# 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 $5 \times 10^7$/ml. Solutions of digitonin were made fresh daily by dissolving 5 mg into 20 ml distilled water (250 μg/ml). Standard solutions of cytochrome *c* at 1 mM were quantitated by the use of the molar extinction coefficients at

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

550 nm of $8.9 \times 10^3$ for the oxidized form and $29.9 \times 10^3$ 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 μl of a granulocyte suspension added to a 1-ml cuvette containing 50 μl $Fe^{+++}$ cytochrome *c* (50 nmol) and 0.86 ml KRP, pH 7.4. After we allowed time for temperature equilibration, 40 μl of digitonin (10 μg) 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 μl of SOD (10 μg). 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*, $21 \times 10^3$ $cm^2/M$ (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 μl 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 *A*/min which is equivalent to 0.04 nm $O_2^-$/min per $10^6$ cells with a standard assay of $2.5 \times 10^6$ cells/ml.

TABLE I

*Comparison of Various Stimulators on $O_2^-$ Generation*

| Stimulator | $O_2^-$ generation |
|---|---|
| | *nmol $O_2^-$/10 min/ $10^7$ cells* |
| Experiment A | |
| — | 0.71 |
| Digitonin (10 μg/ml) | 6.70 |
| Deoxycholate (25 μg/ml) | 5.41 |
| Opsonized zymosan (4.5 mg/ml) | 5.98 |
| Experiment B | |
| — | 0.83 |
| Digitonin (10 μg/ml) | 7.47 |
| Opsonized zymosan (4.5 mg/ml) | 5.71 |
| Digitonin (10 μg/ml) + opsonized zymosan (4.5 mg/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.

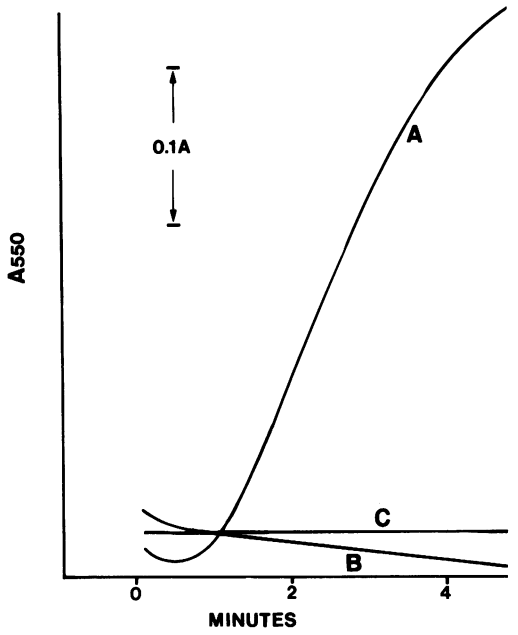


FIGURE 1 Continuous assay for $O_2^-$ production in a single-beam spectrophotometer. Cells at $2.5 \times 10^6$/ml were incubated at 37°C in KRP with cytochrome *c* (50 nmol). For A digitonin (10 μg) was added at zero time. For B digitonin (10 μg) was added together with SOD (10 μg). For C no additions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

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 (±SD) are 1.79 (±0.43) nmol $O_2^-$/min per $10^6$ cells and 44 (±15) 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 μg/ml), deoxycholate (25 μg/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-$^{14}$C]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 $O_2^-$ generation.* Because digitonin is an effective stimulator of $O_2^-$ 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 $O_2^-$-dependent cytochrome *c* reduction is calculated by subtracting the linear rate obtained in the presence of 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.

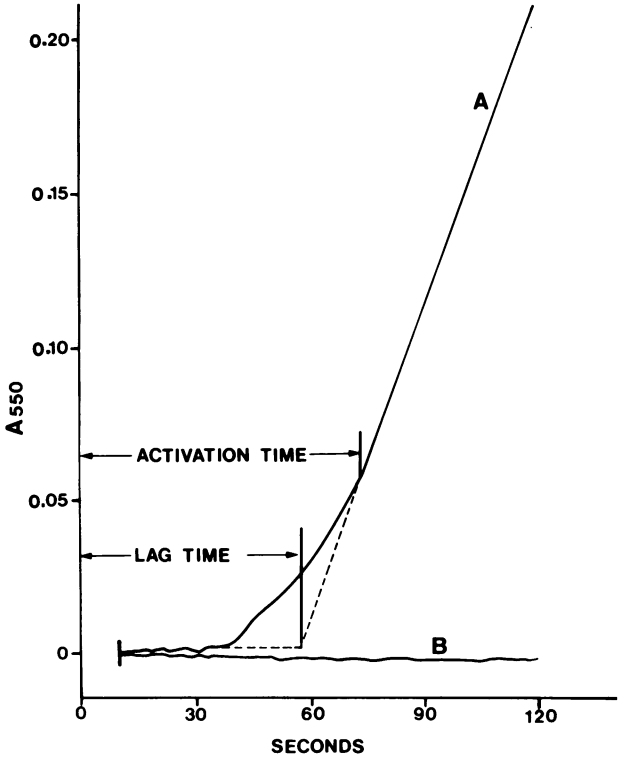


FIGURE 2 Continuous assay for $O_2^-$ production in a double-beam spectrophotometer. Cells at 2.5 × $10^6$/ml were added at zero time to a cuvette containing KRP and cytochrome *c* (50 nmol) in the sample compartment and the same plus SOD (10 μg) in the reference compartment. (A) in the presence of digitonin (10 μg) and (B) in the absence of digitonin. The rate of $O_2^-$ 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.

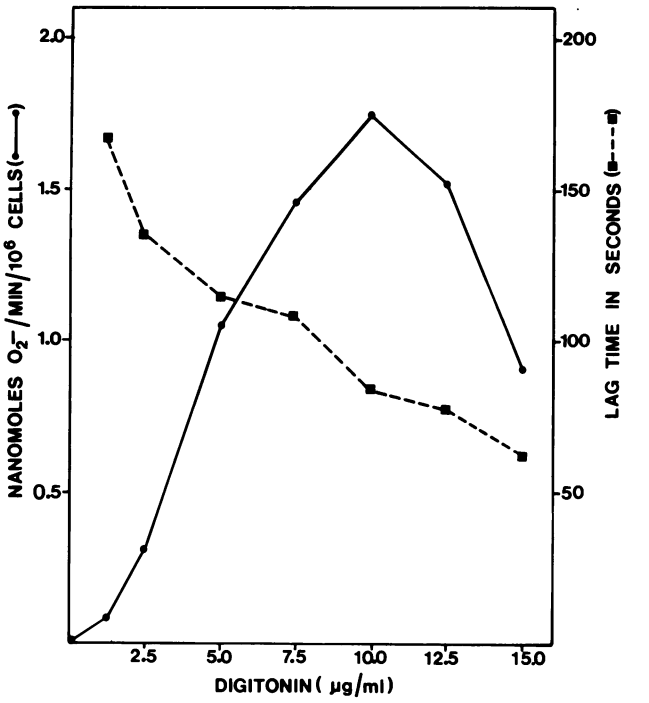


FIGURE 3 The effect of digitonin concentration on the rate of $O_2^-$ generation (●) and the lag time (■). Standard assay conditions were employed with the concentrations of digitonin as given on the abscissa. Rate of $O_2^-$ production and lag time were determined as shown in Fig. 2.

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 $O_2^-$ generation and the time required for the activation of the generating system. Fig. 3 shows that

optimum digitonin concentration in the assay is 10 $\mu$g/ml (~10 $\mu$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 $8 \times 10^5$ cells/ml to $8 \times 10^6$ 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 independent 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 $2.5 \times 10^6$ cells/ml, 30 $\mu$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.

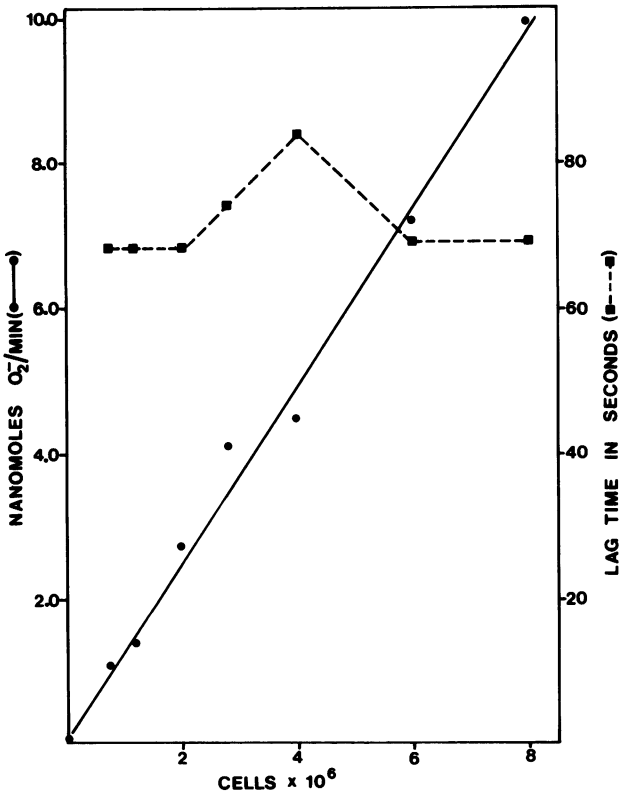


FIGURE 4 Effect of cell concentration on rate of $O_2^-$ generation (●) and lag time (■). Cells at the concentrations noted on the abscissa were added to standard assay mixtures, and the rate of $O_2^-$ generation and the lag time were recorded.

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

| | $O_2^-$ production |
|---|---|
| | *nmol $O_2^-$/min/$10^6$ cells* |
| Control | 2.15 |
| + catalase, 250 U | 2.07 |
| + mannitol, 50 mM | 2.50 |

The amount of SOD required to prevent cytochrome *c* reduction was also determined. With 50 $\mu$M cytochrome *c* and $2.5 \times 10^6$ cells, 7 $\mu$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-

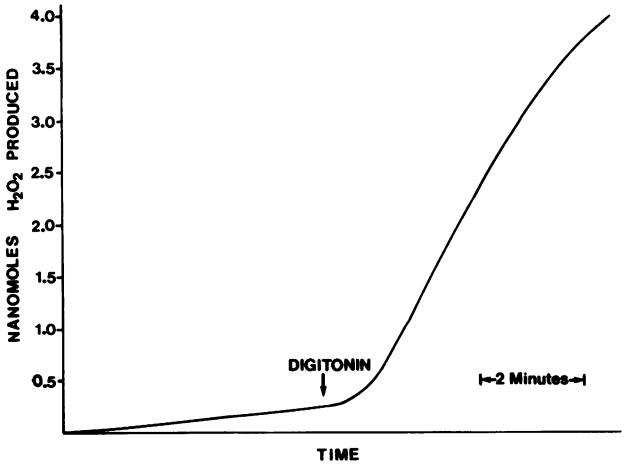


FIGURE 5 The effect of digitonin on $H_2O_2$ production by granulocytes. Continuous monitoring of $H_2O_2$ production was performed as previously described (10). Digitonin (10 $\mu$g) was added at the time noted and the rate of scopoletin oxidation was followed in a fluorometer and related to $H_2O_2$ 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 |
|---|---|---|---|---|
| | *nmol $H_2O_2$/min/$10^6$ cells* | *s* | *nmol $O_2^-$/min/$10^6$ cells* | *s* |
| Digitonin (10 μg/ml) | 1.52±0.02 (3)* | 30.7±2.1 (3) | | |
| Digitonin (10 μg/ml) + SOD (10 μg/ml) | 2.19±0.10 (4)* | 25±2.3 (4) | 4.34±0.56 (5) | 25.8±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 μg/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 μg/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 | *nmol $O_2^-$/min/$10^6$ cells* | *s* |
| 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 ($2.5 \times 10^6$/ml) were incubated in KRP containing cytochrome *c* (50 μM) with and without SOD (10 μg/ml) at the temperatures noted. At zero time digitonin (10 μg/ml) was added to each cuvette and the lag time and rates of $O_2^-$ production were determined.

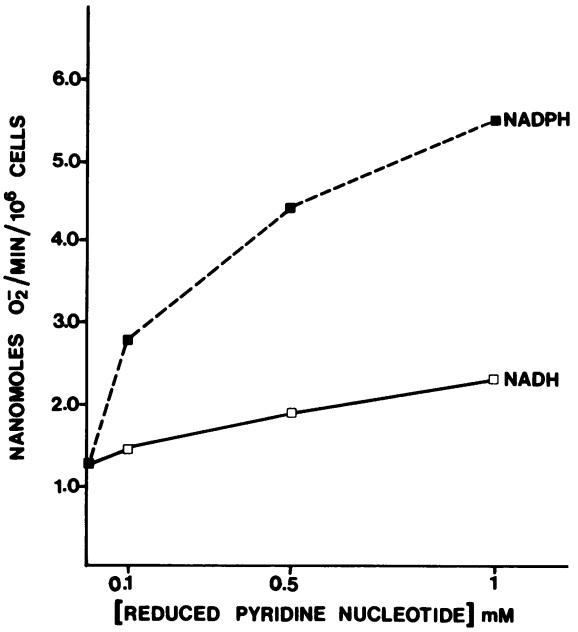


FIGURE 6 The effect of exogenous reduced pyridine nucleotides on $O_2^-$ production by digitonin-stimulated granulocytes. Digitonin was added to standard assay mixtures in the presence of either NADPH (■) or NADH (□) at the concentrations noted on the abscissa. In the absence of digitonin, no appreciable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