase (6-8) which is activated during the phagocytic process. Studies on oxygen consumption (9) and hydrogen peroxide (H_2O_2) production (10) with continuous

monitoring have demonstrated a lag time between the addition of particles to cells and the metabolic changes observed. Assays for O_2^- generation by phagocytes have suggested a possible lag time (11), but because these assays are discontinuous point assays accurate observations on the time course of activation have not been made. O_2^- -dependent cytochrome *c* reduction seemed to be the best basis for designing a continuous assay. Because particles and bacteria interfere with spectrophotometric observations, a nonparticulate method for stimulating O_2^- production was sought. Negatively charged detergents have been shown to stimulate oxidative metabolism in neutrophils (12, 13). A continuous assay for O_2^- generation has been developed with the use of digitonin. The nature of the assay and certain factors that affect the activation of the enzyme and the rate of production of O_2^- will be described.

METHODS

Cytochrome *c* Type VI, superoxide dismutase (SOD), horseradish peroxidase, and scopoletin were obtained from Sigma Chemical Co., St. Louis, Mo., and digitonin was obtained from Fisher Scientific Co., Medford, Mass. These and all other compounds were obtained at the highest rate of purity and used without further purification.

Granulocytes were harvested from the peritoneal cavity of 800-g male guinea pigs, 18 h after an injection of 30 ml of a 12% casein solution as previously described (14). Contaminating erythrocytes were lysed with distilled water. Cells were collected and kept in plastic tubes and transferred with siliconized Pasteur pipettes. Final cell suspension was in Krebs-Ringer phosphate (KRP), pH 7.4, at a final cell concentration of 5×10^7 /ml. Solutions of digitonin were made fresh daily by dissolving 5 mg into 20 ml distilled water (250 μ g/ml). Standard solutions of cytochrome *c* at 1 mM were quantitated by the use of the molar extinction coefficients at

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¹Abbreviations used in this paper: KRP, Krebs-Ringer phosphate; O_2^- , superoxide; SOD, superoxide dismutase.

550 nm of 8.9×10^3 for the oxidized form and 29.9×10^3 for the reduced form (15).

Spectrophotometric assays for O_2^- generation were performed in both a Gilford model 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a thermostatted cell compartment and a Perkin-Elmer model 571 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) equipped with a thermoelectrically controlled temperature regulator. Fluorometric assays were performed in a Perkin-Elmer model 512 double-beam spectrofluorometer (Perkin-Elmer Corp.) using a water-jacketed cell and a thermostatted compartment. All solutions with the exception of enzymes and cells were preheated at 37°C in a Temp-Block Module Heater model 2090 (Lab-Line Instruments, Inc., Melrose Park, Ill.)

Two assays for measuring O_2^- generation were utilized. For a single-beam spectrophotometer, a standard assay consists of $50 \mu\text{l}$ of a granulocyte suspension added to a 1-ml cuvette containing $50 \mu\text{l}$ Fe^{+++} cytochrome *c* (50 nmol) and 0.86 ml KRP, pH 7.4. After we allowed time for temperature equilibration, $40 \mu\text{l}$ of digitonin ($10 \mu\text{g}$) was added and the absorbance change at 550 nm was recorded. To subtract any O_2^- -independent change in absorbance, the same assay was carried out in the presence of $10 \mu\text{l}$ of SOD ($10 \mu\text{g}$). The rate of O_2^- generation was then determined by subtracting the linear rates of absorbance change in the presence of SOD from the rate in its absence, and then dividing by the extinction coefficient for the difference between the absorption of reduced cytochrome *c* minus oxidized cytochrome *c*, $21 \times 10^3 \text{ cm}^2/\text{M}$ (15). For a double-beam spectrophotometer the two cuvettes were prepared as described above, with the reference assay containing SOD, and after temperature equilibration, $40 \mu\text{l}$ of digitonin was added to both cuvettes. Here the rate of O_2^- generation was derived by dividing the linear absorbance change by the molar extinction coefficient for reduced cytochrome *c* minus oxidized cytochrome *c*. The sensitivity of this assay is such that it will detect an absorbance change of $0.002 \text{ A}/\text{min}$

TABLE I
Comparison of Various Stimulators on O_2^- Generation

Stimulator	O_2^- generation <i>nmol O_2^-/10 min/ 10⁷ cells</i>
Experiment A	
—	0.71
Digitonin ($10 \mu\text{g}/\text{ml}$)	6.70
Deoxycholate ($25 \mu\text{g}/\text{ml}$)	5.41
Opsonized zymosan ($4.5 \text{ mg}/\text{ml}$)	5.98
Experiment B	
—	0.83
Digitonin ($10 \mu\text{g}/\text{ml}$)	7.47
Opsonized zymosan ($4.5 \text{ mg}/\text{ml}$)	5.71
Digitonin ($10 \mu\text{g}/\text{ml}$) + opsonized zymosan ($4.5 \text{ mg}/\text{ml}$)	7.77

Assays for O_2^- production were performed as described previously (1); the stimulators were incubated with and without SOD. The reaction was allowed to proceed to 37°C for 10 min, stopped with cold KRP, and after centrifuging the cells the absorbance at 550 nm was recorded. Calculations were based on a millimolar difference extinction coefficient for cytochrome *c* of 21.

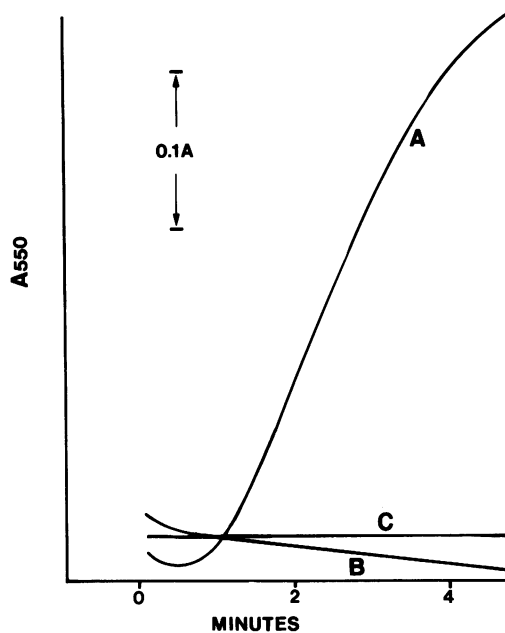


FIGURE 1 Continuous assay for O_2^- production in a single-beam spectrophotometer. Cells at $2.5 \times 10^6/\text{ml}$ were incubated at 37°C in KRP with cytochrome *c* (50 nmol). For A digitonin ($10 \mu\text{g}$) was added at zero time. For B digitonin ($10 \mu\text{g}$) was added together with SOD ($10 \mu\text{g}$). For C no additions were made. The absorbance at 550 nm was recorded continuously. The vertical axis is in absorbance units.

which is equivalent to $0.04 \text{ nm } O_2^-/\text{min}$ per 10^6 cells with a standard assay of 2.5×10^6 cells/ml.

The rate of H_2O_2 generation was followed continuously by monitoring the decrease in fluorescence accompanying the horseradish peroxidase-catalyzed oxidation of scopoletin as previously described (10). Quantitation was performed with known amounts of exogenous H_2O_2 as previously described (16). Unless otherwise indicated all reactions were performed at 37°C .

All results given are the average of two determinations and are within 10% of each other. Both the rate of O_2^- production and lag time vary in different preparations. For granulocytes obtained from 20 guinea pigs the averages ($\pm\text{SD}$) are $1.79 (\pm 0.43) \text{ nmol } O_2^-/\text{min}$ per 10^6 cells and $44 (\pm 15) \text{ s}$, respectively.

RESULTS

Superoxide generation by detergents. To determine whether anionic detergents could stimulate O_2^- generation by granulocytes, a standard assay was performed as described previously (1) with digitonin ($10 \mu\text{g}/\text{ml}$), deoxycholate ($25 \mu\text{g}/\text{ml}$), and opsonized zymosan. Experiment A in Table I shows that digitonin and deoxycholate stimulate O_2^- production from guinea pig granulocytes as well as opsonized zymosan. Experiment B shows that the addition of opsonized zymosan and digitonin together yield no further increase in O_2^- generation, suggesting that the same enzyme system is activated by each stimulator.

Furthermore, [1-¹⁴C]glucose oxidation was not augmented by the addition of both stimulators together. Trypan blue exclusion by granulocytes was greater than 95% after 15 min of incubation with digitonin.

Continuous assays for O₂⁻ generation. Because digitonin is an effective stimulator of O₂⁻ generation at a relatively low concentration and is translucent, it was employed in the continuous assay system. Fig. 1 shows typical tracings obtained in a single-beam instrument. In the absence of digitonin no appreciable change in absorbance occurs over the time course followed. With 10 μg/ml digitonin, after an initial decrease, the absorbance increases and becomes linear after about 70 s and remains linear until most of the cytochrome *c* is reduced. In the presence of 10 μg SOD, there is also an initial rapid decrease in absorbance which finally becomes linear. The O₂⁻-dependent cytochrome *c* reduction is calculated by subtracting the linear rate obtained in the presence of

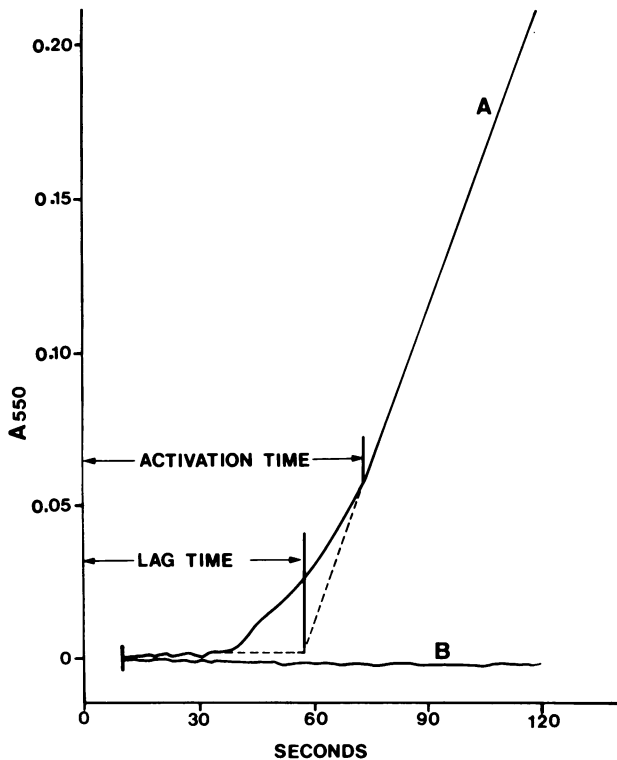


FIGURE 2 Continuous assay for O₂⁻ production in a double-beam spectrophotometer. Cells at 2.5 × 10⁹/ml were added at zero time to a cuvette containing KRP and cytochrome *c* (50 nmol) in the sample compartment and the same plus SOD (10 μg) in the reference compartment. (A) in the presence of digitonin (10 μg) and (B) in the absence of digitonin. The rate of O₂⁻ generation is determined from the slope of the linear portion of the curve, and the lag time from the extrapolation of the curve to zero absorbance change (dashed lines). The activation time is the time to development of a linear rate of cytochrome *c* reduction.

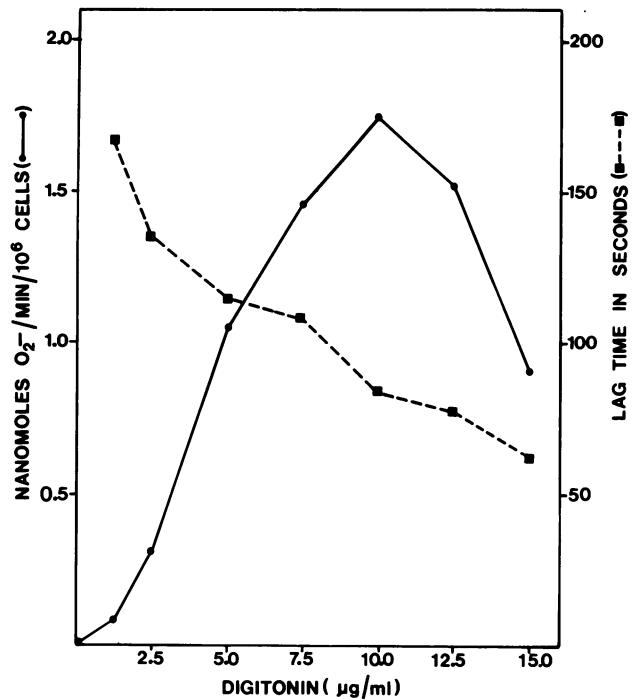


FIGURE 3 The effect of digitonin concentration on the rate of O₂⁻ generation (●) and the lag time (■). Standard assay conditions were employed with the concentrations of digitonin as given on the abscissa. Rate of O₂⁻ production and lag time were determined as shown in Fig. 2.

SOD from that obtained in its absence. The initial decrease in absorbance that occurs when digitonin is added to cells (or when cells are added to cuvettes containing digitonin) is independent of the presence of cytochrome *c* and probably represents an effect of digitonin on light scattering by the granulocytes.

Because this initial decrease occurred in both the presence and absence of SOD and because at times this initial time period was of interest, a double-beam spectrophotometric assay was also utilized. Fig. 2 shows tracings obtained. Again in the absence of digitonin, no change in absorbance occurs. In the presence of 10 μg/ml digitonin, after an initial delay, absorbance increases until a linear rate occurs. It is clear from this tracing that the initial decreases in absorbance seen in the single-beam assay are equal in both sample and reference. We define the activation time as that time between the addition of cells to the digitonin and the onset of the linear rate of cytochrome *c* reduction. In addition, for kinetic purposes, we define the lag time as that time when the linear rate extrapolates to zero absorbance change.

Dose curve for digitonin. Varying the concentration of digitonin has marked effects on the rate of O₂⁻ generation and the time required for the activation of the generating system. Fig. 3 shows that

optimum digitonin concentration in the assay is 10 $\mu\text{g/ml}$ ($\sim 10 \mu\text{M}$) and that the dose-response curve is quite steep. The lag time for the activation process is also dependent upon the digitonin concentration. The effect of digitonin on the rate of activation is different from its effect on the final rate of O_2^- generation because the lag time continues to decrease even at concentrations of digitonin that produce less than optimal final rates.

Effect of cell concentration on O_2^- generation. Fig. 4 shows that for digitonin the rate of O_2^- generation by granulocytes is linear with cell concentration from 8×10^5 cells/ml to 8×10^6 cells/ml, thus allowing comparison of results obtained at different concentrations of granulocytes. The time required for activation of this system, however, is independent of cell concentrations. The small variations seen in Fig. 4 are within the experimental error for calculating the lag time. Thus, the rate of activation of the enzyme system is a function of the concentration of stimulant but not of the number of cells being stimulated, and the rate of production of O_2^- is a function of both.

Cytochrome c and SOD concentrations. In this assay, although the rate of O_2^- generation is in-

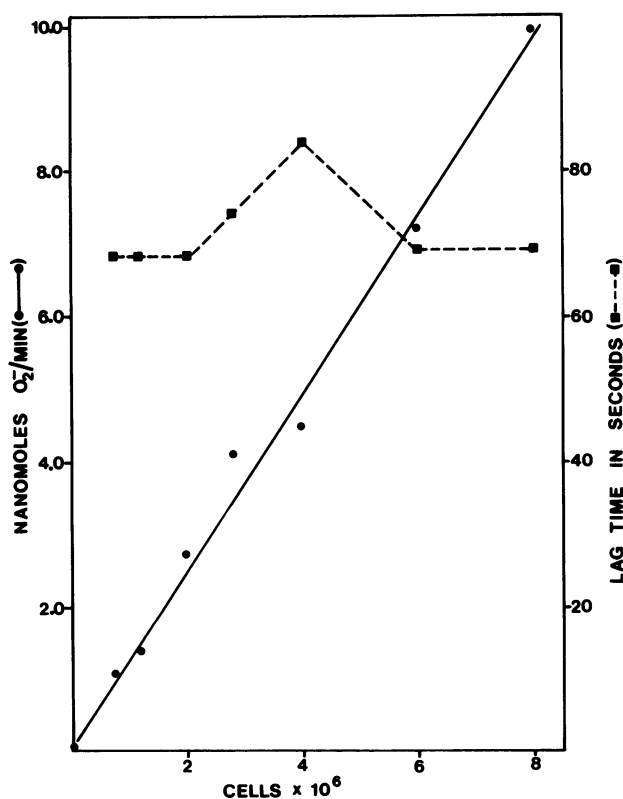


FIGURE 4 Effect of cell concentration on rate of O_2^- generation (\bullet) and lag time (\blacksquare). Cells at the concentrations noted on the abscissa were added to standard assay mixtures, and the rate of O_2^- generation and the lag time were recorded.

TABLE II
Effects of Catalase and Mannitol on O_2^- Production by Granulocytes

	O_2^- production
	nmol $\text{O}_2^-/\text{min}/10^6$ cells
Control	2.15
+ catalase, 250 U	2.07
+ mannitol, 50 mM	2.50

dependent of cytochrome c concentration, the ability to detect O_2^- depends upon whether there is enough cytochrome c present to interact with the O_2^- generated. With 2.5×10^6 cells/ml, $30 \mu\text{M}$ cytochrome c will give maximum rates of absorbance change. It must be noted that the amount of cytochrome c required will depend upon the rate of O_2^- generated, and if markedly increased rates are obtained higher concentrations of cytochrome c need to be used.

The amount of SOD required to prevent cytochrome c reduction was also determined. With $50 \mu\text{M}$ cytochrome c and 2.5×10^6 cells, $7 \mu\text{g/ml}$ SOD caused complete inhibition of cytochrome c reduction and additional SOD has no further effect. However, since SOD competes with cytochrome c for O_2^- , the amount of SOD required varies with the amount of cytochrome c used and with the rate of O_2^- production (17).

Specificity of the assay system for O_2^- . Although SOD can completely inhibit cytochrome c reduction by guinea pig granulocytes (Fig. 1), it was necessary to determine if scavengers of H_2O_2 (catalase) and hydroxyl radical (mannitol) had any effect on the sys-

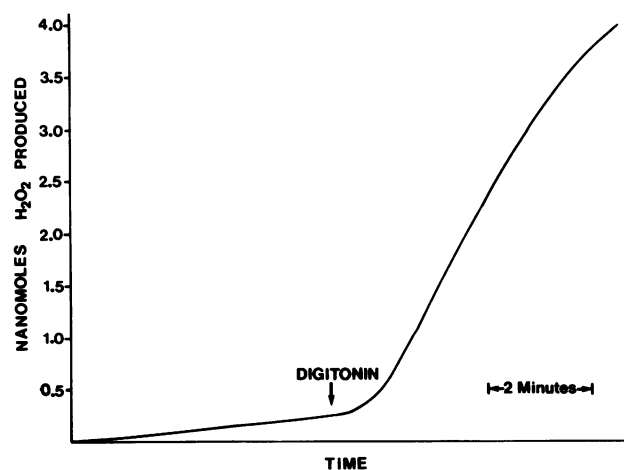


FIGURE 5 The effect of digitonin on H_2O_2 production by granulocytes. Continuous monitoring of H_2O_2 production was performed as previously described (10). Digitonin ($10 \mu\text{g}$) was added at the time noted and the rate of scopoletin oxidation was followed in a fluorometer and related to H_2O_2 production as described previously (15).

TABLE III
The Relationship between O_2^- and H_2O_2 Production by Digitonin-Stimulated Granulocytes

	H_2O_2 production	Lag time	O_2^- production	Lag time
	<i>nmol H_2O_2/min/10⁶ cells</i>	<i>s</i>	<i>nmol O_2^-/min/10⁶ cells</i>	<i>s</i>
Digitonin (10 μ g/ml)	1.52 \pm 0.02 (3)*	30.7 \pm 2.1 (3)		
Digitonin (10 μ g/ml) + SOD (10 μ g/ml)	2.19 \pm 0.10 (4)*	25 \pm 2.3 (4)	4.34 \pm 0.56 (5)	25.8 \pm 1.2 (5)

The numbers in parentheses represent the number of determinations of each point.

* $P < 0.005$.

tem. Table II shows that neither catalase nor mannitol had any effect on O_2^- -dependent cytochrome *c* reduction in this assay system.

Relationship of H_2O_2 production to O_2^- production. With the scopoletin oxidation assay (10), H_2O_2 production was monitored continuously. Fig. 5 shows that the addition of digitonin to granulocytes results in H_2O_2 production after a short lag time. The time to complete activation was similar to that obtained for O_2^- production, and the linear rate of H_2O_2 production was less than one-half that of O_2^- . Cytochalasin B at concentrations up to 50 μ g/ml had no effect on either rate. It has been previously described that SOD stimulates the rate of H_2O_2 production by activated granulocytes (18). As can be seen in Table III, the presence of 10 μ g/ml SOD causes more H_2O_2 to be produced by granulocytes stimulated by digitonin. In addition, only in the presence of SOD is the rate of H_2O_2 production equal to one-half the rate of O_2^- production. In the absence of digitonin no stimulation of H_2O_2 production by SOD was seen. The lag times for O_2^- production and H_2O_2 production were similar, and although the rate of H_2O_2 production was increased in the presence of SOD, the lag time was unaffected. Similar effects of SOD were seen for H_2O_2 production in the presence of latex particles.

The effect of temperature on O_2^- generation and lag time. With the double-beam spectrophotometric method, O_2^- -dependent cytochrome *c* reduction was monitored at temperatures between 20° and 40°C. Table IV shows that the rate of O_2^- generation increases with increasing temperature, and that the lag time is also temperature dependent. There was no appreciable change in the extinction coefficients for cytochrome *c* at the temperatures studied nor on the ability of cytochrome *c* to scavenge the O_2^- formed.

Effect on pH on O_2^- generation. KRP buffer was made at various levels of pH between 6.0 and 8.4, and cells that were suspended in saline were used to measure the effect of pH on the O_2^- producing system. The pH optimum for this system is 7.4; at both acid and alkaline pH there is less O_2^- generation. We

have also examined the effect of pH on activation, and in the pH range examined there was no major effect of pH on the lag time. Again there was no appreciable change in the extinction coefficients for cytochrome *c* and on the ability to scavenge O_2^- over the pH range examined.

The effect of reduced pyridine nucleotides on O_2^- production. The enzyme system responsible for O_2^- generation in phagocytic cells is believed to be a reduced pyridine nucleotide oxidase (6–8). Fig. 6 shows the effect of exogenous NADH and NADPH on O_2^- generation by digitonin-stimulated granulocytes. Both pyridine nucleotides caused additional O_2^- generation but at the concentrations used, NADPH was more effective than NADH. These pyridine nucleotides produced very little O_2^- when added to granulocytes in the absence of digitonin.

DISCUSSION

Little if any O_2^- is produced by resting granulocytes, and with the addition of stimulants a change occurs in an enzyme system which results in altered intracellular metabolism (19) and O_2^- formation (1). To measure this change and determine the factors af-

TABLE IV
Effect of Temperature on the Rate of O_2^- Generation and the Lag Time

Temp	O_2^- generation	Lag time
°C	<i>nmol O_2^-/min/10⁶ cells</i>	<i>s</i>
20	0.207	227
25	0.423	126
30	0.678	77
35	0.922	61
37	1.180	54
40	1.492	48

Cells (2.5×10^6 /ml) were incubated in KRP containing cytochrome *c* (50 μ M) with and without SOD (10 μ g/ml) at the temperatures noted. At zero time digitonin (10 μ g/ml) was added to each cuvette and the lag time and rates of O_2^- production were determined.

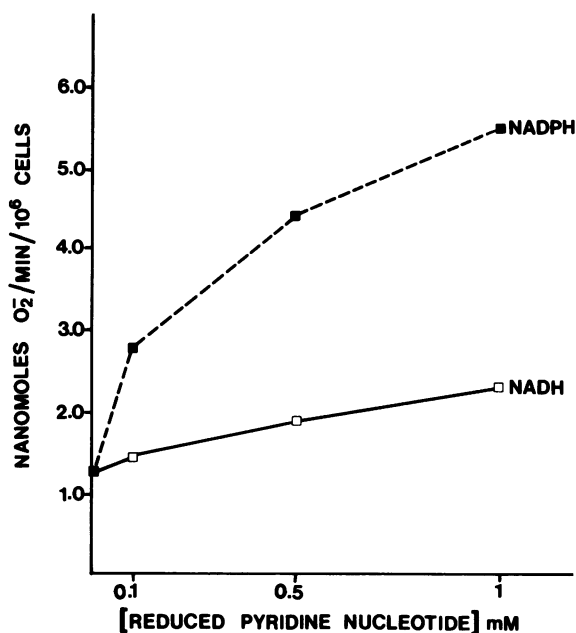


FIGURE 6 The effect of exogenous reduced pyridine nucleotides on O_2^- production by digitonin-stimulated granulocytes. Digitonin was added to standard assay mixtures in the presence of either NADPH (■) or NADH (□) at the concentrations noted on the abscissa. In the absence of digitonin, no appreciable O_2^- production was seen with these amounts of reduced pyridine nucleotides.

fecting the activation of this enzyme system within the cell, we designed an assay for continuous monitoring of O_2^- production. Because particles and bacteria optically interfere with the ability to measure the spectral change that accompanies the reduction of cytochrome *c*, we sought a translucent stimulator of O_2^- generation. Cytochalasin E, a nonparticulate stimulator of oxidative metabolism by granulocytes, has been used for continuous monitoring of O_2^- production (20) but is both difficult to obtain and needs to be dissolved in a nonaqueous solvent. We, therefore, examined the use of detergents as stimulators of O_2^- production. O_2^- production by granulocytes that were stimulated by negatively charged detergents was expected. It has been previously shown that they stimulate both oxygen consumption and H_2O_2 production (12, 13), and it is probably true that the first step of oxygen metabolism in stimulated phagocytic cells is the generation of the one-electron reduction compound, O_2^- . The sensitivity of this assay for O_2^- was such that the number of granulocytes used did not optically interfere with the ability to follow cytochrome *c* reduction.

It is difficult to compare the relative effectiveness of detergents vs. particles as O_2^- generating stimulators. Assays for O_2^- production only measure the extracellular species. For detergents, probably all of the O_2^- produced is extracellular, while for particles much is

formed within phagocytic vesicles. This O_2^- may then spontaneously dismutate, enzymatically dismutate, or react with cellular constituents before reaching the external milieu. The two:one ratio of O_2^- produced to H_2O_2 produced by digitonin-treated cells supports the hypothesis that all O_2^- generated in the presence of digitonin is extracellular.

The stimulation of H_2O_2 production by SOD has been seen before, and the explanation offered was that this was due to a more rapid conversion of O_2^- to H_2O_2 in the presence of SOD (18). There are two reasons why this is probably not the correct explanation. (a) It has previously been shown that SOD has no effect on H_2O_2 production from xanthine-xanthine oxidase, a known O_2^- generating system (21). We have repeated these studies and have confirmed that at rates of O_2^- production equivalent to the rates obtained with granulocytes, SOD has no effect on H_2O_2 production.² (b) Not only does the presence of SOD result in more H_2O_2 production but also in more oxygen consumption (18). This is inconsistent with the above explanation because SOD would cause equal amounts of oxygen to be regenerated as H_2O_2 produced.

The nature of the intracellular, reduced pyridine nucleotide substrate for this enzyme is controversial, and in fact may not be answerable because both substrates can cause O_2^- generation in particles obtained from stimulated cells (6, 22). Briggs et al. (23) have shown that exogenous NADH but not NADPH can cause increased H_2O_2 production in stimulated granulocytes. The experiments described herein demonstrate that both reduced pyridine nucleotides cause increased O_2^- generation by stimulated cells with NADPH being better than NADH at the concentrations used. The results may not be comparable because we used guinea pig neutrophils and digitonin as a stimulator, and Briggs et al. used human neutrophils with particle stimulation. The point to be made from this experiment is that the cells as assayed under ordinary conditions are operating at less than optimal concentrations of substrate. If so, then the intracellular, reduced pyridine nucleotide concentration may be an additional mode of control of enzyme activity.

The use of a continuous assay has made it possible to dissociate O_2^- production into an activation process and an activity process. Factors can affect either one or both of these processes and can result in altered O_2^- generation by stimulated granulocytes.

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² Cohen, H. J. Unpublished observations.

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