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# Effect of Oxygen on Acetylene Reduction by Photosynthetic Bacteria

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The effect of dissolved oxygen concentration on nitrogenase activity was studied in three species of photosynthetic bacteria. The $O_2$ concentration in the cell suspension was measured with an $O_2$ electrode inserted into the reaction vessel. Acetylene reduction by whole cells of *Rhodopseudomonas capsulata*, *Rhodospirillum rubrum*, and *Chromatium vinosum* strain D was inhibited 50% by 0.73, 0.32, and 0.26 μM $O_2$, respectively. The inhibition of the activity by $O_2$ in *R. capsulata* usually was reversed completely by reestablishing anaerobic conditions. In *R. rubrum* and *C. vinosum* the inhibition was only partially reversible. The respiration rate of *R. capsulata* was the highest of the three, that of *R. rubrum* was intermediate, and that of *C. vinosum* was lowest. *R. capsulata* and *R. rubrum* cells were broken after their acetylene reduction activity in vivo had been completely inhibited by $O_2$, and nitrogenase was found to be active in vitro. A concentration of cyanide that did not affect acetylene reduction activity, but which inhibited 75 to 90% of the $O_2$ uptake by whole cells of *R. capsulata*, shifted the $O_2$ concentration causing 50% inhibition of nitrogenase activity from 0.73 μM to 2.03 μM. These results are in accordance with the assumption that within a limited range of $O_2$ concentrations, the respiratory activity of the cells is enough to scavenge the $O_2$ and to keep the interior of the cells essentially anaerobic. It is suggested that $O_2$ inhibits nitrogenase activity by competing for a limited supply of electrons. When cyanide is present, respiration is slower but is adequate to keep the nitrogenase environment in the cell anaerobic. The lower respiration rate may allow a greater proportion of the electrons to be used for acetylene reduction.

It is generally accepted that $N_2$ fixation is an anaerobic process in aerobic organisms as well as in facultative and obligate anaerobes (2, 13). Several lines of evidence indicate the necessity for excluding $O_2$ both in vivo and in vitro so that nitrogenase can function. Both components of nitrogenase are susceptible to damage by $O_2$. The in vitro half-life of the nitrogenase enzyme activity in air is seconds or at the most a few minutes (7, 18, 21). Growth under $N_2$-fixing conditions and nitrogenase activity in vivo are inhibited by excess $O_2$ (5). Many attempts have been made to study quantitatively the effects of $O_2$ on nitrogenase activity in vivo. In reported experiments acetylene reduction activity by whole cells often has been measured under different partial pressures of $O_2$ in the gas phase (14, 15, 22, 23). In other cases (11) the $pO_2$ was kept constant, and the shaking rate of the culture was varied to alter the dissolved $O_2$ concentration. The relationship between the actual $O_2$ concentration in the cell suspension and nitrogenase activity is difficult to determine by these methods, because the steady-state concentration of dissolved $O_2$, which is dependent on the respiration rate of the cells and on the rate of solution of $O_2$, is unknown.

This paper describes the effect of dissolved $O_2$ concentration on acetylene reduction by photosynthetic bacteria in a vessel designed to permit simultaneous determination of the steady-state concentration of $O_2$ in the cell suspension and the rate of acetylene reduction.

## MATERIALS AND METHODS

**Methods.** *Rhodopseudomonas capsulata* B10 was a gift from the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington, and *Rhodospirillum rubrum* ATCC 11170 was from the American Type Culture Collection. Both strains were grown on Ormerod medium (16). For *R. capsulata* the medium contained 0.002% thiamine-hy-

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drochloride, the carbon source DL-lactate (30 mM), and the nitrogen source L-glutamate (7 mM). *R. rubrum* was grown with malate (30 mM) and glutamate (27 mM). *Chromatium vinosum* (strain D) was grown on Hendley's (9) medium with 27 mM glutamate as the sole nitrogen source. All of the cultures were grown anaerobically in the light in 1-liter bottles filled to the top with medium. In vivo experiments were run with cells in the exponential growth phase (absorbance of 0.8 to 1.2 at 660 nm).

Ethylene, the product of acetylene reduction, was monitored with an Aerograph model D-600 gas chromatographic unit equipped with a flame ionization detector. Ethylene was separated from acetylene on a column of Porapak R at 53°C. In vivo assays were performed with the apparatus described in Fig. 1. A sample (3 to 4 ml) of a growing culture was transferred to a reaction vessel which previously had been sparged with $O_2$-free argon. The reaction was started by injecting 10% acetylene and was run in the light (200 $\mu$Einsteins $m^{-2}$ $s^{-1}$) at 30°C.

Crude extracts were prepared by passing cells through a French pressure cell at 18,000 lb/in$^2$. The extract was centrifuged for 10 min at 15,000 × *g* to remove unbroken cells and then for 90 min at 140,000 × *g* to remove the chromatophores. The supernatant was the crude extract used. Acetylene reduction activity in crude extracts was measured in 21-ml vaccine bottles at 30°C. The reaction mixture had 0.1 ml of crude extract and a total volume of 1 ml. The concentrations of other components were: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.7), 5 mM ATP, 25 mM creatine phosphate, 0.05 mg of creatine phosphokinase, 20 mM $MgCl_2$, and 0.5 mM $MnCl_2$. The gas phase was 90% argon and 10% acetylene (atmospheric pressure about 740 mmHg [ca. 98.6 kPa]). The reaction was started by the addition of 5 mM $Na_2S_2O_4$.

Respiration rates were measured at 30°C by following $O_2$ consumption with a Clark type $O_2$ electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). With an improved electronic circuit (19), dissolved $O_2$ concentrations in the range of 0.1 $\mu$M to 0.3 mM could be measured reliably. The light intensity was 200 $\mu$Einsteins $m^{-2}$ $s^{-1}$, the same intensity as used for the acetylene reduction assays.

Total cell protein was measured after extraction of the pigments with acetone-methanol by the method of Clayton (3). The pellet then was boiled for 20 min in 1 N NaOH and then centrifuged, and the protein in the supernatant was determined by Hartree's modification of the Lowry method (8); bovine serum albumin served as a standard.

**Materials.** ATP, creatine kinase (EC 2.7.3.2), and HEPES were obtained from Sigma Chemical Co. Phosphocreatine was from Pierce Chemical Co., Rockford, Ill., and dithionite was obtained from J. T. Baker Co., Phillipsburg, N.J. Acetylene was generated from calcium carbide. $N_2$ and argon were purified by passing over hot (115°C) BASF catalyst R3-11 from Chemical Dynamics Corp., South Plainfield, N.J. All other chemicals were of analytical grade.

## RESULTS

It has been suggested (4, 6, 12) that respiration protects nitrogenase against $O_2$ damage by consuming $O_2$ at a rate sufficient to maintain the $O_2$ concentration at the cell surface near zero, and thus to create an anaerobic intracellular environment. Although there is experimental support for this concept, direct measurement of the dependence of nitrogenase activity and respiratory rates on the dissolved $O_2$ concentration have not been reported. We have devised a vessel that permits the assay of acetylene reduction and the measurement of dissolved $O_2$ concentrations si-

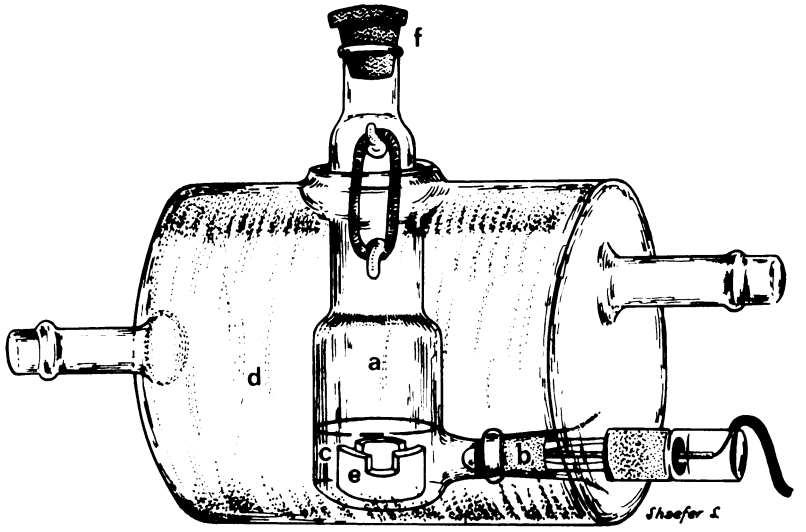


FIG. 1. *Reaction vessel for measurement of acetylene reduction and simultaneously monitoring the dissolved oxygen concentration in the cell suspension. (a) Head space; (b) $O_2$ electrode; (c) cell suspension; (d) water jacket; (e) stirring bar; (f) serum stopper.*

multaneously (Fig. 1). The vessel holds several milliliters of cell suspension and has a head space 10 to 15 times the liquid volume. The stirring rate was fast enough to establish the steady-state concentration of dissolved $O_2$ 50 to 150 s after the injection of air into the vessel.

We used this method to compare the effect of dissolved $O_2$ on acetylene reduction in vivo by three strains of photosynthetic bacteria. Figure 2 shows typical results obtained with *R. capsulata*. $O_2$ at 0.5 $\mu$M inhibited acetylene reduction by 29% relative to anaerobic conditions, whereas 4.17 $\mu$M $O_2$ inhibited acetylene reduction completely. After removal of the $O_2$, complete recovery of activity occurred almost immediately; the nitrogenase was not irreversibly damaged by $O_2$. This type of experiment was repeated with different $O_2$ concentrations, and the effects of $O_2$ on nitrogenase activity and recovery are shown in Fig. 3. $O_2$ at 0.73 $\mu$M inhibited acetylene reduction activity by 50% in whole cells of *R. capsulata*; when anaerobic conditions were reestablished, most of the activity was restored. *R. rubrum* (Fig. 4) and *C. vinosum* (Fig. 5) exhibited the same general pattern. $O_2$ inhibits acetylene reduction activity by cell suspensions to different degrees in different organisms, but even when the $O_2$ concentration was high enough to block nitrogenase activity completely, some acetylene reduction activity was restored after the $O_2$ was removed. Plots of the percentage of residual activity versus the dissolved $O_2$ concentration revealed that in *R. rubrum* 0.32 $\mu$M $O_2$ inhibited nitrogenase activity by 50% (Fig. 6), whereas 0.26 $\mu$M $O_2$ inhibited *C. vinosum* by 50% (Fig. 7). The fraction of activity restored under anaerobic conditions after the treatment with $O_2$ was lower for *C. vinosum* than for *R. rubrum*.

To examine the hypothesis that respiration protects nitrogenases, we compared the respiratory rates of the bacterial species. It has been shown (20) that light inhibits respiration in *Rhodospirillaceae*, and therefore we measured respiration in light of the same intensity used for acetylene reduction assays. Figure 8 records the respiratory activity of *R. capsulata* cells as a function of $O_2$ concentration. Maximum activity of 12 mmol of $O_2$ consumed per mg of cell protein per h was reached at a concentration of 5 to 6 $\mu$M dissolved $O_2$. Cells of *R. rubrum* (Fig. 9) and *C. vinosum* (Fig. 10) had maximal specific activities of respiration of 0.42 and 0.101 mmol of $O_2$ consumed per mg of cell protein per h, respectively. Respiration by all three organisms approached saturation near 6 $\mu$M dissolved $O_2$.

The data presented in Fig. 2, 4, and 5 show that although $C_2H_2$ reduction was inhibited by $O_2$, the activity was restored as soon as $O_2$ was removed from the cell suspension. This implies that the $O_2$ treatment did not damage nitrogenase. For verification we prepared cell-free extracts from *R. capsulata* and from *R. rubrum* by breaking the cells in the presence of $O_2$ concen-

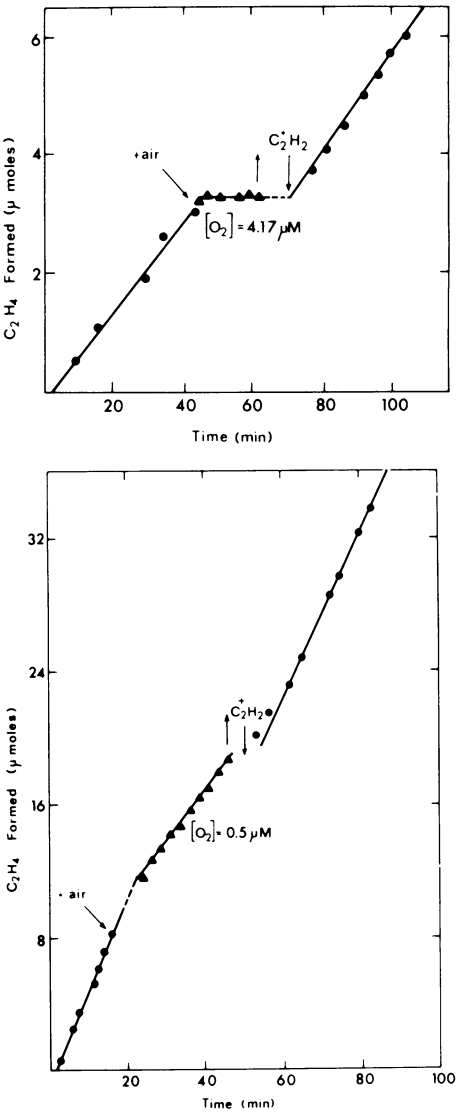


FIG. 2. *Effect of $O_2$ on acetylene reduction activity of whole cells of R. capsulata. The assays were run in the apparatus described in Fig. 1, as detailed in the text. The first part of the graph shows the control rate of acetylene reduction assayed under anaerobic conditions. The ↓ designates the addition of a small volume of the indicated gas, and the figure shows the steady-state concentration of $O_2$ in the cell suspension obtained after this addition; ↑, sparging with argon. The third part of the graph represents the "reversibility," i.e., acetylene reduction activity under anaerobic conditions after the treatment with $O_2$.*

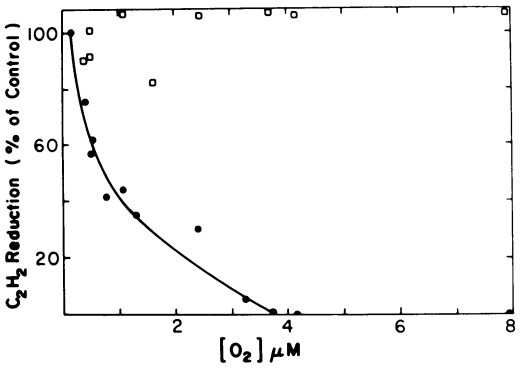


FIG. 3. *Effect of different dissolved $O_2$ concentrations on the acetylene reduction activity of whole cells of R. capsulata. The values were calculated from data obtained from experiments run under the same conditions as those described in Fig. 2. The control rate of 100% was the value obtained for each reaction mixture before the treatment with $O_2$.* ●, *Activity in the presence of $O_2$;* □, *activity observed after anaerobic conditions were restored.*

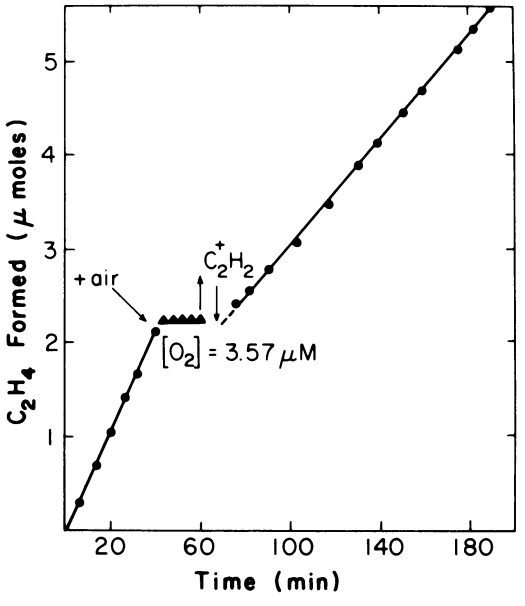


FIG. 4. *Effect of $O_2$ on acetylene reduction activity by whole cells of R. rubrum. The experimental details are as described for Fig. 2 and in the text.*

trations that completely inhibited acetylene reduction. Table 1 shows that nitrogenase preparations from *R. capsulata* after treatment with 80 μM $O_2$ for 10 min were active in vitro when supplied with ATP and a reductant, in spite of the fact that there was no activity in vivo. *R. rubrum* cells broken after 10 min in the presence of 11.4 μM $O_2$ yielded a fully active enzyme, but incubation of *R. rubrum* cells with 49 μM $O_2$ for 10 min damaged nitrogenase; only 25.3% of the control activity was then exhibited in vitro. In control in vivo experiments (not shown) with *R. capsulata* (80 μM $O_2$) and *R. rubrum* (11.4 μM $O_2$) the activity was recovered completely after

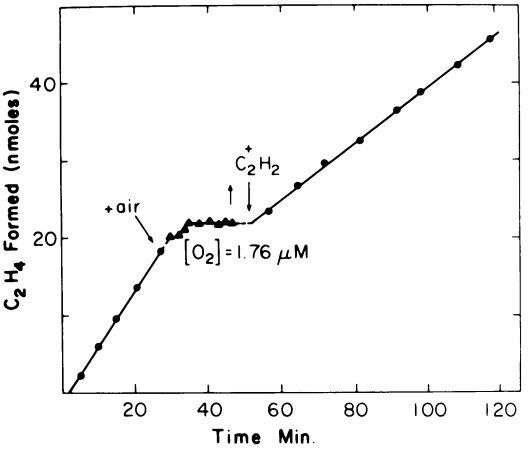


FIG. 5. *Effect of $O_2$ on acetylene reduction activity by whole cells of C. vinosum. The experimental details are as described for Fig. 2 and in the text.*

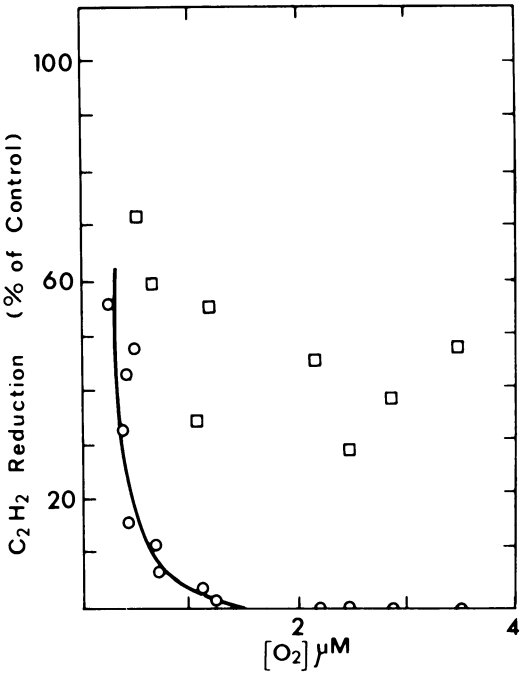


FIG. 6. *Effect of different dissolved $O_2$ concentrations on the acetylene reduction activity of whole cells of R. rubrum. The points were calculated from data obtained from experiments such as those described in Fig. 4.* ○, *Activity in the presence of $O_2$;* □, *activity after anaerobic conditions were restored.*

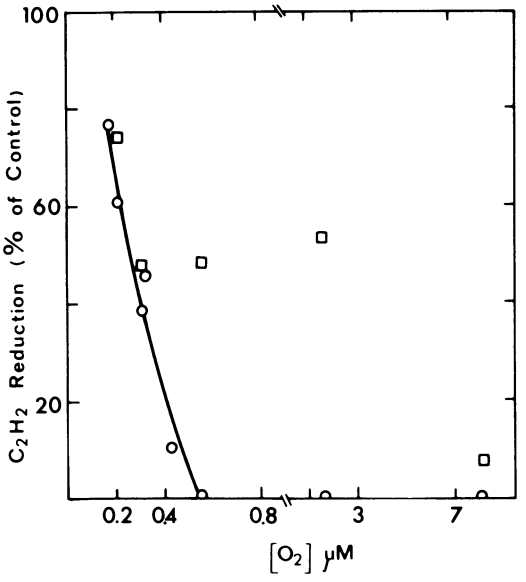


FIG. 7. *Effect of different dissolved $O_2$ concentrations on the acetylene reduction activity of whole cells of C. vinosum. The figures were calculated from data obtained from experiments such as those described in the legend to Fig. 5. ○, Activity in the presence of $O_2$; □, activity after anaerobic conditions were restored.*

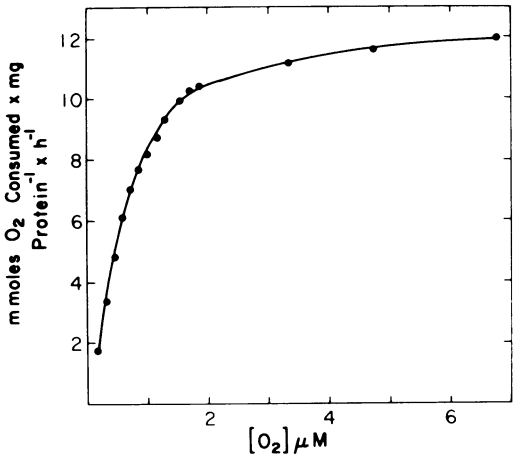


FIG. 8. *Respiration rates in the light by whole cells of R. capsulata under different $O_2$ concentrations. Experimental conditions were as described in the text. Each point on the graph was calculated by drawing a tangent to the progress curve for $O_2$ consumption at the indicated $O_2$ concentration.*

restoration of anaerobic conditions, whereas *R. rubrum* cells exposed to 49 μM $O_2$ recovered only 44% of their initial activity.

These data indicate that the three species of bacteria can be treated with low levels of $O_2$ that inhibit nitrogenase activity, without irreversibly damaging nitrogenase. We propose that nitrogenase was inhibited because a vigorous respiratory system competed with nitrogenase for the cell's limited pool of electrons. To examine this hypothesis we added cyanide to a cell suspension of *R. capsulata* at a concentration (0.2 mM) that had a negligible effect on in vivo nitrogenase activity, i.e., it did not compete effectively with acetylene as a substrate for nitrogenase, although it inhibited respiration by 75 to 90% (data not shown). Figure 11 illustrates a typical result of an experiment in which the effect of $O_2$ on acetylene reduction by *R. capsulata* was examined in the presence of 0.2 mM $CN^-$. The responses resemble those obtained in the absence of the inhibitor (Fig. 2, 4, and 5). Addition of $O_2$ to the cell suspension caused inhibition of acetylene reduction activity, depending on the $O_2$ concentration added. When $O_2$ was removed, the original activity was restored. A plot of the dissolved $O_2$ concentration versus acetylene reduction activity in the presence of 0.2 mM $CN^-$ (Fig. 12) reveals that for each $O_2$ concentration,

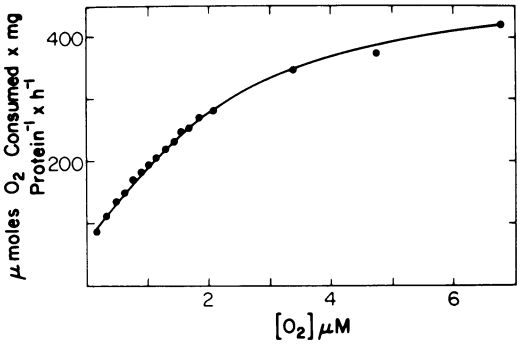


FIG. 9. *Respiration rates in the light by whole cells of R. rubrum under different $O_2$ concentrations. Experimental conditions were as described in the text, and the data were calculated as described for Fig. 8.*

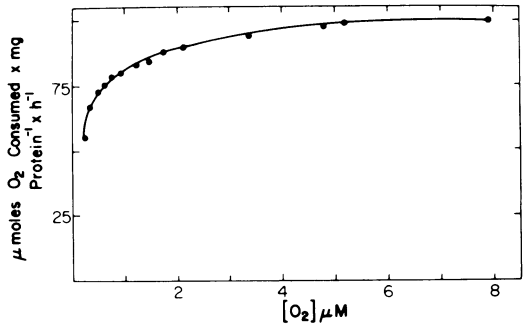


FIG. 10. *Respiration rates in the light by whole cells of C. vinosum, under different $O_2$ concentrations. Experimental details were as described for Fig. 8.*

TABLE 1. *Effect of $O_2$ on acetylene reduction activity by whole cells and crude extracts of R. capsulata and R. rubrum*[a]

| Species | $O_2$ treatment in vivo | | Activity in vivo (% of control) | Sp act in crude extracts[b] |
|---|---|---|---|---|
| | Concn ($\mu$M) | Duration | | |
| *R. capsulata* | None | | 100 | 11.61 |
| | 80 | 10 min | 0 | 9.62 (82.9) |
| *R. rubrum* | None | | 100 | 28.14 |
| | 11.4 | 10 min | 0 | 27.2 (96.7) |
| *R. rubrum* | None | | 100 | 25.12 |
| | 49 | 10 min | 0 | 6.36 (25.3) |

[a] Before the assay, cells were concentrated by centrifugation and resuspension in their growth medium. The cell suspension was placed in the acetylene reduction assay vessel, and acetylene reduction activity was followed for 20 min (control). Air was injected, and acetylene reduction was followed for 10 min more. The cells then were broken immediately, the crude extracts were prepared. Acetylene reduction activity in crude extracts was measured as described in the text.

[b] Nanomoles of $C_2H_4$ per milligram of protein per minute; percentage in parentheses.

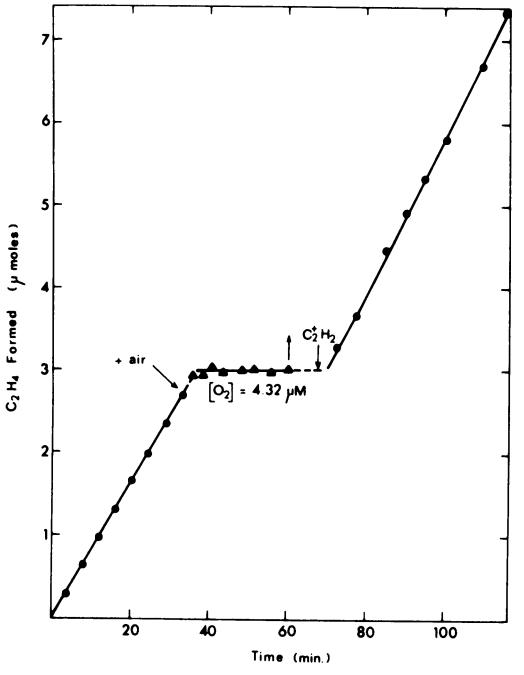


FIG. 11. *Effect of dissolved $O_2$ on acetylene reduction activity by whole cells of R. capsulata in the presence of 0.2 mM $CN^-$. The experimental details were described in the legend to Fig. 2.*

acetylene reduction activity was higher in the presence than in the absence of $CN^-$ relative to the control. $O_2$ at 2.03 mM was required for 50% inhibition of activity, as compared to 0.73 $\mu$M $O_2$ in the absence of $CN^-$ (Fig. 3); the broken line in Fig. 12 reproduces the data of Fig. 3. However, one must note that acetylene reduction was abolished by about 4 $\mu$M $O_2$ both with and without $CN^-$.

## DISCUSSION

Parker and Scutt (17) measured the effect of $O_2$ on $N_2$ fixation by *Azotobacter vinelandii* and concluded that $O_2$ was a competitive inhibitor, despite the fact that one of their figures was indicative of uncompetitive inhibition. Wong and Burris (21) examined $O_2$ as an inhibitor of nitrogenase from *A. vinelandii* and concluded that it inhibited uncompetitively. The suggestion of Parker and Scutt (17) that "nitrogen fixation is a form of respiration" has not gained general acceptance.

Most reports in the literature on responses to $O_2$ express activity for reduction of acetylene or other nitrogenase substrates as a function of the $pO_2$ in the gas phase; however, it is dissolved $O_2$ in contact with the cells that affects the nitrogenase. Because cellular respiration may consume dissolved $O_2$ rapidly, dissolved $O_2$ may not be replaced in solution from the head space rapidly enough to maintain a constant concentration. Thus it is advantageous to measure dissolved $O_2$ directly.

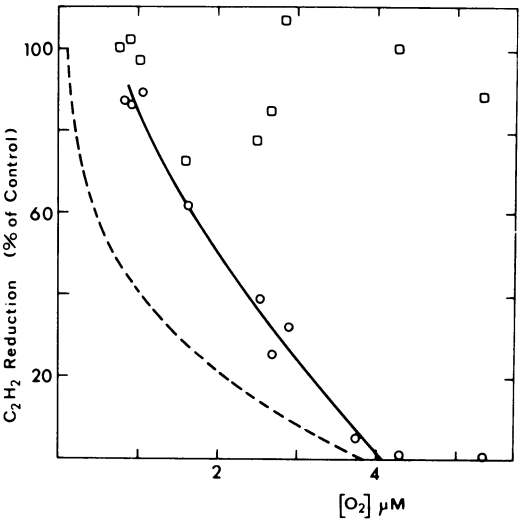


FIG. 12. *Effect of dissolved $O_2$ on acetylene reduction activity by whole cells of R. capsulata in the presence of 0.2 mM $CN^-$. The values were calculated from data obtained from experiments such as those described in Fig. 11. ○, Activity in the presence of $O_2$; □, activity after anaerobic conditions were restored. The dashed line represents data taken from Fig. 3 and is given for comparison of the activity without $CN^-$.*

In this paper we describe studies of the effect of dissolved $O_2$ on nitrogenase activity of whole cells of the photosynthetic bacteria *R. capsulata*, *R. rubrum*, and *C. vinosum* D. Measurements of acetylene reduction were made on samples from an assay vessel that contained an $O_2$ electrode. In general, nitrogenase was inhibited by $O_2$, and its activity returned when $O_2$ was removed. This "switch-off-switch-on" phenomenon for acetylene reduction has been observed for other strains of $N_2$-fixing bacteria, e.g., *Azotobacter chroococcum* (11), *Derxia gummosa* (10), and *Mycobacterium flavum* (1). In our experiments, reduction of acetylene by *R. capsulata* was inhibited 50% by 0.73 $\mu$M $O_2$. Meyer et al. (14) reported that the same strain was inhibited 50% by 13.7% $O_2$ in the gas phase, a concentration that would be equivalent to 159 rather than 0.73 $\mu$M $O_2$ (30°C). We found 50% inhibition of acetylene reduction by *R. rubrum* in 0.32 $\mu$M $O_2$, whereas Neilson and Nordlund (15) reported 50% inhibition of *R. rubrum* by 0.48% $O_2$, equivalent to 5.59 $\mu$M at 30°C. A change in the shaking rates, the density of cells, the light intensity, or the growth phase of the cells used might have altered their results.

Respiratory protection is one of the most frequently suggested hypotheses for protection of nitrogenase from $O_2$ damage; this implies that respiratory activity scavenges $O_2$ from the vicinity of the enzyme. To test this hypothesis we measured respiration rates of the bacterial strains under light intensities identical to those used in our acetylene reduction assay. As anticipated, *C. vinosum*, which had the highest sensitivity to $O_2$, had the lowest $O_2$ consumption rate. *R. capsulata*, with the highest respiration rate, was least sensitive to $O_2$, whereas *R. rubrum* was intermediate in both respects.

The inhibition of acetylene reduction by low levels of $O_2$ was relieved essentially without a lag when anaerobic conditions were restored. Protein synthesis did not appear necessary for the recovery of activity, as added chloramphenicol had no effect (data not shown). Thus, the slower rate of acetylene reduction in the presence of $O_2$ apparently did not result from direct damage to nitrogenase. Indeed, when we broke *R. capsulata* and *R. rubrum* cells that did not exhibit acetylene reduction activity because of the presence of $O_2$, the nitrogenase proved fully active. With *R. rubrum* exposed to a higher $O_2$ concentration, only 75% of the activity was recovered in vitro; this response is consistent with the finding that under these conditions in vivo only part of the activity was restored after $O_2$ was sparged from the cell suspension. The respiration rate in *R. rubrum* probably was inadequate to prevent $O_2$ penetration to the site of nitrogenase.

A similar effect was observed with *C. vinosum* (Fig. 7). After switch-on of the enzyme after its inhibition by $O_2$, acetylene reduction activity was lower than the initial control rate, and recovery was less complete when the cells had been exposed to higher $O_2$ concentrations. This response contrasted with that of *R. capsulata*, which has a higher respiration rate.

Our results can be interpreted to mean that at low concentrations of dissolved $O_2$ in the culture, the respiratory activity of the cells was capable of scavenging sufficient $O_2$ to keep the interior of the cells essentially anaerobic. On the other hand, there was a range of $O_2$ concentration for each bacterial strain which inhibited acetylene reduction activity, although nitrogenase was undamaged and the respiration rate was increasing. We suggest that this lower rate of acetylene reduction was caused by a shortage of electrons available for acetylene reduction when an increasing proportion of the electrons were diverted to support respiration with increasing $O_2$ concentration. When $CN^-$ was present, the residual respiration rate still was adequate to keep the nitrogenase environment essentially anaerobic while sparing sufficient electrons to maintain rapid acetylene reduction. Similarly, Hill et al. (11) found that when the $O_2$ concentration was increased to cell suspensions of *A. chroococcum* the nitrogenase activity disappeared; when the $O_2$ concentration was lowered the activity was restored. They explained this response in terms of the conformational protection suggested by Dalton and Postgate (5); i.e., they believe that the enzyme is capable of adopting a conformation in which it is $O_2$ tolerant but inactive. Our observations do not rule out the operation of a conformational or perhaps other protective mechanisms. It also is quite possible that *A. chroococcum* and *R. rubrum* employ different mechanisms for protection of nitrogenase against $O_2$.

Although our data suggest that competition for electrons between nitrogenase and the respiratory system may lower $N_2$ fixation as the concentration of dissolved $O_2$ increases, and that respiration in turn can protect nitrogenase against inhibition by dissolved $O_2$, one must not assume too simplistic an explanation for the interactions of $O_2$, nitrogenase, and the respiratory system in intact organisms. The interactions may be complex and may vary among different bacterial species.

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