Alveolar macrophages can be stimulated by concanavalin A to produce extracellular superoxide. Conflicting opinions exist, however, concerning the relative importance of the oxidation of either NADPH or NADH in the generation of O$_2^-$ by surface membrane-stimulated phagocytic cells.

Alveolar macrophages were obtained from adult male rats by lavage with phosphate-buffered saline. Cells (~10^6/ml) were incubated in Krebs-Ringer phosphate 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and ferricytochrome c for 15 min at 37°C before addition of concanavalin A. Release of O$_2^-$ was detected as the difference in cytochrome c reduction, followed at 550 nm, in the absence and presence of superoxide dismutase.

Superoxide production by concanavalin A-stimulated alveolar macrophages was markedly increased in the presence of glucose but fructose, lactate, and pyruvate were without effect. Paraquat (methylviologen), an oxidation-reduction dye, significantly reduced concanavalin A-stimulated O$_2^-$ production when incubated at 1 mM with alveolar macrophages in the absence of glucose. The effect of paraquat was reversed by glucose, but fructose, lactate, and pyruvate could not reverse paraquat inhibition. Paraquat enhanced oxidation of NADPH (but not NADH) by cell supernatant and increased pentose phosphate shunt activity in resting macrophages, but did not affect mitochondrial respiration or ATP content of alveolar macrophages. These results suggest that paraquat is able to specifically deplete NADPH in alveolar macrophages while not affecting NADH or ATP. Our conclusion is that NADPH is essential for the production of O$_2^-$ by concanavalin A-stimulated alveolar macrophages.

Alveolar macrophages, as well as other phagocytic cells, can be stimulated by surface membrane reactive agents to release O$_2^-$ into the surrounding medium (1, 2). Agents such as concanavalin A, opsonized zymosan, and digitonin have been used to provoke the superoxide producing activity (3-5). That the superoxide production is extracellular has been suggested by the reduction of exogenous cytochrome c and its inhibition by exogenous superoxide dismutase, proteins which do not cross the cell membrane.

A debate concerning the nature of the O$_2^-$-producing reaction has focused on whether it is NADH- or NADPH-dependent. Sharra and Karnovsky (6) first showed that glucose utilization through the pentose phosphate pathway increases in phagocytic cells during the "respiratory burst," with which O$_2^-$ release is associated, but that glycolysis was insignificantly affected. This was subsequently verified in alveolar macrophages (3, 7, 8). Nevertheless, both NADH and NADPH oxidases have been identified and proposed as the source of O$_2^-$. (9-12).

Paraquat, also known as methylviologen, is an oxidation-reduction dye, which has been shown to be reducible to paraquat radical by a NADPH-dependent microsomal preparation from lungs (13). Oxidized paraquat is rapidly regenerated by auto-oxidation of the paraquat radical (14). In the course of our studies, we found that paraquat incubation with alveolar macrophages inhibited Con A-stimulated O$_2^-$ production by these cells. We hypothesized that this was due to depletion of NADPH.

We will show in this paper that (a) Con A-stimulated O$_2^-$ production by alveolar macrophages depends specifically on glucose rather than other glycolytic intermediates; (b) paraquat can deplete NADPH in the absence of glucose and thereby inhibit the respiratory burst; (c) paraquat inhibition can be reversed by glucose which restores NADPH; and (d) other substrates, which could supply NADH and ATP, cannot reverse paraquat inhibition. Although we have not ruled out roles for NADH or ATP in the respiratory burst or associated phenomena, we believe that a more specific role for NADPH in O$_2^-$ production has been demonstrated.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cytochrome c (type VI), Con A, paraquat, and oligomycin were obtained from Sigma Chemical Co., St. Louis, MO. Superoxide dismutase was obtained from Diagnostic Data, Inc., Mountainview, CA. [methyl-^14C]Paraquat was obtained from Amer sham, Inc., Arlington Heights, IL. [U-^14C]Glucose, [^3H]polyethylene glycol, and Hyamine hydroxide were obtained from New England Nuclear, Inc., Boston, MA.

Paraquat solutions were adjusted to pH 7.4 before use. Preparation of Alveolar Macrophages—Alveolar macrophages were prepared from Sprague-Dawley rats weighing approximately 250 g. Animals were euthanized by intraperitoneal injection with pentobarbital (100 mg/kg). Alveolar macrophages were prepared by alveolar lavage (15). Lungs were filled with KRH buffer gently massaged and allowed to drain through the trachea. The fluids from 5 separate lavages were combined. The total lavage volume for each animal was 50 ml. The suspending medium containing the cells was filtered sequentially through HC-3-37 and HC-3-15 nylon bolting cloth and
then centrifuged at 4°C in plastic tubes for 5 min at 280 X g. The macrophage pellet was resuspended in KRH. Cells were counted with a hemocytometer (American Optical Corp., Buffalo, NY). The concentration of cells in the final suspension was approximately 1.5 X 10^7 cells/ml. Cells were maintained on ice for 1 to 2 h prior to use. This temporary storage had no significant effect on the respiratory burst.

**Assay of Stimulated \( O_2^- \) Production**—The reduction of 5 X 10^-5 M cytochrome c in the presence and absence of superoxide dismutase (followed at 550 nm) was used to measure stimulated \( O_2^- \) production. The single-beam spectrophotometric assay of Cohen and Chovancie (16) was used with the following modifications. Con A (250 \( \mu \)g/ml) rather than digitonin was used to stimulate approximately 1 X 10^6 alveolar macrophages per assay. The small nonstimulated baseline rate of cytochrome c reduction was subtracted. All rates were expressed as the amount of \( O_2^- \) produced in 4 min following the end of the lag phase (16).

Routine ly, the cells, cytochrome c, and buffer were incubated for 15 min in the presence or absence of various substances before the addition of Con A.

**Measurements of Oxygen Uptake**—In preparation for measurement of \( O_2 \) uptake, the cell suspension was warmed to 37°C and equilibrated with room air. Oxygen uptake was measured polarographically using a Clark electrode in a stirred cuvette with a volume maintained at 37°C. Oxygen utilization was calculated from the continuously monitored decrease in PO2 of the suspending medium. The O2 solubility of the suspending buffer was assumed to be 220 nmol of O2/ml.

**Measurement of ATP**—ATP measurements were made on alveolar macrophages after incubation in the absence or presence of glucose and paraquat. The cells were lysed with perchorolic acid, neutralized, and then centrifuged. Supernatants were assayed with glucose, hexokinase, NADP, and glucose-6-phosphate dehydrogenase as described by Williamson and Corkey (17).

**Measurement of Paraquat Uptake**—2 X 10^6 alveolar macrophages were incubated in 1 ml with 1 mM \[^{14}C\]paraquat (specific activity, 0.26 mCi/mmol) and 1.4 X 10^-4 M \[^{3}H\]polyethylene glycol (specific activity, 0.7 mCi/mmole, average molecular weight = 4000) in KRH at 37°C in the presence or absence of 5 mM glucose for the times indicated. The cells were centrifuged at 4°C, washed, and counted. Intracellular [methyl-\(^{14}\)C]paraquat counts were calculated using non-permeable \[^{3}H\]polyethylene glycol to correct for the extracellular counts. Using the mean internal water space of alveolar macrophages (0.36 \( \mu \)l/10^6 cells (18), a value could be derived for the intracellular concentration.

**Measurement of \(^{14}CO_2\) Production from \[^{1-^{14}}C\]Glucose**—One-milliliter aliquots of cell suspension (7.6 X 10^6 alveolar macrophages) in KRH were equilibrated at 37°C for 15 min in a Dubnoff shaking incubator at 60 cycles/min in the presence or absence of 1 mM paraquat. \[^{1-^{14}}C\]Glucose was added to each flask to give a glucose concentration of 5 mM with a specific activity of 0.4 mCi/mmol. At the end of an 15-min incubation, 0.5 ml of perchloric acid (12%) was added to the cell suspension and 0.3 ml of Hymine hydroxide was injected into a plastic disposable center well in each flask. Shaking was continued for 30 min to trap the liberated \(^{14}CO_2\), and the entire center well was then transferred to scintillation fluid (2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazolyl) (POPOP) in toluene). Counts were made in a Packard scintillation counter, and disintegrations per min were calculated using a quench curve.

**NADPH-dependent Paraquat Reduction**—Cells were sonicated with a Sonic Dismembrator (Artrek, Farmingdale, NY) with a microtip and spun at 5000 X g for 5 min. Aliquots of supernatant were incubated in KRH with varying amounts of paraquat, NADPH, or other substances indicated in the experimental section. To demonstrate reduction of paraquat, buffers and other materials were equilibrated with N2, oil was layered on top, and the absorbance at 600 nm was followed. To measure NADPH oxidation, absorbance at 340 nm was followed during aerobic incubation.

**Statistics**—Results are given as mean ± S.E. Student t test for independent or dependent variables were used as indicated (19).

### RESULTS

**Effect of Glucose on \( O_2^- \) Production**—Superoxide production caused by addition of Con A to alveolar macrophages was enhanced by the addition of glucose to the incubation medium either at the beginning of the 15-min incubation or when added simultaneously with Con A. The first column in Table I indicates the rate for \( O_2^- \) production stimulated by addition of Con A in the presence and absence of glucose. Although the rate in the absence of glucose was quite variable, the range obtained with 5 mM glucose was considerably narrower. This suggests that the endogenous concentration of some product from glucose metabolism which was required for the respiratory burst was variable in non-glucose-incubated cells and could reach a saturating level in the presence of glucose.

**Effect of Paraquat on \( O_2^- \) Production**—Alveolar macrophages incubated in the absence of glucose to which 1 mM paraquat was added 15 min prior to Con A became even more dependent on glucose addition for Con A-stimulated \( O_2^- \) production (Table I). The percentage of inhibition by paraquat incubation in the absence of glucose varied from 43 to 100%; however, in the presence of 5 mM glucose, paraquat had no effect. Whether glucose was added either to the 15 min incubation or simultaneously with Con A made no difference. Thus, it appears that paraquat can deplete some substance from cells which is required for Con A-stimulated \( O_2^- \) production but that glucose can rapidly restore that substance.

**TABLE I**

| Glucose | Nanomoles \( O_2^- \) produced/4 min/10^6 cells |
|---------|---------------------------------------------|
| Paraffin absent | Paraffin incubated |
| None | 0.184 ± 0.003a | 0.068 ± 0.015a |
| In assay only | 0.437 ± 0.022a | 0.409 ± 0.017a |
| In incubation | 0.412 ± 0.019b | 0.346 ± 0.034c |

*Results are mean ± S.E.

b \( p < 0.001 \) in paired t test with alveolar macrophages incubated and assayed in the absence of glucose and paraquat.

Numbers in parentheses, number of experiments.
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The effect of paraquat incubation was time dependent. No inhibition was observed when paraquat was added simultaneously with Con A (five experiments), but the time required to develop inhibition was quite variable. The minimum time observed for 100% inhibition was 10 min. A typical experiment is shown in Fig. 1. The results suggest that the effect of paraquat is on metabolism rather than on the surface O₂⁻ generation that may occur in our measurements.

Effect of Glycolytic Intermediates—Lactate, pyruvate, and fructose were not able to enhance the rate of stimulated O₂⁻ production. These substances were also unable to reverse or prevent paraquat inhibition. The lack of effect was observed whether these substances were incubated with the cells as shown in Table II or added simultaneously with Con A (data not shown). These results in comparison with the positive effects of glucose suggest that glucose rather than one of its glycolytic products can provide the necessary substances for the respiratory burst and that probably neither NADH nor ATP was the paraquat-depleted substance.

Effect of Paraquat on Metabolism—To further substantiate that the effect of paraquat was on metabolism, paraquat uptake by isolated cells was measured. The intracellular concentration of paraquat rapidly approached saturation when alveolar macrophages were incubated with 1 mM paraquat in the presence of 1 mM paraquat (see Table I). 5 mM lactate, 5 mM pyruvate, or 5 mM fructose were added at the beginning of incubation. The effect of lactate, pyruvate, or fructose was compared with a paired control (absence of lactate, pyruvate, or fructose) in the presence or absence of paraquat inhibition.

| % control activity | −Paraquat | +Paraquat |
|--------------------|----------|----------|
| Lactate            | 92.3 ± 6.8b | 99.1 ± 7.5 |
| Pyruvate           | 103.9 ± 3.7 | 98.6 ± 8.7 |
| Fructose           | 106.7 ± 3.5 | 87.0 ± 6.8 |

† *p < 0.05 for all comparisons.
‡ Results are mean ± S.E.
§ Numbers in parentheses, number of trials.

Table III

Effect of paraquat on alveolar macrophages metabolism

The effect of paraquat incubation and glucose on O₂ consumption, ATP content, and CO₂ production were measured as described in "Experimental Procedures."

| O₂ uptake | ATP content | CO₂ production from carbon-1 of glucose |
|-----------|-------------|----------------------------------------|
| Glucose   | −Glucose    | +Glucose | −Glucose | +Glucose | −Glucose | +Glucose |
| −Paraquat | 3.62 ± 0.43* | 3.88 ± 0.20 | 0.92 ± 0.07 | 1.02 ± 0.24 | 0.055 ± 0.006 |
| +Paraquat | 3.57 ± 0.18  | 3.49 ± 0.46 | 0.81 ± 0.14  | 0.89 ± 0.21  | 0.083 ± 0.003* |

* Results are mean ± S.E.
§ Numbers are mean ± S.E.
§ Numbers in parentheses, number of experiments.
| p < 0.02 compared with alveolar macrophage incubated in the absence of paraquat; p > 0.05 for other comparisons. |
the presence or absence of glucose (Fig. 2). At 15 min, the intracellular concentration was approximately 0.7 mM. Although the rate of paraquat uptake was slightly lower in the presence of glucose, this would not explain the reversal of paraquat inhibition by glucose; glucose added either 15 min after paraquat or with paraquat was equally effective in reversing the inhibition (see above).

Resting alveolar macrophages showed the same endogenous rate of O2 consumption after incubation with or without 1 mM paraquat for 15 min (Table III). Addition of oligomycin caused the same percentage of inhibition of O2 consumption (46%) with both paraquat- and minus-paraquat-incubated cells (Fig. 3) indicating that paraquat did not uncouple oxidative phosphorylation. The rate of O2 consumption due to paraquat autooxidation was too small to be accurately measured even in the presence of antimycin A, which abolished mitochondrial O2 consumption. The ATP content of alveolar macrophages was also unaffected by incubation with paraquat in the presence or absence of glucose (Table III). Paraquat did, however, cause a 51% increase in [14C]glucose production from [1-14C]glucose in resting alveolar macrophages (Table III). The results suggest that paraquat specifically increased pentose phosphate shunt activity in resting alveolar macrophages. Oxidation of NADPH by paraquat would have caused stimulated pentose phosphate shunt activity.

**NADPH-dependent Paraquat Reduction**—Reduced paraquat is blue (absorption maximum at 603 nm) whereas oxidized paraquat is colorless. Cell sonicates incubated with paraquat and NADPH anaerobically became blue whereas no reaction was observed with either cell sonicate alone or NADPH alone or when NADPH was replaced with NADH (Fig. 4). It therefore appears that paraquat reduction is specific for NADPH.

The oxidation of 0.1 mM NADPH added to cell supernatant could be increased five-fold with paraquat and was a saturable reaction (Km = 3.2 × 10^-4 M for paraquat) (Fig. 5). However, the rate of oxidation of NADH (0.1 mM) added to cell supernatant was not affected by 1 mM paraquat.

**DISCUSSION**

Previous studies showed that surface stimulation of alveolar macrophages increases glucose utilization through the pentose phosphate shunt but not through glycolysis (3, 7, 8). Nevertheless, a debate still continues concerning the relative importance of the oxidation of either NADH or NADPH in generating O2 during the respiratory burst. The results presented here suggest that NADPH rather than NADH is essential for Con A-stimulated O2 production. Our conclusions were based on several lines of evidence.

First, glucose but not lactate, pyruvate, or fructose can increase the baseline rate of Con A-stimulated O2 production (Tables I and II). The rate in the presence of 5 mM glucose appeared to be consistent regardless of the baseline rate suggesting a dependence on a substance such as NADPH which can be rapidly produced at a saturating concentration by glucose but not by the other metabolites.

Our observation that paraquat incubation caused alveolar macrophages to become more dependent than usual on the presence of glucose led us to propose that paraquat depleted alveolar macrophages of NADPH and that glucose could maintain NADPH concentration through the pentose phosphate shunt. Because initiation, but not continuance of the respiratory burst, appears to depend on ATP (5), it was also important to show that alterations of ATP content of alveolar macrophages were not a factor in these studies. Although O2 could possibly have been produced within AM due to auto-oxidation of reduced paraquat (13, 14), paraquat incubation did not cause a detectable increase in reduction of extracellular cytochrome c. Hassan and Fridovich (20) have recently shown that paraquat reduced intracellularly in Escherichia coli B can subsequently cross the cell membrane and generate O2 extracellularly; this apparently does not occur with alveolar macrophages. However, the intracellular reduction of paraquat by a NADPH-dependent paraquat diaphorase, previously observed in E. coli (21), does seem to occur in alveolar macrophages.

Evidence which supports our hypothesis of specific depletion of NADPH by paraquat was that: (a) paraquat appeared to be reducible by NADPH but not by NADH (Fig. 4); (b) NADPH oxidation was saturable with paraquat, but NADH oxidation was unaffected by paraquat (Fig. 5); (c) paraquat enhanced pentose phosphate shunt activity in cells (Table III); (d) mitochondrial respiration and the effect of glucose on respiration were unaffected by paraquat (Fig. 3; Table III); and (e) ATP concentration in alveolar macrophages was unaffected by paraquat (Table III).

When cells were incubated in the presence of paraquat but in the absence of glucose the significantly lower O2 production upon addition of CoA (Table I) was therefore due to the specific depletion of NADPH rather than to any effect on NADH or ATP. The reversal by glucose of the paraquat effect (Table I) would then have involved an increase in pentose phosphate shunt activity to maintain or restore the NADPH concentration (Table III). Fructose, lactate and pyruvate, which would be expected to reverse the effect of paraquat were it due to depletion of ATP or NADH, were ineffective (Table II).

Despite the evidence that NADH generation through glycolysis is not stimulated during the respiratory burst (3, 7, 8), the existence of a NADH-dependent O2 producing enzyme still remains as a support for the involvement of NADH in stimulated O2 production (12). It is important that the results of the present studies not be misinterpreted to suggest that NADH and ATP may not also be required for the respiratory burst; rather, the major point is that NADPH appears to be an essential requirement for O2 production whereas NADH and ATP are not sufficient.

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