SUMMARY

1. Light induces a decrease in the concentration of violaxanthin in the algae, Scenedesmus and Chlorella.

2. The specific radioactivity of a number of pigments has been determined for algae fed $C^{14}O_2$ for varying periods of time.

3. The specific radioactivity of chlorophyll a is nearly 3 times that of chlorophyll b; therefore these 2 substances are not involved in a rapid, reversible conversion.

4. The specific radioactivity of a group of carotenoids varies from 1000 for the carotenes, down to a value less than 10 for xanthophyll-epoxide.

5. The specific radioactivity of violaxanthin and xanthophyll are very nearly equal at 75.

6. It is suggested that violaxanthin and xanthophyll are rapidly interconverted in a light dark reaction, and that this reaction may be part of the O_2 evolution sequence in photosynthesis.

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STUDIES ON NITROGEN FIXATION AND PHOTOSYNTHESIS OF RHODOSPIRILLUM RUBRUM [1, 2]

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Nitrogen fixation in the photosynthetic bacterium *Rhodospirillum rubrum* has been described as an anaerobic, light dependent process (4, 7, 8, 9). Nevertheless, N_2 fixation by *R. rubrum* has not been studied in sufficient detail to permit one to answer the question as to how soon N_2 fixation stops after a period of illumination, or whether one might find conditions which would permit N_2 fixation to occur in the dark at rates that could be measured quite readily. Consequently, it was of interest to re-investigate the light requirement for N_2 fixation in greater detail with the help of a recording mass spectrometer (2) which permits one to follow N_2 uptake as well as photosynthetic and respiratory gas exchanges in an almost continuous manner. With this method it was thus possible to study the effect of light intensity on rates of N_2 fixation and photosynthesis in the same sample, and to investigate the effect of a number of substances on CO_2 and N_2 uptake in the light.

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Materials and Methods

Rhodospirillum rubrum (Esmarch) Molisch (Sl isolated by Dr. C. B. van Niel) was maintained in stabs and in liquid cultures in a manner previously described (3, 4, 5). For experimental purposes, cultures were grown anaerobically in the light in 100 ml culture tubes containing 35 ml nitrogen-free, sterile culture medium (autoclaved for 20 min at 15 p.s.i.). The medium was that described by Gest, Kamen and Bregoff (4) except that it was neutralized with KOH instead of NaOH and that sources of combined nitrogen (glutamic acid and yeast extract) were omitted. Trace elements were supplied to give final concentrations in parts per million as follows: B, 0.5; Mn, 0.5; Mo, 0.3 (growth was extremely limited in nitrogen-free media containing only 0.01 ppm); Zn, 0.05; Cu, 0.02; Co, 0.02.

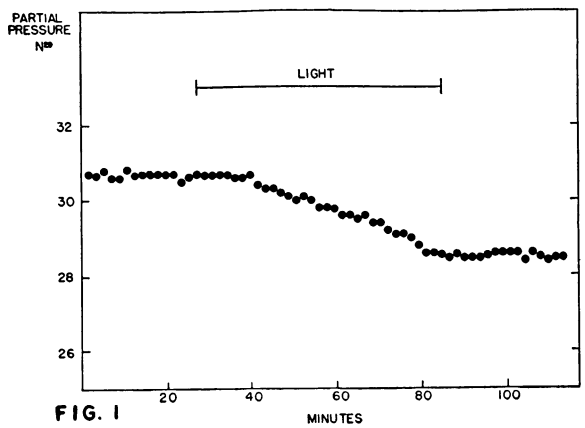
A gas mixture of 5 % CO_2 and 95 % N_2 was supplied under slight pressure at the inlet of the culture tube and was dispersed into a fine stream of bubbles by a sintered glass tube. Slow bubbling (5 to 10 ml gas/min) was found to be most satisfactory to avoid foaming. Inoculations were made with 3 ml of a culture grown in the above nitrogen-free medium for 48 to 72 hours. Little or no growth occurred in cultures which were not continuously supplied with CO_2. Since cultures supplied with glutamic acid under otherwise identical conditions also failed to develop in the absence of CO_2, this CO_2 requirement was not restricted to cultures dependent on N_2 as a nitrogen source.

Cultures grown on N_2 were incubated at 25 to 28° C and were illuminated by four 60-watt bulbs 30 cm from the center of the culture tube. For experimentation, 48-hour-old cultures (cell density 0.8 to 1.0 mg dry wt/ml) were harvested at 1000 × gravity in a refrigerated centrifuge and resuspended in 3 to 4 ml of fresh, nitrogen-free medium at pH 7.0 with added potassium phosphate giving a final concentration of 0.01 M phosphate. Two ml of this suspension were placed in a manometer vessel which could be attached to the leak housing of the mass spectrometer and immersed in a constant temperature bath at 28° C. The vessel was illuminated by a 1000-watt tungsten lamp, focused by 2 condensing lenses. Light intensities, varied with screens, were measured with a calibrated Weston light meter.

The method used for gas analysis in the present investigation was an adaptation of the one developed by Brown and coworkers (1, 2, 6) for the simultaneous measurement of gas exchange in respiration and photosynthesis. The mass spectrometer employed (Consolidated Engineering Company Model 21–201) was modified, as previously described, to permit periodic determinations of the partial pressure of gases in a manometer vessel attached to the mass spectrometer through an appropriate leak assembly. The manometer vessel itself could be shaken in a bath and maintained at constant temperature. The following masses were analyzed: 29 (N^{14}-N^{15}), 40 (A), and 44 (CO_2), by automatically cycling the ion accelerating voltage through the proper values. The cycling time was adjusted to obtain a reading for each mass every 1.8 or 3.6 minutes.

The use of mass 28 for the detection of changes in the partial pressure of N_2 was complicated by the fact that CO formed as a degradation product of CO_2 within the mass spectrometer contributed to the mass 28 peak. Rather than correct for this effect due to the presence of CO_2, N_2 enriched with N^{15} (30 atom % N^{15}) was used in the gas phase, and mass 29 determinations were employed to follow changes in the partial pressure of N_2.

Argon (mass 40) was included in the gas phase to provide a means for detecting any variations in the readings due to non-biological causes. Argon had



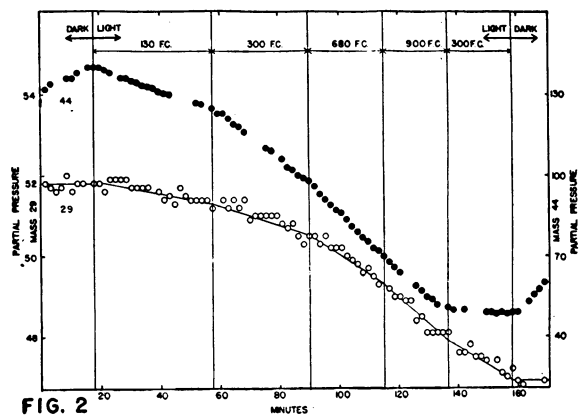


FIG. 1. The effect of light on uptake of N_2 by *R. rubrum*. Light intensity: 1750 ft-c. The numbers on the ordinate of this figure and subsequent figures are in relative units. The following conversion factor must be applied to relate the ordinate for this figure to absolute units: partial pressure of mass 29 × 6.0 equals microliters of N_2.

FIG. 2. The effect of light intensity on uptake of CO_2 and N_2 by *R. rubrum*. Ordinate values: partial pressure of mass 29 × 11 equals microliters N_2; partial pressure of mass 44 × 6.0 equals microliters of CO_2.

been used for a similar purpose by Sisler and Zobell (12) in long term experiments designed to detect biological N_2 fixation.

Gas pressure in the manometer vessel was adjusted to atmospheric pressure (approximately 740 mm/Hg) at the start of each experiment.

Results

Light Relations: The results of one of the first attempts to follow N_2 fixation with a mass spectrometer are shown in figure 1. The suspension of Rhodospirillum was incubated in the manometer vessel with a gas phase consisting of 2 % N_2, 2 % CO_2, 2 % A and 94 % Helium. N_2 uptake is indicated by the decrease in the partial pressure of N_2 in the light; uptake of N_2 in the dark could not be established by the experimental method employed in any of the experiments which were performed.

It is apparent from figure 2 that the rates of N_2 and CO_2 uptake were related directly to light intensity. The metabolism of CO_2 in the latter part of the experiment was complicated by an effect which also was observed in suspensions kept at constant light intensity. Such suspensions showed an initial high rate of CO_2 uptake which decreased after a period of illumination (cf. fig 4, 6); this decrease in net CO_2 consumption could have been due to an increase in the rate of photofermentation (photoproduction of CO_2), however, the change in the photometabolism of CO_2 appeared to have little or no effect on the rate of N_2 uptake.

Inhibitors of Nitrogen Fixation: Kamen and Gest (7) reported that ammonium chloride inhibits N_2 fixation by *R. rubrum* and such inhibition also could be demonstrated with the mass spectrometer technique. An example is given in figure 3, where the addition of 0.2 ml of 0.05 M NH_4Cl (to give a final concentration of 4.5×10^{-3} M NH_4Cl) to a suspension of cells actively fixing molecular nitrogen produced complete inhibition after a lag period of 15 to 20 minutes; in different experiments this time lag could always be observed varying from 6 to 20 minutes.

With the analytic method described here it could be demonstrated that molecular hydrogen inhibits N_2 fixation by *R. rubrum*. A typical experiment is depicted in figure 4; a manometer vessel with a Rhodospirillum suspension was flushed with a gas mixture of 4 % N_2, 5 % CO_2, 89 % H_2 and 2 % Argon. Upon illumination N_2 uptake did not occur even though active CO_2 uptake could be observed. The atmosphere in the vessel then was displaced by a gas mixture of the same composition except for the replacement of H_2 by He. Upon subsequent illumination, active N_2 uptake could now be observed but there was little or no change in the rate of CO_2 uptake compared with the previous light period. Inhibition of N_2 fixation was complete at the H_2 and N_2 partial pressures used in this experiment. Partial inhibitions could be observed at lower tensions of H_2, for instance with a gas mixture of 45 % H_2 and 2.5 % N_2 (the remaining gases being CO_2, A and He) an inhibition of 60 % was observed against a suitable control. The details of the kinetics of the effect of H_2 on N_2 fixation by this organism remain to be worked out.

Molecular oxygen completely inhibits N_2 fixation by *R. rubrum* at relatively low partial pressures. An example is given in figure 5; in this experiment the initial gas phase contained 4 % N_2, 5 % CO_2, 2 % A and 89 % He with molecular oxygen present only in trace amounts, and under these conditions active N_2 uptake could be observed in the light. This gas phase was then displaced with a gas mixture containing approximately the same proportions of N_2, CO_2, A and He and in addition 4.1 % O_2 (the O_2 tension dropped to 3.8 % by the end of the light period as a result of respiration); upon subsequent illumination of the cell suspension N_2 uptake was completely suppressed. In other experiments, comparable to the one described here, the inhibition of N_2 uptake by 4 % O_2 could be reversed completely by making the system anaerobic again. Figure 6 demonstrates a partial suppression of N_2 fixation in the presence of a gas mixture containing 1.8 % O_2 giving an inhibition of about 45 %. The experiment also shows the reversal of this inhibition upon reduction of the partial pressure of O_2.

Discussion

The experimental findings of this paper support the observation that N_2 fixation by *Rhodospirillum rubrum* is essentially light dependent. Dark fixation of N_2 was not detectable by the methods employed in this study even in the period immediately after illumination.

The rates of N_2 fixation were strongly influenced by light intensity (fig 2). At low light intensities, rates of N_2 fixation and photosynthesis both were directly related to light intensity. Unfortunately, we were not able to obtain a good saturation curve for photosynthetic CO_2 uptake under the experimental conditions because of what appeared to be photofermentative production of CO_2. As a consequence a study of N_2 uptake at still higher light intensities, to establish a complete saturation curve, was not pursued at this time. However, at high light intensities the rate of N_2 fixation did not increase in a linear fashion with increase in light intensity and N_2 uptake most likely was saturating near or somewhat above the highest light intensity employed here. Further studies on the effect of light intensity on the rates of photosynthesis and N_2 fixation most likely should be carried out in some detail.

Although N_2 fixation by *R. rubrum* is essentially light dependent, Gest, Kamen and Bregoff (4) and Lindstrom, Newton and Wilson (9) have reported small amounts of fixation aerobically in the dark. In the experiments of these investigators, suspensions of *R. rubrum* were incubated for long periods of time (5 to 6 days) in a closed atmosphere containing N_2 enriched with N^{15}. Under these conditions the O_2 partial pressure undoubtedly dropped to a very low level. It is possible that the N_2 fixation which they observed took place at partial pressures of O_2 which were sufficient for oxidative metabolism, but insufficient to

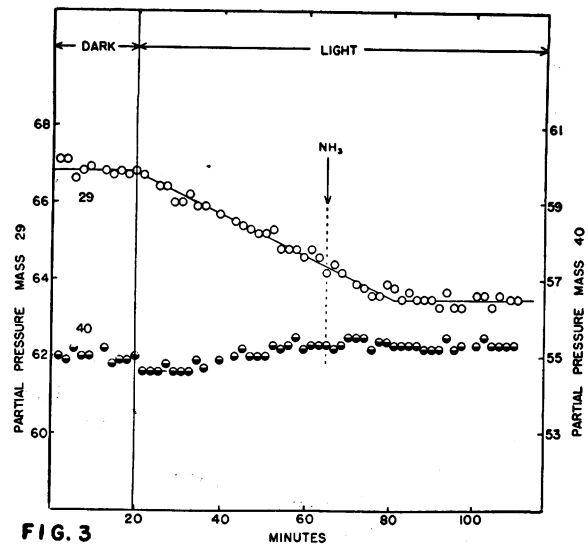


FIG. 3

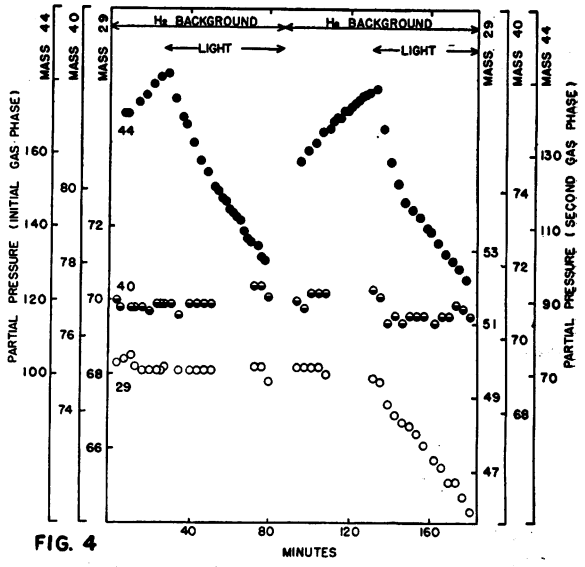


FIG. 4

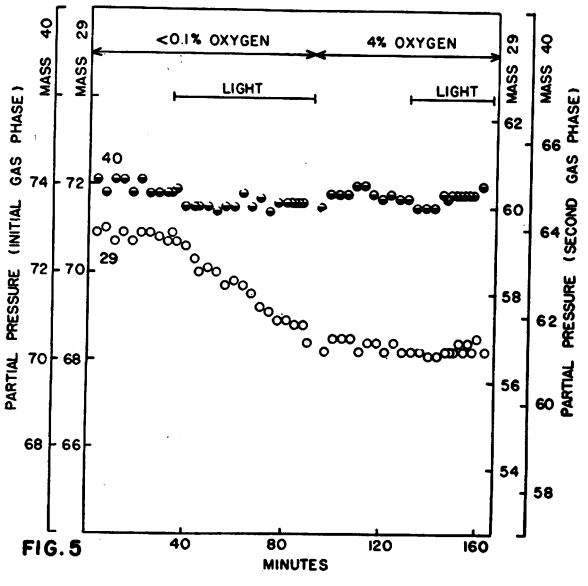


FIG. 5

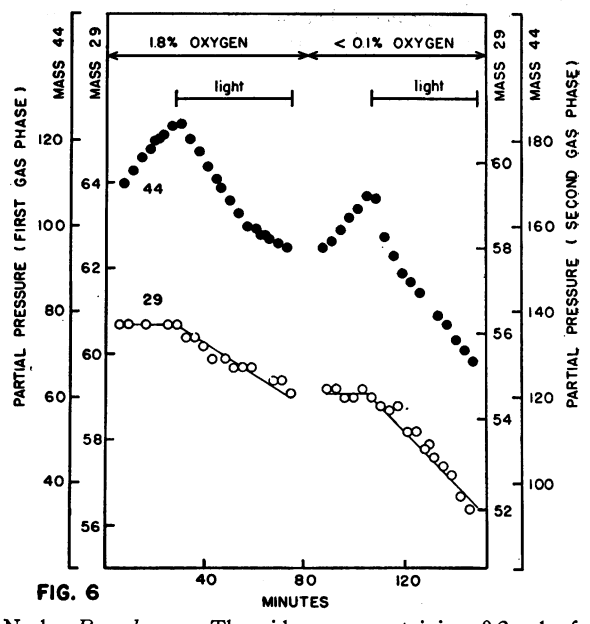


FIG. 6

FIG. 3. The effect of ammonium chloride on uptake of N_2 by *R. rubrum*. The side arm, containing 0.2 ml of 0.05 M NH_4Cl, was tipped at the time indicated. Light intensity: 1250 ft-c. The data for argon (mass 40) are included to indicate the type of variation commonly found in readings for the inert gas. Ordinate values: partial pressure of mass 29 × 11 equals microliters of N_2; partial pressure of mass 40 × 4.5 equals microliters of argon.

FIG. 4. The effect of molecular hydrogen on uptake of N_2 by *R. rubrum*. Light intensity: 1600 ft-c. Ordinate values: partial pressure of mass 29 × 6.9 equals microliters of N_2; partial pressure of mass 44 × 5.0 equals microliters of CO_2; partial pressure of mass 40 × 2.9 equals microliters of argon.

FIG. 5. The effect of low partial pressures of oxygen on uptake of N_2 by *R. rubrum*. Light intensity: 1150 ft-c. Ordinate values: partial pressure of mass 29 × 6.9 equals microliters of N_2; partial pressure of mass 40 × 2.9 equals microliters of argon.

FIG. 6. The effect of low partial pressures of oxygen on uptake of N_2 by *R. rubrum*. Light intensity 1600 ft-c. Ordinate values: partial pressure of mass 29 × 6.7 equals microliters of N_2; partial pressure of mass 44 x 5.0 equals microliters of CO_2.

inhibit N_2 fixation completely. Such low rates of N_2 fixation would be difficult to pick up with the method described here and the incorporation of N^{15} derived from N_2 over several days most likely is a more sensitive method for the detection of slow N_2 fixation in the dark.

When ammonium chloride was added to a cell suspension actively fixing N_2 a lag was noticed before inhibition of N_2 fixation became apparent. It may be of interest to study this lag time as a function of inhibitor and hydrogen ion concentration to determine whether the rate of penetration of ammonia was an important factor in this phenomenon.

Molecular hydrogen is a well known competitive inhibitor of N_2 fixation in a number of organisms (13), but attempts to demonstrate inhibition in the anaerobic Clostridium have not been successful (11). Therefore it has been assumed by some investigators that molecular hydrogen is not inhibitory to N_2 uptake by anaerobic N_2 fixers (13, 14). Pengra and Wilson, however, have shown recently that H_2 is inhibitory to the anaerobic N_2 fixing system of *Aerobacter aerogenes* (10). Nitrogen fixation by *R. rubrum* also is inhibited by molecular hydrogen, although the nature of this inhibition (competitive or non-competitive) has not been established.

It is of interest that N_2 fixation by Rhodospirillum is comparable to that of many organisms carrying out dark fixation of N_2 in its behavior toward inhibitors, pointing to the likelihood that the basic mechanism of N_2 fixation in these organisms must be rather similar. The fact, however, that N_2 fixation in *R. rubrum* is so strongly light dependent favors the assumption that the intermediate required for the initial fixation of N_2, or for the elaboration of early products of N_2 fixation, must be close to the primary photochemical products and that these intermediates never accumulate in sufficient amounts to bring about an appreciable dark fixation of N_2.

Summary

1. Metabolic gas exchanges involving CO_2, N_2 and O_2 by *Rhodospirillum rubrum* have been studied using a recording mass spectrometer. This technique is convenient for following N_2 fixation over short experimental periods. The effect of changes in environmental conditions which may affect N_2 fixation can be observed within 5 to 10 minutes.

2. Earlier reports on the light dependence of N_2 fixation by *Rhodospirillum rubrum* have been confirmed, and it also has been demonstrated that fixation stops abruptly once illumination ceases. The rate of N_2 fixation is an almost linear function of light intensity at low light intensities, paralleling photosynthetic CO_2 uptake which proceeds at about 13 times the rate of N_2 uptake.

3. It has been established that molecular hydrogen reversibly inhibits the light dependent N_2 fixation in this organism.

4. Molecular oxygen also brings about a reversible inhibition of N_2 fixation. Four percent O_2 brings about complete inhibition of N_2 uptake.

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