Termination of the Respiratory Burst in Human Neutrophils

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ABSTRACT Recent evidence has suggested that a particulate O_2^--forming system is responsible for the respiratory burst in activated neutrophils. The respiratory burst is normally a transient event, lasting only 30–60 min. To investigate the mechanism by which the burst is terminated, we examined the O_2^--forming activity of neutrophil particles as a function of time in the presence and absence of agents known to affect the function of intact cells. Measurements of the O_2^--forming capacity of the particles against time of exposure of neutrophils to opsonized zymosan, a potent stimulating agent, revealed a rapid fall in activity when exposure was continued beyond 3 min. Exposure to zymosan under conditions in which the myeloperoxidase system was inactive (i.e., in the presence of myeloperoxidase inhibitors, or in the absence of oxygen) resulted in a substantial increase in the initial O_2^--forming activity of particles from the zymosan-treated cells, but did not prevent the sharp fall in activity seen when zymosan exposure exceeded 10 min. The fall in activity was, however, prevented when activation took place in the presence of cytochalasin B (1.5 μg/ml), an agent thought to act largely by paralyzing the neutrophil through an interaction with its microfilament network.

We conclude from these findings that the termination of the respiratory burst results at least in part from the inactivation of the particulate O_2^--forming system. This inactivation involves at least two processes which probably act simultaneously. One is the destruction of the system through the action of myeloperoxidase. The other appears to require active cell motility and is independent of oxygen. The current view holds that the O_2^--forming system of the neutrophil is located in the plasma membrane. It may be that the second process involves the internalization and degradation of this membrane-bound system.

Received for publication 22 March 1977 and in revised form 15 December 1977.

INTRODUCTION

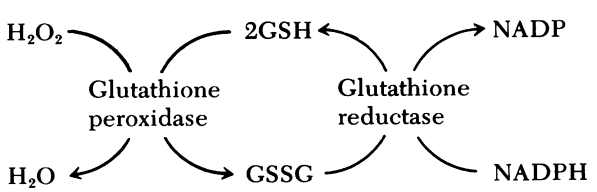
The exposure of neutrophils to suitable stimuli results in the activation of an oxygen-consuming metabolic pathway which is dormant in the unstimulated cell (1, 2). The outward manifestations of this pathway include increases in oxygen uptake (3–6), superoxide production (6–9), hydrogen peroxide production (10–12), and glucose consumption by way of the hexosemonophosphate shunt (3, 5), a group of events which taken together are designated the "respiratory burst". It now appears that the respiratory burst is a consequence of the activation of a particulate enzyme which catalyzes the reduction of oxygen to superoxide (O_2^-) according to the following reaction (13):

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

Most of the hydrogen peroxide released during the burst appears to originate from the dismutation of the superoxide produced by that enzyme (12, 14):

$$2\ O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2,$$

whereas the increase in the hexosemonophosphate shunt activity reflects an increase in NADPH turnover which is attributable in part to the pyridine nucleotide requirement for the O_2^--forming reaction, and in part to the activity of the glutathione peroxidase-glutathione reductase cycle, a metabolic pathway whose function seems to be to detoxify any H_2O_2 which diffuses back into the cytosol of the neutrophil (15, 16):



 J. Clin. Invest. © *The American Society for Clinical Investigation, Inc., 0021-9738/78/0501-1176 $1.00*

Studies to date demonstrate that the respiratory burst is a self-limited phenomenon. Both oxygen uptake and O_2^- production rates in stimulated neutrophils begin to decline 20–30 min after the activation event, and by 60 min, have generally fallen to small fractions of the initial rates (3, 7, 17). The limitation in the duration of the respiratory burst probably has little adverse effect on the bactericidal activity of the neutrophil, since studies have shown that bacterial killing requires only a very brief exposure of the microorganism to the toxic agents produced during the burst (18, 19). On the other hand, the damage inflicted by these toxic agents on surrounding tissues would be limited by the termination of the burst after a defined interval of time.

A number of explanations can be offered for the decline in burst activity over time. These include depletion of substrate, inactivation of an essential enzyme, or general loss of integrity of the cell. In this paper, we report studies suggesting that at least one cause for the decline in the burst is a loss in activity of the particulate O_2^--forming enzyme. Evidence is also presented concerning the mechanisms responsible for the disappearance of this activity.

METHODS

NADPH (preweighed vials), cytochrome *c* (type VI), methimazole, diethyldithiocarbamate, dianisidine·2HCl, *N*-ethylmaleimide (NEM),[1] cytochalasin B, and zymosan were purchased from Sigma Chemical Co., St. Louis, Mo. Superoxide dismutase was obtained from Truett Laboratories, Dallas, Tex. Phosphate-buffered saline, with and without Ca^{++} and Mg^{++}, was prepared as described elsewhere (13). Hanks' balanced salt solution, 1X without phenol red, was purchased from the Grand Island Biological Co., Grand Island, N. Y. Other reagents were the best grade commercially available, and were used without further purification. Cytochalasin B was stored at −70°C as a stock solution in dimethylsulfoxide (50 mg/ml); cytochrome *c* and diethyldithiocarbamate were also stored at −70°C. Solutions of inhibitors were prepared fresh each day. Diethyldithiocarbamate was dissolved in water. Other inhibitor solutions were prepared as described elsewhere (20).

Neutrophils, particles, and O_2^- production. Human neutrophils were prepared as described previously (13). Activation with opsonized zymosan was accomplished by the method of Hohn and Lehrer (21), except for alterations in the duration of exposure to zymosan and the addition of various inhibitors as indicated below. 27,000-*g* particles from both resting and activated neutrophils were obtained as described by Hohn and Lehrer (21). The particles were washed once with 0.34 M sucrose adjusted to pH 7.4 with $NaHCO_3$, and suspended in the same medium to a final concentration of 0.8–1.0 mg particle protein/ml. O_2^- production by particles was determined at pH 7.0 according to a previously described method (13), using 0.1 mM NADPH as electron donor.

Anerobic incubations. Anerobic incubations were conducted in Warburg flasks fitted in such a way that gassing, equilibration, and incubation could be conducted while the vessel and fittings were completely submerged in a 37°C water bath. For these experiments, siliconized 15-ml Warburg flasks with single sidearms were used. The cells (2.5×10^7 in 1.5 ml phosphate-buffered saline) were placed in the main compartment of the flask, and zymosan (6 mg in 0.5 ml phosphate-buffered saline) in the sidearm. The mouth of the flask was stoppered with a syringe port, and the sidearm stoppered with a venting plug cut sufficiently short so the outlet would be under water during the incubation. The venting plug was attached to a nitrogen source by means of a rubber tube, and the syringe port was pierced by a 20-gauge needle connected to a short length of plastic intravenous tubing. The whole assembly was placed on a low rocking platform designed specifically for this purpose which was placed in the bottom of a Thelco model 83 water bath (Thelco Corp., Englewood, Colo.) and driven by means of an Ames aliquot mixer (Ames Co., Div. of Miles Lab, Inc., Elkhart, Ind.) to which it was connected by a long rod. The water level was such that the needle hub and the venting plug outlet were submerged, and the plastic tubing was arranged so that its remote end was under water as well. The rocking motor was turned on, and the flask was gassed with nitrogen for 7 min. Gassing was terminated by first closing the venting plug, then removing the needle from the syringe port; this procedure ensured that the flask would contain nitrogen at atmospheric pressure. After incubating for 3 more min to establish equilibrium between the constituents in the flask and the overlying atmosphere, the contents of the sidearm were tipped into the main compartment, taking care to keep the reaction vessel completely submerged. The flask was placed back on the rocking platform and the incubation continued for the desired length of time. The incubation was terminated by removing the flask from the water bath and immediately placing it in melting ice. The syringe port was then removed and the contents of the flask, now chilled, was transferred to a homogenizer for further work.

To confirm that anerobiosis was maintained in these experiments, measurements were made of O_2^- production by neutrophils incubated under these conditions for 60 min.[2] With opsonized zymosan as the respiratory burst stimulus, O_2^- production by whole cells was negligible under nitrogen, whereas values obtained under room air were comparable to those previously reported (6). These results indicate that this technique is suitable for conducting incubations under defined atmospheres.

Phagocytosis. Phagocytosis was measured using opsonized zymosan as the ingestible particle. A reaction mixture containing neutrophils, opsonized zymosan, and other constituents as noted was incubated at 37°C in a Dubnoff metabolic shaking incubator. At the desired time, a 0.1-ml portion was withdrawn and added to 0.1 ml of ice-cold NEM solution (2 mM, in Hanks' balanced salt solution) to stop phagocytosis; the remainder of the reaction mixture was discarded. To the portion in NEM was added 2 ml of a 0.25-M sucrose solution containing 0.1 M EDTA, 5 μM colchicine, and 1% (wt/vol) bovine serum albumin. The sucrose solution was at room temperature when added, and subsequent steps were carried out at room temperature. After agitating the diluted sample (Vortex Omnimixer, Scientific Industries, Inc., Bohemia, N. Y.) to disrupt clumps, the cells in a 0.4-ml portion were deposited on a slide by cytocentrifuge. The slide was dried in air, and the cells fixed for 10 min in absolute ethanol:37% aqueous formaldehyde (9:1 [vol/vol]). The zymosan was stained with periodic acid-Schiff, and the cells were counter-stained with

[1] *Abbreviation used in this paper*: NEM, *N*-ethylmaleimide.

[2] Gabig, T. G., S. I. Bearman, B. J. McMurrich, and B. M. Babior. Manuscript in preparation.

methyl green (2% aqueous solution for 5 min) followed by fast green (2% aqueous solution for 5 min). The number of zymosan particles in contact with each of 50 cells was then determined microscopically.

For electron microscopy, the same procedure was followed through the termination of phagocytosis by NEM. At this point, instead of sucrose solution, glutaraldehyde (0.2 ml of 2.5% glutaraldehyde in 1.0 M sodium phosphate buffer [pH 7.2]) was added to the sample. The cells were fixed for 30 min at 4°C, then prepared for electron microscopy as described by André-Schwartz et al. (22). Ultrathin sections were examined with an Hitachi HU-11E electron microscope (Hitachi America, Ltd., San Francisco, Calif.) operated at 75 kV.

Other assays. Myeloperoxidase activity in particles in the presence or absence of inhibitors was assayed by a modification of the method of Bretz and Baggiolini (23). Particles from resting neutrophils (0.39 mg protein in 1.0 ml sucrose) were preincubated for 2 min at 37°C with 0.01 ml of a 0.1-M solution of the agent to be tested. Control particles were incubated with 0.01 ml of water. Myeloperoxidase was then assayed by adding a 0.1-ml portion of the preincubation mixture to 1.0 ml of a solution containing the agent to be tested (1 mM), dianisidine·HCl (0.32 mM), H_2O_2 (0.08 mM), and Triton X-100 (0.05%) in 0.1 M sodium citrate buffer (pH 5.5). After incubating for 1 min at room temperature, the reaction was stopped with 1.0 ml of 35% $HClO_4$. The precipitate was removed by centrifugation, and the absorbance read at 560 nm. Under these conditions, the absorbance measured in the absence of inhibitor was linear with particle concentration.

Protein was measured by the method of Lowry et al. (24).

RESULTS

Studies published to date have been performed with particles obtained from neutrophils activated for 3 min with opsonized zymosan. We have now found that longer periods of activation result in particles with progressively diminishing O_2^--forming activity (Fig. 1). This reduction in activity does not represent decay of the O_2^--forming enzyme after preparation of the particles, because (*a*) the activity in particles from cells exposed to zymosan for 3 min and then stored on ice for various periods before homogenization is equal to that from cells homogenized immediately after activation, and (*b*) the O_2^--forming activity of the isolated particles is stable for several hours at 0°C. It appears rather that the activity of the O_2^--forming enzyme declines in the intact cells when they are incubated with zymosan for periods longer than 3 min.

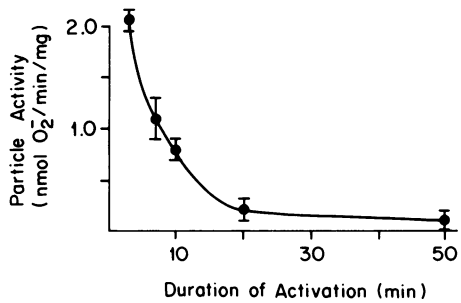


FIGURE 1 O_2^- formation by particles from neutrophils incubated with zymosan for various periods of time. Neutrophils were prepared as described in the text. For each experimental point, a reaction mixture containing 6×10^7 of these cells plus 8 mg opsonized zymosan in a total volume of 4 ml was incubated at 37°C for the time indicated. The reaction mixture was then placed on ice, and particles were prepared and assayed as described in Methods. The data shown represent the mean±1 SE of three experiments, each conducted with a different neutrophil preparation.

Evidence regarding a possible mechanism for this loss in O_2^--forming activity has been provided by studies performed in Klebanoff's laboratory with myeloperoxidase-deficient neutrophils (25, 26). Stimulation of these cells leads to substantially greater rates of oxygen uptake, H_2O_2 formation, and O_2^- production as compared to normal cells. With O_2^-, it has been shown that initial rates of formation are comparable in the two cell types, but that the termination of the respiratory burst is delayed in myeloperoxidase-deficient cells (25). This finding suggests that the myeloperoxidase system, known to be important in bacterial killing by the neutrophil (1, 2, 26), is somehow involved in the late decline of the respiratory burst. If this decline is caused by a loss in the activity of the O_2^--forming enzyme, then the elimination of the myeloperoxidase system should preserve the O_2^--forming activity just as it preserves the respiratory burst. This possibility was tested by examining the effect of NaN_3, a powerful inhibitor of myeloperoxidase (25), on the O_2^--forming activity of particles from zymosan-treated neutrophils. O_2^- formation by particles from cells activated for 7 min in the presence of 1 mM NaN_3 was 13.2±2.1 SE nmol/min per mg protein, as compared with 4.5±0.9 nmol/min per mg protein for particles from the same cells similarly activated in the absence of azide ($P < 0.05$ by Student's paired t test; $n = 4$). This does not appear to be due to an effect of azide on the interaction between the neutrophils and the particles, since kinetics of phagocytosis were the same in the presence and absence of 1 mM NaN_3 (Fig. 2). The presence of 1 mM NaN_3 during the entire experiment (i.e., during neutrophil isolation, incubation with zymosan, and the preparation and assay of the particles) resulted in particulate O_2^--forming activity similar to that seen when NaN_3 was present only during the incubation with zymosan. Thus, the O_2^--forming activity of particles from zymosan-activated neutrophils is substantially increased when activation takes place in the presence of a myeloperoxidase inhibitor.

NaN_3, however, affects enzymes other than myeloperoxidase and similar heme proteins. It is known to inhibit a number of copper oxidases, including laccase (27), benzylamine oxidase (28), and ceruloplasmin (29). To confirm that the effect of NaN_3 on the particulate O_2^--forming activity is mediated through the inhi-

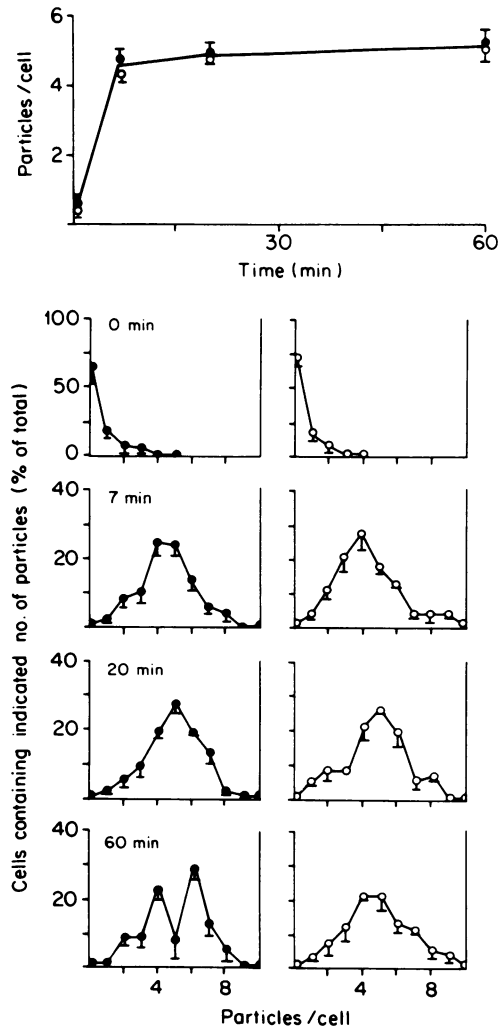


FIGURE 2 Effect of azide on phagocytosis by neutrophils. Phagocytosis was measured as described in Methods. The reaction mixtures contained 6×10^7 cells plus 8 mg of opsonized zymosan in 4 ml of Hanks' balanced salt solution. NaN_3 (1 mM) was also present where indicated. The uppermost panel shows the average number of zymosan particles per cell (mean±1 SE) as a function of time. In the lower panels are shown the distributions of the neutrophil population (mean±1 SE) with respect to the number of cell-associated zymosan particles at the times chosen for assay. The results shown represent three experiments, each using cells from a different subject. ●, N_3^- present; ○, no N_3^-.

bition of myeloperoxidase and not by way of an effect on a copper oxidase, other inhibitors with various specificities were examined. Two of these agents—namely, cyanide and methimazole—are recognized as inhibitors of myeloperoxidase (26), but like azide they are also antagonists of certain copper oxidases (30–32). The other two agents, 1,10-phenanthroline and diethyldithiocarbamate, are known to inhibit copper oxidases (33), the latter recently having been shown to be a highly effective inhibitor of Cu-Zn superoxide dismutase (34), but neither is known to inhibit myeloperoxidase. The activity of the particulate O_2^--forming system isolated from neutrophils incubated for 7 min with zymosan in the presence of each of these inhibitors is shown in Table I. Also shown is the effect of each agent on myeloperoxidase. Large increases over control O_2^--forming activity are seen with azide, cyanide, and methimazole, each a known inhibitor of myeloperoxidase. A similar increase is seen with diethyldithiocarbamate, an agent which on direct examination proved to be a powerful inhibitor of myeloperoxidase. With 1,10-phenanthroline, the only one of all the agents tested which had no effect on myeloperoxidase, the activity of the particles was similar to control. The ability of these agents to augment the O_2^--forming activity of the particles thus correlates with their ability to inhibit myeloperoxidase.

These experiments indicate that myeloperoxidase is involved in the decline in particulate O_2^--forming activity that occurs after activation. That myeloperoxidase is not the only factor responsible for this decline is shown by the results of an experiment in which the O_2^--forming system from cells activated in the presence of azide was measured at various intervals after activation (Fig. 3). Comparison with the curve in Fig. 1 shows a minor but reproducible difference in the early time-course, in that the activity of the particles from the azide-treated cells was relatively constant for 10 min, whereas the particles from cells incubated without azide lost activity rapidly after 3 min. The major finding, however, is that the late fall in activity took place under both sets of conditions, little O_2^--forming enzyme being detected in particles from either azide-treated or control cells after 50 min.

TABLE I
O_2^- Formation by Particles from Neutrophils Activated in the Presence of Inhibitors

Agent	Particle O_2^- production	Myeloperoxidase activity
	nmol/min/mg protein	A_{560}
None	3.2	0.069
KCN	14.0	0.006
NaN_3	10.1	0.006
Methimazole	19.2	0.006
Diethyldithiocarbamate	11.8	0.004
1, 10-Phenanthroline	2.4	0.073

Neutrophils were activated for 7 min as described in Fig. 1, but in the presence of the agents indicated (1 mM final concentration). The particles were prepared and assayed as described in the text. Myeloperoxidase activity in the presence of these agents was determined by the procedure outlined in Methods. The data shown are representative of four experiments.

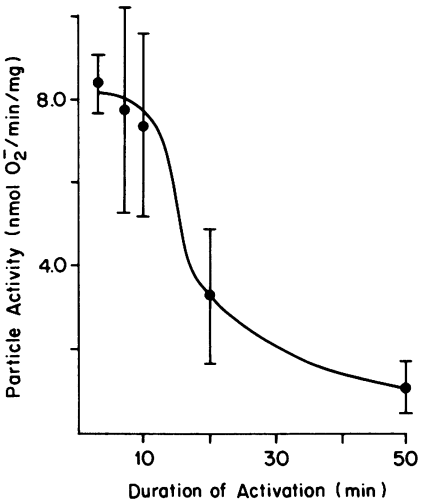


FIGURE 3 O_2^- formation by particles from neutrophils activated in the presence of azide. The experiment was carried out exactly as described in Fig. 1, except that 1.0 mM NaN_3 was present during activation. The data shown represent the mean±SE for three experiments.

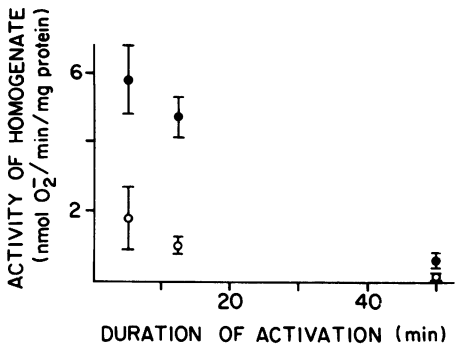


FIGURE 4 O_2^- formation by whole neutrophil homogenates. Neutrophils were activated with zymosan for the times indicated, in the presence (●) or absence (○) of 1 mM N_3^-. Other conditions for activation were as described in Fig. 1. Homogenates were obtained by the method used for preparing particles, except that the final centrifugation at 27,000 *g* was omitted. The whole homogenates (0.1–0.2-ml portions) were assayed by the technique used for determining the O_2^--forming activity of particles, as described in Methods. The data shown represent the mean±1 SE of three experiments, each conducted with a different neutrophil preparation.

Thus, the particulate O_2^--forming activity decayed even under conditions in which myeloperoxidase was largely nonfunctional.

Although myeloperoxidase is not essential for the late decline in the activity of the O_2^--forming enzyme, oxygen may still be required. Evidence is accumulating (1, 2, 9, 35–39) that neutrophils may be able to generate from oxygen certain extremely reactive substances, including hydroxyl radical and singlet oxygen, and it is conceivable that these substances are responsible for the inactivation of the O_2^--forming system by means which are independent of myeloperoxidase. To investigate this possibility, measurements were made of O_2^- production by particles isolated from neutrophils incubated with zymosan under an atmosphere of nitrogen. The results are shown in Table II. Particles isolated after a 7-min period of activation under nitrogen produced much more O_2^- than particles activated in air. This finding provides further evidence that the elimination of the myeloperoxidase system preserves the O_2^--forming activity of the granules; in this particular experiment, the system was eliminated by preventing the formation of

TABLE II
O_2^- Formation by Particles from Neutrophils Activated under Nitrogen

Atmosphere	Particle O_2^- production	
	7-min activation	60-min activation
	nmol/min/mg protein	
Air	2.0±0.4*	0.9±0.6
Nitrogen	8.6±1.7	1.9±0.5

Neutrophils were activated for the times indicated under defined atmospheres by the procedure described in Methods. This procedure was employed for both anerobic and aerobic activation, gassing aerobic reaction vessels with air, and anerobic vessels with nitrogen. Particles from activated neutrophils were prepared and assayed as described in the text. The data shown represent the results of five experiments, each conducted with a different neutrophil preparation.
* Mean±1 SE.

TABLE III
O_2^- Formation by Particles from Neutrophils Activated in the Presence of Cytochalasin B

Activation conditions	Particle O_2^- production	
	Experiment 1	Experiment 2
	nmol/min/mg protein	
Cytochalasin B present		
Activated 7 min	21.2	12.1
Activated 60 min	26.3	7.7
No cytochalasin B		
Activated 7 min	31.7	26.3
Activated 60 min	4.9	2.8

Neutrophils were activated for the times indicated in the presence or absence of cytochalasin B (final concentration 1.5 μg/ml). Controls were supplemented with dimethylsulfoxide equivalent to the amount present in the cytochalasin-containing incubations (see Methods). NaN_3 (1 mM) was present in all incubations. Particles from activated neutrophils were prepared and assayed as described in the text.

one of its substrates (i.e., H_2O_2) rather than by inhibiting the enzyme itself. However, even under nitrogen most of the activity was gone by 60 min. Thus, oxygen is not required for the process leading to the loss of particle O_2^--forming activity after 60 min.

It is possible that the late decline in particulate O_2^--forming activity results from the death of the cell. However, 95% of the cells excluded trypan blue after a 60-min incubation with opsonized zymosan, indicating that, at least by this criterion, the cells were able to survive under the conditions of the experiment.

Another explanation for the late decline would be the transfer of the O_2^--forming activity to a subcellular fraction which is not sedimentable at 27,000 *g*. This possibility was tested by examining the O_2^--forming capacity of whole homogenates from cells activated with zymosan. These experiments (Fig. 4) showed that the O_2^--forming activity of whole homogenates fell with time in a manner similar to the O_2^--forming activity of particles. The fall was seen whether or not N_3^- was present during activation. Thus, the decline in O_2^- production by particles represents a true loss in the activity of the O_2^--forming system, not a transfer of the system from one subcellular compartment to another.

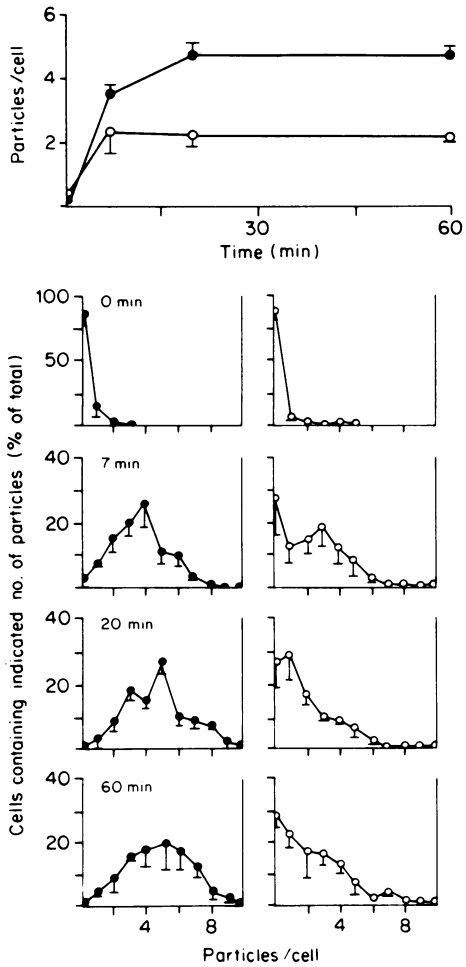


FIGURE 5 Effect of cytochalasin B on phagocytosis by neutrophils. The experiments were conducted exactly as described in Fig. 2. NaN_3 (1 mM final concentration) was present in all incubations. Cytochalasin B plus dimethylsulfoxide (final concentration 1.5 μg cytochalasin B/ml), or an equivalent volume of dimethylsulfoxide alone, were present as indicated. The data shown represent the mean ±SE for three experiments. ○, cytochalasin B; ●, dimethylsulfoxide only.

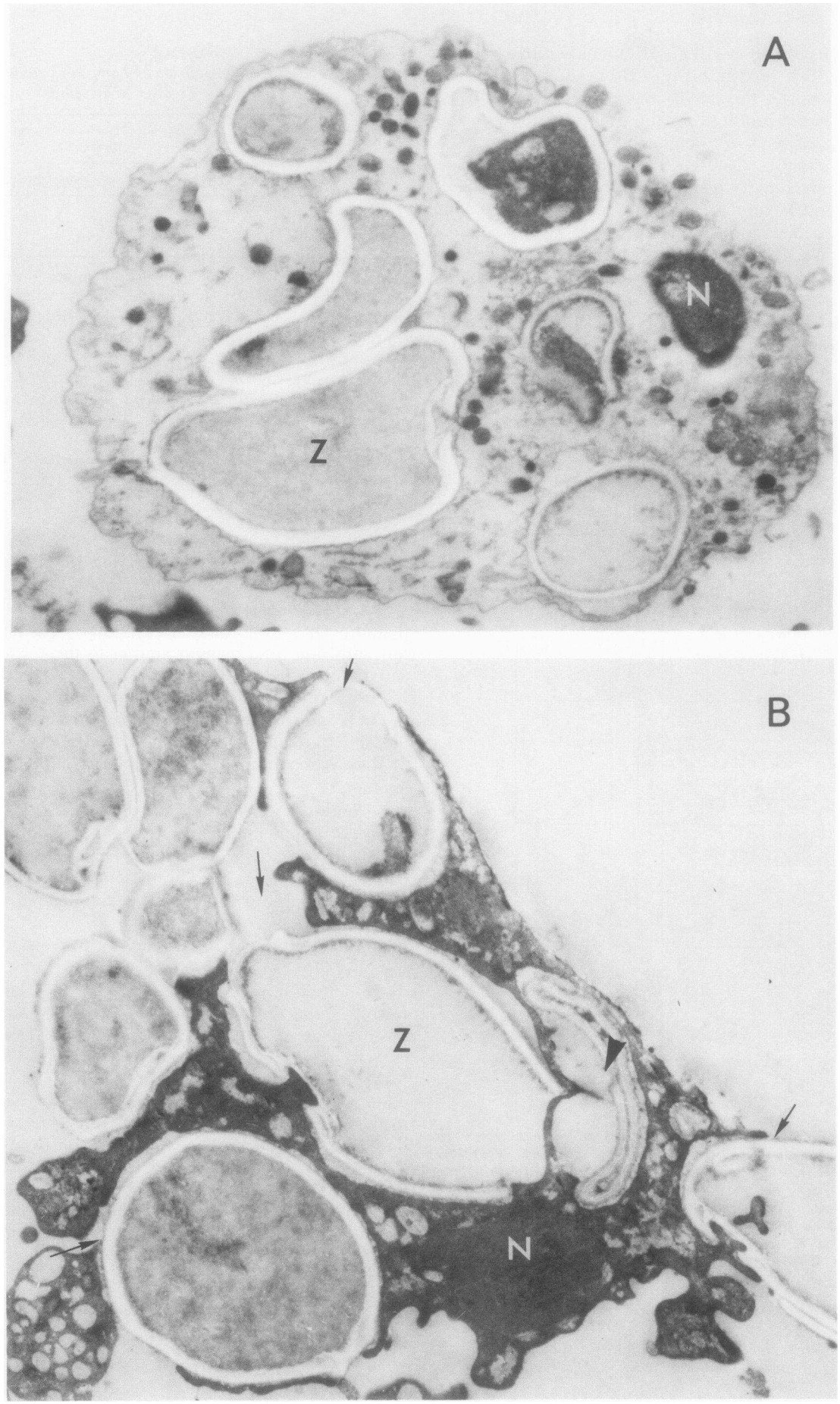
TABLE IV
Electron Microscope Analysis of Zymosan Uptake by Cytochalasin B-Treated Neutrophils

Agent	Zymosan profiles/cross section (Mean±1 SE)	
	Total	Completely enclosed
None	2.7±0.2	2.1±0.2
Cytochalasin B	2.1±0.2	0.7±0.1

The reaction mixtures contained 6×10^7 neutrophils plus 8 mg opsonized zymosan in 4 ml of Hanks' balanced salt solution. Cytochalasin B plus dimethylsulfoxide (final concentration 1.5 μg cytochalasin B/ml), or an equivalent volume of dimethylsulfoxide alone, were present as indicated. Incubations were conducted at 37°C for 7 min. Phagocytosis was then stopped with NEM and the samples prepared for electron microscopy as described in Methods. For analysis of phagocytosis, zymosan profiles in 50 consecutive neutrophil cross sections were counted and classified as to whether or not they were completely surrounded by cytoplasm.

To understand the mechanism by which this loss occurs, a number of maneuvers were undertaken to try to preserve the O_2^--forming system in cells incubated with zymosan for 60 min. Incubating the cells with glucose (1 mg/ml) to replenish substrates, or in the presence of colchicine (25 μM), an inhibitor of microtubule polymerization, did not prevent the loss in the ability of the particles to generate O_2^-. O_2^- production at 60 min was not restored by the addition of freshly opsonized zymosan 7 min before terminating the incubation. However, as shown in Table III, cytochalasin B at low concentrations prevented the loss of the O_2^--forming activity at 60 min. In this experiment, O_2^- production by particles from cells incubated in the presence of cytochalasin B was approximately the same after 60 min as after 7 min of exposure to zymosan. By contrast, control particles showed the expected fall in activity.

In the hope of providing an explanation for the effect of cytochalasin B on the O_2^--forming system, the interaction between cytochalasin B-treated neutrophils and opsonized zymosan was analyzed in detail. This anal-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ysis showed that a 1.5-μg/ml dose of cytochalasin B reduced the number of cell-associated particles by ≅50% (Fig. 5). This reduction did not appear to be due to a decrease in the rate of interaction of neutrophils with opsonized zymosan, since both the average number of particles per cell (uppermost panel) and the distribution of cells with respect to the number of associated particles (lower panels) remained roughly constant from 7 min on. Rather, there seemed to be a decrease in the maximum number of particles with which the cell could become associated.

Close inspection of the cytochalasin B-treated cells suggested not only that the number of associated zymosan particles was reduced, but also that the nature of the association was abnormal. Specifically, it appeared as if the cytochalasin B-treated cells were unable to internalize particles which had attached to their surface. This impression was confirmed by electron microscopy. Cross sections of cytochalasin B-treated neutrophils exposed to opsonized zymosan for 7 min showed fewer zymosan profiles than normal, and most of these were exposed to the environment. This contrasts with the particles associated with normal cells, most of which were entirely surrounded by cytoplasm (Table IV, Fig. 6).

DISCUSSION

The course of the respiratory burst in neutrophils incubated with opsonized zymosan (3, 6) is mimicked by that of the O_2^--generating capacity of particles isolated from such cells. Both reach maximum levels rapidly, then decline to substantially lower values later in the incubation. These correlations between the behavior of whole cells on one hand and particles on the other suggests that the changes with time in the activity of the particulate O_2^--forming system may to a considerable extent explain the time-course of the respiratory burst.

The decline in particulate O_2^--forming activity which takes place during the incubation of neutrophils with opsonized zymosan seems at least in part to result from inactivation of the O_2^--forming system by the peroxide-halide-myeloperoxidase mechanism, which has been shown by Klebanoff and others to be a powerful effector of bacterial killing in the neutrophil (26). This is indicated by the protection conferred on the O_2^--forming system by anerobic incubation conditions and by inhibitors of myeloperoxidase. The studies with myeloperoxidase-deficient neutrophils referred to above are also consistent with this notion, and suggest that the supranormal H_2O_2 production seen in myeloperoxidase-deficient cells (26) may be due to improved survival of the O_2^--forming system in these cells.

The action of myeloperoxidase, however, does not account for all of the decline in the O_2^--forming capacity of neutrophil particles. This is evident from the experiments carried out for 60 min, in which a sharp fall in particle activity is seen even when the incubations were carried out in the presence of azide, or under nitrogen. These findings indicate the presence of an oxygen-independent mechanism which acts to destroy the O_2^--forming system in neutrophil particles.

Evidence concerning the nature of this oxygen-independent mechanism is provided by the finding that its effect is suppressed by cytochalasin B. Cytochalasin B is an agent with far-reaching effects on cell motility[3] which are thought to be mediated through an interaction with the microfilament network of the cell (42). With neutrophils, these effects are expressed in a number of ways. At very low doses, cytochalasin B eliminates spontaneous locomotion in these cells, while paradoxically stimulating chemotaxis (43, 44). Higher doses abolish chemotaxis and depress phagocytosis; at these doses, bacterial killing is also inhibited (45–47). Of particular interest in terms of the present study is the inability of neutrophils treated with this agent to internalize surface membrane. This was first shown by Ryan et al. (48), who found an impairment in the ability of cytochalasin B-treated cells to internalize and (presumably) degrade surface-bound concanavalin A, a process that normally takes place over the 30 min following exposure of the cells to the lectin. The present study extends those observations to surface-bound particles, showing that an association between opsonized zymosan and cytochalasin B-treated neutrophils sufficient to initiate the respiratory burst can take place without internalization of the particle (and the region of membrane to

[3] Cytochalasin B also inhibits the active transport of glucose (40). It is unlikely that glucose starvation by this mechanism explains the present results, however, because glucose itself did not prevent the loss in activity of the O_2^--forming enzyme, in addition to which neutrophils contain large stores of glycogen on which they can drawn when exogenous glucose is lacking (3, 41).

FIGURE 6 Electron micrographs of zymosan uptake by cytochalasin B-treated and control neutrophils, showing impairment in internalization caused by cytochalasin B treatment. (A) Control (×10,200). All zymosan granules (Z) are surrounded by cytoplasm. A section of nucleus (*N*) is also seen. (B) Cytochalasin B (×11,600). Only one zymosan particle is entirely surrounded by cytoplasm (thick arrow). Other particles (Z) in contact with the cell are exposed to the extracellular environment (thin arrows). Part of the nucleus (*N*) is shown.

which it is attached). It may be that, like the receptor to which surface-bound concanavalin A is attached, the O_2^--forming system of the neutrophil is situated in the plasma membrane (49, 50). If so, the result with cytochalasin B suggests that the oxygen-independent inactivation of this system involves its internalization and degradation by a process related to phagocytosis.

ACKNOWLEDGMENTS

We thank Ms. Margarett Baker for her excellent technical assistance and Dr. H. Q. Campwala for his helpful advice concerning the staining of neutrophils.

This work was supported in part by U. S. Public Health Service grant AI-11827.

REFERENCES

1. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin. Hematol.* **12:** 117–142.
2. Cheson, B. D., J. T. Curnutte, and B. M. Babior. 1977. The oxidative killing mechanisms of the neutrophil. *Prog. Clin. Immunol.* **3:** 1–65.
3. Sbarra, A. J., and M. L. Karnovsky. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.* **234:** 1355–1362.
4. Weening, R. S., D. Roos, and J. A. Loos. 1974. Oxygen consumption of phagocytizing cells in human leukocyte and granulocyte preparations: a comparative study. *J. Lab. Clin. Med.* **83:** 570–576.
5. Oren, R., A. E. Farnham, K. Saito, E. Milofsky, and M. L. Karnovsky. 1963. Metabolic patterns in three types of phagocytizing cells. *J. Cell Biol.* **17:** 487–501.
6. 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.
7. 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.
8. Weening, R. S., R. Wever, and D. Roos. 1975. Quantitative aspects of the production of superoxide radicals by phagocytizing human granulocytes. *J. Lab. Clin. Med.* **85:** 245–252.
9. Johnston, R. B., B. B. Keele, H. P. Misra, J. P. Lehmeyer, L. S. Webb, R. L. Baehner, and K. V. Rajagopalan. 1975. The role of superoxide anion generation in phagocytic bactericidal activity: studies with normal and chronic granulomatous disease leukocytes. *J. Clin. Invest.* **55:** 1357–1372.
10. Iyer, G. Y. N., M. F. Islam, and J. H. Quastel. 1961. Biochemical aspects of phagocytosis. *Nature (Lond.).* **192:** 535–541.
11. Root, R. K., J. Metcalf, N. Oshino, and B. Chance. 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation and some regulating factors. *J. Clin. Invest.* **55:** 945–955.
12. Homan-Müller, J. W. T., R. S. Weening, and D. Roos. 1975. Hydrogen peroxide production by phagocytosing human granulocytes. *J. Lab. Clin. Med.* **85:** 198–207.
13. Babior, B. M., J. T. Curnutte, and B. J. McMurrich. 1976. The particulate superoxide-forming system from human neutrophils. Properties of the system and further evidence supporting its participation in the respiratory burst. *J. Clin. Invest.* **58:** 989–996.
14. Root, R. K., and J. Metcalf. 1976. Initiation of granulocyte (PMN) $O_2^-\cdot$ and H_2O_2 formation by stimulation of membrane phagocytic receptors (PR). *Clin. Res.* **24:** 352A. (Abstr.)
15. Reed, P. W. 1969. Glutathione and the hexosemonophosphate shunt in phagocytizing and hydrogen peroxide-treated rat leukocytes. *J. Biol. Chem.* **244:** 2459–2464.
16. Baehner, R. L., S. K. Murrmann, J. Davis, and R. B. Johnston, Jr. 1975. The role of superoxide anion and hydrogen peroxide in phagocytosis-associated oxidative metabolic reactions. *J. Clin. Invest.* **56:** 571–576.
17. Malawista, S. E., and P. T. Bodel. 1967. The dissociation by colchicine of phagocytosis from increased oxygen consumption in human leukocytes. *J. Clin. Invest.* **46:** 786–796.
18. 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.
19. Simmonds, S. R., and M. L. Karnovsky. 1973. Iodinating ability of various leukocytes and their bactericidal activity. *J. Exp. Med.* **138:** 44–63.
20. Curnutte, J. T., and B. M. Babior. 1975. Effects of anerobiosis and inhibitors on O_2^- production by human granulocytes. *Blood.* **45:** 851–861.
21. Hohn, D. C., and R. I. Lehrer. 1975. NADPH oxidase deficiency in X-linked chronic granulomatous disease. *J. Clin. Invest.* **55:** 707–713.
22. André-Schwartz, J., R. S. Schwartz, M. S. Hirsch, S. M. Phillips, and P. H. Black. 1973. Activation of leukemia viruses by graft-versus-host and mixed-lymphocyte-culture reactions: electron microscopic evidence of C-type particles. *J. Natl. Cancer Inst.* **51:** 507–518.
23. Bretz, U., and M. Baggiolini. 1974. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. *J. Cell Biol.* **63:** 251–269.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
25. Rosen, H. S., and S. J. Klebanoff. 1976. Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. *J. Clin. Invest.* **58:** 50–60.
26. Klebanoff, S. J., and C. B. Hamon. 1972. Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. *J. Reticuloendothel. Soc.* **12:** 170–196.
27. Morpurgo, L., G. Rotilio, A. Finazzi-Agro, and B. Mondovi. 1974. Anion binding to *Rhus vernicifera* laccase. *Biochim. Biophys. Acta.* **336:** 324–328.
28. Lindstrom, A., B. Olsson, and G. Pettersson. 1974. Effect of azide on some spectral and kinetic properties of pig-plasma benzylamine oxidase. *Eur. J. Biochem.* **48:** 237–243.
29. Gunnarsson, P. O., U. Nylen, and G. Pettersson. 1972. Inhibition of ceruloplasmin by inorganic anions. *Eur. J. Biochem.* **27:** 572–577.
30. Speyer, B. E., and G. Curzon. 1969. The inhibition of ceruloplasmin by cyanide. *Biochem. J.* **106:** 905–911.
31. Rotilio, G., R. C. Bray, and E. M. Fielden. 1972. A pulse radiolysis study of superoxide dismutase. *Biochim. Biophys. Acta.* **268:** 605–609.
32. Hanlon, D. P., and S. Shuman. 1975. Copper ion binding and enzyme inhibitory properties of the antithyroid drug methimazole. *Experientia (Basel).* **31:** 1005–1006.
33. Bardsley, W. G., R. E. Childs, and M. J. C. Crabbe. 1974.

Inhibition of enzymes by metal ion-chelating reagents. The action of copper-chelating reagents on diamine oxidase. *Biochem. J.* **137:** 61–66.
34. Heikkila, R. E., F. S. Cabbat, and G. Cohen. 1976. *In vivo* inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J. Biol. Chem.* **251:** 2182–2185.
35. Babior, B. M., J. T. Curnutte, and R. S. Kipnes. 1975. Biological defense mechanisms. Evidence for the participation of superoxide in bacterial killing by xanthine oxidase. *J. Lab. Clin. Med.* **85:** 235–244.
36. Gregory, E. M., and I. Fridovich. 1974. Oxygen metabolism in *Lactobacillus plantarum*. *J. Bacteriol.* **117:** 166–169.
37. 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.
38. Krinsky, N. I. 1974. Singlet excited oxygen as a mediator of the antibacterial action of leukocytes. *Science (Wash. D.C.).* **186:** 363–365.
39. Maugh, T. H., II. 1973. Singlet oxygen: a unique microbicidal agent in cells. *Science (Wash. D.C.).* **182:** 44–45.
40. Lin, S., and J. A. Spudich. 1974. Biochemical studies on the mode of action of cytochalasin B. Cytochalasin B binding to red cell membrane in relation to glucose transport. *J. Biol. Chem.* **249:** 5778–5783.
41. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leukocytes. I. Observations on the requirements and consequences of particle ingestion. *J. Exp. Med.* **111:** 667–687.
42. Wessels, N. K., B. F. Spooner, J. T. Ash, M. O. Bradley, M. A. Luduena, E. L. Taylor, J. T. Wrenn, and K. M. Yamada. 1971. Microfilaments in cellular and developmental processes. Contractile microfilament machinery of many cell types is reversibly inhibited by cytochalasin B. *Science (Wash. D.C.).* **171:** 135–143.
43. Zigmond, S. H., and J. G. Hirsch. 1972. Effects of cytochalasin B on polymorphonuclear leukocyte locomotion, phagocytosis and glycolysis. *Exp. Cell Res.* **73:** 383–393.
44. Becker, E. L., A. T. Davis, R. D. Estensen, and P. G. Quie. 1972. Cytochalasin B. IV. Inhibition and stimulation of chemotaxis of rabbit and human polymorphonuclear leukocytes. *J. Immunol.* **108:** 396–402.
45. Davies, P., R. I. Fox, M. Polyzonis, A. C. Allison, and A. D. Haswell. 1973. The inhibition of phagocytosis and facilitation of exocytosis in rabbit polymorphonuclear leukocytes by cytochalasin B. *Lab. Invest.* **28:** 16–22.
46. Davies, A. T., R. Estensen, and P. H. Quie. 1971. Cytochalasin B. III. Inhibition of human polymorphonuclear leukocyte phagocytosis. *Proc. Soc. Exp. Biol. Med.* **137:** 161–164.
47. Malawista, S. E., J. B. L. Gee, and K. Bensch. 1971. Cytochalasin B reversibly inhibits phagocytosis: functional, metabolic and ultrastructural effects in human blood leukocytes and rabbit alveolar macrophages. *Yale J. Biol. Med.* **44:** 286–300.
48. Ryan, G. B., J. Z. Borysenko, and M. J. Karnovsky. 1974. Factors affecting the redistribution of surface-bound concanavalin A on human polymorphonuclear leukocytes. *J. Cell Biol.* **62:** 351–365.
49. Goldstein, I. M., D. Roos, H. B. Kaplan, and G. Weissmann. 1975. Complement and immunoglobulin stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* **56:** 1155–1163.
50. Goldstein, I. M., M. Cerquiera, S. Lind, and H. B. Kaplan. 1977. Evidence that the superoxide-generating system of human leukocytes is associated with the cell surface. *J. Clin. Invest.* **59:** 249–254.