EFFECT OF OXYGEN ON PHOTOSYNTHETIC CO_2 FIXATION IN *CHROOMONAS* SP.

KENSAKU SUZUKI

1984

Submitted in partial fulfillment of the requirements for the degree of Doctor of Science, in Doctoral Program in Biological Sciences, University of Tsukuba.

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GENERAL INTRODUCTION

Photosynthesis in terrestrial C_3 plants is inhibited considerably by oxygen even in ambient air condition (21% O_2, 0.03% CO_2). The oxygen inhibition has been attributed mainly to photorespiration derived from the oxygenase activity of ribulose-1,5-bisphosphate (RuBP) carboxylase-oxygenase which has two competitive gaseous substrates, CO_2 and O_2 (Beck 1979, Canvin 1979). The lack of oxygen inhibition in C_4 plants seems to be caused by a combination of the Hatch-Slack pathway and the leaf anatomy which cooperate to raise the CO_2/O_2 ratio at the reaction site of RuBP carboxylase-oxygenase and suppress the oxygenase activity (Raven and Glidewell 1978, Ray and Black 1979, Ku and Edwards 1980).

Oxygen inhibition of photosynthesis has been also observed in many species of algae (Warburg 1920, Wassink et al. 1938, Gaffron 1940, Turner et al. 1956, Coleman and Colman 1980, Kremer 1980, Shelp and Canvin 1980, Birmingham et al. 1982). However, there seems considerable differences in their photosynthetic responses to oxygen, although the major pathway of photosynthetic CO_2 fixation appears to be the reductive pentose phosphate cycle in most of such algae (Bean and Hassid 1955, Coombs and Volcani 1968, Holdsworth and Colbeck 1976, Kremer and Küppers 1977, Kremer and Berks 1978). Earlier investigators observed considerable oxygen

inhibition of photosynthesis in certain species of algae, such as the green alga Chlorella ellipsoidea (Tamiya and Huzisige 1949) and the diatom Phaeodactylum tricornutum (Beardall and Morris 1975); the inhibition is dependent upon CO_2 concentration as in the case of terrestrial C_3 plants. On the other hand, Lloyd et al. (1977) reported that there was little effect of oxygen on photosynthesis in several species of algae at the O_2 concentrations below 50%. A lack of oxygen inhibition has been also reported in the blue-green alga Coccochloris peniocystis (Coleman and Colman 1980). In "air-grown" Chlorella pyrenoidosa, in which photorespiration appears to be absent, the inhibition of photosynthesis by 100% O_2 is not dependent upon the CO_2 concentration (Shelp and Canvin 1980). Furthermore, oxygen enhancement of photosynthetic $^{14}CO_2$ fixation has been observed in the blue-green alga Anacystis nidulans, which occurred only under CO_2-limiting conditions (Miyachi and Okabe 1976).

Thus, several reports have shown a wide variety of photosynthetic responses to oxygen in algae. However, most of these studies have been performed on a limited species of green or blue-green algae. And, even in these species, it has been still unclear whether the differences in the photosynthetic responses to oxygen are attributed to the differences in species or in the experimental conditions or in the culture conditions.

The author has shown that there are considerable

differences among the species of several algal divisions in the effect of oxygen on photosynthetic $^{14}CO_2$ fixation, even under the same experimental conditions using the algae which had been grown in the same CO_2 concentration (ordinary air containing 0.03% CO_2) (Suzuki and Ikawa 1978, 1981, 1983, Suzuki et al. 1980). In these algae, the effect of oxygen on photosynthetic CO_2 fixation can be classified roughly into four types: (1) the oxygen inhibition which is dependent upon CO_2 concentration as in the case of terrestrial C_3 plants, e.g. in Cryptomonas sp. (Cryptophyta); (2) the oxygen inhibition which is independent of CO_2 concentration, e.g. in Chromulina nebulosa (Chrysophyceae, in Chromophyta); (3) little or no oxygen inhibition which is independent of CO_2 concentration, e.g. in Nitzschia sp. (Bacillariophyceae, in Chromophyta); and (4) the oxygen enhancement, e.g. in Chroomonas sp. (Cryptophyta), Nitzschia ruttneri and Phaeodactylum tricornutum (Bacillariophyceae, in Chromophyta), Heterosigma akashiwo (Rhaphidophyceae, in Chromophyta), Pavlova sp. (Haptophyta), and Tetraselmis sp. (Prasinophyceae, in Chlorophyta).

Although the lack of CO_2-dependent oxygen inhibition has not yet been explained well, it has been suggested that RuBP oxygenase activity may be suppressed due to the formation of a high CO_2-concentration state at the reaction site of RuBP carboxylase-oxygenase by a "CO_2-concentrating mechanism" mediated by carbonic anhydrase or active HCO_3^--trans-

port system (Raven and Glidewell 1978, Badger et al. 1980, Coleman and Colman 1980, Shelp and Canvin 1980, Imamura et al. 1981). However, little has been reported about the detailed characteristics of the CO_2-independent oxygen inhibition and the oxygen enhancement of photosynthetic CO_2 fixation, and their mechanisms remain to be resolved.

In the Cryptophyta, the oxygen enhancement of photosynthesis in Chroomonas sp. presents a striking contrast to the CO_2-dependent oxygen inhibition in Cryptomonas sp. However, no other investigation has been reported on the characteristics of photosynthetic CO_2 fixation and related carbon metabolism in the cryptomonads.

The purpose of this thesis is to characterize the effect of oxygen on photosynthetic CO_2 fixation and to elucidate the mechanisms of the inhibitions by oxygen and by anaerobiosis in Chroomonas sp. cells.

In Chapter I, the author shows the general characteristics of the oxygen effect on the photosynthetic CO_2 fixation. Oxygen seems to exert a dual effect on photosynthesis in Chroomonas cells; an inhibitory and an enhancing effect. The detailed investigations on the enhancing effect on photosynthesis revealed that the "enhancement" by oxygen is, in fact, the "inhibition" of photosynthesis by anaerobiosis under light-saturating conditions. This inhibition is a new type of photosynthetic response to oxygen in plants.

Effect of oxygen on the photosynthetic carbon metabolism

is dealt with in Chapter II, after showing that the reductive pentose phosphate cycle (the Calvin cycle) is the major pathway of photosynthetic CO_2 fixation in Chroomonas sp. cells.

In Chapter III, relationship between the anaerobic inhibition of photosynthetic CO_2 fixation and photochemical reactions is investigated, using some inhibitors and uncouplers, as well as an artificial electron mediator. The inhibition of photosynthetic CO_2 fixation by anaerobiosis in Chroomonas sp. cells is explained in terms of ATP deficiency which may result from over-reduction of photosynthetic electron transport under high light conditions.

ABSTRACT

The effect of oxygen on the photosynthetic $^{14}CO_2$ fixation in the air-grown freshwater flagellate Chroomonas sp. (the Cryptophyta) was studied. Considerable inhibition by anaerobiosis was observed only under light-saturated conditions and was not affected by the CO_2 concentration. This inhibition was reversed by 2% O_2. Increase in O_2 concentration above 2% inhibited the rate of $^{14}CO_2$ fixation; the inhibition was about 20% at 100% O_2 and was released by 2% O_2. The degree of inhibition was only slightly higher at low concentrations (less than 0.43 mM $NaHCO_3$) than at high CO_2 concentrations, indicating that photorespiration is not the main cause of this inhibition. Possible causes of the inhibitions by anaerobiosis and by oxygen are discussed.

CHAPTER I

SOME CHARACTERISTICS OF THE OXYGEN EFFECT ON PHOTOSYNTHETIC $^{14}CO_2$ FIXATION IN *CHROOMONAS* SP. CELLS

ABBREVIATIONS

Chl, chlorophyll; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; RuBP, ribulose-1,5-bisphosphate.

INTRODUCTION

Only a limited number of species have been used for the studies concerned with effect of oxygen on photosynthetic CO_2 fixation in algae; such as green algae Chlorella and Chlamydomonas, and a blue-green alga Anacystis. These observations suggest that the effect of oxygen on photosynthesis is different among algae (Miyachi and Okabe 1976, Lloyd et al. 1977, Coleman and Colman 1980, Shelp and Canvin 1980). Inspite of such difference, the major pathway of photosynthetic CO_2 fixation appears to be the reductive pentose phosphate cylce in most of these algae (Bean and Hassid 1955, Coombs and Volcani 1968, Holdsworth and Colbeck 1976, Kremer and Küppers 1977, Kremer and Berks 1978).

Chroomonas sp. is one of the cryptomonads, which occupy a unique position in algal phylogeny. However, there is no information on the photosynthetic characteristics of this group. In this chapter, the author describes some of the features of the oxygen effect on photosynthetic $^{14}CO_2$ fixation in this alga.

MATERIALS AND METHODS

Algal culture

Chroomonas sp. (CHR-1A) cells were grown axenically in 2-liter Erlenmeyer flasks containing 1 liter of "YT medium" (Ichimura 1979), an enriched "Volvox medium" (Starr 1971) with 0.1 g of yeast extract and 0.2 g of Bacto-tryptone (Difco), at 18°C and using a 12-h light 12-h dark cycle. Illumination was provided by cool-white fluorescent tubes at an intensity of about 12 $W \cdot m^{-2}$ at the culture flask level. Cultures were bubbled continuously with filtered air without supplementary CO_2.

Cells in the late log phase of growth (12-14 days old) were harvested by centrifugation at 180 × *g* for 5 min and resuspended in 20 mM HEPES-KOH buffer (pH 7.6) containing the inorganic components of the growth medium at a concentration of less than 5 µg Chl *a* /ml.

Experimental procedures

Photosynthetic $^{14}CO_2$ fixation was carried out at 20°C in a small spitz-type test tube (16 × 150 mm). The cell suspension (1 ml) was placed in the test tube and bubbled with CO_2-free air (21% O_2), N_2, O_2, or their mixture, introduced with a long hypodermic needle. The flow rate was adjusted to 100 $ml \cdot min^{-1}$ by a thermal mass flow control system (SEC-L, Standard Technology Co., Kyoto, Japan). Test tubes

were illuminated from one side at 200 $W \cdot m^{-2}$, unless otherwise specified, with a halogen lamp (EYE halogen lamp, JCD 100V/650W/B, Iwasaki Electric Co., Tokyo, Japan). After 10 min of illumination, $^{14}CO_2$ fixation was started by injecting $NaH^{14}CO_3$, and 5 min later, stopped by adding ethanol to give 80% (v/v). The ethanol suspensions were then acidified by adding 50 µl of acetic acid and an aliquot of 50 µl was put on a small disc of filter paper and dried under an infra-red lamp.

For time course experiments, 1.2 ml of algal suspension was placed in a test tube. After injecting $NaH^{14}CO_3$, an aliquot of 25 µl of algal suspension was quickly taken out at intervals with a micropipette (Pippetman P-200, Gilson), mixed with 50 µl of acetic acid on a disc of filter paper and then dried under an infra-red lamp. The sample was placed in a scintillation vial containing 10 ml of a toluene scintillation liquid (4 g 2,5-diphenyloxazole and 0.25 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene). Radioactivity was determined using a liquid scintillation spectrometer (Beckman LS-8100, or LS-3155T).

Pigments in algal suspension were extracted with 80% ethanol or 90% methanol, and Chl *a* in the extract was determined spectrophotometrically using the Talling and Driver (Iwamura et al. 1970) formula:

Chl *a* ($\mu g \cdot ml^{-1}$) = 13.9 × A_{665}, at the light path of 1 cm.

RESULTS

Effect of oxygen concentration

The effect of oxygen concentration on the rate of photosynthetic $^{14}CO_2$ fixation was determined at low (40 $W \cdot m^{-2}$) and high (200 $W \cdot m^{-2}$) light intensities. As shown in Fig. 1, the highest rate was obtained under 2% O_2 both at 40 and 200 $W \cdot m^{-2}$. Increase in O_2 concentration above 2% decreased the rate of photosynthesis. The rate under 100% O_2 was 20 to 30% lower than that under 2% O_2. Under anaerobic conditions, the rate of photosynthesis was inhibited to about 70% of that under 2% O_2 at high light intensity, but was not inhibited when the light intensity was low. The magnitude of the inhibition by anaerobiosis at high light intensity shows differences from one culture of this alga to another, although the qualitative features are consistent.

Effect of light intensity

Fig. 2 shows the rate of photosynthesis under 2% O_2 and N_2 as a function of light intensity. The rate of photosynthetic $^{14}CO_2$ fixation under 2% O_2 was saturated at about 40 $W \cdot m^{-2}$, and was not inhibited by higher light intensities up to 320 $W \cdot m^{-2}$. The rate under N_2 was almost the same as that under 2% O_2 at light intensities below 40 $W \cdot m^{-2}$, but decreased with increasing the light intensity to reach a

steady state level above 200 $W \cdot m^{-2}$. Thus, the anaerobic inhibition of photosynthesis was light-dependent and oxygen was required for normal photosynthetic CO_2 fixation in this alga under light-saturating conditions.

Time course of photosynthetic $^{14}CO_2$ fixation

The amount of photosynthetic $^{14}CO_2$ fixation increased linearly for about 20 min without a lag under 2% and 100% O_2 (Fig. 3). On the other hand, under N_2 the rate was depressed from the beginning of $^{14}CO_2$ fixation and decreased slightly with time after 10 min (Fig. 3A). These results indicate that the anaerobic inhibition of photosynthetic $^{14}CO_2$ fixation was not a transitory phenomenon; it persisted at least for 25 min.

Effect of changing the composition of the bubbling gas on the rate of photosynthetic $^{14}CO_2$ fixation

When the bubbling gas was changed from N_2 to 2% O_2 during the course of $^{14}CO_2$ fixation, the rate of $^{14}CO_2$ fixation increased rapidly, and after a few minutes it attained the same rate as that under 2% O_2 (Fig. 3A). This indicates that the inhibitory effect of anaerobiosis on photosynthetic $^{14}CO_2$ fixation in Chroomonas cells is fully reversible. The rapid response to the gas indicates that 2% O_2 is sufficient to relieve the anaerobic inhibition. Photosynthesis was inhibited within several minutes after increasing

the O_2 concentration in the bubbling gas from 2 to 100%, and the inhibition was released by decreasing the O_2 concentration from 100 to 2% (Fig. 3B). Thus, the inhibition by high O_2 concentration is also reversible.

By contrast, the anaerobic inhibition was observed 15 min after changing the bubbling gas from 2% O_2 to N_2 (Fig. 3A).

Similar results to those in Fig. 3 were obtained when the bubbling gas was changed during the preillumination period (Fig. 4). Cells were preilluminated for 20 min, and then $NaH^{14}CO_3$ was injected to the cell suspension to determine the rate of photosynthesis by measuring the radioactivity fixed during 5 min of illumination. The bubbling time with N_2 (Fig. 4A) or with 100% O_2 (Fig. 4B) during the preillumination period was changed as indicated. The bubbling with N_2 or 100% O_2 was continued during the $^{14}CO_2$ fixation. Before the bubbling with N_2 or 100% O_2, cells bubbled with 2% O_2 under illumination.

The inhibition of $^{14}CO_2$ fixation by 100% O_2 required almost no pre-bubbling with 100% O_2 (bubbling with 100% O_2 in the preillumination period); the inhibition was about 20% after 30 s of pre-bubbling, and increased with the time to reach about 30% after 20 min (Fig. 4B). On the other hand, the inhibition by anaerobiosis required the pre-bubbling with N_2 gas; no detectable inhibition was observed after 30 s. Pre-bubbling with N_2 decreased the rate of

$^{14}CO_2$ fixation under N_2; about 40% decrease was observed after 15 min and no further decrease after 20 min (Fig. 4A).

When the preillumination period was changed from 5 to 20 min without changing the composition of the bubbling gas, preillumination under 2% O_2 did not affect the rate of the subsequent $^{14}CO_2$ fixation under 2% O_2 (data not shown).

Effect of $NaHCO_3$ concentration

Fig. 5 shows the effect of $NaHCO_3$ concentrations on the rate of photosynthetic $^{14}CO_2$ fixation under N_2, 2% O_2 and 100% O_2. The rate of photosynthesis under 2% O_2 was saturated at about 0.4 mM $NaHCO_3$ (about 10 μM CO_2) and a half-saturation for $NaHCO_3$ was about 0.1 mM (3 μM CO_2). The degree of inhibition by anaerobiosis was not affected by $NaHCO_3$ concentrations, and neither was that by 100% O_2 at $NaHCO_3$ concentrations above 0.45 mM. However, the degree of inhibition by 100% O_2 increased slightly at $NaHCO_3$ concentrations below 0.45 mM (Fig. 5). These results suggest that the inhibition of photosynthesis by oxygen at high $NaHCO_3$ concentrations cannot be attributed to a direct effect of oxygen on RuBP carboxylase-oxygenase. The increase in the degree of oxygen inhibition at low $NaHCO_3$ concentrations suggests that the inhibition is partly due to photorespiration. In fact, the amount of ^{14}C-glycolate in this alga increased with increasing the O_2 concentration under low

$NaHCO_3$ concentrations (data not shown).

DISCUSSION

Photosynthetic $^{14}CO_2$ fixation in "air-grown" Chroomonas sp., a freshwater cryptomonad, was inhibited by both anaerobiosis and oxygen at concentrations higher than 2%, which was the optimum concentration for $^{14}CO_2$ fixation (Fig. 1 and 2).

Oxygen inhibition

The rate of $^{14}CO_2$ fixation under 100% O_2 was about 20% lower than that under 2% O_2 both at low (40 $W \cdot m^{-2}$) and at high (200 $W \cdot m^{-2}$) light intensities (Fig. 1). The rate was rapidly inhibited by 100% O_2 (Fig. 3B and 4B) and reversed by 2% O_2 (Fig. 3B). The inhibition was hardly affected by $NaHCO_3$ concentrations (Fig. 5). In this alga, the products of short term photosynthetic $^{14}CO_2$ fixation resembled those of C_3 plants (cf. Fig. 2 in Chapter II). Although ^{14}C-glycolate was detected at high O_2 concentrations as in terrestrial C_3 plants, the amount was much less than that expected from the degree of inhibition (cf. Table 1 in Chapter II). These results, together with the low CO_2 concentration of about 10 μM for saturating the rate of photosynthesis (Fig. 5), suggest that a CO_2-concentrating mechanism operates to depress RuBP oxygenase activity by raising the CO_2/O_2 ratio at the reaction site of RuBP carboxylase-oxygenase in this alga, as suggested in some species

of green algae (Raven and Glidewell 1978, Badger et al. 1980, Imamura et al. 1981).

CO_2-independent oxygen inhibition of photosynthesis has been observed in "air-grown" Chlorella pyrenoidosa (Shelp and Canvin 1980). They suggested that the CO_2-independent oxygen inhibition may be caused by the interaction of H_2O_2, a product of the Mehler reaction (Mehler 1951), with the light-generated sulfhydryl groups of the enzymes in the Calvin cycle. On the other hand, Radmer et al. reported light-induced uptake of oxygen in Scenedesmus obliquous, Chlorella vulgaris and Anacystis nidulans (Radmer and Kok 1976, Radmer et al. 1978, Radmer and Ollinger 1980). They suggested that oxygen inhibits the photo-reduction of $NADP^+$ competitively by the Mehler reaction. The oxygen inhibition in Chroomonas cells also seems to be mainly caused by the interaction of oxygen with photosynthetic electron transport via the Mehler reaction, and partly by the photorespiratory reaction.

Anaerobic inhibition

Anaerobic inhibition of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. cells was observed only under high light intensities, and no inhibition was detected when the light intensity limited the rate of photosynthesis (Fig. 2). This inhibition was continued for at least 25 min and was fully reversed by 2% O_2 (Fig. 3A).

Miyachi and Okabe (1976) reported that photosynthetic $^{14}CO_2$ fixation in a blue-green alga A. nidulans was enhanced by oxygen and was not inhibited by anaerobiosis. The enhancement occurs at low CO_2 concentrations under high light intensities. They suggested that the enhancement might be caused by the transport of CO_2 from outside the cells to the site of RuBP carboxylase, which would be facilitated by the presence of oxygen. In Chroomonas cells, this explanation appears inappropriate, since the rate of $^{14}CO_2$ fixation under N_2 decreased with increasing light intensity (Fig. 2) and the inhibition by anaerobiosis was not affected by CO_2 concentrations (Fig. 5).

The rate of photosynthesis in several species of terrestrial C_3 plants decreased when the O_2 concentration was reduced from 21 to 2% at high light intensities (Viil and Pärnik 1974, Viil et al. 1977, Canvin 1978, McVetty and Canvin 1981). This inhibition was only a transient effect (McVetty and Canvin 1981), in contrast to the inhibition by anaerobiosis in Chroomonas sp. (Fig. 3A). But the cause of the inhibition by "low oxygen" seems to be similar to that of the inhibition by "anaerobiosis" in Chroomonas sp; the inhibition by "low oxygen" was observed under high light and high CO_2 conditions, and was suggested to be associated with the interaction of oxygen with photochemical reactions (McVetty and Canvin 1981).

On the other hand, light-induced oxygen uptake unrelated

to photorespiration has been observed in intact chloroplasts and isolated cells from higher plants (Egneus et al. 1975, Marsho et al. 1979, Ziem-Hanck and Heber 1980, Furbanck et al. 1982) and in algae (Bunt and Heeb 1971, Glidewell and Raven 1975, Radmer and Kok 1976, Radmer et al. 1978, Radmer and Ollinger 1980). Under aerobic conditions, even if linear electron flow from water to $NADP^+$ exceeds the capacity to reduce $NADP^+$ or to consume NADPH, oxygen can act as another electron acceptor to prevent the over-reduction of electron transport carriers. Under anaerobic conditions, on the other hand, excess electrons might not be consumed. This could cause the over-reduction, which suppresses cyclic electron flow (Kaiser and Urbach 1976, Heber et al. 1978, Ziem-Hanck and Heber 1980).

In Chroomonas cells, photosynthetic $^{14}CO_2$ fixation was inhibited by anaerobiosis only at high light intensities and the inhibition was released by 2% O_2 (Fig. 1, 2 and 3A). These results suggest that the inhibition is caused by over-reduction of electron transport carriers. Increase in the pre-bubbling time did not result in complete inhibition of photosynthetic $^{14}CO_2$ fixation under anaerobic and high light conditions (Fig. 4A). This suggests that non-cyclic photophosphorylation is not inhibited significantly by anaerobiosis under these conditions, although it is not clear whether cyclic photophosphorylation is inhibited completely. The role of oxygen appears to be in poising electron car-

riers of the cyclic electron flow or in acting as an electron acceptor of pseudo-cyclic electron flow from water (Egneus et al. 1975, Kaiser and Urbach 1976, Heber et al. 1978, Ziem-Hanck and Heber 1980, McVetty and Canvin 1981, Furbanck et al. 1982). Supply of the additional ATP under anaerobic conditions by cyclic photophosphorylation and under aerobic conditions by cyclic and/or pseudo-cyclic photophosphorylation seems to be necessary for normal operation of photosynthetic CO_2 fixation in Chroomonas cells. The additional ATP seems to be provided by cyclic photophosphorylation even under anaerobic conditions, only if the rate of the electron flow from water to $NADP^+$ does not exceed its consumption rate, since the rate of photosynthetic $^{14}CO_2$ fixation was not inhibited by anaerobiosis under light-limiting conditions (Fig. 1 and 2).

Remaining for further clarification is whether mitochondrial respiration affects photosynthetic activity in this organism. Further studies are needed to clarify the mechanism of the oxygen effect on photosynthetic CO_2 fixation in Chroomonas sp. cells.

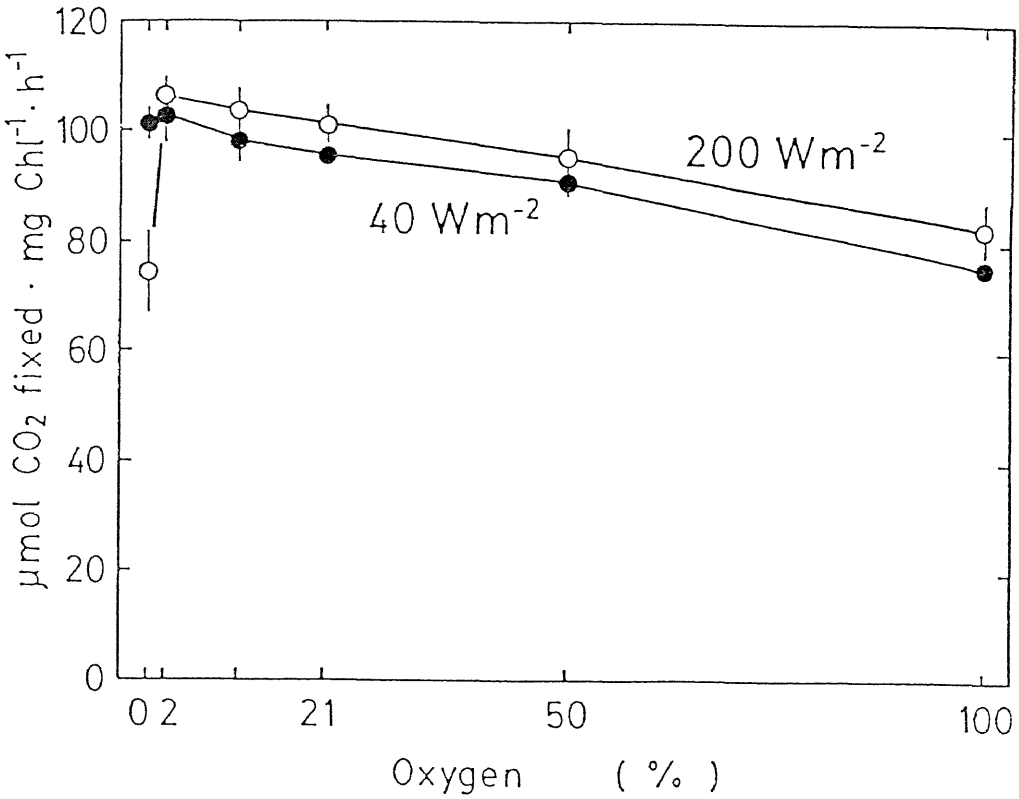

Fig. 1 Effect of oxygen concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. at 40 and 200 $W \cdot m^{-2}$. The rate was calculated from the amount of ^{14}C fixed for 5 min after 10-min preillumination. Three series of experiments were carried out with cells from different batch cultures to give the average of three measurements. Standard deviations are indicated by the vertical bars. Chl a contents and $NaHCO_3$ concentrations in the three experiments were; 4.1 $\mu g \cdot ml^{-1}$ and 1.4 mM; 5.0 $\mu g \cdot ml^{-1}$ and 0.71 mM; 4.9 $\mu g \cdot ml^{-1}$ and 0.71 mM.

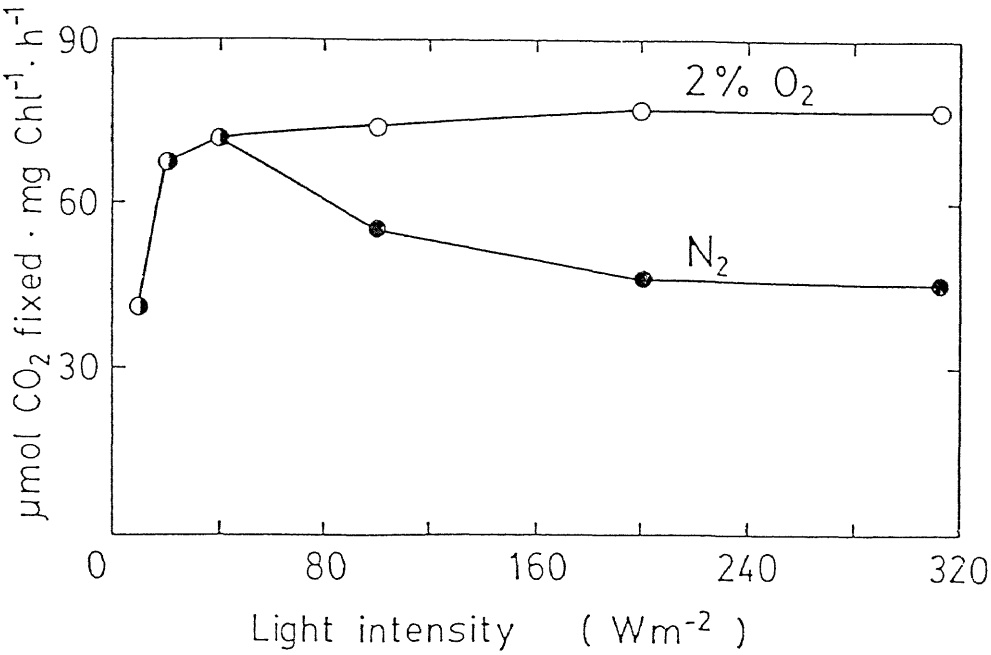

Fig. 2 Effect of light intensity on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2. Chl a content and $NaHCO_3$ concentration were 2.8 μg·ml^{-1} and 0.71 mM, respectively.

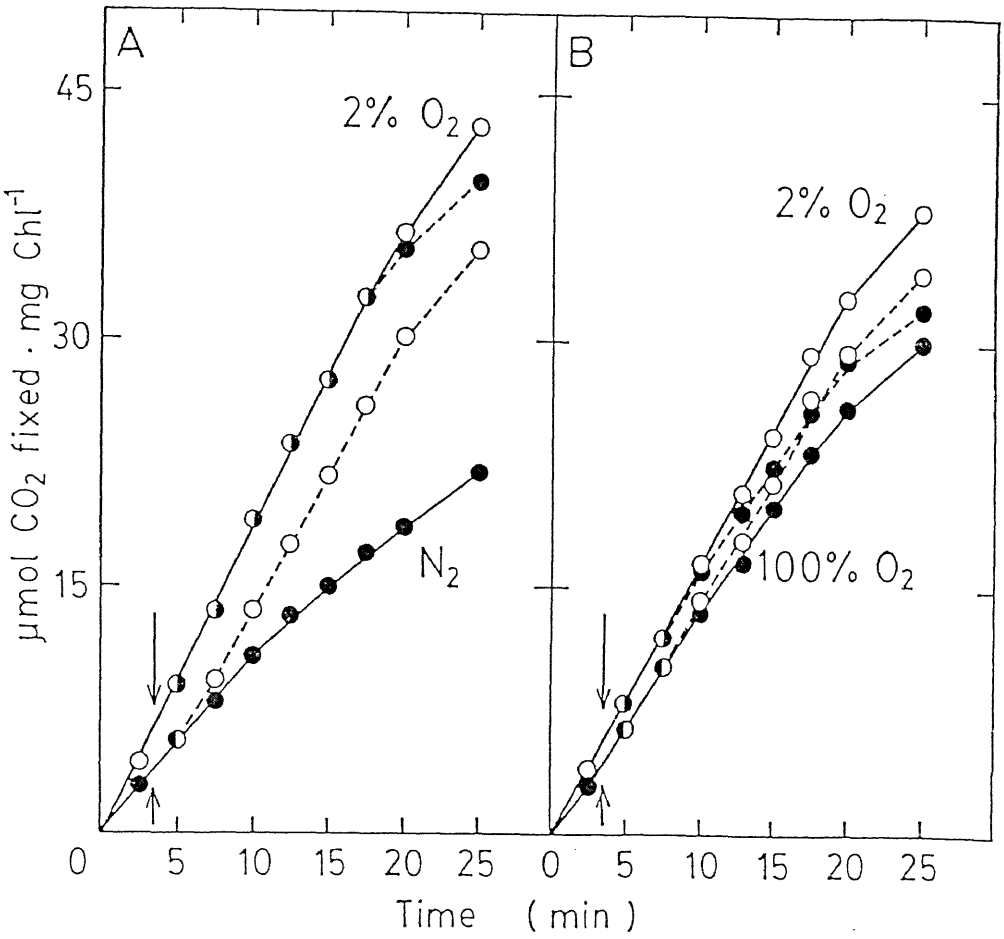

Fig. 3 Effect of changing the composition of the bubbling gas on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. Solid lines; the O_2 concentration was constant throughout the experiment. Broken lines; the O_2 concentration was changed 3.5 min after the injection of $NaH^{14}CO_3$ as indicated by the arrows. Chl a contents were 3.4 μg·ml^{-1} (A) and 4.2 μg·ml^{-1} (B). $NaHCO_3$ concentration and light intensity were 0.76 mM and 200 W·m^{-2}, respectively.

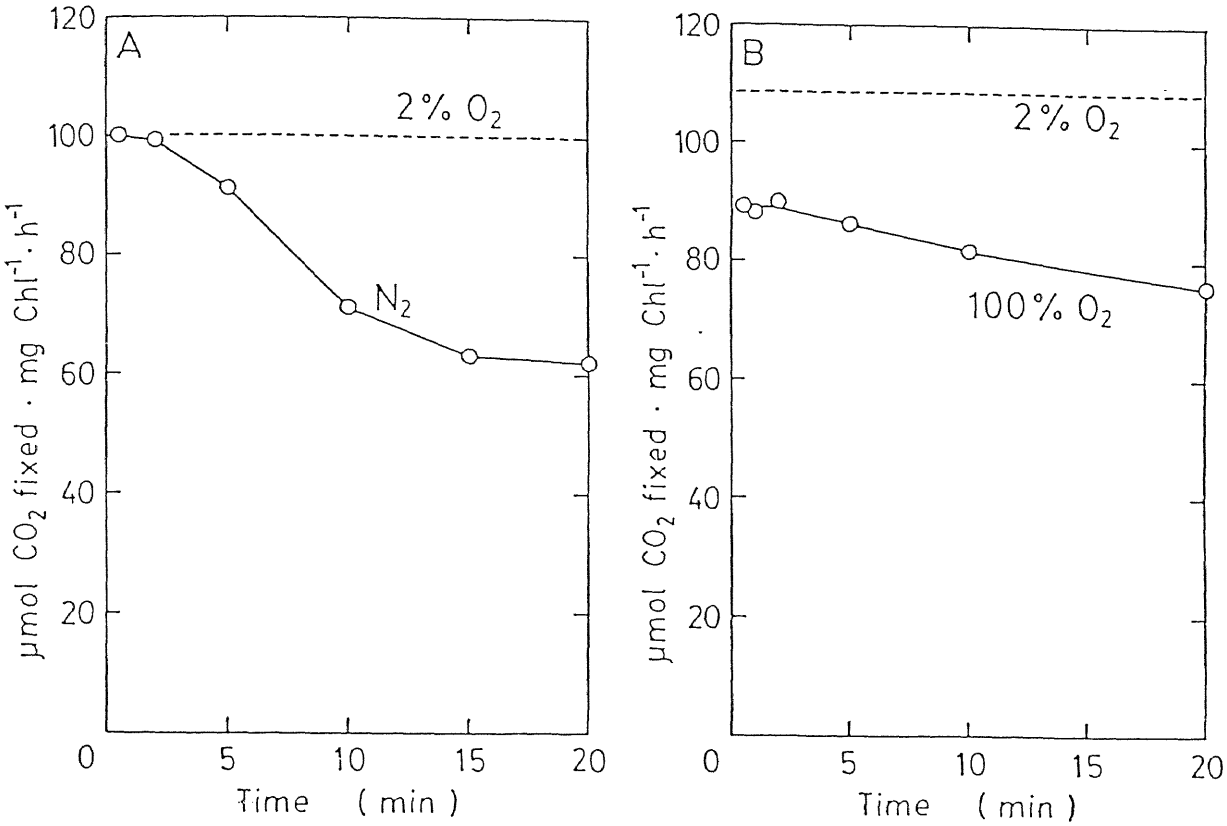

Fig. 4 Effect of changing the composition of the bubbling gas during the preillumination period on the rate of subsequent photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. Broken lines; the rate of photosynthetic $^{14}CO_2$ fixation under 2% O_2 measured after 20-min preillumination under 2% O_2. The cell suspension was preilluminated for 20 min, during which the bubbling gas was changed from 2% O_2 to N_2 (A) or to 100% O_2 (B), and then $NaH^{14}CO_3$ was injected. The pre-bubbling time with N_2 (A) or with 100% O_2 (B) was changed as indicated. The rate of photosynthesis was calculated from the amount of ^{14}C fixed during 5 min of illumination under N_2 (A) or under 100% O_2 (B). (A) Chl a content, 3.8 $\mu g \cdot ml^{-1}$; $NaHCO_3$, 0.71 mM. (B) Chl a content, 4.4 $\mu g \cdot ml^{-1}$; $NaHCO_3$, 0.73 mM. The light intensity was 200 $W \cdot m^{-2}$, for A and B.

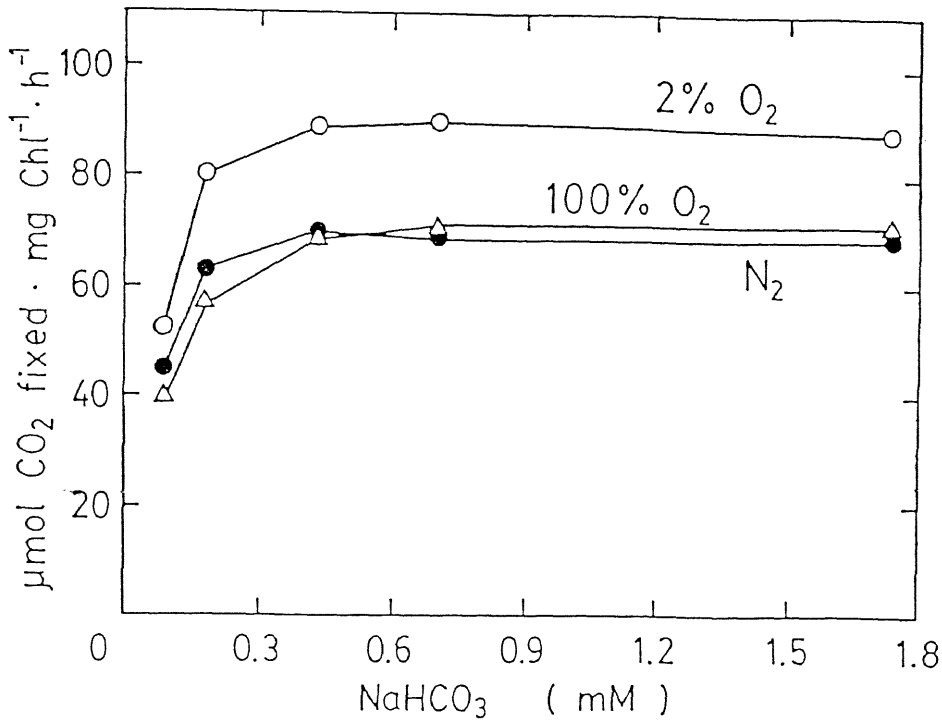

Fig. 5 Effect of $NaHCO_3$ concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2, 2% O_2 and 100% O_2. Chl a content and the light intensity were 4.8 $\mu g \cdot ml^{-1}$ and 200 $W \cdot m^{-2}$, respectively.

CHAPTER II

PATH OF CARBON AND THE OXYGEN EFFECT IN PHOTOSYNTHESIS OF *CHROOMONAS* SP. CELLS

ABSTRACT

Time courses of ^{14}C-incorporation into individual products during photosynthesis under 21% O_2 at 200 $W \cdot m^{-2}$ revealed that 3-phosphoglycerate was the initial product of photosynthetic CO_2 fixation in Chroomonas sp. cells; about 70% of the fixed ^{14}C in the ethanol soluble fraction was found in 3-phosphoglycerate after 10 s of photosynthetic $^{14}CO_2$ fixation and then the percent of ^{14}C incorporated into 3-phosphoglycerate rapidly decreased with the rest of time.

During 5 min of photosynthetic $^{14}CO_2$ fixation, a considerable amount of ^{14}C was incorporated into the insoluble fraction (mostly starch), and the effect of oxygen was observed predominantly in ^{14}C-incorporation into this fraction.

Although ^{14}C-incorporation into the intermediates of photorespiratory pathway increased with increasing O_2 concentration, the amounts were much less than that expected from the degree of oxygen inhibition; 3.6% in glycolate and 0.8% in glycine and serine, after 5 min of photosynthetic $^{14}CO_2$ fixation under 100% O_2.

It is noteworthy that ^{14}C-dihydroxyacetone phosphate accumulated during photosynthesis only under anaerobic condition, whereas the levels of the other phosphate esters were scarcely affected by the O_2 concentration.

ABBREVIATIONS

Chl, chlorophyll; EDTA, ethylenediaminetetraacetic acid; RuBP, ribulose-1,5-bisphosphate.

INTRODUCTION

Photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. cells was inhibited by anaerobiosis, as well as by oxygen at high concentrations, as shown in Chapter I. Although the oxygen inhibition of photosynthesis in terrestrial C_3 plants has been attributed mainly to photorespiration derived from the oxygenase activity of RuBP carboxylase-oxygenase (Beck 1979, Canvin 1979), the inhibition by both anaerobiosis and high concentrations of oxygen in Chroomonas cells appear to be mainly caused by changes in the rates of photochemical reactions, rather than by the regulation of RuBP carboxylase-oxygenase via changing the ratio of the CO_2 and O_2 concentrations (Chapter I).

In the Cryptophyta, the accumulation of starch and lipids was observed cytochemically and electron-microscopically in Chroomonas salina under both heterotrophic and autotrophic conditions (Antia et al. 1973, 1974). And the chemical properties of the starch produced heterotrophically have been characterized in Chilomonas paramecium (Archibald et al. 1960) and in Chroomonas salina (Antia et al. 1979). However, no investigation has been reported on the photosynthetic carbon metabolism, much less on the effect of oxygen on the carbon metabolism.

In this chapter, the author presents the evidence which indicates the operation of the reductive pentose phos-

phate cycle (the Calvin cycle) during photosynthesis, and characterizes the effect of oxygen on the photosynthetic carbon metabolism in Chroomonas sp. cells.

MATERIALS AND METHODS

Algal culture

Chroomonas sp. (CHR-1A) cells were grown axenically in "YT medium" at 18°C, aerated continuously with air as described in Chapter I.

Photosynthetic $^{14}CO_2$ fixation

Photosynthetic $^{14}CO_2$ fixation was carried out using a 1 ml series of cell suspensions placed in small spitz-type test tubes at 20°C, and bubbled with CO_2-free air, N_2 gas, O_2 gas, or 2% O_2 (in N_2 gas), from a long hypodermic needle at a flow rate of 100 $ml \cdot min^{-1}$. The tubes were illuminated from one side at 200 $W \cdot m^{-2}$ with a halogen lamp. After 10 min of illumination, $^{14}CO_2$ fixation was started by injecting $NaH^{14}CO_3$, and 5 min later, stopped by adding ethanol to give 80% (v/v). The ethanol suspensions were then acidified by adding 50 µl of acetic acid and an aliquot of 50 µl was put on a small disc of filter paper, and dried under an infra-red lamp. The amount of ^{14}C was determined using a liquid scintillation spectrometer as described in Chapter I.

For time course experiments, 6 ml of algal suspension was placed in a test tube. After injecting $NaH^{14}CO_3$, an aliquot of 1 ml of algal suspension was quickly taken out at intervals with an automatic pipette into ethanol (final

concentration, 80%, v/v). The subsequent procedures were the same as described above.

Analysis of $^{14}CO_2$ fixation products

The ethanol suspension was filtered through a Millipore membrane filter (pore size, 0.45 μm) with a suction pump. The cells on the membrane filter were extracted six times with 3 ml of 80% ethanol. Extracts were combined and a portion was taken out to determine the radioactivity in the 80% ethanol soluble fraction. The radioactivity of the residue on the filter paper (the 80% ethanol insoluble fraction) was determined with a liquid scintillation spectrometer. The rest of the ethanol extract was concentrated in vacuo at about 37°C and chromatographed two-dimensionally on Whatman 3 MM filter paper, first with phenol–acetic acid–water–0.1 M EDTA (369.5 : 5 : 80.7 : 50, v/v), and then in second dimension with the solvent which was freshly prepared from equal volumes of n-butanol–water (249 : 16, v/v) and propionic acid–water (207 : 263, v/v) (Ashino-Fuse and Ikawa 1981). After radioautograms were prepared, each radioactive area was cut off and its radioactivity was determined with a liquid scintillation spectrometer. The individual compounds were identified on paper by co-chromatography with authentic compounds. Amino acids, organic acids, free sugars and phosphate esters were identified as described by Akagawa et al. (1972). Glycolate was identified by co-

chromatography with authentic ^{14}C-glycolate. Dihydroxy-acetone phosphate was identified as the free sugar after removing the phosphate group by enzymic hydrolysis with wheat germ acid phosphatase (Type I, Sigma Chemical Co.) (Ogasawara and Miyachi 1970). Paper co-chromatography of organic acids was also carried out with n-butyl formate–formic acid–water (10 : 4 : 1, v/v)(Blurdstone 1963).

Other details were described in "MATERIALS AND METHODS" in Chapter I.

RESULTS

Time course of photosynthetic $^{14}CO_2$ fixation

Fig. 1 shows that the total amount of photosynthetic $^{14}CO_2$ fixation products increased linearly for 5 min under 21% O_2. The percent of ^{14}C incorporated into the 80% ethanol soluble fraction was about 86% after 10 s of $^{14}CO_2$ fixation, and decreased with the time of photosynthesis. On the other hand, the percent of ^{14}C incorporated into the insoluble fraction increased with the time and was about 67% after 5 min of $^{14}CO_2$ fixation. About 96% of ^{14}C in the insoluble fraction after 5-min photosynthesis was solubilized in 80% ethanol after incubation with α-amylase in 0.1 M acetate buffer (pH 6.0) at 30°C for 48 h, and about 97% after hydrolysis with 1 N H_2SO_4 at 100°C for 4 h. These results indicate that almost all the ^{14}C in this fraction after 5 min of photosynthetic $^{14}CO_2$ fixation was incorporated into a starch-type polysaccharide, which seems to be equivalent to the "cryptomonad starch" in *C. salina* (Antia et al. 1979).

Time courses of the changes in percentage distributions of ^{14}C incorporated into individual compounds in the 80% ethanol soluble fraction during photosynthetic $^{14}CO_2$ fixation under 21% O_2 are shown in Fig. 2. Most of ^{14}C incorporated into the 80% ethanol soluble fraction during the first 10 s was found in 3-phosphoglycerate, which decreased

rapidly during the rest of the time period. On the other hand, the percentage of ^{14}C incorporated into C_4-compounds such as aspartate and malate was very small and increased with the time. These results indicate that photosynthetic CO_2 fixation in Chroomonas sp. cells is mainly carried out through the reductive pentose phosphate cylce, at least under our experimental conditions. The percentage of ^{14}C incorporated into lipids rapidly increased with the time and attained more than 50% after 5 min of photosynthetic $^{14}CO_2$ fixation under 21% O_2. On the other hand, the percentages of ^{14}C incorporated into glycolate, glycine and serine, which are intermediates of glycolate pathway, were very small.

Effect of oxygen

Effect of oxygen on the distributions of ^{14}C in the 80% ethanol soluble and the insoluble fractions during 5 min of photosynthesis was presented in Fig. 3. As described in Chapter I, the rate of photosynthetic $^{14}CO_2$ fixation was inhibited under N_2 and 100% O_2, and the maximal rate was obtained under 2% O_2 at high light condition (200 $W \cdot m^{-2}$). A considerable amount of ^{14}C was fixed in the insoluble fraction under each O_2 concentrations, and the effect of oxygen on the rate of total $^{14}CO_2$ fixation in Chroomonas cells is represented by the change in the amount of ^{14}C incorporated into the insoluble fraction much more than

that into the 80% ethanol soluble fraction (Fig. 3).

Table 1 shows that the effect of oxygen on the percentage of ^{14}C incorporated into individual compounds during 5 min of photosynthetic $^{14}CO_2$ fixation. Although the percentage distributions of the radioactivity in glycolate, glycine and serine increased with increasing O_2 concentration, they were very small (less than 0.5%, respectively) under O_2 concentrations up to 21%, and that in glycolate was only 3.6% even under 100% O_2. These results suggest that photorespiration occurs during photosynthesis under O_2 concentrations higher than 21% at a saturating $NaHCO_3$ concentration (0.7 mM), but its contribution to the oxygen inhibition of photosynthesis is considerably low. The percentage distributions of radioactivity in glutamate and fumarate were also increased with increasing O_2 concentration and were higher than that in photorespiratory pathway intermediates such as glycolate, glycine and serine. The percentage radioactivity in phosphate esters except for dihydroxyacetone phosphate were scarcely affected by the O_2 concentration. However, the percentage in dihydroxyacetone phosphate was more than 8 times under N_2 as large as under 2% O_2, whereas it was scarcely affected when the O_2 concentration in the bubbling gas was 2% or higher.

DISCUSSION

Photosynthesis in terrestrial C_3 plants is inhibited considerably by oxygen even in ambient air condition (21% O_2, 0.03% CO_2); about 30% inhibition is observed (cf. Raven and Glidewell 1978). The inhibition is mainly associated with photorespiration derived from the oxygenase activity of RuBP carboxylase-oxygenase, and is released by increasing the CO_2 concentration (Beck 1979, Canvin 1979).

In Chroomonas cells, the pattern of photosynthetic $^{14}CO_2$ fixation closely resembled those of C_3 plants (Fig. 2). The author, therefore, concluded that the reductive pentose phosphate cycle is the major pathway of photosynthetic CO_2 fixation in Chroomonas sp. cells. However, the effect of oxygen on photosynthesis in Chroomonas cells seems to be different from that in terrestrial C_3 plants. Photosynthetic $^{14}CO_2$ fixation was scarcely inhibited by 21% O_2, and the degree of the inhibition was only about 25% even under 100% O_2 (Fig. 3), and was scarcely affected by CO_2 concentration (Chapter I). Therefore, the inhibition seems to be attributed only partly to the oxygenase activity of RuBP carboxylase-oxygenase, and the low sensitivity of photosynthesis to oxygen in Chroomonas cells seems to be attributed to the suppression of photorespiration. The percent radioactivity incorporated into photorespiratory pathway intermediates, such as glycolate,

glycine and serine, was almost negligible at O_2 concentrations up to 21%, and was very small even under 100% O_2 (Table 1). These results indicate that photorespiration is severely limited during photosynthesis in Chroomonas cells. The apparent half-saturation constant for CO_2 in the light in Chroomonas cells was relatively small (about 3 μM) (cf. Fig. 5 in Chapter I) and high activity of carbonic anhydrase, which is suggested to play an important role in a CO_2-concentrating mechanism (Imamura et al. 1981), was detected in this algal cells (data not shown). These results suggest that photorespiration is considerably suppressed in Chroomonas cells by raising the CO_2/O_2 ratio at the reaction site of RuBP carboxylase-oxygenase due to a CO_2-concentrating mechanism as suggested in some species of green algae (Raven and Glidewell 1978, Badger et al. 1980, Imamura et al. 1981).

On the other hand, the percentage radioactivity in glutamate and fumarate increased with increasing O_2 concentration (Table 1). CO_2 release in the tricarboxylic acid cycle can also decrease the rate of $^{14}CO_2$ fixation under aerobic conditions. However, it is doubtful that the tricarboxylic acid cycle operates completely during photosynthesis in photoautotrophs (Benedict 1978). Under photosynthetic conditions, the intermediates of the tricarboxylic acid cycle can be formed by β-carboxylation of phosphoenolpyruvate, which is derived from 3-phosphoglycerate, a re-

ductive pentose phosphate cycle intermediate, and by the generation of acetyl CoA from pyruvate even if the tricarboxylic acid cycle is blocked by repressing α-ketoglutarate dehydrogenase as observed in many obligate photoautotrophs (Benedict 1978). In that case, the magnitude of the CO_2 release in a "incomplete tricarboxylic acid cycle" during photosynthesis can be estimated to be less than 2% of the total ^{14}C fixed under 100% O_2. It is much less than the degree of the oxygen inhibition of photosynthetic $^{14}CO_2$ fixation under 100% O_2 in Chroomonas cells.

Under anaerobic conditions, the rate of photosynthetic $^{14}CO_2$ fixation was inhibited to about 70% of that under 2% O_2 at high light intensity (Fig. 3). Anaerobiosis did not significantly affect the percent distributions of ^{14}C in individual compounds, except those in dihydroxyacetone phosphate and the insoluble fraction (Table 1). ^{14}C-dihydroxyacetone phosphate accumulated only under anaerobic condition. Dihydroxyacetone phosphate, the isomer of glyceraldehyde-3-phosphate, is formed from 3-phosphoglycerate by the reaction which is the only reductive reaction in the main pathway of photosynthetic CO_2 assimilation. Therefore, this reaction utilizes all of the NADPH which consumed in the reductive pentose phosphate cycle. And the regeneration of RuBP via a series of reactions so-called "sugar phosphate shuffle" from dihydroxyacetone phosphate requires ATP. Anaerobiosis depressed the incorporation of ^{14}C into

the starch-type polysaccharide. Starch synthesis also requires ATP. Thus, the accumulation of ^{14}C-dihydroxy-acetone phosphate and the depression of ^{14}C-incorporation into the insoluble fraction seem to be closely related to the deficiency of ATP and the excess supply of NADPH. These results are consistent with the assumption that the anaerobic inhibition of photosynthetic $^{14}CO_2$ fixation in Chroomonas cells is caused by ATP deficiency which is a result of the over-reduction of electron transport carriers (Chapter I).

Table 1 Effect of oxygen on the percent incorporation of ^{14}C into products during 5 min of photosynthetic $^{14}CO_2$ fixation

Compounds	Concentration of atmospheric oxygen (%)			
	0	2	21	100
PGA	2.4	2.4	2.1	1.9
Sugar phosphates	6.2	2.3	2.1	2.1
UDPG	0.6	0.7	0.7	0.6
DHAP	4.4	0.5	0.6	0.6
others	1.2	1.1	0.8	0.9
Alanine	0.9	0.6	0.8	0.5
Aspartate	2.8	3.7	2.3	1.1
Asparagine	0.4	0.4	0.3	0.3
Malate	0.9	1.1	0.5	0.6
Succinate	0.4	0.6	0.3	0.1
Fumarate	0.4	0.5	0.7	3.8
Glutamate	0.2	0.4	1.4	6.0
Glutamine	0.0	0.0	0.0	0.2
Glycolate	0.0	0.1	0.3	3.6
Glycine + Serine	0.3	0.3	0.4	0.8
Lipids	18.9	17.5	17.3	13.2
Unidentified (C)	1.5	0.8	1.1	0.6
Unidentified (D)	0.7	0.4	0.5	0.3
Insoluble	63.7	68.5	68.9	62.1

Symbols: DHAP, dihydroxyacetone phosphate; UDPG, uridine-5'-diphospho-glucose; PGA, 3-phosphoglycerate.

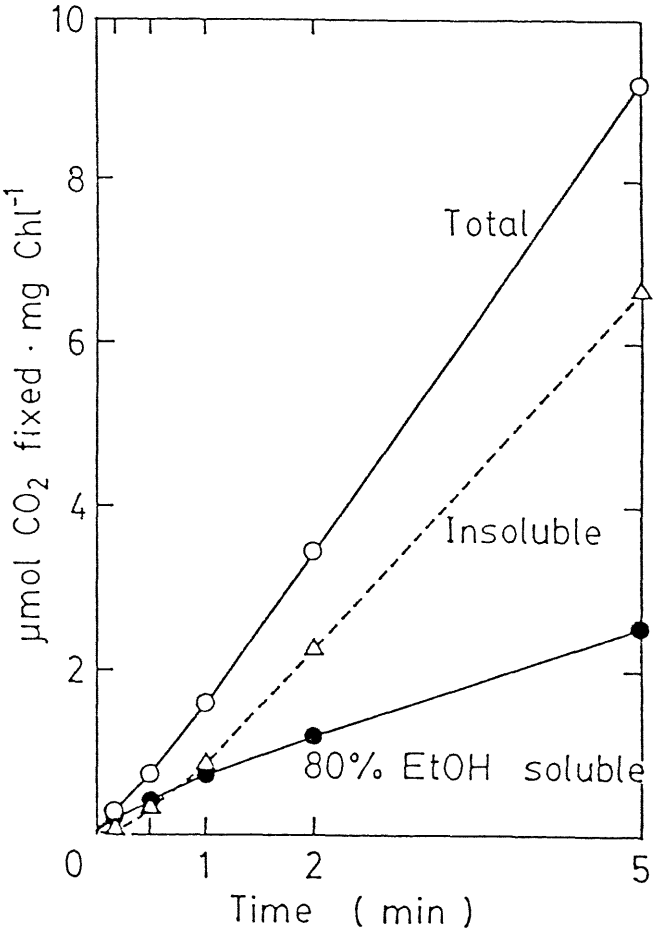

Fig. 1 Time course of ^{14}C-incorporation into the 80% ethanol soluble and the insoluble fractions during photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under 21% O_2. Chl a content and $NaHCO_3$ concentration were 7.8 $\mu g \cdot ml^{-1}$ and 0.70 mM, respectively.

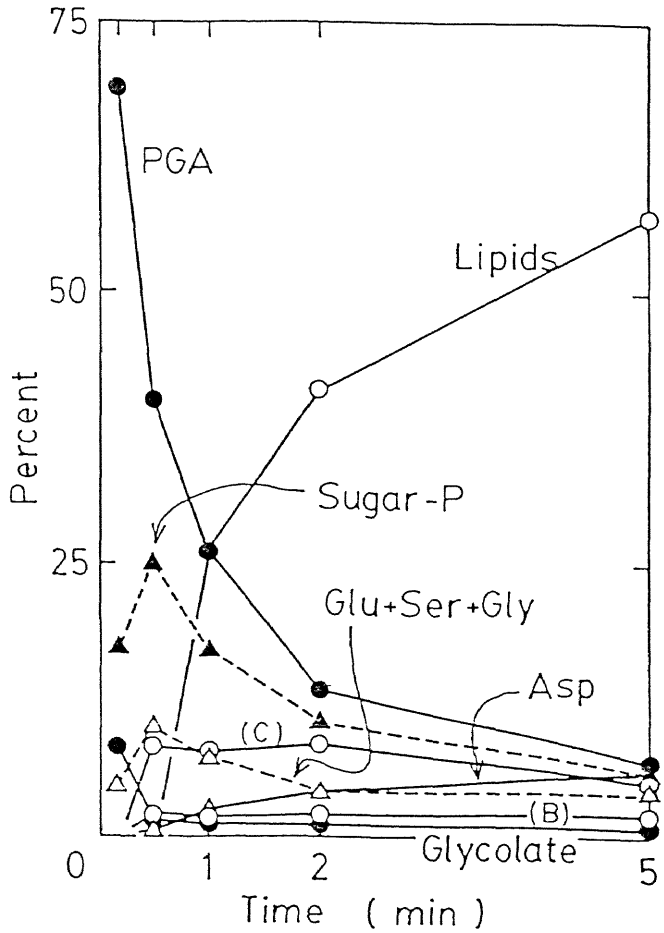

Fig. 2 Percentage distribution of ^{14}C in individual products in the 80% ethanol soluble fraction versus time of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. Data are from the same experiment as described in Fig. 1. (B), fumarate; (C), an unidentified product.

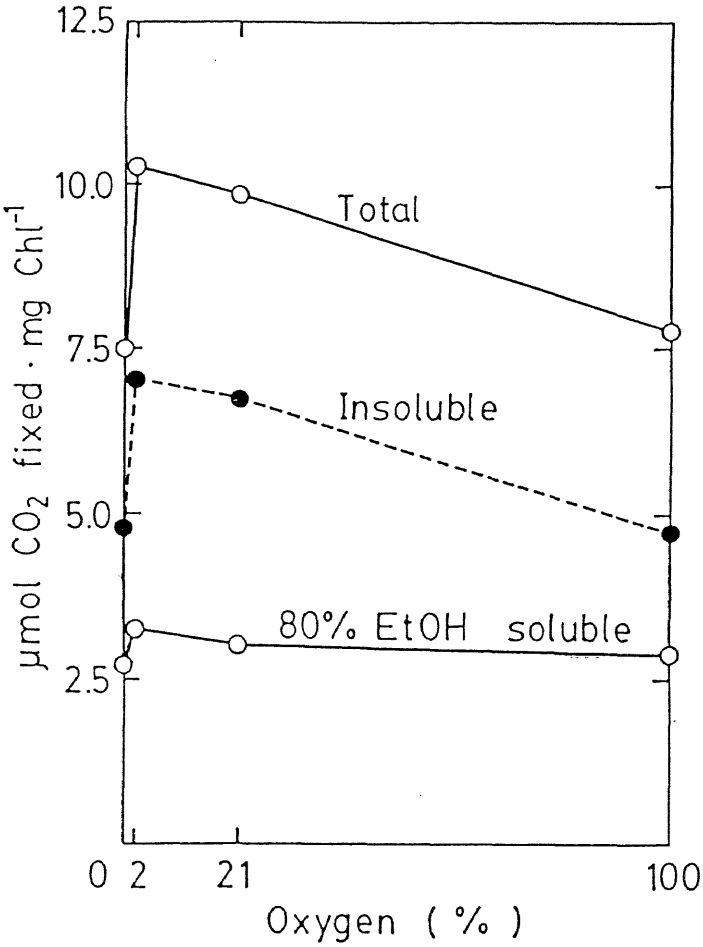

Fig. 3 Effect of oxygen on the rate of photosynthetic $^{14}CO_2$ fixation and the distribution of ^{14}C in the 80% ethanol soluble and the insoluble fractions in Chroomonas sp. Chl a content and $NaHCO_3$ concentration were the same as in the experiment shown in Fig. 1. Data are from the same experiment as described in Table 1.

CHAPTER III

EFFECTS OF INHIBITORS, UNCOUPLERS AND AN ARTIFICIAL ELECTRON MEDIATOR ON THE INHIBITION OF $^{14}CO_2$ FIXATION BY ANAEROBIOSIS IN *CHROOMONAS* SP. CELLS

ABSTRACT

In "air-grown" Chroomonas sp. cells, low concentrations of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (less than 0.1 μM) could prevent the inhibition of photosynthetic $^{14}CO_2$ fixation by anaerobiosis at light-saturating conditions (more than 40 $W \cdot m^{-2}$), and phenazine methosulfate showed similar effect. Antimycin A, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and N,N'-dicyclohexylcarbodiimide strongly inhibited anaerobic photosynthesis at concentrations which did not significantly inhibit the rate under 2% O_2 at high light intensity (200 $W \cdot m^{-2}$), although 0.2 μM CCCP stimulated the rate under 2% O_2 to some extent. On the other hand, KCN inhibited the rate much more strictly under 2% O_2 than N_2, although it inhibited the rate very strongly at concentrations above 5 μM under both N_2 and 2% O_2. These results suggest that the inhibition of photosynthetic $^{14}CO_2$ fixation by anaerobiosis in this alga would result from ATP deficiency which would be caused by an over-reduction of electron carriers of cyclic electron flow, and that the role of oxygen would be in preventing the over-reduction. Cyclic electron flow seems to be necessary to provide additional ATP for CO_2 reduction at least under anaerobic conditions in this alga, although it seems to be less necessary under aerobic conditions.

ABBREVIATIONS

CCCP, carbonyl cyanide m-chlorophenylhydrazone; Chl, chlorophyll; Cyt, cytochrome; DCCD, N,N'-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PMS, phenazine methosulfate; PS I, photosystem I; PS II, photosystem II; RuBP, ribulose-1,5-bisphosphate.

INTRODUCTION

Photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. cells was inhibited remarkably by anaerobiosis (bubbled with N_2 gas), compared with 2% O_2 (Chapters I and II). This was observed only under light-saturating conditions (above 40 $W \cdot m^{-2}$), and the degree of inhibition under N_2 increased with an increase of light intensity up to 200 $W \cdot m^{-2}$, but it was not affected by CO_2 concentration. This inhibitory effect by anaerobiosis seems to be mainly concerned with the decrease in the level of RuBP regenerated, rather than the decrease in the availability of CO_2 at the site of carboxylation reaction. It is very likely that the regeneration of RuBP is limited by the level of ATP and/or NADPH produced in photosynthetic electron transport systems and photophosphorylation.

Anaerobiosis appears to inhibit cyclic photophosphorylation in intact spinach chloroplasts, by causing an "over-reduction" of electron carriers of cyclic eletron transport system under high light conditions (Kaiser and Urbach 1976, Ziem-Hanck and Heber 1980, Steiger and Beck 1981). Under aerobic conditions, in those studies, it has been proposed that excess electrons are consumed to reduce oxygen at the reducing side of PS I (the Mehler reaction), and thus over-reduction is prevented.

Although the role of cyclic photophosphorylation in vivo

has been argued in some species of algae (Klob et al. 1973, Simonis and Urbach 1973), it has not been shown whether enough over-reduction can occur to inhibit CO_2 fixation in algal cells *in vivo*. It is possible, however, to explain the inhibition of photosynthetic $^{14}CO_2$ fixation by anaerobiosis in *Chroomonas* cells in terms of ATP deficiency which may result from over-reduction of electron carriers (cf. Chapter I and II). In this chapter, the author presents several lines of evidence for this assumption, using some inhibitors and uncouplers, as well as an artificial electron mediator.

MATERIALS AND METHODS

Algal culture

Chroomonas sp. (CHR-1A) cells were grown axenically in "YT medium" at 18°C, aerated without supplementary CO_2 (see "MATERIALS AND METHODS" in Chapter I).

Determination of photosynthetic $^{14}CO_2$ fixation

Photosynthetic $^{14}CO_2$ fixation was carried out using a 1 ml series of cell suspensions placed in small spitz-type test tubes at 20°C, and bubbled with N_2 gas or 2% O_2 (in N_2 gas), using CO_2-free gas, from a long hypodermic needle at a flow rate of 100 $ml \cdot min^{-1}$. Test tubes were illuminated from one side at 200 $W \cdot m^{-2}$, unless otherwise specified, with a halogen lamp. After 10 min of illumination, $^{14}CO_2$ fixation was started by injecting $NaH^{14}CO_3$, and stopped by adding ethanol to give 80% (v/v) after 5-min photosynthesis. The ethanol suspensions were acidified by adding 50 µl of acetic acid, and portions were put on small discs of filter paper, and then dried under an infra-red lamp. The amount of ^{14}C was determined using a liquid scintillation spectrometer.

Extraction and assay of ATP

The concentration of ATP in Chroomonas cells was measured by the luciferin-luciferase method (Strehler 1974).

After 10 min of preillumination, photosynthesis was started by adding $NaH^{12}CO_3$ (0.7 mM), and stopped by the addition of 0.2 ml of 2.5 M $HClO_4$, followed by allowing the mixture to stand for 5 min at 0°C. The mixture was neutralized by adding 1 ml of 0.5 M KOH and allowed to stand for 15 min at 0°C. The aliquot of the supernatant was diluted appropriately with 25 mM HEPES-$MgCl_2$-KOH buffer (pH 7.7) (St. John 1970), and was used for ATP determination. The reaction mixture (0.55 ml) for ATP determination contained 25 mM HEPES-KOH buffer (pH 7.7), 25 mM $MgCl_2$, 0.1 mM D-luciferin, 20 µg of luciferase preparation (Sigma, type VI) and the sample. The reaction was started by adding 50 µl of luciferase solution. After 15 s of mixing, the luminescence with ATP for 60 s was integrated using an integrating photometer (Sai Technology Co.).

Other details were described in "MATERIALS AND METHODS" in Chapter I.

RESULTS

Effect of DCMU

Under aerobic (2% O_2) conditions, the rate of photosynthetic $^{14}CO_2$ fixation at high light (200 $W \cdot m^{-2}$) and HCO_3^--saturating (0.71 mM) conditions was inhibited by DCMU at concentrations above 0.05 μM, and more than 60% inhibition was observed at 0.5 μM (Fig. 1, cf. Fig. 2 and 3 in Chapter I). In contrast, the rate under anaerobic (N_2) condition increased with an increase of DCMU concentration up to 0.1 μM, although it decreased with the further increase of DCMU concentration (Fig. 1).

Fig. 2 shows the relationship between the light intensity and the rate of $^{14}CO_2$ fixation under N_2, in the presence and absence of 0.05 μM DCMU. The depression of the rate of $^{14}CO_2$ fixation by anaerobiosis at high light intensities could not be observed in the presence of 0.05 μM DCMU, and the rate was almost restored to the level which was previously observed under 2% O_2 (cf. Fig. 2 in Chapter I). These results show that low concentration of DCMU can apparently substitute for oxygen under anaerobic condition.

Effect of antimycin A

Antimycin A at concentrations of 0.5 to 5 μM inhibited $^{14}CO_2$ fixation under aerobic and anaerobic conditions at high light intensity (200 $W \cdot m^{-2}$) (Fig. 3). Under 2% O_2,

about 20% inhibition of the rate was observed in the presence of 5 μM antimycin A. On the other hand, the rate under N_2 was strictly inhibited by antimycin A, and about 70% inhibition was observed even at a concentration of 1 μM. Antimycin A did not increase the percentage inhibition at concentrations higher than 1 μM (Fig. 3).

Similar experiments on the effect of antimycin A were performed at low light intensity (40 $W \cdot m^{-2}$), in which the inhibition of $^{14}CO_2$ fixation by anaerobiosis was hardly observed without the inhibitor (Fig. 4, cf. Fig. 2 in Chapter I). Under 2% O_2, similar magnitude of inhibition to that at 200 $W \cdot m^{-2}$ (Fig. 3) was observed. Under N_2, however, the inhibition by lower concentrations of antimycin A was not as strong as that at 200 $W \cdot m^{-2}$, although the percentage inhibition by 5 μM antimycin A was almost the same as that at 200 $W \cdot m^{-2}$ (Fig. 4).

Effect of PMS

Fig. 5 shows the effect of PMS, an artificial electron mediator of cyclic electron transport (Trebst 1974, 1980), on the rate of photosynthetic $^{14}CO_2$ fixation under N_2 and 2% O_2 at 200 $W \cdot m^{-2}$. Under 2% O_2, PMS scarcely affected the rate at concentrations lower than 2 μM, but inhibited it remarkably at higher concentrations. In contrast, under N_2, the rate of $^{14}CO_2$ fixation was stimulated by increasing the concentration of PMS up to 2 μM, and 4 μM PMS recovered

the rate almost to the same level as that under 2% O_2, although it inhibited the rate similarly to that observed under 2% O_2 at concentrations above 4 μM.

Effect of CCCP

To determine whether the inhibition of $^{14}CO_2$ fixation by anaerobiosis is concerned only with decreasing production of ATP coupled to electron transport, the effect of an uncoupler, CCCP (McCarty 1980), on the rate of $^{14}CO_2$ fixation was examined under N_2 and 2% O_2 at 200 $W \cdot m^{-2}$ (Fig. 6). The rate under 2% O_2 increased to some extent with increasing CCCP concentration up to 0.2 μM. At higher concentrations, CCCP inhibited the rate under 2% O_2; about 30% at 1 μM. In contrast, under anaerobic conditions, CCCP inhibited the rate about 90% at 0.5 μM and almost completely at 1 μM, although it did not affect the rate at 0.1 μM (Fig. 6).

Gramicidin S was also tested as another uncoupler (data not shown). However, no effect was observed at concentrations below 0.1 μM. Higher concentrations of this uncoupler caused a burst of the cells both under N_2 and 2% O_2.

Effect of DCCD

Fig. 7 shows the effect of DCCD, an energy transfer inhibitor (McCarty 1980), on the rate of photosynthetic

$^{14}CO_2$ fixation under N_2 and 2% O_2. The rate under 2% O_2 was scarcely inhibited by DCCD even at 10 μM. However, the rate under N_2 was inhibited markedly by DCCD above 1 μM, and about 60% inhibition was observed at 10 μM. DCCD caused a burst of the cells at concentrations above 100 μM (data not shown).

ATP levels in the cells illuminated under N_2 and 2% O_2

The ATP levels in Chroomonas cells during photosynthesis under 2% O_2 in the presence of 0.7 mM $NaHCO_3$ was about 70 to 80 nmol·mg Chl^{-1} and did not change significantly during photosynthesis. On the other hand, the ATP level under N_2 was about 35% lower than that under 2% O_2 at 30-sec photosynthesis, then decreased with time, and was less than the half of that under 2% O_2 after 10-min photosynthesis (Fig. 8)

Effect of KCN

Photosynthetic $^{14}CO_2$ fixation was inhibited considerably by KCN at concentrations below 50 μM both under N_2 and 2% O_2 (Fig. 9), and completely at 200 μM (data not shown). However, the degree of inhibition by KCN was much greater under 2% O_2 than that under N_2 (Fig. 9).

DISCUSSION

In Chroomonas cells, the rate of photosynthetic $^{14}CO_2$ fixation under 2% O_2 was inhibited by DCMU. In contrast, the rate under N_2 was considerably stimulated by adding low concentrations (below 0.1 μM) of DCMU under light- and HCO_3^--saturating conditions (Fig. 1), and the photosynthetic light response curve under N_2 with 0.05 μM DCMU (Fig. 2) is similar to that under 2% O_2 without DCMU (cf. Fig. 2 in Chapter I). These results suggest that excess eletron flow from PS II to PS I depresses photosynthetic $^{14}CO_2$ fixation under anaerobic and high light conditions, and that electron supply and its consumption can be properly poised by oxygen under 2% O_2, via a reaction such as the Mehler reaction (Mehler 1951).

Although it is still unclear whether non-cyclic photophosphorylation can satisfy energy requirement of CO_2 fixation in vivo, many investigators have suggested that cyclic (Schürman et al. 1972, Heber et al. 1978, Mills et al. 1978, Slovacek et al. 1978, Ziem-Hanck and Heber 1980, Steiger and Beck 1981, Woo 1983) or pseudo-cyclic photophosphorylation (Forti and Gerola 1977, Furbank et al. 1982) would provide additional ATP for CO_2 fixation in isolated chloroplasts from terrestrial C_3 plants, when non-cyclic photophosphorylation cannot provide sufficient ATP.

It has been shown, in intact spinach chloroplasts, that

cyclic photophosphorylation may be inhibited by excess electron flow from PS II under anaerobic conditions, and it can be stimulated by reducing electron pressure from PS II (Kaiser and Urbach 1976, Slovacek et al. 1978, Ziem-Hanck and Heber 1980, Steiger and Beck 1981). The inhibition by anaerobiosis under light-saturating conditions has been explained by over-reduction of electron carriers which would result from that excess electrons could not be drained off, and the role of oxygen is thought to be the redox poising of electron carriers (Kaiser and Urbach 1976, Ziem-Hanck and Heber 1980).

Effect of DCMU on photosynthetic $^{14}CO_2$ fixation under light-saturating conditions in Chroomonas cells (Fig. 1 and 2) is comparable well to that on the following processes in intact spinach chloroplasts; (1) dihydroxyacetone phosphate-dependent $^{14}CO_2$ fixation, which is thought to reflect the rates of cyclic and/or pseudo-cyclic photophosphorylation (Kaiser and Urbach 1976, Woo et al. 1983), (2) proton gradient formation in the absence of electron acceptors (Slovacek et al. 1978, Ziem-Hanck and Heber 1980), and (3) slow Chl a quenching in the absence or presence of a high concentration of HCO_3^- (Mills et al. 1978). These observations suggest that cyclic photophosphorylation is a limiting factor of CO_2 fixation under anaerobic conditions in Chroomonas cells.

In intact spinach chloroplasts, the rate of O_2 evolu-

tion under HCO_3^--saturating conditions is inhibited by anaerobiosis under saturating light, and the rate is stimulated by uncoupler level of NH_4Cl or CCCP, or by antimycin A at concentrations which partially block cyclic electron transport chain between Cyt $\underline{b}_6$ and $\underline{f}$ (Slovacek and Hind 1977, 1980).

In contrast, in Chroomonas cells, antimycin A did not stimulate the rate of photosynthetic $^{14}CO_2$ fixation under N_2 at light intensities of both 40 and 200 $W \cdot m^{-2}$, but strictly inhibited it at concentrations which inhibited the rate under 2% O_2 only less than 20% (Fig. 3 and 4). This suggests that a proton gradient back pressure (Slovacek and Hind 1977, 1980) would not occur in Chroomonas cells under anaerobic conditions and that cyclic electron flow might significantly contribute to provide ATP for CO_2 fixation under N_2, not only at 200 $W \cdot m^{-2}$ but also at 40 $W \cdot m^{-2}$ at which the inhibition by anaerobiosis could not be observed without antimycin A. Antimycin A inhibited the rate more strongly at 200 $W \cdot m^{-2}$ than at 40 $W \cdot m^{-2}$ at lower concentrations (Fig. 3 and 4), suggesting the inhibition of cyclic photophosphorylation by antimycin A accompanied with the over-reduction, or, possibly, a greater demand for cyclic photophosphorylation to provide ATP at higher light intensities. On the other hand, under aerobic conditions, cyclic photophosphorylation may be less necessary to provide the additional ATP, since $^{14}CO_2$ fixation under 2% O_2 was much

less sensitive to antimycin A than that under N_2 even at 40 $W \cdot m^{-2}$. Thus, the additional ATP appears to be predominantly provided by pseudo-cyclic photophosphorylation under 2% O_2.

PMS stimulated photosynthetic $^{14}CO_2$ fixation under N_2 at 200 $W \cdot m^{-2}$ at concentrations which scarcely affected the rate under 2% O_2 (Fig. 5). This result suggests that sufficient ATP cannot be provided for CO_2 fixation under N_2 at high light intensities. The inhibition of $^{14}CO_2$ fixation by higher concentrations of PMS under both atmospheric conditions (Fig. 5) may be due to an excess reoxidation of the primary reductant of PS I by PMS, because PMS must compete with NADPH regeneration, or due to the inhibition of linear electron flow to $NADP^+$ by a proton gradient back pressure which result from excessive "artificial" cyclic electron flow.

Effect of CCCP (Fig. 6), as well as that of DCCD (Fig. 7), also supports above conclusions. CCCP is known to inhibit ATP generation by dissipating the proton gradient by carrying the proton across the membrane, and DCCD is thought to inhibit ATP generation by reacting with a membrane proteolipid to prevent the flow of protons to the chloroplast coupling factor 1 (McCarty 1980). Both CCCP and DCCD significantly inhibited photosynthetic $^{14}CO_2$ fixation under N_2 at concentrations which inhibit it only slightly under 2% O_2. This, like the effect of antimycin A, suggests that a proton gradient back pressure did not occur under N_2 under high light

condition in Chroomonas cells and ATP supply would limit the rate of $^{14}CO_2$ fixation. Instead, CCCP stimulated $^{14}CO_2$ fixation under 2% O_2 (Fig. 6). This suggests that the back pressure did occur more or less under 2% O_2; namely ATP seems not to be rate-limiting under 2% O_2. On the other hand, DCCD scarcely inhibited $^{14}CO_2$ fixation under 2% O_2 at concentrations which significantly inhibited under N_2 (Fig. 7). This also suggests that ATP supply was not rate-limiting under 2% O_2 at high light intensities.

In fact, intracellular ATP level was much lower under N_2 than that under 2% O_2 during photosynthesis (Fig. 8). This strongly supports the assumption that ATP deficiency causes the anaerobic inhibition of photosynthetic CO_2 fixation in Chroomonas cells.

Photosynthetic $^{14}CO_2$ fixation in Chroomonas cells was considerably sensitive to KCN (Fig. 9) and was completely inhibited by 200 μM KCN under both N_2 and 2% O_2 (data not shown). Spinach RuBP carboxylase is reversibly inhibited by cyanide at low concentrations; more than 90% by 100 μM (Wishnick and Lane 1969) and its oxygenase activity was also inhibited (Lorimer et al. 1973), although photosynthetic electron transport is not significantly inhibited by cyanide below 1 mM (Trebst 1980). Therefore, in Chroomonas cells, the inhibition of $^{14}CO_2$ fixation by KCN under N_2 may be mainly caused by the inhibition of RuBP carboxylase. However, it must be noted that $^{14}CO_2$ fixation in Chroomonas

cells was much more strictly inhibited by KCN under 2% O_2 than that under N_2 (Fig. 9). This O_2-dependent inhibition by KCN cannot be explained by the inhibition of RuBP carboxylase-oxygenase.

Oxygen reduction by PS I, which was found by Mehler (1951), is known to produce H_2O_2 (Asada et al. 1977, Elstner 1979). H_2O_2 production during photosynthesis was observed in Anacystis cells (Patterson and Myers 1973) and in intact spinach chloroplasts (Egneus et al. 1975, Forti and Gerola 1977, Steiger and Beck 1981). CO_2 fixation in intact chloroplasts was inhibited by the addition of H_2O_2 (Kaiser 1976, 1979) and was stimulated by the addition of catalase (Egneus et al. 1975). In spinach chloroplasts, H_2O_2 appears to be scavenged by ascorbate-specific peroxidase (Nakano and Asada 1980, 1981). Both the "scavenging" enzymes, peroxidase and catalase, are known to be strongly inhibited by cyanide (Forti and Gerola 1977, Nakano and Asada 1980). These observations suggest that the O_2-dependent inhibition by KCN in Chroomonas cells would be due to the accumulation of H_2O_2, which is a consequence of the inhibition of peroxidase or catalase by KCN.

There still remains another explanation of the O_2-dependent inhibition by KCN, because Cyt c oxidase in mitochondria is strongly inhibited by cyanide. Excess NADPH can be easily reoxidized and its reducing power can be transported into mitochondria, as NADH, by a combination

of shuttle systems, and thus excess NADPH may provide additional ATP via mitochondrial oxidative phosphorylation even if the tricarboxylic acid cycle could not operate. But this may be rather unlikely, and be inconsistent with the effects of antimycin A and uncouplers on $^{14}CO_2$ fixation in Chroomonas cells, even though the interaction of respiration and photosynthesis in this alga cannot be denied. Thus, the main role of oxygen seems to be in poising electron transport carriers in chloroplasts via the Mehler reaction. However, the relative contribution of cyclic versus pseudo-cyclic electron flow to provide additional ATP during photosynthetic CO_2 fixation under aerobic conditions in Chroomonas cells remains to be resolved.

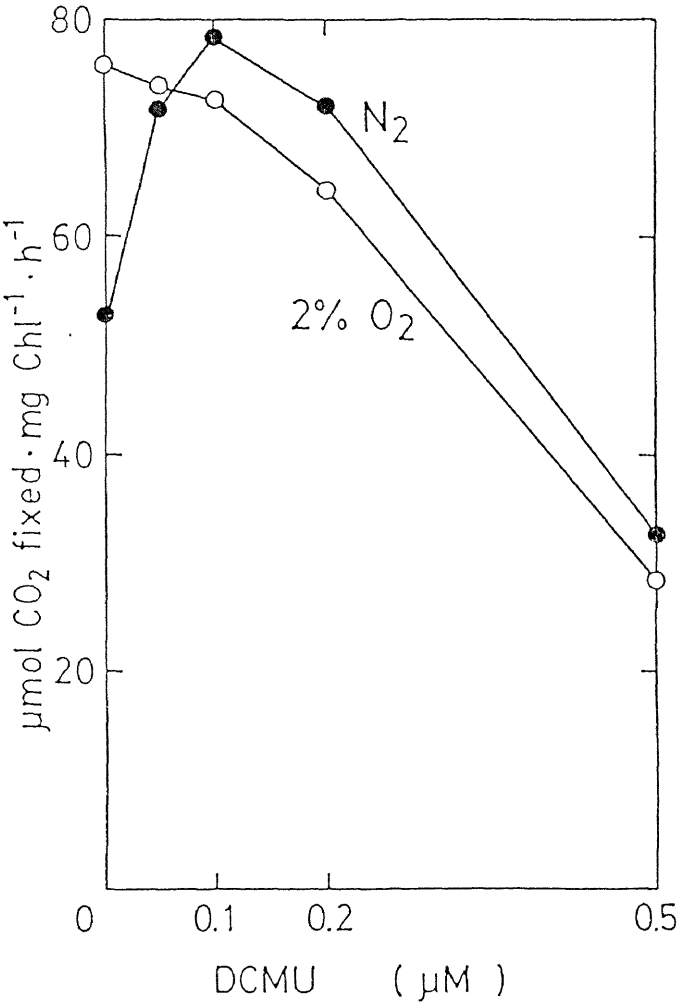

Fig. 1 Effect of DCMU concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2 atmospheres, at 200 $W \cdot m^{-2}$. Chl a content and $NaHCO_3$ concentration were 4.9 $\mu g \cdot ml^{-1}$ and 0.71 mM, respectively. DCMU in ethanol was added where indicated to give a final concentration of 1% ethanol. The controls also contained 1% ethanol.

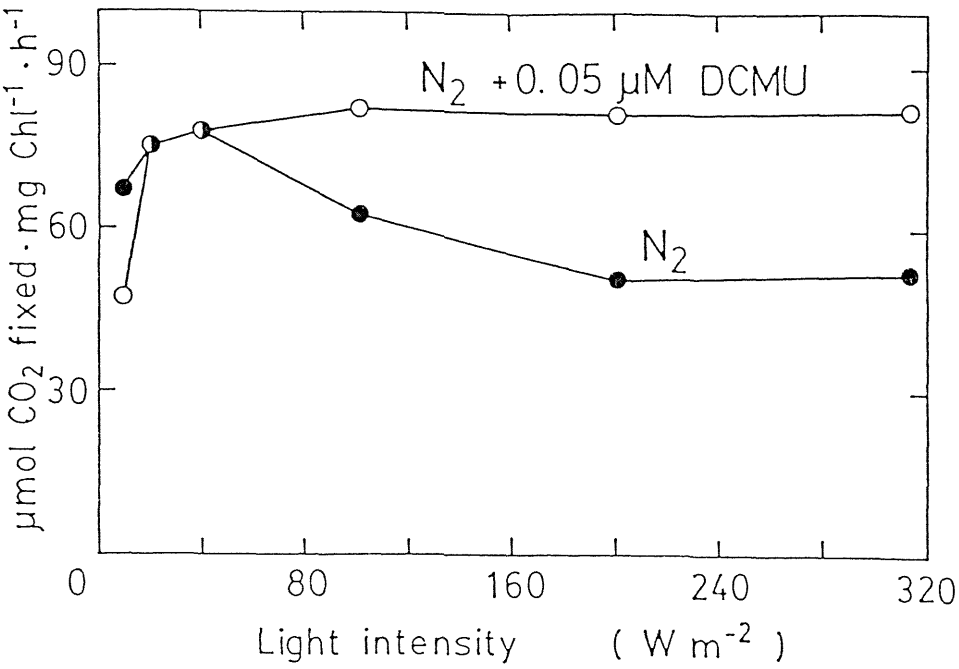

Fig. 2 Effect of light intensity on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 in the presence or absence of 0.05 μM DCMU. Chl a content and $NaHCO_3$ concentration were 4.8 $\mu g \cdot ml^{-1}$ and 0.73 mM, respectively. DCMU was added as Fig. 1.

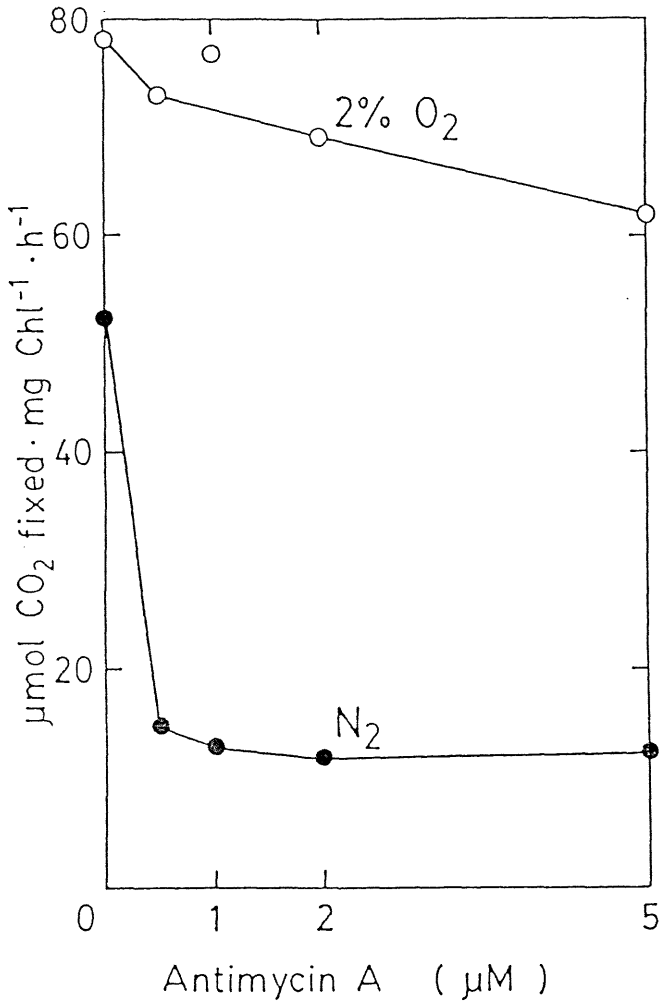

Fig. 3 Effect of antimycin A concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2, at 200 $W \cdot m^{-2}$. Chl a content and $NaHCO_3$ concentration were 3.8 $\mu g \cdot ml^{-1}$ and 0.73 mM, respectively. Antimycin A in ethanol was added where indicated to give a final concentration of 1% ethanol. The controls also contained 1% ethanol.

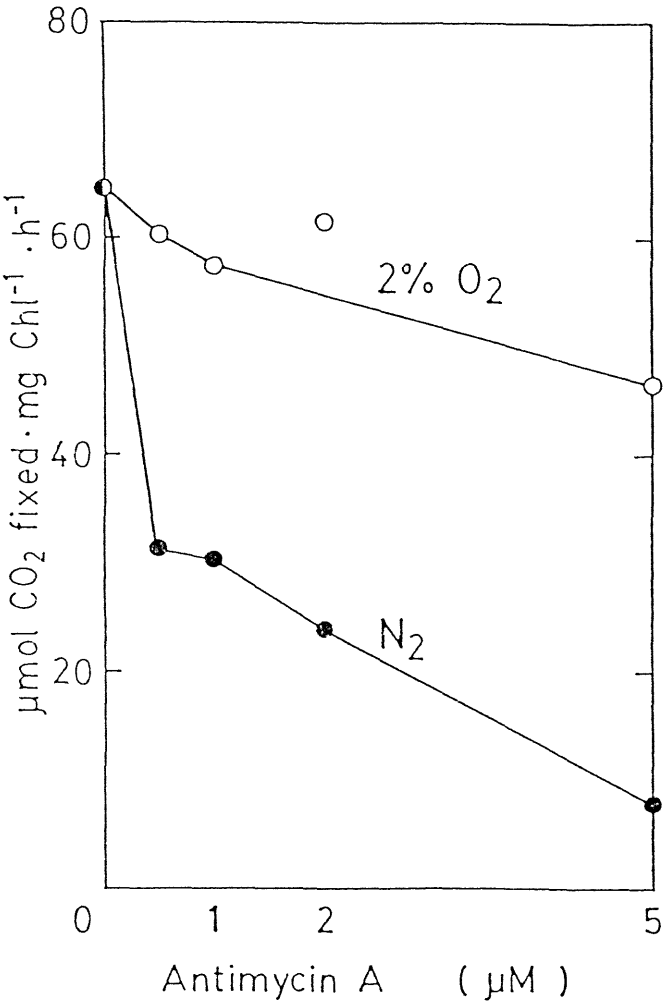

Fig. 4 Effect of antimycin A concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2, at 40 $W \cdot m^{-2}$. Chl a content and $NaHCO_3$ concentration were 3.9 $\mu g \cdot ml^{-1}$ and 0.70 mM, respectively. Antimycin A was added as Fig. 3.

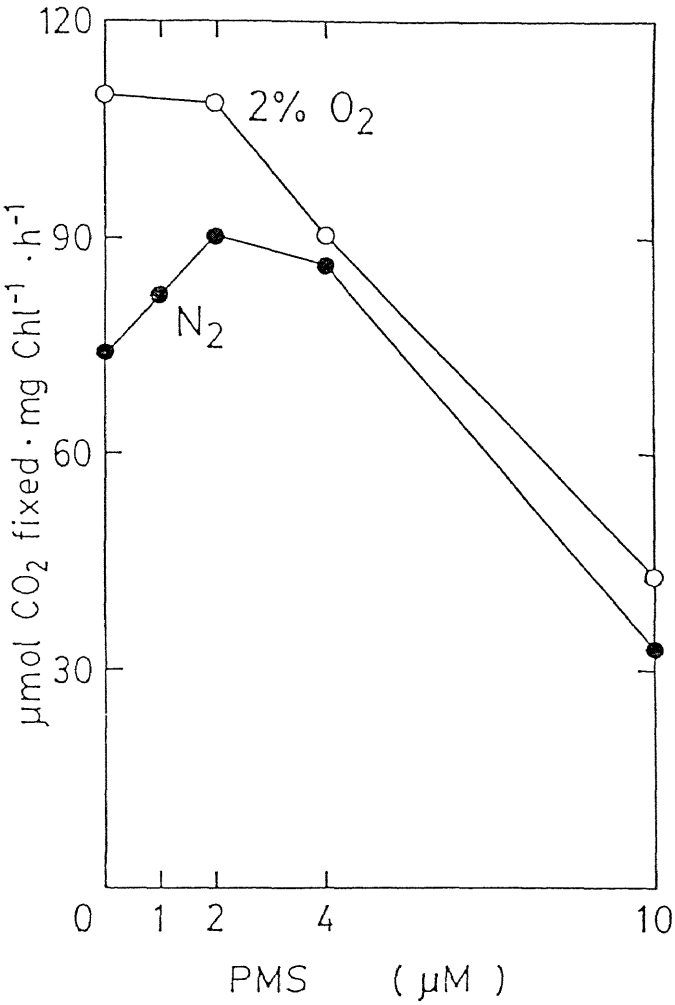

Fig. 5 Effect of PMS concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2, at 200 $W \cdot m^{-2}$. Chl a content and $NaHCO_3$ concentration were 3.7 $\mu g \cdot ml^{-1}$ and 0.71 mM, respectively.

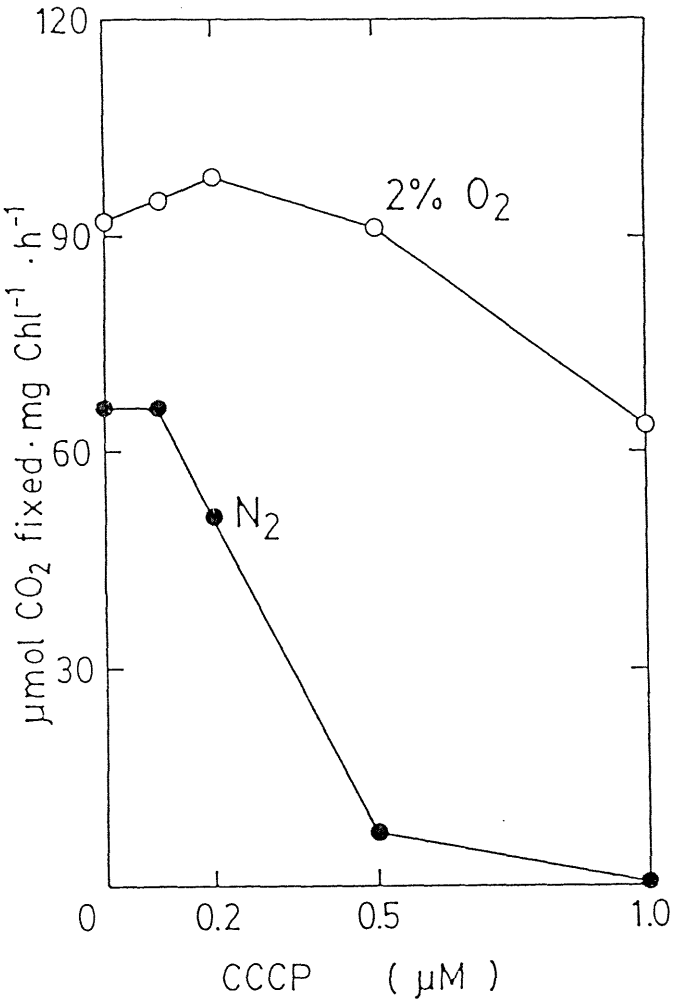

Fig. 6 Effect of CCCP concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2, at 200 $W \cdot m^{-2}$. Chl a content and $NaHCO_3$ concentration were 4.4 $\mu g \cdot ml^{-1}$ and 0.70 mM, respectively. CCCP in ethanol was added where indicated to give a final concentration of 1% ethanol. The controls also contained 1% ethanol.

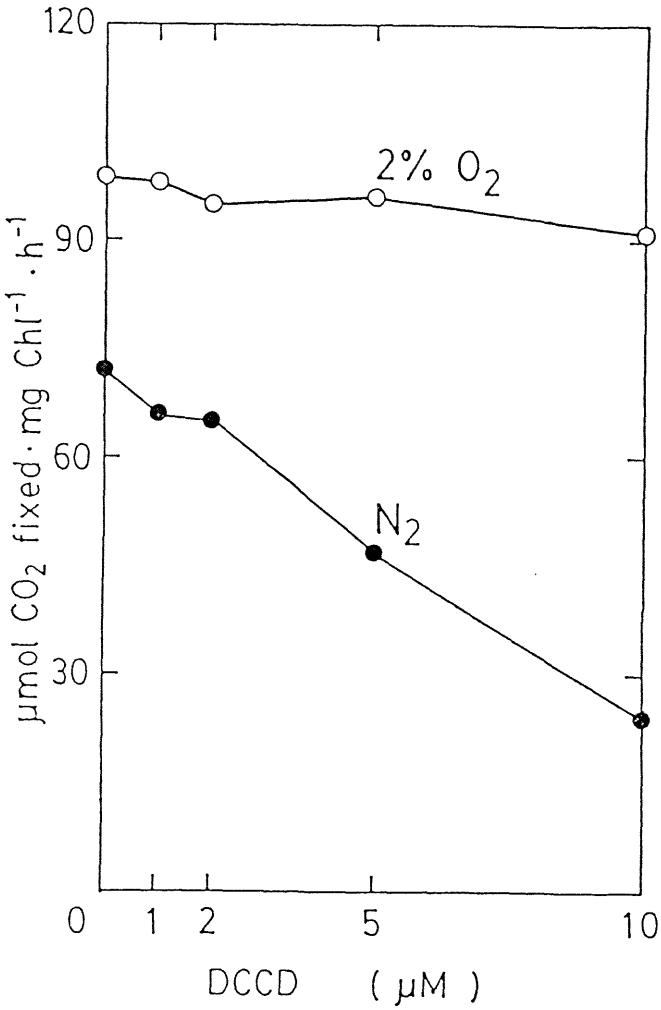

Fig. 7 Effect of DCCD concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2, at 200 $W \cdot m^{-2}$. Chl a content and $NaHCO_3$ concentration were 5.7 $\mu g \cdot ml^{-1}$ and 0.73 mM, respectively. DCCD in ethanol was added where indicated to give a final concentration of 1% ethanol. The controls also contained 1% ethanol.

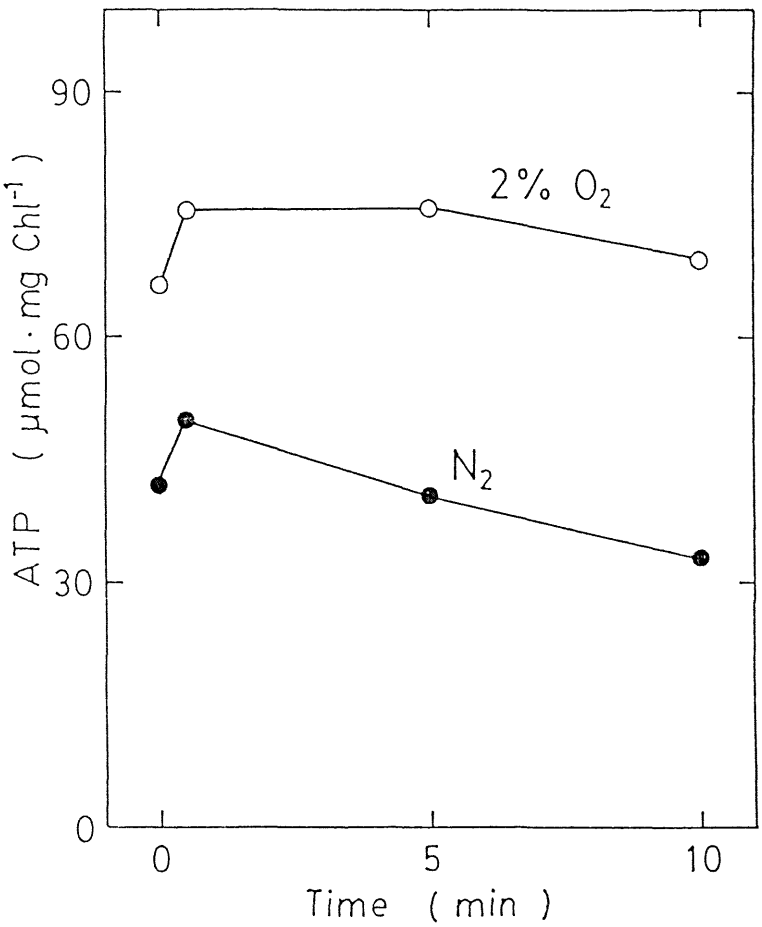

Fig. 8 Intracellular ATP levels during photosynthetic CO_2 fixation in Chroomonas sp. under N_2 and 2% O_2. Chl a content and $NaHCO_3$ concentration were 6.1 μg·ml^{-1} and 0.70 mM, respectively.

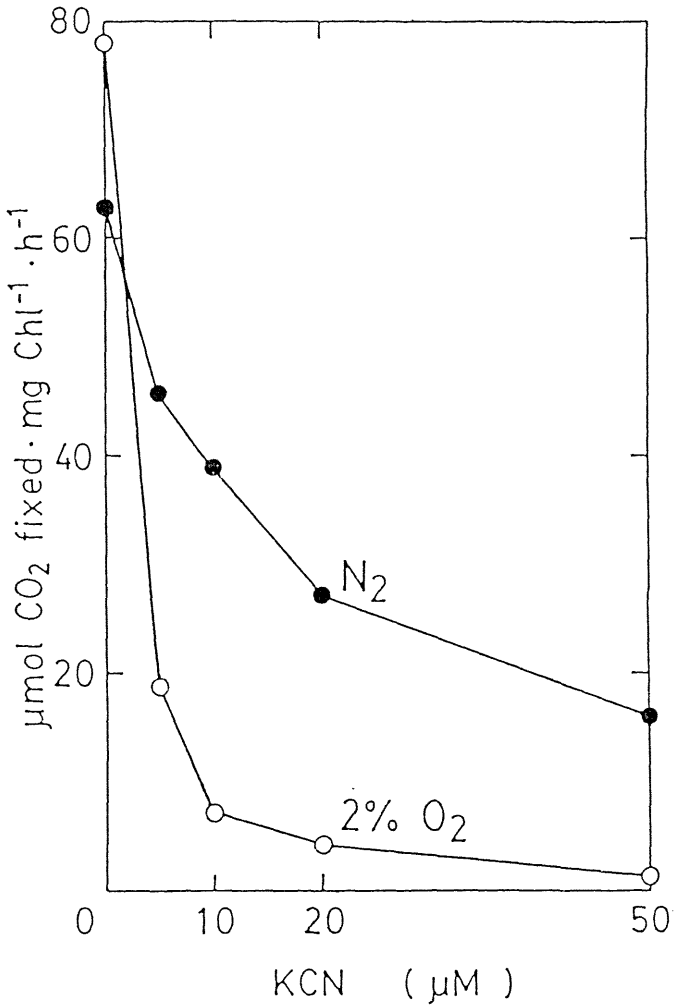

Fig. 9 Effect of KCN concentration on the rate of photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. under N_2 and 2% O_2, at 200 $W\cdot m^{-2}$. Chl a content and $NaHCO_3$ concentration were 4.1 $\mu g\cdot ml^{-1}$ and 0.71 mM, respectively.

CONCLUSIONS

Photosynthetic $^{14}CO_2$ fixation in Chroomonas sp. cells was inhibited by both anaerobiosis and oxygen at concentrations above 2%.

In Chroomonas cells, the labeling pattern of photosynthetic $^{14}CO_2$ fixation closely resembled those of C_3 plants. The author, therefore, concluded that the reductive pentose phosphate cycle is the major pathway of photosynthetic CO_2 fixation in Chroomonas sp. However, the effect of oxygen on photosynthesis seems to be different from that in terrestrial C_3 plants.

Photorespiratory activity seems to be considerably low in Chroomonas cells. During photosynthetic $^{14}CO_2$ fixation, the incorporation of ^{14}C into the intermediates of photorespiratory pathway was very low even under 100% O_2. The oxygen inhibition was much less than that observed in terrestrial C_3 plants, and was scarcely affected by CO_2 concentration. Then, the oxygen inhibition of photosynthetic CO_2 fixation in Chroomonas cells seems to be attributed mainly to the interaction of oxygen with the reducing side of photosystem I in photosynthetic electron transport systems (the Mehler reaction).

On the other hand, the anaerobic inhibition of photosynthesis was observed only under light-saturating condition, and was not affected by CO_2 concentration. This

inhibition was released by 3-(3,4-dichlorophenyl)-1,1-dimethylurea at suitable concentrations and by oxygen at very low concentrations (less than 2%). These results indicate that the anaerobic inhibition is due to excessive electron flow from water to $NADP^+$ in photosynthetic electron transport. The accumulation of ^{14}C-dihydroxyacetone phosphate and the depression of starch synthesis during photosynthetic $^{14}CO_2$ fixation under light-saturating and anaerobic conditions suggest that the inhibition is closely concerned with ATP deficiency and with excessive supply of NADPH. The ATP deficiency was also confirmed by the experiments using carbonyl cyanide m-chlorophenylhydrazone and N,N'-dicyclohexylcarbodiimide, as well as by the measurement of the intracellular ATP levels. The effects of antimycin A and phenazine methosulfate suggest that the additional ATP supply via cyclic photophosphorylation is necessary for photosynthetic CO_2 fixation under anaerobic conditions, and that the ATP deficiency is caused by the inhibition of cyclic photophosphorylation by over-reduction of the electron transport carriers. Under aerobic conditions, on the other hand, oxygen can act as another electron acceptor, and the excess electrons can be consumed to reduce oxygen at the reducing side of photosystem I (the Mehler reaction). Therefore, the additional ATP can be provided via cyclic and/or pseudo-cyclic photophosphorylation under aerobic conditions. The effect of

KCN suggests the occurrence of oxygen reduction at the reducing side of photosystem I.

Thus, oxygen is necessary for the normal operation of photosynthetic CO_2 fixation in Chroomonas sp. cells. The anaerobic inhibition of photosynthetic CO_2 fixation could occur, even in other photosynthesizing organisms, if sufficient ATP cannot be provided by non-cyclic photophosphorylation alone for photosynthetic CO_2 fixation. This idea may be applicable to the other algae which show the oxygen enhancement of photosynthetic CO_2 fixation, such as Nitzschia ruttneri, Phaeodactylum tricornutum, Heterosigma akashiwo, Pavlova sp., and Tetraselmis sp.

ACKNOWLEDGEMENTS

The author wishes to express his considerable appreciation to Professor Tomoyoshi Ikawa of the University of Tsukuba for his continuing guidance and encouragement for the completion of this thesis.

The author also wishes to acknowledge helpful advices of Professor Hiroshi Suzuki, Associate Professor Takashi Yamashita and Dr. Yoshiaki Hara of the University of Tsukuba.

The author is very grateful to Professor Mitsuo Chihara and Dr. Isao Inouye of the University of Tsukuba for supplying the strain of Chroomonas sp. (CHR-1A).

Thanks are due to all members of the laboratory for their useful discussions and assistance.

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