NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

HCO_3^- TRANSPORT IN THE CYANOBACTERIUM *Synechococcus leopoliensis* UTEX 625

Ramani A. Kandasamy

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

January 1991

ISBN 0-315-64729-9

ABSTRACT

HCO_3^- TRANSPORT IN THE CYANOBACTERIUM *Synechococcus leopoliensis* UTEX 625

Ramani A. Kandasamy

In the absence of Na^+, cells of the cyanobacterium *Synechococcus* UTEX 625 grown in standing culture photosynthesized at a rate 7 to 8 -fold greater than the rate of CO_2 supply. This photosynthesis was presumably supported by Na^+ - independent HCO_3^- uptake and accumulation. Simultaneous measurements of Chl *a* fluorescence yield, which provided an indirect means of monitoring the accumulation of internal DIC (i.e. CO_2 + HCO_3^-; Miller *et al*. 1988), indicated the presence of a large intracellular DIC pool in the absence of Na^+. The occurrence of Na^+ - independent HCO_3^- uptake was subsequently confirmed by directly measuring HCO_3^- uptake using silicone fluid centrifugation experiment. Intracellular DIC accumulation by the cells occurred at a rate 10 to 12 -fold faster than the rate of HCO_3^- dehydration to CO_2. The cells were capable of accumulating HCO_3^- up to a concentration 2000 times greater than the external medium, indicating Na^+ - independent HCO_3^- uptake involved an active transport process.

In contrast to the standing culture, cells grown with air bubbling (air-grown cells) required extracellular Na^+ (25 mM) for active HCO_3^- uptake. These results are consistent with

previous observations made by Espie et al. (1988) and Miller et al. (1984). The Na^+ - dependent HCO_3^- transport system was inhibited by Li^+. Studies with monensin, indicated that a Na^+ gradient ($[Na^+]out > [Na^+]in$) facilitated HCO_3^- uptake in air-grown cells. Inhibition of Na^+ -dependent HCO_3^- transport by amiloride further suggests the direct requirement of Na^+ for HCO_3^- uptake in air-grown cells. In contrast, Na^+ -independent HCO_3^- transport was not inhibited by Li^+ or monensin. Amiloride had no effect on Na^+ -independent HCO_3^- uptake over a short term exposure (15 min) but long term exposure caused a general metabolic deterioration.

Ethoxyzolamide (EZA), an inhibitor of carbonic anhydrase (CA), drastically reduced Na^+ -independent HCO_3^- transport with little apparent effect on Na^+ -dependent HCO_3^- uptake. The addition of 25 mM Na^+ to standing culture cells resulted in recovery of EZA -inhibited fluorescence quenching and photosynthesis to a level above the CO_2 supply rate, through Na^+ -dependent HCO_3^- uptake. This reversed Na^+ - dependent photosynthesis and fluorescence quenching was inhibited by monensin. These results indicated the standing culture cells possess Na^+ - dependent and Na^+ -independent HCO_3^- transport systems. Upon illumination, the standing culture cells rapidly removed almost all the CO_2 from the medium. Addition of CA revealed that CO_2 depletion was due to selective uptake of CO_2 from the medium. Analysis of CO_2 efflux data indicated active HCO_3^- transport occurring simultaneously with CO_2 uptake.

This work is dedicated to

Sri Sathya Sai Baba

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. George Espie for his valuable advice, assistance and patience in directing this research work.

I would like to thank my supervisory committee, Dr. C. Hill and Dr. B. Mangat for their suggestions; Dr. P. Gullick, for judging the thesis.

I would also like to thank Prassad Aysola for assistance in the operation of the atomic absorbtion spectroscopy. Thanks are also extended to the members of the research group and fellow graduate student, Johnny Basso for their advice and suggestions.

Finally, I am most grateful to my parents for all their love, constant encouragement, patience and support throughout all my studies.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Cont'd)

LIST OF FIGURES (Cont'd)

LIST OF FIGURES (Cont'd)

LIST OF TABLE

ABBREVIATIONS

DIC, Dissolved inorganic carbon ($CO_2 + HCO_3^- + CO_3^{2-}$); BTP, 1,3 -bis(tris [hydroxymethyl] methylamine)- propane; CA, carbonic anhydrase; EZA, ethoxyzolamide; F_M, maximum fluorescence yield; F_V, variable fluorescence; Chl *a*, Chlorophyll a; Rubisco, ribulose bisphosphate carboxylase/ oxygenase; RuBP, ribulose bisphosphate; DCMU, 3- (3,4 - dichlorophenyl) -1 dimethyl urea: DMSO, dimethyloxysulfide

INTRODUCTION

Cyanobacteria are photosynthetic procaryotes which inhabit a wide variety of illuminated environments ranging from the hypersaline, marine and fresh water to terrestrial habitats. The cyanobacteria as a group, however, are characteristically alkalotolerant with growth optima usually between pH 8.5 to 11. In an alkaline aquatic environment, cyanobacteria are frequently found to be the predominant photosynthetic microorganism and thus contribute significantly to the primary productivity of that environment.

Cyanobacterial photosynthesis is similar in many respects to the photosynthesis carried out by terrestrial plants. Like all land plants, Chl a is the primary light harvesting pigment and H_2O serves as the electron donor for the reduction of $NADP^+$. The light dependent oxidation of H_2O results in the evolution of molecular oxygen and it is this process which distinguishes cyanobacterial photosynthesis from all other forms of bacterial photosynthesis. Carbon dioxide fixation occurs through the classical Calvin or C_3 cycle of photosynthetic carbon reduction. The first reaction of the Calvin cycle is mediated by the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) and involves the addition of CO_2 to the five-carbon substrate ribulose-1,5-bisphosphate (RuBP). The resulting six-carbon enzyme-bound intermediate is subsequently hydrolysed to form two molecules of 3-phosphoglyceric acid (PGA), the first detectable product

of photosynthetic carbon dioxide fixation. Rubisco is a bifunctional enzyme which also catalyzes the oxidation of RuBP by molecular O_2, resulting in the production of one molecule of PGA and one molecule of phosphoglycollic acid (PG). The PGA is metabolized by enzymes of the Calvin cycle ultimately producing carbohydrate. The PG, however, is further oxidize by the enzymes associated with the photosynthetic carbon oxidation (PCO) cycle. For every two molecules of PG which enter the PCO cycle, one molecule of PGA is reformed and one molecule of CO_2 is released. Thus, Rubisco catalyzes the first reaction of two diametrically opposed metabolic pathways. The flow of carbon through these two pathways is controlled by the kinetic properties of Rubisco and by the $[CO_2]/[O_2]$ ratio.

Although Rubisco is the primary carboxylating enzyme in both terrestrial plants and cyanobacteria, distinct differences in the kinetic properties exist between the enzyme obtained from the two sources. In particular, the K_m (CO_2) of cyanobacterial Rubisco (250 μM) is more than 30-fold higher than its terrestrial plant counterpart (8 μM) while V_{max} is about 2-fold higher(Andrews & Abel, 1981; Badger, 1980). Furthermore, the substrate specificity factor, which is a measure of the number of carboxylations per oxygenation, is two-fold lower for cyanobacterial Rubisco (40:1) compared to the higher plant enzyme (80:1). Thus, given the kinetic properties of cyanobacterial Rubisco, it can be readily calculated (Turpin et al., 1985) that little net carbon

dioxide fixation or growth would occur if the intracellular [CO_2] was only as high as the 10-12 μM typically found in air-equilibrated aquatic environments. To over come this limitation in their photosynthetic apparatus cyanobacteria have evolved a mechanism to allow them to concentrate CO_2 at the site of carboxylation.

THE ROLE AND SUBCELLULAR COMPONENTS OF THE DIC CONCENTRATING MECHANISM IN CYANOBACTERIA

Using the silicone fluid filtering centrifugation technique, direct measurements of the intracellular pool of DIC ($CO_2 + HCO_3^- + CO_3^{2-}$) have shown that cyanobacteria such as Anabaena variabilis (Kaplan et al., 1980), Coccochloris peniocystis (Miller & Colman, 1980a) and Anacystis nidulans (Shelp & Canvin, 1984) are able to concentrate DIC internally to levels greater than 1,000 times that present in the external medium. Intracellular DIC pools as large as 40 to 60 mM are commonly found when the extracelluar [DIC] is only 40 to 60 μM. The intracellular CO_2 (1 to 2 mM) in equilibrium with this DIC pool is sufficient to saturate Rubisco, even though the extracellular CO_2 concentration is less than 1 μM. Moreover, the high internal CO_2 concentration means that the [CO_2]/[O_2] ratio is shifted to greatly favour the flow of carbon through the photosynthetic carbon reduction (Calvin) cycle rather than the photosynthetic carbon oxidation cycle. Thus, these organisms are able to photosynthesize efficiently at low external [DIC].

The essential components of the CO_2 concentrating mechanism are: a) transport systems for the translocation of inorganic carbon from the external medium to the cytosol against a large concentration gradient. Cyanobacteria have the ability to transport CO_2 and HCO_3^- from the external medium (Badger & Andrew, 1982; Espie et al.,1988a). b) An input of energy is required for the porter to translocate inorganic carbon against a large concentration gradient. CO_2 and HCO_3^- transport in Synechococcus (Badger & Andrew, 1982; Miller & Colman 1980a; Miller et al., 1988a) could be inhibited by DCMU or darkness. This suggest that inorganic carbon uptake is energised by energy supply from light dependent electron transport system. c) Back leak control device for CO_2. The CO_2 concentrating mechanism should have a means of preventing the back flux of CO_2 accumulated internally against a large concentration gradient for the efficient utilization of energy. The permeability of the cyanobacterial cell membrane to CO_2 ($P_{CO2} < 10^{-7} m.s^{-1}$) is apparently low compared to condensed monolayers of long chain fatty acids (Badger 1987). Low DIC grown cells in which the CO_2 - concentrating mechanism is fully induced had a thicker cell wall/membrane structure which was more resistant to lysozyme treatment compared to the high CO_2 grown cells in which the CO_2 concentrating mechanism was repressed (Badger 1987). The presence of the more resistant cell wall/membrane structure in low DIC grown cells is linked to the greater ability to concentrate CO_2. d) Presence of

intracellular CA. The primary carboxylating enzyme, Rubisco, utilizes CO_2 as the substrate. At alkaline pH HCO_3^- is the predominant inorganic carbon species transported by cyanobacteria. For Synechococcus calculations have indicated that a build up of internal [HCO_3^-] to 2 M would be necessary to support CO_2 saturated rates of photosynthesis in the absence of CA (Badger, 1987). But maximum intracellular pool formation of 30 - 60 mM has been found to be sufficient to saturate photosynthesis, inferring the presence of intracellular CA. Studies with O^{18} labelled HCO_3^- indicated rapid loss (faster than the uncatalyzed rate of HCO_3^- dehydration) of O^{18} to H_2O. This further support the presence of intracellular CA.

DIC SPECIES TRANSPORTED BY CYANOBACTERIA

The CO_2 - concentrating mechanism in cyanobacteria involves the active transport of inorganic carbon across the cell membrane (Badger and Andrew, 1982; Kaplan et al., 1980; Miller & Colman 1980a). Though initially there was controversy over the inorganic carbon species transported, subsequent studies have shown that cyanobacteria have the ability to transport both CO_2 and HCO_3^- (Badger & Andrew 1982; Espie et al., 1988a).

Mass spectrometer studies have provided strong evidence that cyanobacteria are capable of removing CO_2 from the medium

by an active transport process (Badger & Andrew, 1982; Espie et al., 1988a; Miller et al., 1988a). Upon illumination of the cells the [CO_2] in the medium dropped to a near zero level. Addition of CA following the drop in [CO_2] resulted in the rapid rise in [CO_2] to equilibrium level. These results were interpreted that the cells took up CO_2 rapidly and selectively from the medium. The rise in [CO_2] following the addition of CA indicated that the CO_2:HCO_3^- system was removed far from equilibrium by cells and that during the initial uptake phase most of the HCO_3^- remained in the medium. The addition of CA restored chemical equilibrium to the system and thus a rise in [CO_2] was observed.

Isotopic disequilibrium studies gave further evidence for the presence of CO_2 transport system in cyanobacteria (Badger & Andrew, 1982: Espie et al., 1984; Miller et al., 1988a; Volokita et al., 1984). Rapid accumulation and fixation of ^{14}C DIC without a lag period was observed following the addition of $^{14}CO_2$ to the cells. Theoretically, if the cells were only capable of only transporting and accumulating HCO_3^- then a 15 - 20 s lag period would be observed before substantial DIC accumulated. The absence of this lag phase indicates that the DI^{14}C species taken up was $^{14}CO_2$. Presence of CO_2 transport in cyanobacteria has also been demonstrated by the CO_2 pulsing experiments (Espie et al., 1989; Miller et al., 1988a). In these experiments aliquots of CO_2 saturated H_2O was added to buffer solution in the presence and absence cells and the

[CO_2] in the medium was monitored. In the presence of cells a much lower [CO_2] in the medium was observed compared to the control (in the absence of cells) and the CO_2 was rapidly depleted to zero level. This further confirmed the presence of CO_2 uptake mechanism in cyanobacteria.

The presence of active HCO_3^- transport was first conclusively shown by Miller & Colman (1980a) in the cyanobacterium C. peniocystis. This was accomplished by demonstrating that these organism photosynthesized much faster than CO_2 could be supplied to the cells from the slow dehydration of HCO_3^-. Thus, external HCO_3^- must also be taken up to support photosynthesis. No extracellular CA which would catalyse the HCO_3^- conversion to CO_2 was detected in C.peniocystis. Later studies provided further evidence for the presence of HCO_3^- transport in number of other cyanobacteria (Badger & Andrew, 1982; Espie et al., 1984; Shelp & Canvin, 1984).

Another convincing line of evidence for the presence of HCO_3^- transport comes from recent studies with the mass spectrometer (Espie et al., 1989; Miller et al., 1989). Under conditions where the cells ability to transport CO_2 was inhibited by H_2S or COS, the extracellular [CO_2] in the medium rose dramatically above the equilibrium level when DIC was added. However simultaneous measurements of O_2 evolution showed a high rate of photosynthesis. This rise above equilibrium indicated that the increase in the [CO_2] in the

medium was due to the back leak of CO_2 from the intracellular compartment. Since photosynthesis was causing a net draw down in [DIC] while [CO_2] was increasing, the only possible source of inorganic carbon for both of these processes was HCO_3^-. Thus, the cells must actively transport HCO_3^-, which is subsequently converted to CO_2 within the cells. The CO_2 leaks from the cells into the medium, but since the CO_2 transport system is inhibited (by COS or H_2S), the [CO_2] rises. The slow extracellular conversion of CO_2 to HCO_3^- in the medium plus leakage of CO_2 from a region of high [CO_2] combined to drive the external [CO_2] above equilibrium.

An elegant experiment conducted by Espie et al., (1988a) provided evidence for the suggestion that HCO_3^- and CO_2 are transported simultaneously in the cyanobacterium, S. leopoliensis. In this experiment the mass spectrometer was used to monitor the [CO_2] and [O_2] in the medium simultaneously. Upon illumination of the cells the [CO_2] dropped to near zero and was maintained at that level in the medium indicating the occurrence of CO_2 transport. Under these conditions the rate of transport of CO_2 was equal to it's production in the medium from HCO_3^- dehydration and photosynthesis proceeded at a rate which could be supported by CO_2 uptake alone. HCO_3^- transport was initiated by the addition of Na^+ and evidence for it's occurrence was obtained by comparing the Na^+ - stimulated rate of photosynthesis to the CO_2 supply rate. Initiation of HCO_3^- transport appear to have

have no effect on the on going CO_2 uptake as the level of CO_2 in the medium was still maintained close to zero. This indicated that both species were transported simultaneously and the uptake of one species had no inhibitory effect on the other. Although cyanobacteria rapidly takes up CO_2 (Badger & Gallagher, 1987; Volokita et al., 1984) and have high apparent affinity for CO_2 transport mechanism ($K_{0.5}$ (CO_2) = 17 μM & $K_{0.5}$ (HCO_3^-) = 60 μM) than for HCO_3^- (Volokita et al. 1984), at the optimum growth pH range (8 - 10; Miller & Colman 1980b) HCO_3^- transport provides the bulk of the DIC to the intracellular pool and photosynthesis.

INFLUENCE OF GROWTH [DIC] ON HCO_3^- AND CO_2 UPTAKE

The level of [DIC] experienced during growth of cyanobacteria plays an important role in determining the photosynthetic affinity for DIC (Kaplan et al. 1980; Shiraiwa & Miyachi, 1985; Badger & Andrew, 1982; Abe et al., 1987; Mayo et al. 1987; Badger & Gallagher 1987). S. leopoliensis grown under low (<50 μM) and high (>2 mM) [DIC] had a photosynthetic $K_{0.5}$ (DIC) of 1 - 3 μM and 200 μM respectively (Badger & Gallagher 1987). Growth under intermediate levels of DIC produced cells with intermediate values of $K_{0.5}$ (DIC) (Badger & Gallagher, 1987; Mayo et al., 1986). This difference was not due to variation in the primary carboxylating enzyme, Rubisco, as the turn over number and kinetics characteristics

of the enzyme from high and low DIC grown cells was found to be similar (Kaplan et al., 1980; Badger & Gallagher, 1987). Studies relating the rate of DIC transport as a function of external [DIC], found that low DIC grown cells had a greater ability to form a larger intracellular DIC pool than high DIC grown cells at a comparable extracellular [DIC] (Kaplan et al., 1980; Badger & Gallagher 1987). Active species experiment, which provide a measure of the extent to which the inorganic carbon species (either CO_2 or HCO_3^-) could be utilized (Badger & Andrew 1982) indicated that the reduced ability to accumulate inorganic carbon in high DIC grown cells was due to the lack of HCO_3^- transport, whereas CO_2 transport was present constitutively in all cell types (Badger & Gallagher 1987; Miller & Canvin, 1987).

MEASUREMENT OF INTRACELLULAR DIC ACCUMULATION

The accumulation of inorganic carbon within the cells can be measured directly using silicone fluid filtering centrifugation technique (Badger et al., 1978; Espie et al., 1988b; Kaplan et al., 1980; Miller & Colman, 1980a). Although this method gives a precise measure of the accumulated DIC it is extremely time consuming, labour intensive and tedious. However, recent studies by Miller & Canvin, (1987) and Miller et al., (1988b) have shown that measurements of Chl a fluorescence yield from S.leopoliensis UTEX 625 could be used

as an alternate, rapid but indirect means of monitoring the ability of the cells to accumulate DIC (CO_2 and/or HCO_3^- Espie et al., 1989). The fluorescence yield was near maximum in the absence of DIC. Addition of DIC (CO_2 or HCO_3^-) resulted in rapid fluorescence quenching. The pattern of fluorescence quenching and subsequent recovery correlated well with the size of the internal DIC pool. There was decrease in fluorescence yield with increase in intracellular [DIC] with the maximum extent of fluorescence quenching corresponding to the maximum intracellular DIC pool formed. Subsequent decrease in intracellular pool due to C fixation resulted in recovery of fluorescence yield. The observed fluorescence quenching was not due to C fixation alone as fluorescence quenching occurred under conditions where photosynthesis was inhibited with iodoacetamide (Miller & Canvin, 1987). Independent studies have shown that pool formation occurred in the presence of iodoacetamide. This confirmed the fact that the fluorescence quenching in S.leopoliensis is proportional to the magnitude of the internal DIC pool. Their studies also showed that the extent of Chl a fluorescence quenching was highly correlated with the intracellular concentration of DIC. The larger the pool, the greater the extend of fluorescence quenching.

Illumination of the cells with a brief saturating light flash under conditions where the fluorescence was quenched due to intracellular DIC pool formation resulted in the rapid recovery of fluorescence to a near maximum level in a

monophasic fashion (Miller et al. 1991). Whereas in higher plants the fluorescence recovery was biphasic. The first phase of recovery is rapid and is attributed to reduction of Q_A. The second phase is slow and is due to the collapse of the pH gradient across the thylakoid membrane (Miller et al. 1991). The rapid, monophasic recovery of fluorescence in Synechococcus suggested most of the quenching was due to the oxidation of Q_A. The rapid monophasic recovery of fluorescence in Synechococcus was also observed following the addition of DCMU. The DCMU blocks linear electron transport between Q_A and plastoquinone. This further supports the fact that observed quenching was due to oxidation of Q_A.

ROLE OF Na^+ IN DIC TRANSPORT

A role for sodium in the DIC uptake process was first discovered in 1984 (Kaplan et al., 1984; Miller et al., 1984). In the absence of Na^+ at alkaline pH and low [DIC], the rate of photosynthesis by the air-grown (0.03 % CO_2 v/v) cells of S.leopoliensis and A.variabilis was found to be low. In the presence of Na^+ the photosynthetic affinity for DIC increased by 20 - fold at pH 9.6 in S.leopoliensis (Miller et al., 1984). Similar observations were also made for A.variabilis grown with air bubbling (Kaplan et al., 1984). The enhancement of photosynthesis by Na^+, however, was not due to a direct involvement of Na^+ in the carbon fixation process as

measurements of the rate of C fixation as a function of intracellular [DIC] were similar in the presence or absence of Na^+ (Kaplan et al., 1984). The increase in photosynthetic affinity for DIC was due to increase in rate of DIC uptake as the affinity of the transport system for extracellular [DIC] increased with increasing [Na^+] in the mM range (Reinhold et al., 1984). The observed stimulation of photosynthesis and DIC transport by Na^+ was highly specific to Na^+ and could not be mimicked by any other cation.

The Na^+ requirement for photosynthesis was found to be dependent upon the external [DIC] (Espie et al., 1988b; Miller et al., 1984). The $K_{0.5}$ (Na^+) decreased exponentially from about 10 mM to 1 mM with increasing external [DIC] from 5 μM to 140 μM respectively (Espie et al., 1988). In the absence of Na^+ the rate of photosynthesis paralleled the CO_2 supply rate at low [DIC]. Studies of the CO_2 transport using the mass spectrometer indicated that rapid CO_2 uptake occurred in the absence of Na^+ and only μM [Na^+] was required for optimum CO_2 uptake (Espie et al., 1988). Thus, at low [DIC] where the CO_2 supply is limiting the millimolar Na^+ requirement by the air-grown cells for optimum photosynthesis was for HCO_3^- transport. Over coming the requirement for Na^+ at low [DIC] by the addition of CA (to catalyse HCO_3^- dehydration) or by increasing the extracellular [DIC] further supports the above conclusion.

Recent studies by Espie & Canvin (1987) showed that

S.leopoliensis cells grown in cultures which are not aerated nor stirred (standing cultures) could photosynthesize efficiently in the absence of Na^+ at low [DIC]. Unlike air-grown cells, the rate of photosynthesis by the standing culture cells in the absence of Na^+ was found to be several folds greater than the CO_2 supply rate and the affinity for DIC was also high. Lithium which inhibited Na^+ - stimulated HCO_3^- uptake in air-grown cells (Espie et al., 1988b; Espie & Canvin, 1987; Miller & Canvin, 1985), had no effect on the high rate of photosynthesis observed in standing culture cells. This suggested the presence of Na^+ -independent HCO_3^- transport in standing culture cells, distinct from the Na^+ - dependent HCO_3^- transport system present in air-grown cells.

The objectives of this study were to: a) To establish whether or not Na^+ - independent HCO_3^- transport occured in cyanobacteria and to study the rate of this transport system in the CO_2 - concentrating mechanism. b) To provide means to distinguish between Na^+ - dependent and Na^+ - independent mode of HCO_3^- transport and c) Determine if standing culture cells have an active CO_2 transport system.

MATERIALS AND METHODS

ORGANISM

The cyanobacterium Synechococcus leopoliensis UTEX 625, also known as Anacystis nidulans TX20 and Synechococcus PCC 6301 was obtained from University of Texas culture collection at Austin, Tx, U.S.A. The cells were maintained at 30°C on 1.4% (v/v) agar plates, made with Allen's medium, an inorganic salts mixture (Allen, 1968). Illumination was provided at 25 μmol photons.m^{-2}.s^{-1} measured with a LiCOR Li185B light meter.

GROWTH CONDITIONS

For all experiments the organisms were grown in aqueous culture in unbuffered Allen's medium. Synechococcus UTEX 625 was grown at 30°C either as standing cultures or air-grown cultures. Aseptically, 3 - 4 loops of colonies were transferred from plates into 50 mL of Allen's medium, contained in 125 mL foam-stoppered erlenmeyer flask to obtain an initial [Chl a] of 0.3 - 0.4 μg.mL^{-1} and an A_{720} of 0.03 - 0.06. These cultures were grown without supplementary aeration or stirring and were termed standing cultures. The cells were harvested and used for experiments when the [Chl a] reached 3.5 - 4 μg.mL^{-1}, after 8 to 10 days of growth.

Cells were also grown in 20 x 2.5 cm (i.d) cylindrical glass culture tubes and bubbled continuously with 0.03% (v/v) CO_2 in air. These cultures are referred to as the air - grown cultures. These cultures were started by adding an appropriate volume of standing culture cells to 50 - 55 mL Allen's medium to give an initial [Chl a] of 0.3 - 0.5 μg.mL^{-1}. The air flow

was regulated by a flow meter (Cole-Parmer) and was supplied at 71 $mL.min^{-1}$.

Illumination for the growth of standing culture and air - grown culture was provided at 25 µmol $photons.m^{-2}.s^{-1}$ and 50 µmol $photons.m^{-2}.s^{-1}$ (PAR) respectively, by a combination of Sylvania Gro-Lux and cool white fluorescent light. Both cultures were grown at 30°C and the pH when harvested was approximately 10.4.

PREPARATION OF LOW DIC BUFFER

To prepare low DIC containing buffer of the appropriate pH, a predetermined volume of 2N HCl was added to 100 mL of distilled H_2O. This mixture was gassed with N_2 for 20 min to remove the CO_2. An amount (0.71 g) of solid Bis Tris Propane buffer was then quickly added to the acidified H_2O to give a final concentration of 25 mM. The mouth of the flask was closed with a rubber stopper immediately after addition of base. Routinely 1 mL of buffer was drawn with a syringe and the pH was checked. Buffer prepared in this way typically contained about 15 - 20 µM DIC. The concentration of Na^+ as a contaminant was determined by atomic absorption spectroscopy at 295 nm and was found to be around 8 µM.

EXPERIMENTAL CONDITIONS

Prior to the experiments cells were harvested from growth medium by centrifugation, washed 3 times by centrifugation (1 min at 12,000 x g, Beckman Microfuge E) with 25 mM BTP/HCl buffer and resuspended in the same buffer. All experiments

were conducted at 30°C.

Chlorophyll concentration of the cell samples was measured as described by Mackinney (1941). Cells were pelleted by centrifugation, the supernatant removed and the Chl *a* was extracted from the cell pellet with 1.4 mL of methanol and incubated in the freezer for 30 min in the dark for maximum extraction. Subsequently, the cell debris was separated from the Chl *a* extract (supernatant) by centrifugation for 30 seconds at 12,000 x g. The [Chl *a*] was determined spectrophotometrically at 665 nm using the equation

Chl *a* ($\mu g.mL^{-1}$) = 13.42 x A_{665} x 1.4

PHOTOSYNTHESIS

Photosynthesis was measured by the amount of O_2 evolved using a Clark type oxygen electrode unit (Hansatech, Kings Lynn, Norfolk, U.K). The washed cell suspension (1.5 mL) was placed in the electrode chamber, purged with N_2 (to remove O_2), and allowed to consume the residual DIC in the light. The reaction vessel was thermostated at 30°C and the cell suspension was stirred continuously with a magnetic stirrer. All additions to the O_2 electrode chamber were made through a capillary opening in the plunger using a Hamilton syringe.

Once the cells had reached the CO_2 compensation point (zero O_2 evolution), photosynthesis was initiated by adding a defined concentration of DIC from a stock solution made from $KHCO_3$. Inorganic carbon in solution is found as CO_2, HCO_3^- or CO_3^{2-}. These three major forms of inorganic carbon are

interconvertible by the following reaction.

$$CO_2 + H_2O = H^+ + HCO_3^- = H^+ + CO_3^{2-}$$

The pKa of the first reaction is 6.4 and that of the latter is around 10 (Aizawa & Miyachi, 1986). In this study most of the experiments were performed at pH 8. At this pH most of the inorganic carbon exist as HCO_3^-. Only 1.56% of the total DIC is present as CO_2 (taking into account the ionic strength of the buffer as 25 mM). The maximum rate of CO_2 supply from HCO_3^- dehydration in the closed aqueous reaction medium at pH 8 was calculated as described by Espie et al., (1988a) using the formula: $\frac{d[CO_2]}{dt} = K_d [HCO_3^-]$

where $K_d = 0.87 \times 10^{-3}.s^{-1}$. The K_d value was determined using the mass spectrometer following the addition of 1 mM K_2CO_3 to 25 mM BTP/HCl buffer at pH 8 and 30°C. The CO_2 hydration to HCO_3^- was not taken into account since the organisms had an efficient CO_2 uptake mechanism to maintain the $[CO_2]$ close to zero at low DIC concentrations (Espie et al., 1988a).

Light for photosynthesis was provided by a Quartz - halogen projector lamp. The light intensity at the surface of the cuvette, as measured by a quantum photometer (Li-COR model Li-185B), was maintained at 100 µmol photons.$m^{-2}.s^{-1}$ (PAR).

The O_2 electrode was calibrated using the N_2 - air method of Delieu & Walker, (1970). Zero O_2 concentration was established by vigorously gassing BTP buffer with O_2 - free N_2 for 5 minutes at 30°C. This signal was set to zero on the chart recorder. Subsequently the buffer was gassed with air

containing 20.8% (v/v) O_2 (for about 5 min) until equilibrium was reached. The change in the O_2 electrode signal was monitored on a chart recorder and the span was adjusted to an appropriate level. At equilibrium 20.8% (v/v) O_2 yields a dissolved O_2 concentration of 230 µM (Truesdale & Downing, 1956).

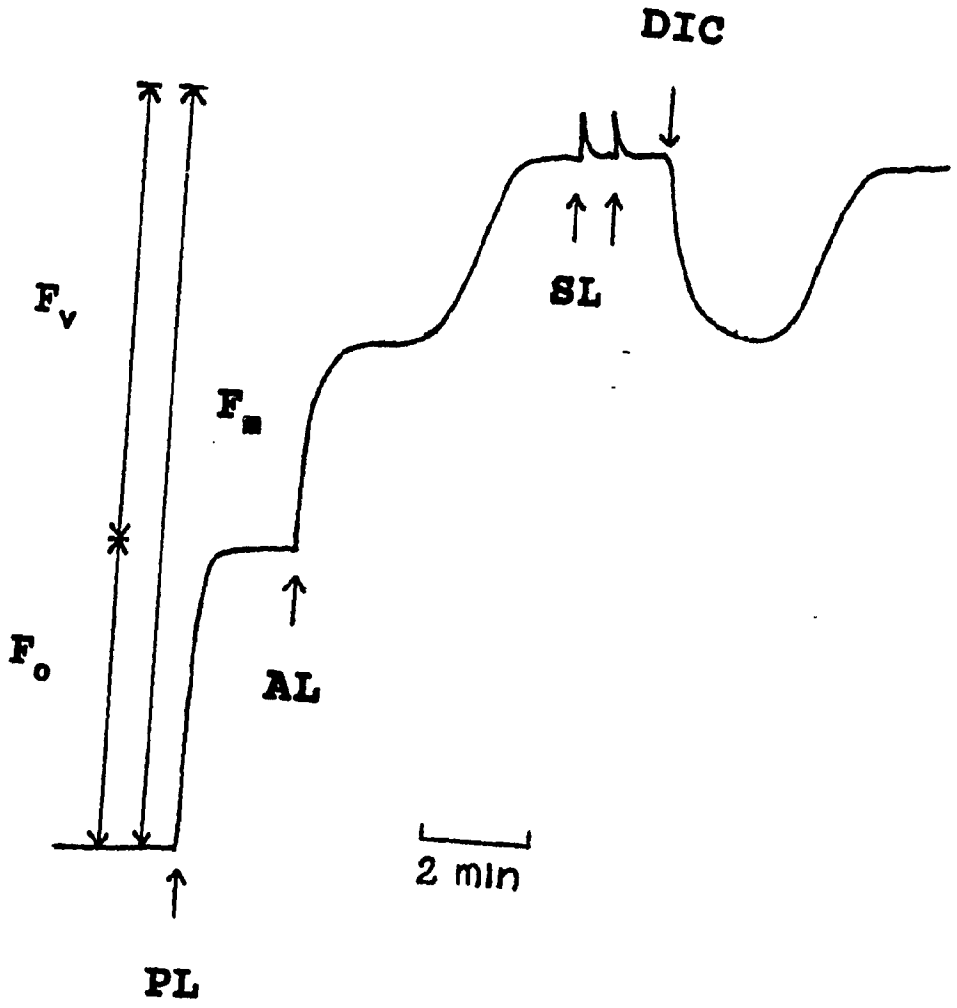
FLUOROMETRY

Chlorophyll a fluorescence yield of cell suspensions was measured using a pulse amplitude modulation fluorometer (H-Walz PAM 101,103) as described by Miller et al., (1988b). Measurements were made with cells suspended in buffer in the O_2 electrode chamber. A fiber optics system connected to the emitter - detector diode of the fluorometer was placed at the surface of the electrode chamber to facilitate simultaneous measurements of O_2 evolution and fluorescence yield. Pulse modulated fluorescence yield was monitored with a weak pulse modulated light beam (5 µmol photons.m^{-2}.s^{-1} at 100 kHz) and actinic light for photosynthesis was provided at 100 µmol photons.m^{-2}.s^{-1}.

Illumination of dark adapted (5 min) Synechococcus leopoliensis cells with the weak pulse modulated light beam resulted in a low level of fluorescence referred to as F_0 (Fig. 1). This low level of illumination was sufficient to cause fluorescence from Chl a but insufficient to cause a significant reduction of Q_A, the primary electron acceptor of PSII nor drive photosynthesis. Subsequent illumination with

FIGURE 1

Induction of Chl *a* fluorescence from intact cells of the cyanobacterium Synechococcus UTEX 625. Fluorescence yield was measured with a pulse amplitude modulation fluorometer (PAM 101). Generally, cells (1.5 mL, 4 - 10 µg Chl *a*.mL^{-1}) were suspended in 25 mM BTP/HCl buffer, pH 8 at 30°C and dark adapted for 5 min in the O_2 electrode chamber. A weak pulse modulated light beam (PL, 4 µmol(photon).$m^{-2}.s^{-1}$ 100 kHz) was first provided to the dark adapted cells and the observed fluorescence was referred to as F_0. Illumination of the cells with actinic (AL) light (100 µmol photons.$m^{-2}.s^{-1}$), resulted in a rise in fluorescence yield to near maximum level at CO_2 compensation point. Once a steady state yield of fluorescence was obtained (at CO_2 compensation point) maximum fluorescence yield (Fmax) was obtained by providing the cells with a brief (1 s) intense (1600 µmol photons.$m^{-2}.s^{-1}$) flash of light (SL). Variable fluorescence, (F_v) was the difference between Fmax and F_0. Routine measurements of fluorescence yield started when the cells were at CO_2 compensation point and fluorescence was near the Fmax level. Quenching of variable fluorescence upon the addition of 20 µM DIC (↓) is also shown in this figure. In all subsequent experiments, measurement of fluorescence yield (Fluorescence quenching) are initiated as for the 20 µM DIC case with cells near Fmax.


DIC
SL
F_v
F_m
F_o
AL
2 min
PL

actinic light, to drive photosynthesis increased the fluorescence yield which reached a near-maximum steady state level at the CO_2 compensation point (Fig. 1). A high intensity light flash (1600 µmol photon.m^{-2}.s^{-1}. for 1 s) at the CO_2 compensation point increased the fluorescence yield very slightly (Fig. 1). This saturating light flash was sufficient to fully reduce Q_A. This value was taken as maximum fluorescence yield (F_m) and could also be obtained by poisoning the cells with DCMU. DCMU blocks electron flow out of the Q_A pool. Thus, in the presence of DCMU Q_A is fully reduced and fluorescence yield is at a maximum as a consequence. Changes in fluorescence yield due to inorganic carbon transport was expressed as % F_v (Fig. 1). This fluorescence yield varied depending upon the oxidation state of the Q_A pool. It is the yield of variable fluorescence which has been found to correlate with the magnitude of the internal DIC pool (Miller et al., 1988b). Continuous illumination with actinic light following dark adaptation of standing culture cells in the presence of DIC resulted in rapid increase in fluorescence yield followed by a gradual increase to near maximum level (Fig. 1). Giving a saturating light flash at this point relieved about 70-80% of the fluorescence quenching indicating that most of the quenching was due to the oxidation of Q_A (i.e. Q quenching).

MASS SPECTROMETRY

Dissolved $^{13}CO_2$ or $^{12}CO_2$ uptake by cell suspensions was

measured using a VG Gas Analysis magnetic sector mass spectrometer (Middlewich, England) connected to a membrane inlet system as described by Miller et al., (1988a). A thin, gas-permeable, dimethyl silicone rubber membrane supported by a metal grid separated the inlet capillary to the mass spectrometer from the cell suspension. Washed cell suspensions (6 mL) containing 6-8 µg Chl. a.ml^{-1}, were placed in a N_2 purged glass cuvette thermostated at 30°C and closed with a plexiglass plug. Additions were made through a small bore in the plug. The cell suspension was continuously stirred with a magnetic stir bar for maximum sensitivity. Light for photosynthesis was provided at 200 µmol photons.$m^{-2}.s^{-1}$ (PAR).

The spectrometer was calibrated for CO_2 by the addition of 50 µM K_2CO_3 to buffer of known pH. The equilibrium [CO_2] was calculated as described by Buch (1960) taking into account the ionic strength of the buffer (Yokota & Kitaoka, 1985).

For "CO_2 pulsing" experiments ice cold acidified distilled H_2O (1 mM HCl) continuously bubbled with 5% CO_2 was used. The [CO_2] in the stock solution was determined from the equilibrium [CO_2] following injection of a known aliquot into the closed mass spectrometer reaction cuvette containing 6 mL of 25 mM BTP/HCl buffer (pH 8) and 25 µg.mL^{-1} carbonic anhydrase (CA).

INORGANIC CARBON UPTAKE AND FIXATION

Direct measurements of the internal inorganic carbon (DIC) pool and photosynthetic CO_2 fixation were made using a

modified version of the silicone fluid filtering centrifugation technique (Espie et al., 1988b). The experiments were conducted in 400 µL polypropylene microcentrifuge tubes containing from the bottom up, 100 µL killing solution (2N KOH in 10% methanol) and 100 µL of a less dense silicone fluid mixture (AR20:AR200 1.75:1 Wacker - Chemie). The layers were compacted by a brief (2 s) centrifugation. Over this was layered 75 µL of 25 mM BTP/HCl buffer containing twice the required [DI^{14}C]. The remaining head space in the tube was flushed with N_2 before and after addition of the incubation medium and the tube was capped.

A washed cell suspension containing twice the required [Chl a] was placed in the O_2 electrode and allowed to photosynthetically utilize the residual DIC in the buffer. Then a 75 µL aliquot of cells at the CO_2 compensation point was placed in the microfuge tube such that there was a N_2 - filled gap between the incubation medium and the cell suspension. Four tubes were placed in the horizontal rotor of a Beckman E microcentrifuge and were illuminated from the top at a light intensity of 450 µmol photons.$m^{-2}.s^{-1}$. The temperature was maintained around 28 - 30°C. The cell suspension was mixed with the incubation medium and the reaction started by quickly turning the centrifuge on and off. This action resulted in the cell suspension mixing with the incubation layer. However, the "spin" was sufficiently brief that the cells did not pass through the silicone fluid. At

timed intervals the reaction was terminated by centrifuging (60 seconds) the cells through the silicone fluid into the killing solution. The tubes were frozen immediately in liquid N_2 and stored in the freezer until processed.

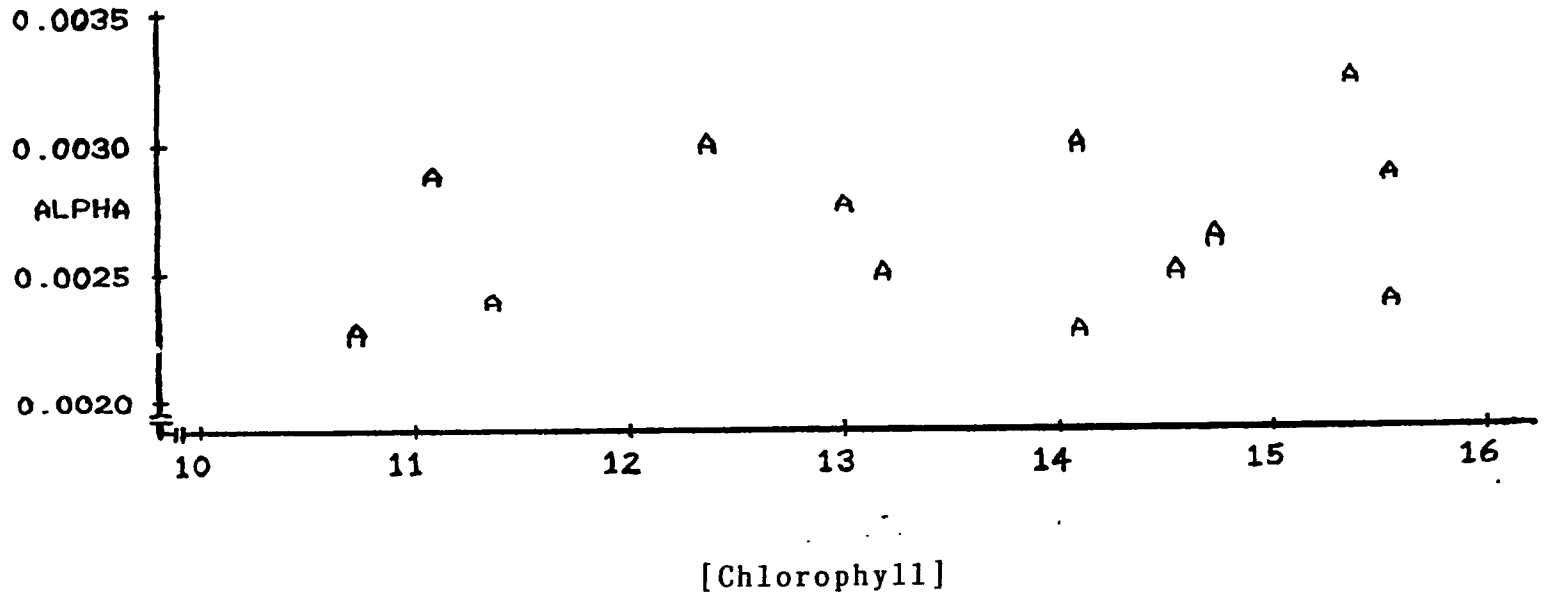
The frozen tubes were cut at the silicone fluid/killing solution interphase and the 100 µL terminating solution and the cell pellet was removed. The tube tip was washed with 100 µL of basic water (10 mM KOH) and combined with the terminating solution. Of this sample 50 µL was suspended in 5 mL scintillation fluid (Universol, ICN) and total ^{14}C activity determined using liquid scintillation counter (LKB 1215 RackBeta). This value, corrected for extracellular contamination, is a measure of total ^{14}C taken up by the cells. Of the remaining sample, 50 µL was acidified by the addition of 350 µL of 4N acetic acid and the acid labile ^{14}C was completely removed by drying the sample at 92°C for 4h. The dried sample was suspended in 200 µL of distilled H_2O and total, fixed, acid stable ^{14}C was counted as described above. The difference between the total intracellular inorganic carbon and acid stable organic carbon is a measure of the intracellular pool of inorganic carbon (DIC).

The intracellular volume of the cyanobacterial cells was measured by first incubating the cells with $^{3}H_2O$ (3.5 µCi/100 µL) for 10 minutes in the dark and spinning (60 s) the cells through silicone fluid into 6% (w/v) sorbitol. The $^{3}H_2O$ permeates to all aqueous regions of the cell and thus the

fraction of the total ^3H_2O inside the cell represents the fractional volume of the suspending medium occupied by the cells in solution. The tubes were frozen in liquid N_2 and stored in the freezer until processed. Processing involved cutting the frozen tubes, removing the thawed cell pellet and termination layer, washing the tip and determining the amount of ^3H_2O present by scintillation counting. The fraction of the total pelletable ^3H_2O that was extracellular (α) was estimated by incubating the cells with non-permeant ^{14}C - sorbitol (0.15 $\mu Ci.100\ \mu L^{-1}$) and spinning the cells through silicone fluid. Since ^{14}C - sorbitol does not enter the cell, ^{14}C -associated with the cell pellet is material not stripped from the cell surface during centrifugation through silicone fluid. This fraction of the total added ^{14}C-sorbitol not removed (α value) was a constant (Fig.2) and was assumed to represent the fraction of the total ^3H_2O or $DI^{14}C$ which would be external to the cells in the previous experiments. The tubes containing ^{14}C - sorbitol were processed as described above. The fraction of the total incubation volume that was transferred to the killing solution extracellular to the cells (mean α value) was 0.00266 (Fig. 2) for the standing culture cells and 0.00212 for the air - grown cells. For figure 2, the correlation coefficient calculated from all the data points was 0.2. The difference between extracellular and total volume transferred was assumed to be the intracellular volume which was 62 $\mu L.mg^{-1}$Chl *a* and 48.5 $\mu L.mg^{-1}$Chl *a* for the standing culture and air -

FIGURE 2

Dependence of the α value upon Chl *a* concentration. Extracellular contaminating fluid not removed during centrifugation of cells through silicone fluid was estimated using the non - permeant radiolabelled solute, ^{14}C-sorbitol. Cells were incubated in the dark for 10 min in the presence of ^{14}C-sorbitol (0.15 μCi.100 μL^{-1}) and subsequently centrifuged through silicone fluid. The value of α was estimated as the ratio of ^{14}C found in the cell pellet to ^{14}C present in the incubation medium prior to centrifugation.


0.0035
0.0030
ALPHA
0.0025
0.0020
10
11
12
13
14
15
16
[Chlorophyll]

grown cell respectively.

The fraction of the cells passing through the silicone fluid following a 60 s centrifugation was determined by comparing the [Chl *a*] of the pellet to that of the cell suspension originally used. Fractional recovery was 95% over a [Chl *a*] range of 7 - 15 $\mu g.mL^{-1}$.

INHIBITORS

Where indicated the appropriate volume of amiloride was added from a stock solution of 50 mM in distilled H_2O. Monensin was prepared as 100 mM stock solution in 95% ethanol. Dilution factor for monensin stock was 1000 fold. Addition of 0.1% ethanol did not have any effect on photosynthetic O_2 evolution nor fluorescence yield. Stock solutions of 50, 100, and 200 mM ethoxyzolamide were prepared by dissolving appropriate quantity in dimethyloxysulfide (DMSO). LiCl was used from a 5 M stock solution. All experiments were repeated 3 times with different batch cultures.

CHEMICALS

Monensin (sodium salt), amiloride, BTP and bovine erythrocyte CA (2500 WA units.mg^{-1} protein) were from Sigma, Chemical Co. (St. Louis). The $K_2{}^{13}CO_3$ (99 atom % ^{13}C) was from MSD isotope, Montreal. Compressed gas mixtures (ie 20.8% O_2 in N_2, O_2 free N_2) were obtained from Union Carbide.

RESULTS

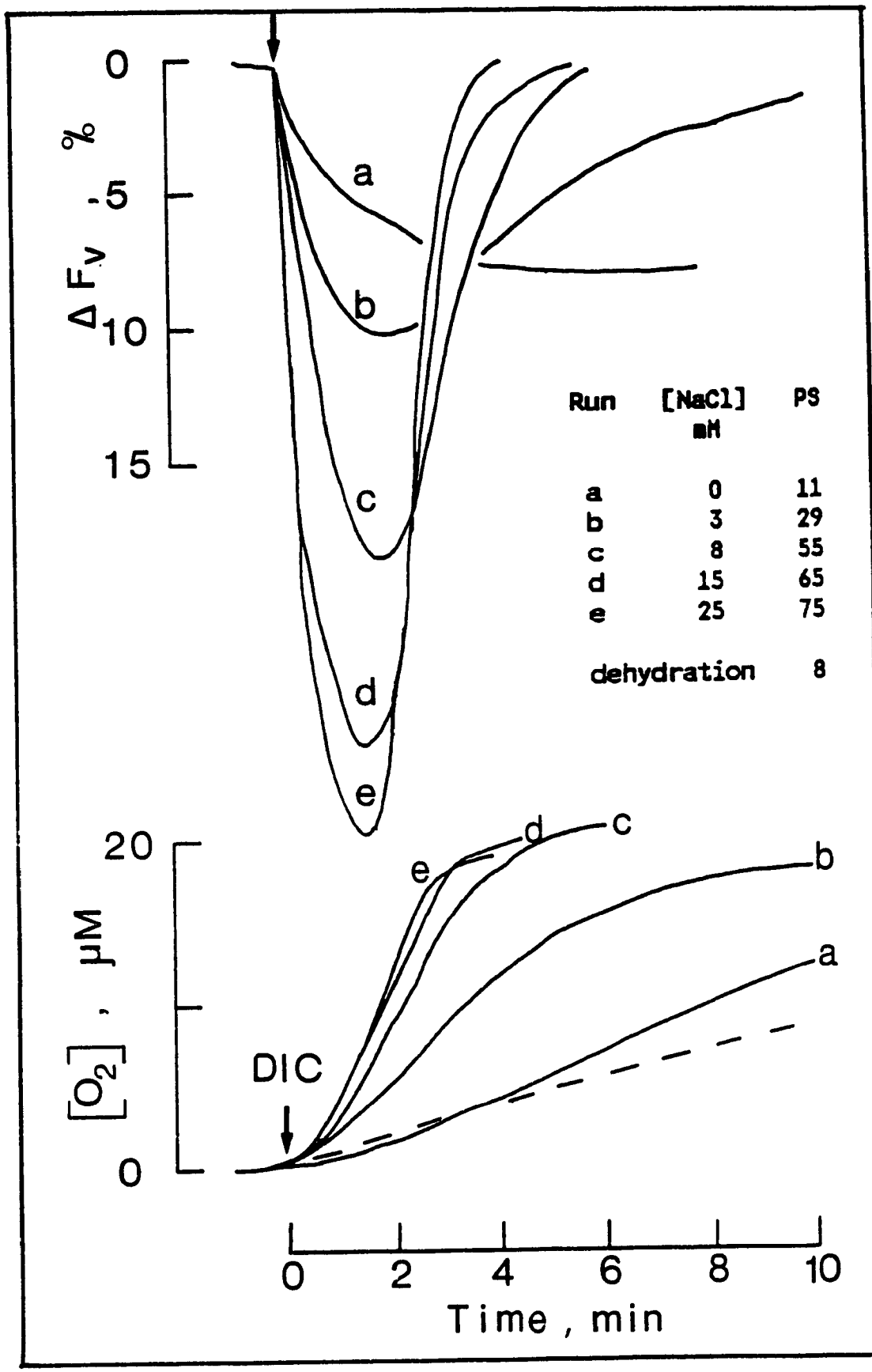
EFFECT OF Na^+ ON PHOTOSYNTHESIS AND FLUORESCENCE YIELD

Air - grown cells of the cyanobacteria Synechococcus leopoliensis UTEX 625 supplied with 20 μM DIC (pH 8), at the CO_2 compensation point photosynthesised at a low rate in the absence of added Na^+ (Fig.3; Espie & Canvin 1987; Miller et al. 1984). The observed rate of photosynthesis was similar to that which could be theoretically supported solely by CO_2 uptake. Similar observations have also been made for another air - grown cyanobacterium, Anabaena variabilis (Kaplan et al. 1984). At low [DIC], increasing the Na^+ concentration in the millimolar range markedly stimulated the rate of photosynthesis by the air - grown cells (Fig. 3). At 25 mM Na^+ the rate of photosynthesis was 6.8 - fold greater than the theoretical rate of CO_2 production, from the dehydration of HCO_3^- in the medium. This observation suggests that most of the observed enhancement of photosynthesis by Na^+ in air - grown cells was mainly due to an enhancement of HCO_3^- uptake from the medium, since the CO_2 supply rate was inadequate to support the observed rate of photosynthesis. Previous studies have shown that the enhancement of photosynthesis in Synechococcus UTEX 625 by NaCl is due to a specific effect of Na^+ (Espie and Canvin 1987; Miller et al. 1984).

Simultaneous measurements of Chl a fluorescence yield with photosynthesis indicated that little fluorescence quenching occurred in the absence of Na^+, following the

FIGURE 3

Effect of [NaCl] on photosynthesis and Chl *a* fluorescence yield of air-grown cells. Chart recorder tracings of Chl *a* fluorescence yield (upper panel) and photosynthetic O_2 evolution (lower panel) were obtained in the absence (a) and presence of (b) 3, (c) 8, (d) 15 and (e) 25 mM Na^+ at pH 8.01. The reaction was started by the addition of 20 µM $KHCO_3$ (↓) to illuminated cells at the CO_2 compensation point. The dashed line (---), indicates the maximum rate at which CO_2 can be produced from the dehydration of HCO_3^- in solution. The observed maximum rate of photosynthesis (ps) is given in µmol $O_2.mg^{-1}Chl.h^{-1}$. The cells (8 µg $Chl.mL^{-1}$) were suspended in 25 mM BTP/HCl buffer, pH 8.01, 30°C and illuminated at 100 µmol $photon.m^{-2}.s^{-1}$ PAR. The results of several runs have been overlaid to facilitate comparison.


0
5
10
15
ΔFv, %
a
b
c
d
e
Run
[NaCl] mM
PS
a 0 11
b 3 29
c 8 55
d 15 65
e 25 75
dehydration 8
20
0
[O2], μM
DIC
0 2 4 6 8 10
Time, min

addition of 20 μM DIC (Fig. 3). Increasing the extracelluar [Na^+], accelerated both the initial rate and the maximum extent of fluorescence quenching. The maximum extent of Chl *a* fluorescence quenching coincided in time with the maximum rate of photosynthesis. The return of fluorescence to a value near F_{max} also coincided with the cessation of O_2 evolution and the rate of this return was more rapid in the presence of Na^+. Previous studies with Synechococcus UTEX 625 (Miller *et al.* 1988b) have shown that the extent of Chl *a* fluorescence quenching was highly correlated with the magnitude of the internal pool of DIC. Furthermore, recent studies have also shown the initial rate of fluorescence quenching to be highly correlated with the initial rate of CO_2 or HCO_3^- transport (G.S. Espie, A.G. Miller and D.T. Canvin, personal communication). Therefore, Chl *a* fluorescence measurements provide a simple, non-invasive means of following the transport and intracellular accumulation of DIC. The present data (Fig. 3) interpreted in this light indicated that a relatively small pool of DIC formed in the absence of added Na^+, but that increasing [Na^+] increased both the rate of transport of DIC and the magnitude of the intracellular DIC pool. As argued previously, the increase in DIC transport must be due to an increase in HCO_3^- transport since the CO_2 supply rate was inadequate to account for the observed rate of photosynthesis (Fig. 3). These cells do not possess an extracellular carbonic anhydrase which would accelerate the

interconversion between HCO_3^- and CO_2 in the medium (Espie and Canvin 1987).

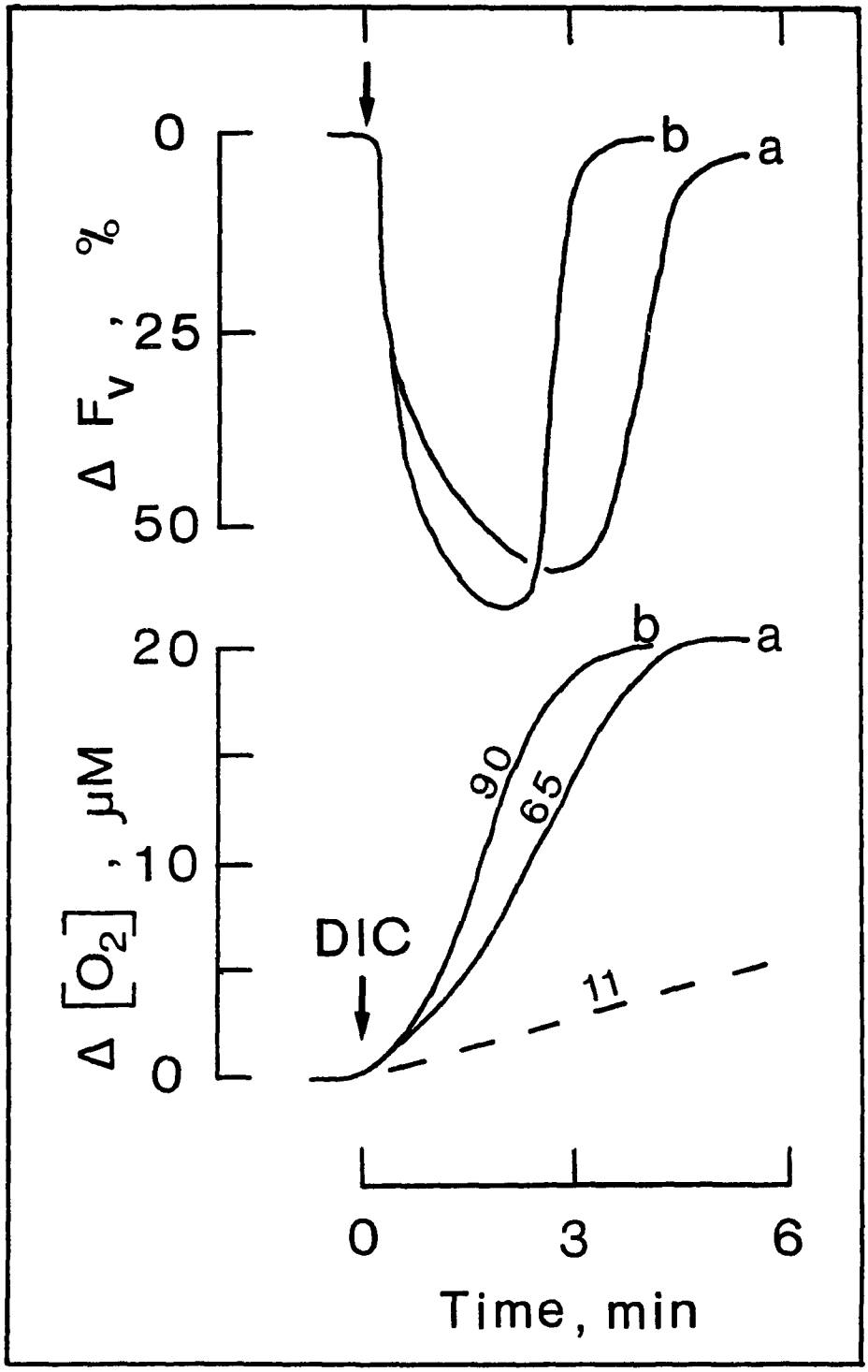
Some apparent DIC uptake and accumulation occurred in the absence of added Na^+ which may be due to either CO_2 or HCO_3^- transport (Fig. 3). Studies using a mass spectrometer to directly measure CO_2 uptake (Espie et al. 1988a) in the absence of Na^+ indicated that air - grown cells of Synechococcus UTEX 625 did, in fact, rapidly take up CO_2 in the medium and maintained the $[CO_2]$ close to zero and far from its chemical equilibrium with HCO_3^- even though there was continuous resupply of CO_2 to the medium from HCO_3^- dehydration. The occurence of CO_2 uptake suggests that most of the observed fluorescence quenching and O_2 evolution (which paralleled the CO_2 supply rate; Fig. 3 tracing a) in the absence of Na^+ was mainly due to CO_2 uptake and assimilation from the medium.

At 20 μM DIC, the rate of photosynthesis increased with increasing $[Na^+]$ up to a saturation level (Fig. 3). Optimum rate of photosynthesis was observed at 25 mM Na^+. The $[Na^+]$ required to elicit one-half the maximum rate of photosynthesis ($K_{0.5}$ (Na^+)) was determined (using more extensive data) from double reciprocal plots and found to be in the range of 5.8 - 7.5 mM (not shown). These results are consistent with previous observations made by Espie et al., (1988b).

In contrast to air - grown cells, addition of 20 μM DIC to standing culture cells in the absence of NaCl resulted in

FIGURE 4

Effect of Na^+ on photosynthesis and fluorescence quenching in cells grown in standing culture. Shown are the chart recorder tracings of Chl *a* fluorescence yield (upper panel) and photosynthetic O_2 evolution (lower panel) in the absence (a) and presence (b) of 25 mM Na^+ at pH 8.03. The measurements were made at 30°C and 100 µmol photons $m^{-2}.s^{-1}$ following the addition of 20 µM $KHCO_3$ (↓) to cells at compensation point. The numbers beside the O_2 tracings indicates the maximum rate of photosynthesis in µmol $O_2.mg^{-1}Chl.h^{-1}$. Also shown (---), the calculated course of O_2 evolution that could be supported solely by the uptake of CO_2. Results of several runs have been overlaid to facilitate comparisons.


0
25
50
Δ F_v , %
b
a
20
10
0
Δ [O_2] , μM
b
a
90
65
11
DIC
0
3
6
Time, min

the significant quenching of Chl a fluorescence and a rapid rate photosynthetic O_2 evolution (Fig. 4 tracing a). The rate of photosynthesis in the absence of Na^+ was 6 to 7 times greater than the rate of HCO_3^- dehydration to CO_2 at 20 µM DIC. At even lower extracelluar [DIC] the difference between the observed rate of photosynthesis and that which could be supported by CO_2 uptake alone was even greater (see Fig. 6). For example, at 2 µM DIC the maximum rate of photosynthesis that would be supported by CO_2 uptake was only 0.8 µmol $O_2.mg^{-1}Chl.h^{-1}$ whereas the observed rate of photosynthesis was 38 - fold greater. The high rate of photosynthesis observed for the standing culture cells in the absence of added Na^+ was supported mainly by uptake of HCO_3^- from the external medium. The significant degree of Chl a fluorescence quenching observed in the absence of Na^+ is characteristic of the formation of a large internal pool of DIC (Miller et al. 1988b) and is consistent with the suggestion that standing culture cells are capable of Na^+ - independent HCO_3^- uptake and accumulation (Espie and Canvin 1987).

As with air - grown cells, addition of 25 mM NaCl to standing culture cells stimulated the rate of photosynthesis, but to a much lesser extent than that observed for air - grown cells. At 20 µM DIC, photosynthesis was typically enhanced by 1.3 to 1.5 -fold (Fig. 4 tracing b) in the presence of 25 mM NaCl. The initial rate of fluorescence quenching and the extent was not greatly stimulated by Na^+ (Fig. 4), but it

accelerated the fluorescence recovery phase. In this case the main effect of Na^+ appears to be one of enhancing the utilization of the internal DIC pool.

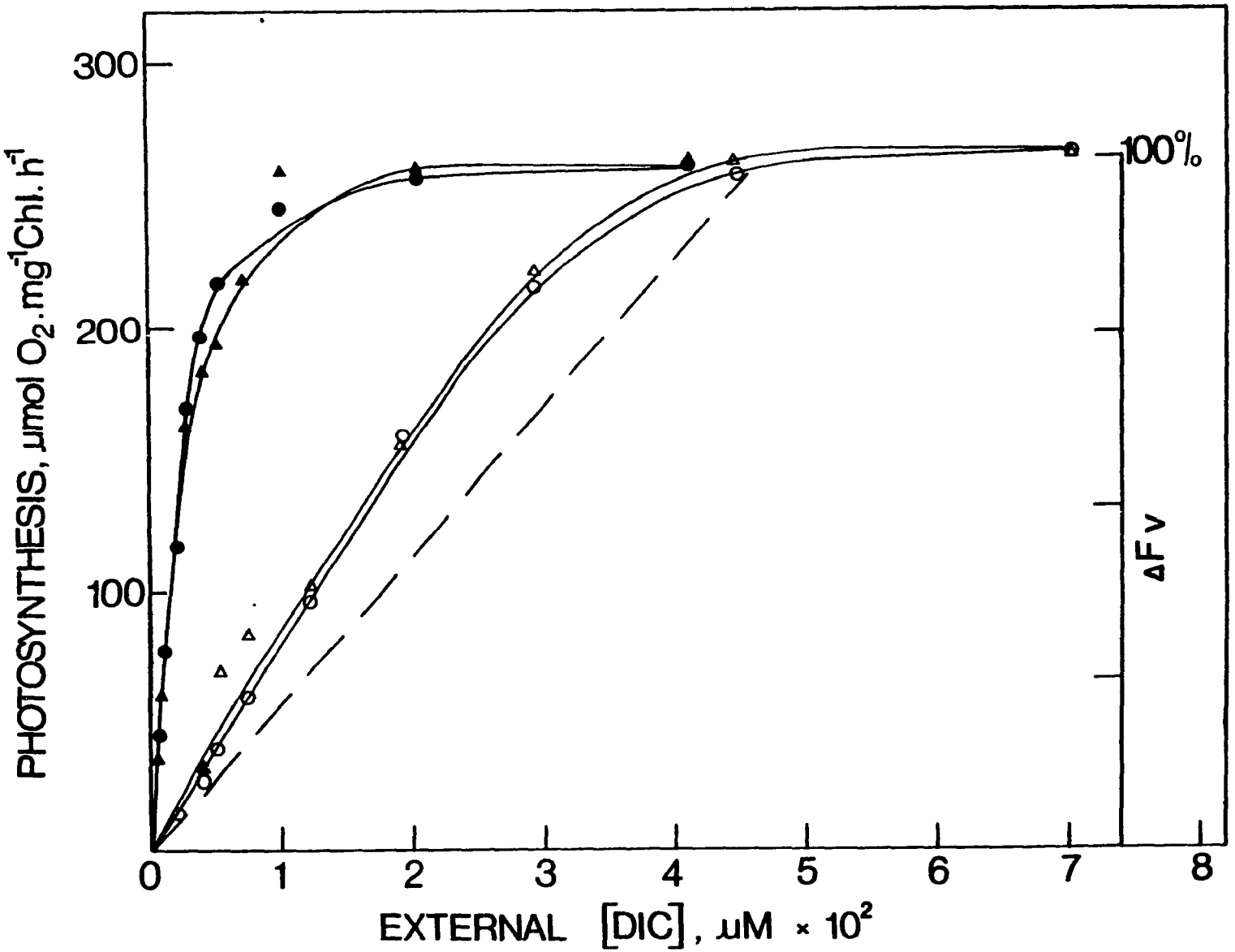
EFFECT OF [DIC] ON PHOTOSYNTHESIS AND FLUORESCENCE YIELDS

Figure 5 & 6 shows the rate of photosynthesis and the extent of Chl a fluorescence quenching at different [DIC] for air - grown and standing culture cells respectively. The extent of fluorescence quenching is expressed as a percentage of the maximum extent observed. The maximum extent of fluorescence quenching has been normalized to the maximum rate of photosynthesis. Over the entire range of [DIC] tested, there was a very close correspondence between the maximum extent of Chl a fluorescence quenching and the rate of photosynthetic O_2 evolution, for any given [DIC]. This was true whether or not Na^+ was present in the medium. This close correspondence would be expected if the rate of photosynthesis was completely dependent upon the size of the internal DIC pool (monitored by fluroescence quenching).

The photosynthetic affinity of the air - grown cells for external DIC was greatly increased by the presence of 25 mM Na^+. In the absence of added Na^+ $K_{0.5}$ (DIC) was 130 µM while in the presence of 25 mM Na^+ it was around 24 µM. In air-grown cells, high rates of photosynthesis could be achieved in the absence of Na^+ at high external [DIC]. In fact, at 500 µM DIC the rate of photosynthesis at ± 25 mM Na^+ was the same (Fig.

FIGURE 5

DIC response curve for air - grown cell photosynthesis and fluorescence quenching in the presence or absence of Na^+. The rate of photosynthetic O_2 evolution (▲,▲) and extent of Chl *a* fluorescence quenching (o,o) was measured as a function of extracellular [DIC], in the absence (▲,o) and presence (▲,o) of 25 mM Na^+ at pH 8 and 30°C. The extent of fluorescence quenching is expressed as a percentage of the maximum extent observed. The maximum extent of fluorescence quenching was normalized to the maximum rate of photosynthesis. The dashed line (---), indicates the rates of photosynthesis that could be supported solely by the use of CO_2. The [Chl *a*] ranged from 4.9 - 5.5 $\mu g.mL^{-1}$. The cells were illuminated at a light intensity of 100 $\mu mol\ photon.m^{-2}.s^{-1}$.


PHOTOSYNTHESIS, µmol O_2.mg^{-1}Chl.h^{-1}
300
200
100
0
1
2
3
4
5
6
7
8
EXTERNAL [DIC], µM × 10^2
100%
ΔF v

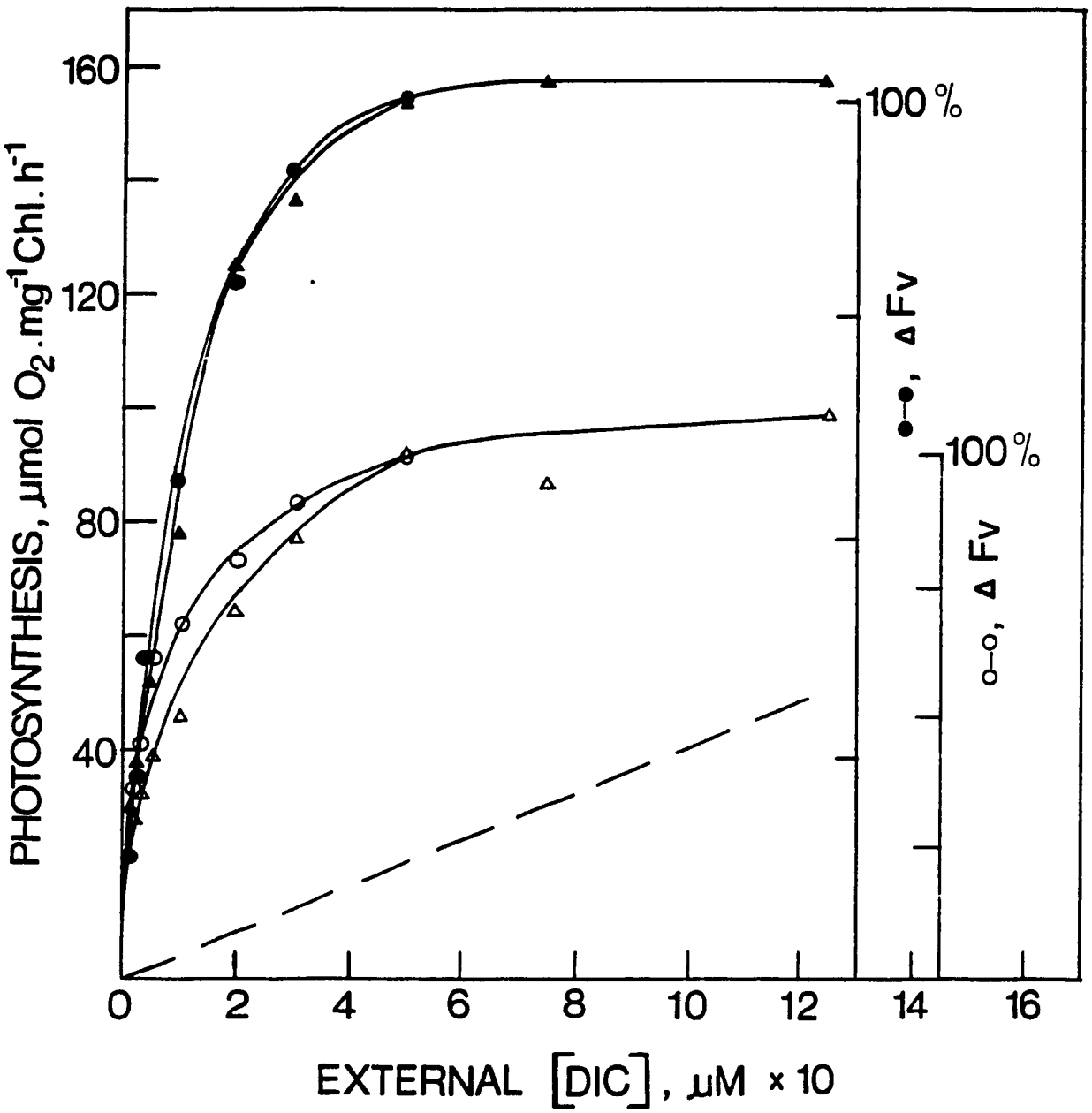
5). This result clearly indicates that the requirement for Na^+ in photosynthesis is not due to a requirement for Na^+ in CO_2 fixation per se. Thus, the increase in the apparent photosynthetic affinity for DIC also can not be ascribed to a direct effect of Na^+ on Calvin cycle enzymes or on photosynthetic electron transport. Rather, the increased apparent affinity for DIC is most likely related to the increased rate of HCO_3^- transport and accumulation brought about by Na^+.

In the absence of Na^+, the photosynthetic rate increased with increasing external [DIC] up to a saturation level (Fig. 5). In this case, however, the increase in the rate was found to parallel the increase in the CO_2 supply rate, which is linearly dependent on the external [DIC] (Miller 1985). Most of the observed rate of photosynthesis can, therefore, be accounted for on the basis of CO_2 uptake and subsequent fixation. This suggests that the Na^+ requirement for photsynthesis (distinct from HCO_3^- transport) can be overcome by increasing the rate of CO_2 supply to the cells. Indeed, when the enzyme carbonic anhydrase (CA) was included in cell suspensions to increase the rate of conversion of HCO_3^- to CO_2, high rates of photosynthesis were observed at 20 μM DIC (Fig. 13; Miller and Canvin 1985). In the presence of 25 $\mu g.mL^{-1}$ CA and absence of Na^+, the $K_{0.5}$ (DIC) was about 26 μM (not shown), compared to 130 μM in the absence of CA (Fig. 5).

In contrast to the air - grown cells the photosynthetic

FIGURE 6

DIC response curve for standing culture cell photosynthesis and fluorescence quenching in the presence and absence of Na^+. The extent of Chl a fluorescence quenching (o,o) and rate of photosynthetic O_2 evolution (▲,▲) at different extracellular [DIC] was obtained in the absence (▲,o) and presence (▲,o) of 25 mM Na^+. Fluorescence quenching was measured simultaneously with photosynthetic O_2 evolution and is expressed as a % of the maximum extent observed. The maximum extent of fluorescence quenching was normalized to the maximum rate of photosynthesis. The cell suspension was illuminated at 100 μmol photon.m^{-2}.s^{-1} at 30°C. The broken line (---), indicates the maximum rates of photosynthesis that could be supported solely by CO_2 uptake.


160
120
80
40
PHOTOSYNTHESIS, µmol O_2.mg^{-1}Chl.h^{-1}
100 %
●—●, ΔFv
100 %
○—○, Δ Fv
0
2
4
6
8
10
12
14
16
EXTERNAL [DIC], µM × 10

affinity for DIC in the standing culture cells was always high and was not affected subtantively by Na^+ (Fig. 6). The $K_{0.5}$ (DIC), ± 25 mM NaCl was in the range of 1.5 - 10 µM DIC. Similar $K_{0.5}$ (DIC) values were obtained for Synechococcus PCC6301 grown in cultures containing less than 50 µM DIC (Gallagher & Badger 1987).

Maximum rates of photosynthesis were in the range of 130 - 160 µmol $O_2.mg^{-1}$ $Chl.h^{-1}$ for standing culture cells and 200 - 250 µmol $O_2.mg^{-1}$ $Chl.h^{-1}$ for air - grown cells. This difference in photosynthetic capacity likely reflects differences in the [DIC] in the growth medium during growth. The inorganic carbon supplied to standing culture cells is through diffusion of atmospheric CO_2 across a foam stopper into the liquid growth medium. For the air - grown cultures there is a continuous supply of inorganic carbon through bubbling of 0.03% (v/v) CO_2 in air into the medium. Thus, there is a higher supply of DIC to the air - grown cultures compared to the standing culture and this is reflected in their respective rates of growth. Previous studies have indicated similar difference in photosynthetic V_{max} with response to growth [DIC] below saturation level (Mayo et al. 1986; Badger & Gallagher 1987). This is because the growth rate of cells growing at low [DIC] is low. The cells adapt to this environment by lowering the Rubisco concentration for a more efficient utilization of nitrogen resources which in turn reduces the maximum photosynthetic ability (Mayo et al. 1989).

EXTRACELLUAR CONTAMINANT Na^+

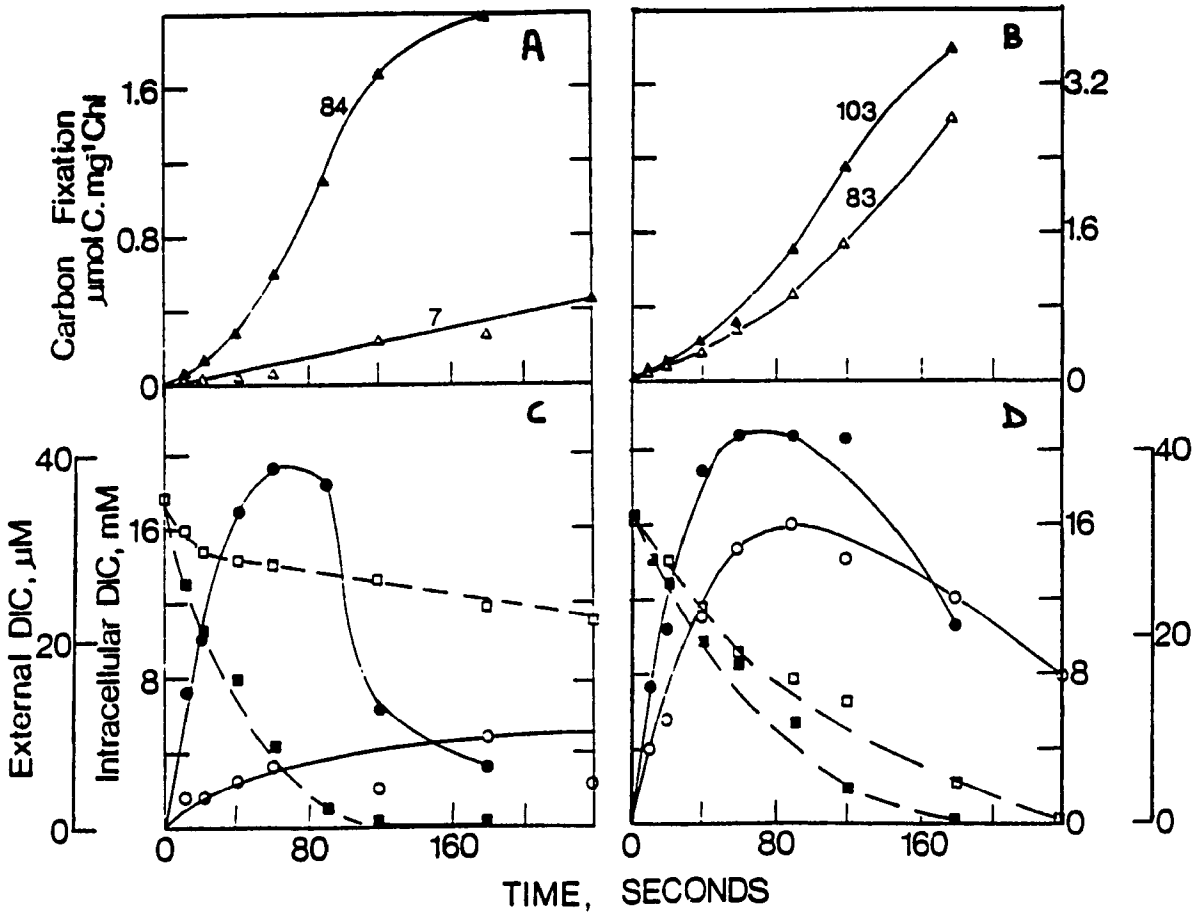
The contaminant [Na^+] in the 25 mM BTP/HCl buffer, determined by atomic absorption spectroscopy was in the range of 8 - 10 μM. This value increased to 25 - 30 μM with incubation of the standing culture cells (6 μg Chl.mL^{-1}) for 40 minutes. In order to test if Na^+ bound to the cell wall was involved in the HCO_3^- transport in the absence of added Na^+, the cells were washed twice with buffer containing 50 mM LiCl and subsequently transferred to Li^+ free buffer. Lithium being more reactive than Na^+ would displace Na^+ bound to the membrane and anionic site on the cell wall. Washing the cells in this manner did not change the observed level of photosynthesis or fluorescence quenching. Thus, neither contaminant nor bound Na^+ can explain the standing culture cells ability for Na^+ - independent HCO_3^- transport.

EFFECT OF Na^+ ON INTRACELLULAR DIC ACCUMULATION

Figure 7 shows time courses for DIC disappearance from the medium, intracellular accumulation of DIC and photosynthetic ^{14}C-fixation for air - grown and standing culture cells in the presence and absence of NaCl. In all cases the flow of DIC in the system appears to proceed initially from the external pool to the internal pool. When the external [DIC] reached zero the internal inorganic concentration decreased indicating that the external inorganic carbon was the direct source of carbon for the internal pool.

FIGURE 7

"Silicone fluid" measurements of DIC uptake and carbon fixation by the air grown and standing culture cells ± of 25 mM Na^+. Shown are the time courses of $DI^{14}C$ depletion from the medium (■ ,■), intracellular $DI^{14}C$ accumulation (o,o) and carbon fixation (▲ ,▲) by the air-grown (A,C) and standing culture (B,D) cells in the absence (■ ,o,▲) and presence (■ ,o,▲) of 25 mM Na^+ at pH 8. The rate of photosynthesis in µmol $C.mg^{-1}Chl.h^{-1}$ is given beside each curve. The [Chl] ranged between 7 - 14 $\mu g.mL^{-1}$. Light was supplied at 200 - 400 µmol photon.m^{-2}.s-1 and the experiment was performed at 28 - 30°C with cells suspended in 25 mM BTP/HCl buffer. The specific activity of ^{14}C in the tube was 40 $\mu Ci.\mu mol^{-1}$.


A
B
C
D
Carbon Fixation
µmol C. mg⁻¹ Chl
84
7
103
83
1.6
0.8
0
3.2
1.6
0
External DIC, µM
Intracellular DIC, mM
40
20
0
16
8
0
80
160
TIME, SECONDS

The internal [DIC] was also found to be considerably higher than the external [DIC] after a few seconds exposure to $DI^{14}C$. The ability of the cells to concentrate DIC internally above the level found in the medium indicates that the accumulation of DIC proceeds through the expenditure of metabolic energy.

The rate of carbon dioxide fixation by standing culture and air - grown cells of Synechococcus UTEX 625 was dependent on the internal DIC concentration (Fig. 7 & 8). As the size of the internal $DI^{14}C$ pool increased, the rate of photosynthetic ^{14}C fixation was also found to increase until a maximum rate was reached which coincided with the largest observed pool of internal DIC. Once the external DIC was exhausted the level of intracellular DIC decreased and the rate of photosynthesis declined (Fig. 7). These findings are consistent with previous observations for other cyanobacteria such as Anabaena variabilis (Kaplan et al. 1980) and Anacystis nidulans (Shelp & Canvin 1984). The requirement to form a large inorganic carbon pool to obtain high rates of photosynthesis is likely due to the very low affinity of the enzyme Rubisco for CO_2 (Kaplan et al. 1980). The Km (CO_2) of cyanobacterial Rubisco is about 200 μM (Badger 1980) or 20 times higher than the [CO_2] present in the air - equilibrated water. In contrast Km (CO_2) of terrestrial plant Rubisco is 7 - 10 μM. Thus, the formation of the internal inorganic carbon pool in Synechococcus acts as an intermediate for C fixation.

There was very slow depletion of inorganic carbon from

the external medium and relatively low intracellular DIC accumulation by the air - grown cells in the absence of Na^+ (Fig. 7C). Under the same conditions there was rapid decrease in external inorganic carbon and a large intracellular pool of inorganic carbon formed by the standing culture cells (Fig. 7D). If it is assumed that the standing culture cells only transport CO_2 and all of the CO_2 in the medium is scavenged as fast as it is being produced from HCO_3^- dehydration, then at the 60 s time point calculated DIC depletion from the medium would be 5.5 μM. But, the measured DIC depletion at this time point was 4.2 - fold greater than the calculated value. If CO_2 alone was taken up from the medium then maximum DIC depletion between 40 and 60 s time point would be 0.4 μM but the observed depletion by the standing culture cells was 5 μM in the absence of Na^+.

The level of carbon fixation by the air - grown cells in the absence of Na^+ can be theoretically accounted for solely by CO_2 uptake (Fig. 7A) whereas only 1/6 of the observed rate of C fixation by standing culture cells could be supported solely by CO_2 transport (Fig. 7B). Electrometric assay did not indicate any extracelluar CA production by the standing culture cells of Synechococcus UTEX 625 (Espie & Canvin 1987) which would have accelerated the production of CO_2 from HCO_3^- dehydration. Collectively, these results conclusively demonstrate direct HCO_3^- uptake by the standing culture cells in the absence of Na^+.

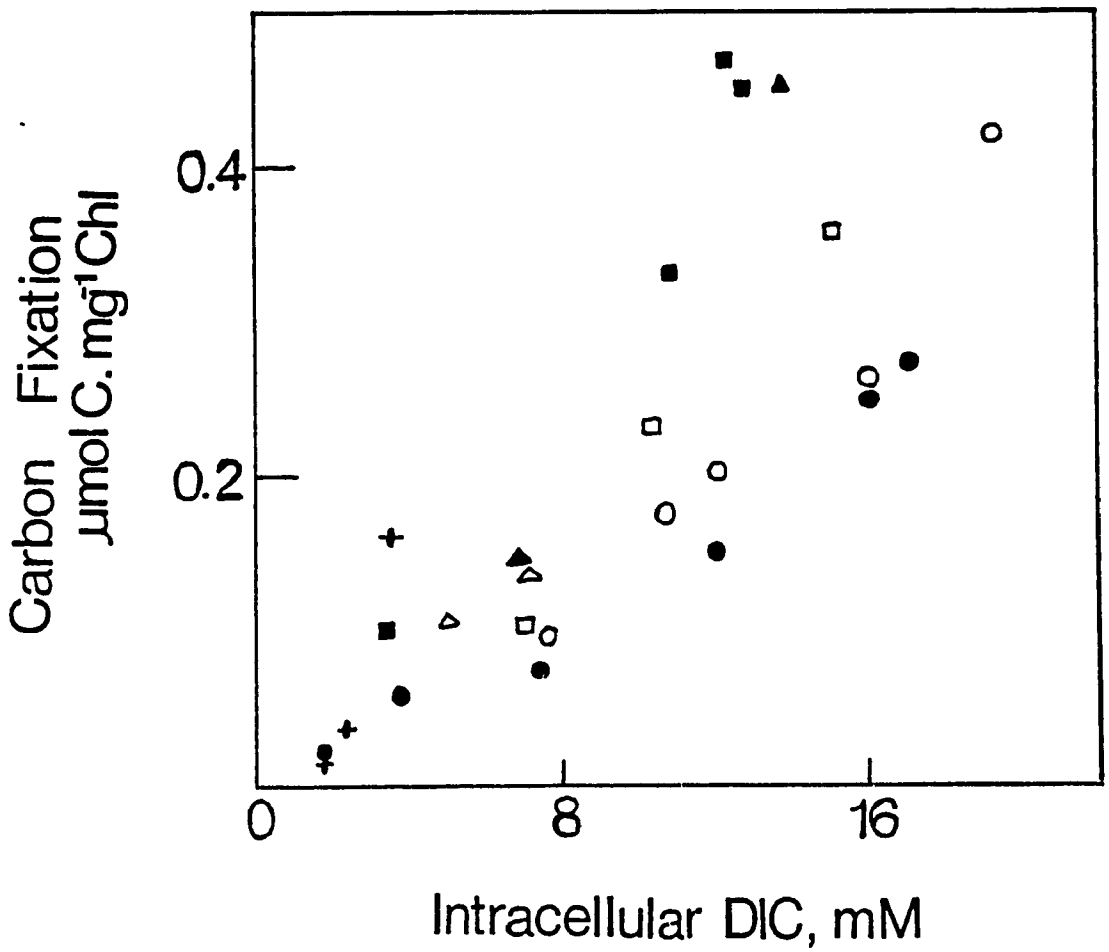
In the presence of 25 mM Na^+, inorganic carbon pool formation by air - grown cells was greatly accelerated. This increase in intracellular [DIC] in the presence of sodium was mainly due to HCO_3^- uptake as the external DIC depletion rate was several fold higher than that would be observed if CO_2 was the sole species taken up. Secondly, the observed rate of C fixation was 6 to 7 -fold greater than that could be supported solely by CO2 transport.

Similarly, 25 mM Na^+ stimulated the size of the internal inorganic carbon pool formed by the standing culture cells (1.3 -fold) but the enhancement was far less than for air - grown cells (5.7 -fold). Paralleling the increased intracellular DIC pool was a 1.2 -fold increase in the rate of photosynthetic carbon fixation (Fig. 7B).

Ongoing intracellular accumulation of inorganic carbon by the standing culture and air - grown cells of Synechococcus UTEX 625 occurred against a large concentration gradient. The accumulation of ratio [DIC]in/[DIC]out in the absence of Na^+ for the standing culture and air - grown cells was 970 and 127 respectively. The higher accumulation ratio by the standing culture cells is due to the presence of Na^+ - independent HCO_3^- uptake mechanism. Accumulation ratios similar to the standing culture cells could only be achieved by air - grown cells in the presence of millimolar levels of Na^+ through a Na^+ - dependent HCO_3^- transport mechanism. Previous studies have reported accumulation ratios of 1000 for air - grown cells of

FIGURE 8

Carbon fixation by the standing culture and air-grown cells as a function of intracellular [DIC]. The results obtained for standing culture and air-grown cells are represented as open and close symbols respectively. Different symbols represent different sets of results obtained from time course "silicone fluid" DIC accumulation experiments. The experiments were conducted at pH 8 and 30°C. The [Chl] ranged from 7 - 14 $\mu g.mL^{-1}$.


Carbon Fixation
μmol C. mg⁻¹ Chl
0.4
0.2
0
8
16
Intracellular DIC, mM

Coccochloris peniocystis (Miller & Canvin 1980), Anacystis nidulans (Shelp & Canvin 1984) and Anabaena variabilis (Kaplan et al. 1980). It is not known if this high accumulation ratio in these organisms was due to Na^+ - dependent or independent HCO_3^- transport as Na^+ was present at high concentration in all these experiments.

Data ~~extracted~~ from a number of "silicone fluid" experiments (e.g. Fig. 7) relating the rate of C-fixation to the level of intracellular DIC are compiled in Fig. 8. It is evident that as internal [DIC] increased so did photosynthesis. The slope of the relationship between CO_2 fixation and internal [DIC] are approximately the same for air-grown and standing culture cells. This result indicates that the apparent affinity of the CO_2 fixing enzyme Rubisco for its substrate was similar in both cell types and unaffected by the presence or absence of Na^+ in the medium. It can now be concluded that the higher apparent photosynthetic affinity for external DIC observed for cells grown in standing culture compared to air-grown cells in the absence of Na^+ (Fig. 5 & 6) was truly due to a superior ability of standing culture cells to form an internal DIC pool.

EVIDENCE FOR HCO_3^- TRANSPORT FROM CO_2 MEASUREMENTS

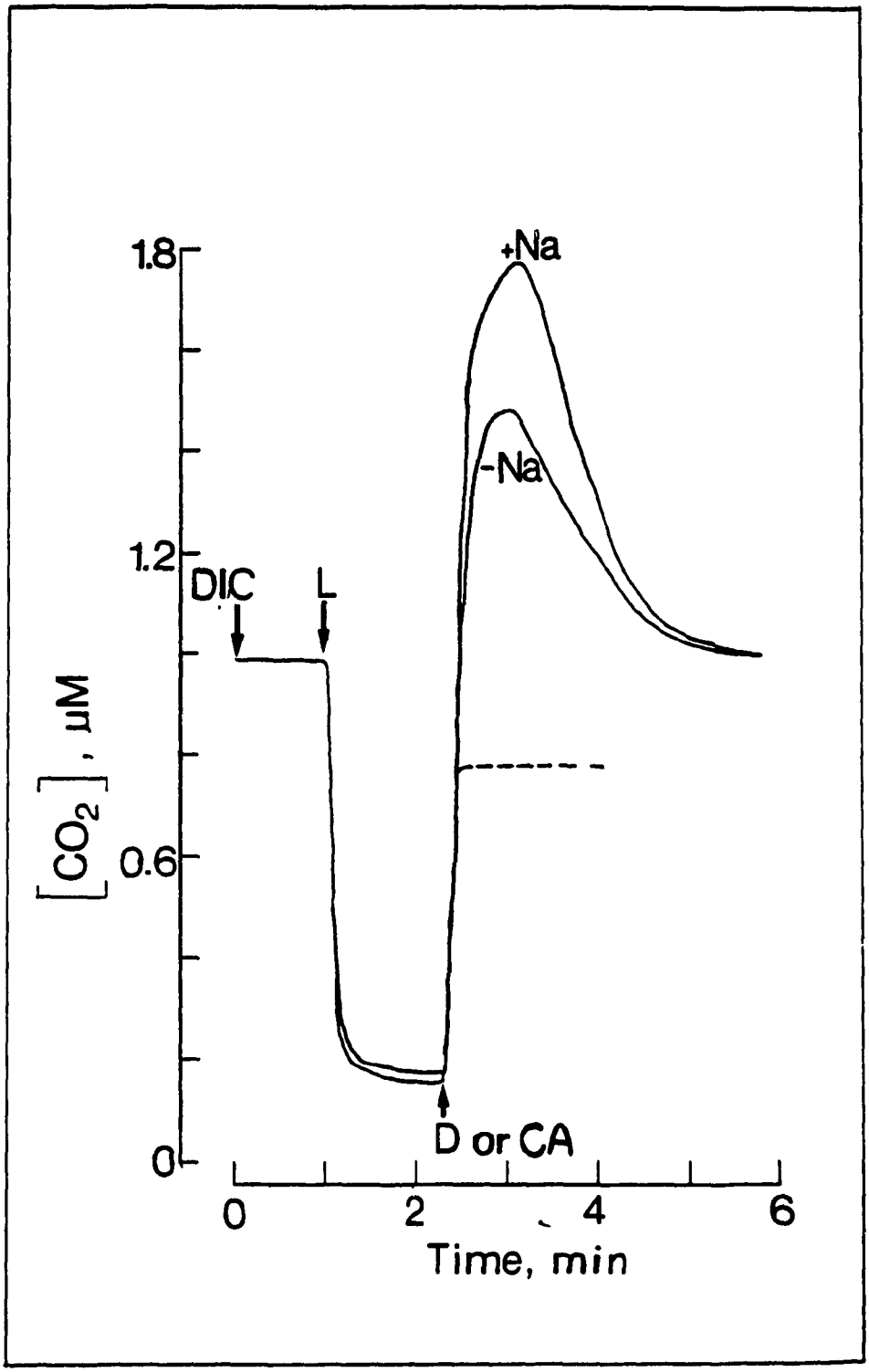
Another convincing line of evidence for Na^+ - independent HCO_3^- uptake by the standing culture cells was obtained from

studies conducted with the mass spectrometer (Fig. 9). Following illumination of the cells there was rapid, light - dependent uptake of $^{13}CO_2$ resulting in the depletion of $^{13}CO_2$ from the medium to a level close to zero. Presumably, the CO_2 which disappeared from the medium was accumulated internally and was thus not measured by the mass spectrometer. When the light was switched off there was rapid reappearance of the internally sequested $^{13}CO_2$ in the medium. Initially the [$^{13}CO_2$] rose to a level well above the starting level but gradually declined to a concentration close to that found prior to illumination. In this particular experiment, cells were illuminated for only a brief period of time and consequently, little net carbon fixation occurred. These phenomenon were completely dependent upon the presence of cells but independent of photosynthetic carbon metabolism because similar observation was made under conditions where carbon fixation was inhibited with iodoacetamide (G.S. Espie, unpublished). Thus, the uptake and intracellular accumulation of CO_2 could be separated from the process of carbon fixation, both of which would contribute to CO_2 disappearence from the medium.

Assuming the cells only transported CO_2, then the maximum DIC that could be taken up by the cells before the lights was switched off would be 5.53 nmol. Taking into account the intracellular pH (8; Coleman & Colman 1981) and the presence of intracellular CA (Badger et al. 1985; Tu et al. 1987) only

FIGURE 9

Mass spectrometer measurement of DIC transport against a concentration gradient by the standing culture cells in the $\pm$ of 25 mM Na^+. The cells (8 $\mu g.mL^{-1}$) were incubated at 30°C in 25 mM BTP/HCl (pH 8) buffer in the presence of 65 μM $K_2{}^{13}CO_3$. DIC transport was initiated by illuminating the cells (L) at 200 μmol photon.$m^{-2}.s^{-1}$. Once the cells established a new steady-state [CO_2] the lights were turned off (D) or CA (25 $\mu g.mL^{-1}$) was added at the time indicated. The amount of DIC accumulated by the cells was estimated by the amount of $^{13}CO_2$ that leaked back in to the medium upon turning out the lights (D) and the total amount of DIC present in the medium (---) was determined by the addition of CA. The CA data adapted from G.Espie unpublished results. The results of several experiments have been overlaid to facilitate comparisons.


1.8
1.2
0.6
0
[CO2], µM
+Na
-Na
DIC
L
D or CA
0
2
4
6
Time, min

0.086 nmol. of the accumulated DIC would be found as $^{13}CO_2$ inside the cells. But, the observed efflux of $^{13}CO_2$ from the cells in the absence of Na^+, was 15 - fold greater. This measured $[CO_2]$ is a underestimate, since some of it is converted to HCO_3^- before it was measured by the mass spectrometer. Evidently, a much larger internal pool of DIC existed in the cells than that could be accounted for by CO_2 uptake alone. This $^{13}CO_2$ efflux could not be due to respiration since ^{13}C - labelled DIC (natural abundance, 1.1 %) was used and the $^{13}CO_2$ efflux occurred within a short period of time (75 seconds). Therefore the only reasonable explanation for this data is active transport and accumulation of $H^{13}CO_3^-$ inside the cell followed by intracellular dehydration to $^{13}CO_2$.

In the presence of 25 mM Na^+, the tracings for $^{13}CO_2$ depletion of the medium were similar to that observed in the absence of Na^+. But, when the lights were switched off the observed $^{13}CO_2$ efflux was greater than that observed in the absence of Na^+. Since the CO_2 depletion from the medium in the $\pm$ Na^+ was similar the higher efflux of CO_2 suggests a larger intracellular pool formation presumably due to higher HCO_3^- uptake by the cells through a Na^+ - dependent mechanism in conjunction with the Na^+ - independent mechanism. The enhancement of intracellular DIC accumulation in standing culture cells by Na^+ suggested by these experiment is consistent with previous results (Fig. 7) obtained using the silicone fluid filtering centrifugation technique to measure

internal DIC accumulation.

Illumination of the cells resulted in rapid depletion of CO_2 from the medium independent of photosynthesis (Fig. 9). This could be due to selective uptake of CO_2 followed by HCO_3^- uptake or due to uptake of all of the DIC from the medium. Previous experiments (Fig. 7D) showed that in the presence of 37 µM DIC and at a [Chl] of 7 $\mu g.mL^{-1}$ it took 2.5 - 3 min to completely deplete all of the DIC from the external medium (Fig. 7). Thus, under the same condition it would take a longer time span to deplete 65 µM DIC. Therefore, the observed depletion of $[CO_2]$ to near zero is not due initially to complete removal of DIC from the medium but to the selective and efficient uptake of CO_2. If, rather than switching the lights off at the indicated time (Fig.9), the enzyme CA was added to the cell suspension, the $[^{13}CO_2]$ was found to rise to a level somewhat below the starting level. This findings indicated that $H^{13}CO_3^-$ was present in the medium but that it was not in chemical equilibrium with $^{13}CO_2$, in the absence of CA, thus confirming our initial conclusion. The occurrence of a disequilibrium between $^{13}CO_2$ and $H^{13}CO_3^-$ additionally suggests that a high affinity carrier systems exists for CO_2 uptake which through the input of metabolic energy is able to create the chemical disequilibrium. Similar observations with regard to CO_2 transport have been obtained for air - grown cells (Miller et al. 1988a; Espie et al. 1988a). Thus it appears as if standing culture cells are capable of both active HCO_3^- and

CO_2 transport.

GROWTH OF STANDING CULTURE AND AIR-GROWN CULTURE CELLS

Figure 10 & 11 display growth curves for standing culture and air - grown cells as increases in A_{720} and Chl *a* concentration. It should be noted that air - grown cultures are initiated by inoculating growth media with cells grown in standing culture. Like typical growth curves the log phase of growth for these cultures was preceded by a lag phase. The lag phase in the air - grown cultures lasted for about 18 hours and the log phase for 65 - 70 hours. In comparison to air - grown cells, the cells in the standing cultures grew very slowly. In the standing cultures, the lag phase lasted for about 2 days and the log for more than 19 days. The doubling time for the air - grown cultures and standing cultures was around 15 hours and 4.8 days respectively. The longer time taken by the standing culture cells to adapt to the new environment and slower growth rate compared to the air - grown cells was due to the limited availability of DIC and lack of mixing or shaking of culture.

The pH of the standing culture cells increased over the first 1.5 days from about 8 to 10, whereas the pH of the air - grown cultures were maintained fairly constant around 8 for the first 15 - 18 hours. This is probably due to the fact that the air - grown cells are continuously bubbled with air containing CO_2 where the inflow of CO_2 acts as a weak buffer.

FIGURE 10

Growth curve for standing culture cells. Growth is indicated as a measure of cell density, OD_{720} (o) and [Chl] (o). Changes of the culture medium pH (▲) with growth is also shown. Cells grown on plates were used as initial inoculum. The culture was grown in Allen's medium, maintained at 30°C and illuminated at 25 - 30 μmol photon.m^{-2}.s^{-1}.

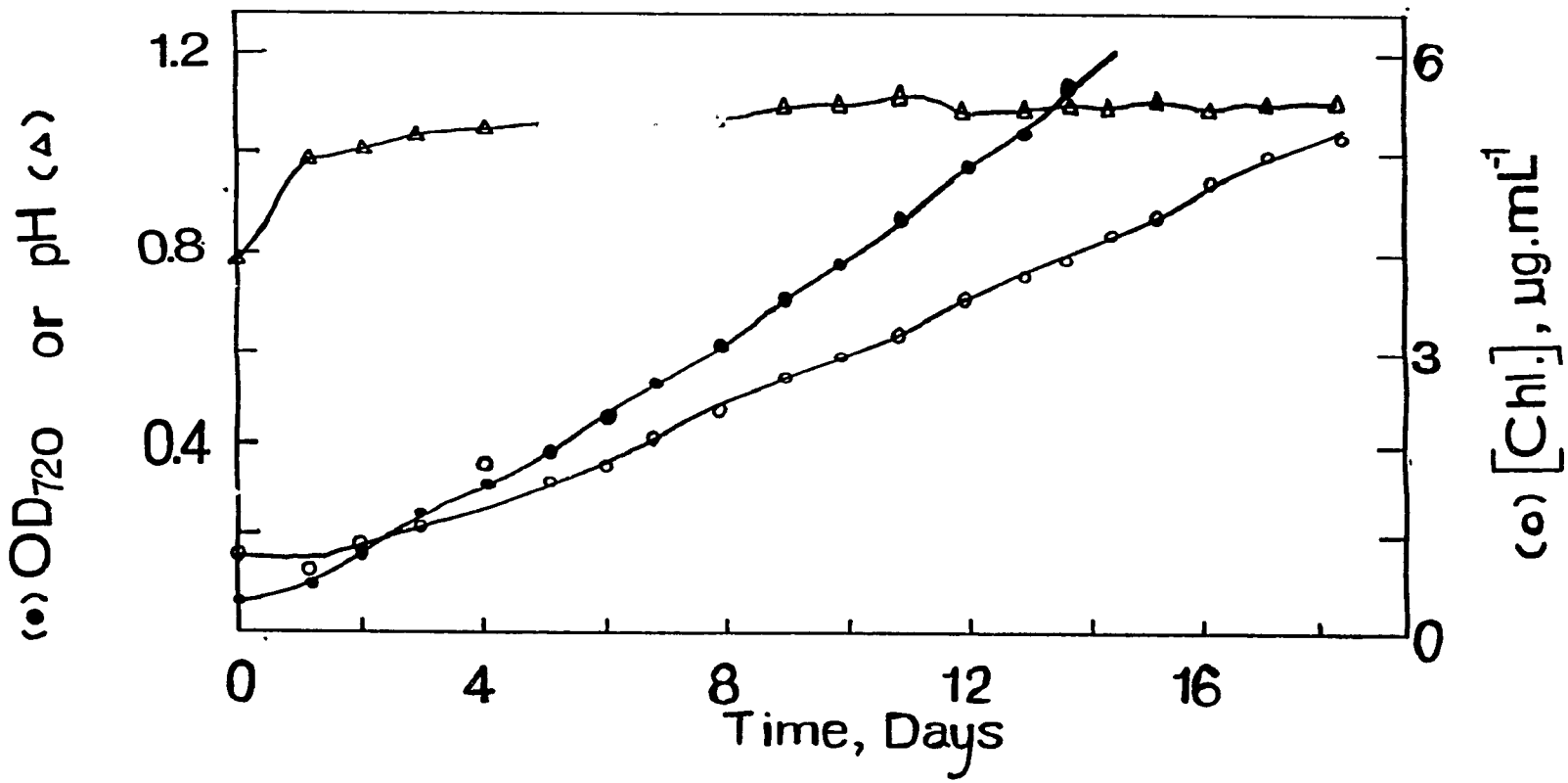
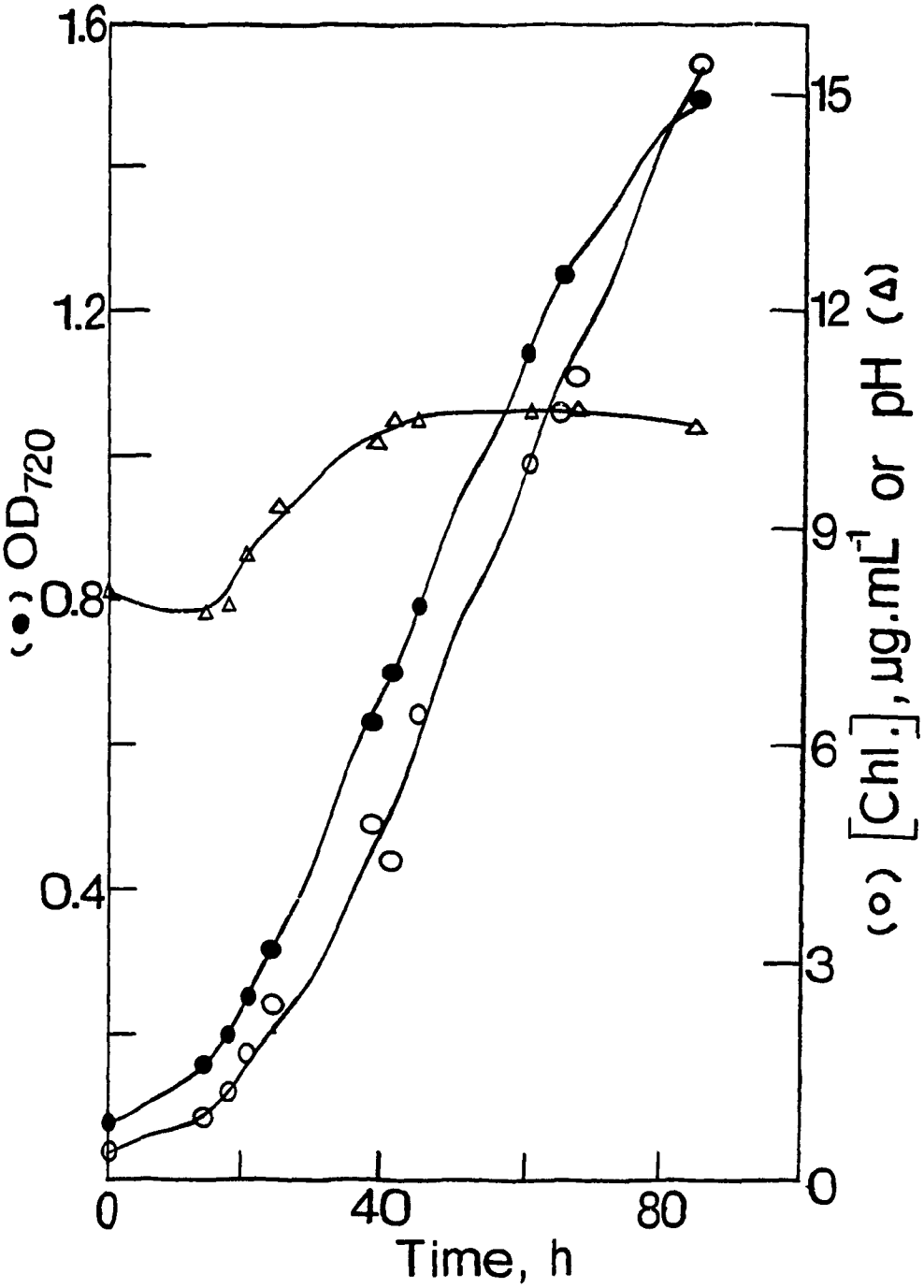

1.2
0.8
0.4
0
4
8
12
16
Time, Days
6
3
0
(•) OD720 or pH (Δ)
(o) [Chl.], µg.mL-1

FIGURE 11

Growth curve for air grown cells. Growth was measured as OD_{720} (o) and [Chl] (o). The changes of the culture medium pH (▲) during growth are also shown. The cultures were started by inoculating standing culture cells into Allen's medium which was continuously bubbled with 0.03% (v/v) CO_2 in air. The cells were illuminated at 50 - 55 μmol photon.m^{-2}.s^{-1} at 30°C.


1.6
1.2
0.8
0.4
(●) OD720
15
12
9
6
3
0
(○) [Chl.], µg.mL-1 or pH (Δ)
0
40
80
Time, h

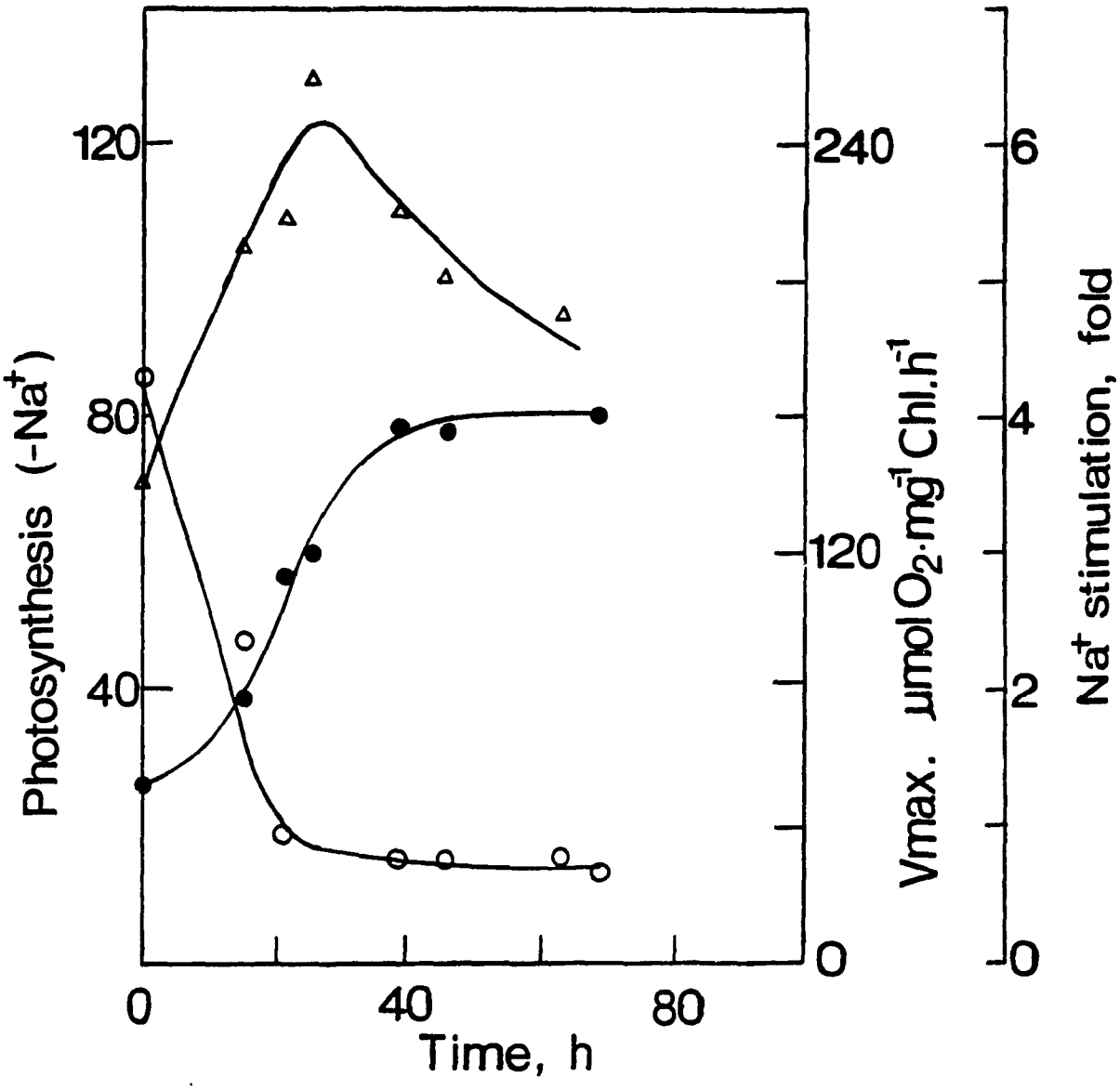
However, the increasing level of photosynthesis (which generates OH^-) with increase in cell number results in the alkalization of the medium. This would account for the gradual rise of the pH during the early log phase to about 10.2. For all experiments the cells were harvested at mid - log phase around 45 - 60 hours and 10 - 14 days for air - grown cultures and standing cultures respectively.

INDUCTION OF Na^+ - DEPENDENT HCO_3^- TRANSPORT

The loss of Na^+ - independent HCO_3^- transport and increase of Na^+ - dependent HCO_3^- uptake (as inferred from measurements of photosynthesis and fluorescence quenching at 20 μM DIC and pH 8) with time after inoculation of standing culture cells into air - grown cultures is depicted in figure 12. The maximum rate of photosynthesis that could be supported solely by CO_2 transport at 20 μM DIC and pH 8 is around 17 μmol $O_2.mg^{-1}Chl.h^{-1}$. But, the observed rate of photosynthesis in the absence of Na^+ at zero time was 5 - fold greater. This Na^+ - independent HCO_3^- transport was reduced by 60 % by late lag phase (16 hours) and by early log phase there was complete loss of Na^+ - independent HCO_3^- transport as inferred from measurements of photosynthesis in the absence of Na^+. Over the same time period a corresponding increase in Na^+ - stimulated photosynthesis and HCO_3^- transport was observed (Fig. 12). Initially 25 mM Na^+ enhanced the rate of photosynthesis (at 20 μM DIC) by 1.3 to 2 -fold. By early log phase (20 hours) this

FIGURE 12

Induction of Na^+ - dependent HCO_3^- transport. Changes in Na^+ - independent (o), Na^+ - dependent (o) and the DIC saturated (▲) rate of photosynthesis were obtained during the growth of the air- grown culture cells. Na^+ - independent rate of photosynthesis and the Na^+ (25 mM) stimulation was measured following the addition of 20 µM $KHCO_3$ to cells (3 - 5 µg $Chl.mL^{-1}$) suspended in 25 mM BTP/HCl buffer at pH 8 and 30°C. The DIC saturated rate of photosynthesis was measured in the presence of 25 mM Na^+ and 1 mM $KHCO_3$. Illumination for the cells was provided at 100 µmol $photon.m^{-2}.s^{-1}$.


Photosynthesis (-Na+)
120
80
40
0
40
80
Time, h
240
120
0
Vmax. µmol O2·mg-1 Chl.h-1
6
4
2
0
Na+ stimulation, fold

had increased to 3 to 5.4 -fold while maximum stimulation (4 to 8 -fold) occurred 35 to 40 hours after inoculation. Figure 12 illustrates the lower extreme of Na^+ - stimulation.

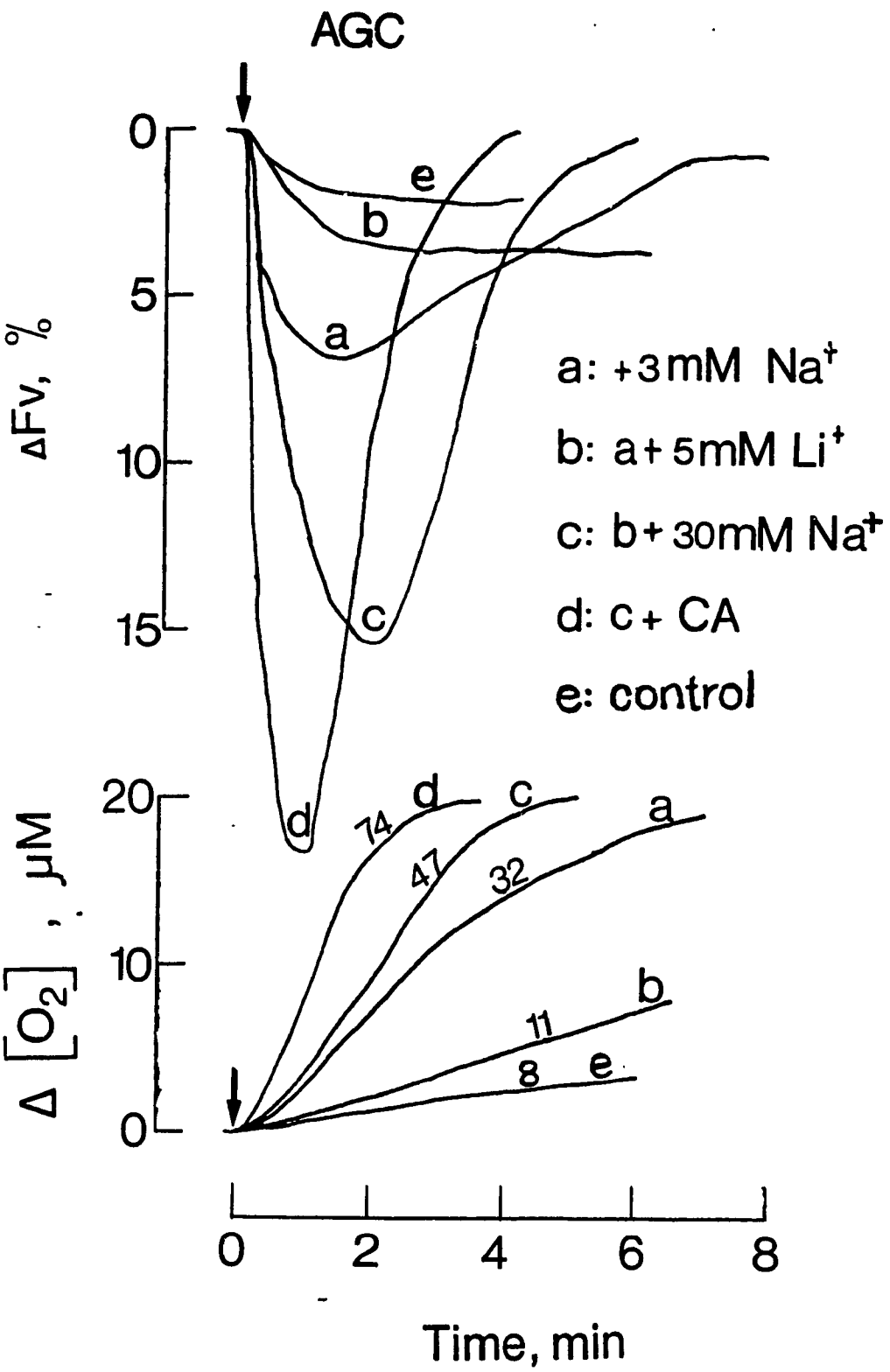
The maximum rate of photosynthesis (Vmax) increased from about 110 to 250 μmol $O_2.mg^{-1}Chl.h^{-1}$ over the first 25 - 28 hours of growth. This may be because the cells experience a higher [DIC] in the air - grown cultures compared to standing cultures thus, increasing their capacity for C fixation probably by increasing the synthesis of more Chl, proteins, Rubisco etc. But as the cell number and the level of photosynthesis increased there would be a draw down in [DIC] in the medium away from equilibrium with the incoming gas. This in turn would have reduced C fixation capacity per unit Chl and the rate of photosynthetic O_2 evolution. Cells growing on plates had a maximum photosynthetic rate of 45 μmol $O_2.mg^{-1}Chl.h^{-1}$. Like high CO_2 grown cells these cells did not have a Na^+ - dependent nor independent HCO_3^- transport. This may be because the CO_2 is the only available carbon source for cells growing on solid media.

EFFECT OF LiCl ON PHOTOSYNTHESIS AND CHLOROPHYLL *a* FLUORESCENCE YIELD

It has been previously established that LiCl inhibits Na^+ - stimulated photosynthesis (Espie & Canvin 1987; Espie *et al.* 1988b; Miller & Canvin 1985; Kaplan *et al.* 1984) and HCO_3^- accumulation (Espie *et al.* 1988b) in the air - grown cells of

FIGURE 13

Effect of LiCl on photosynthesis and Chl *a* fluorescence yield of air-grown cells. Shown are the tracings of lithium inhibition (b) of Na^+ - stimulated rate of photosynthetic O_2 evolution and Chl *a* fluorescence quenching (a) and subsequent recovery by the addition of 30 mM Na^+ (c) and 25µg.mL^{-1} CA (d). The control (e) was obtained in the absence of any added Na^+ or Li^+. The reaction was started by the addition (↓) of 20 µM $KHCO_3$ to the cells (8.5 µg Chl.mL^{-1}). The rate of photosynthesis in µmol O_2.mg^{-1}Chl.h^{-1} is given beside each curve. The measurements were made at 30°C (pH 8.02) and light was supplied at 100 µmol photon.m^{-2}.s^{-1}.


AGC
0
5
10
15
ΔFv, %
e
b
a
c
d
a: +3mM Na+
b: a+5mM Li+
c: b+30mM Na+
d: c+ CA
e: control
20
10
0
Δ [O2], μM
74
47
32
11
8
0
2
4
6
8
Time, min

S.leopoliensis (Espie & Canvin 1987; Espie et al. 1988b; Miller & Canvin 1985) and A.variabilis (Kaplan et al. 1984). Figure 13 shows the results of a typical experiment for air - grown cells. In the absence of Na^+, addition of 20 μM DIC to the cells resulted in a low rate of photosynthesis, which did not exceed the rate at which CO_2 could be supplied to the cells. As shown previously 3 mM Na^+ typically enhanced photosynthesis in air - grown cells by 2.5 to 3 -fold and increased the initial rate and extent of Chl a fluorescence quenching (Fig. 13). This level of Na^+ was sub-optimal for photosynthesis. Addition of 5 mM LiCl in the presence of 3 mM Na^+ resulted in the reduction in rate of photosynthesis as well as in the initial rate and extent of fluorescence quenching. This effect of LiCl on photosynthesis and fluorescence quenching could be reversed by increasing the extracellular [Na^+] (Fig. 13c).

To distinguish between Na^+ - dependent and Na^+ - independent HCO_3^- transport, we examined the effect of [LiCl] on standing culture and air - grown cell photosynthesis and Chl a fluorescence yield at pH 8 and 20 μM DIC (Fig. 14). With air - grown cells fluorescence quenching and photosynthesis stimulated by 3 mM Na^+ was drastically reduced at 15 mM LiCl concentration. In contrast, at the same concentration of LiCl very little effect was noted on Na^+ - independent photosynthesis or fluorescence quenching by the standing culture cells. At higher [LiCl] there is some decline in

FIGURE 14

Effect of [LiCl] on air-grown and standing culture cell photosynthesis and Chl *a* fluorescence yield as a percentage of the control rates. The extent of fluorescence quenching (■,■) and the rate of photosynthesis (o,o) for the standing culture (o,■) and air-grown (o,■) cells were obtained following the addition of 20 µM DIC to cell suspensions (6.2 - 10 µg $Chl.mL^{-1}$) at pH 8 and 30°C. The experiments were conducted in the presence of 3 mM Na^+ for air-grown cells (in order to stimulate Na^+-dependent HCO_3^- uptake) and in the absence of any added Na^+ for the standing culture cells. The control rate of photosynthesis and the extent of fluorescence quenching was 28 µmol $O_2.mg^{-1}Chl.h^{-1}$ and 10% Fv for air-grown cells & 72 µmol $O_2.mg^{-1}Chl.h^{-1}$ and 55.8% Fv for standing culture cells.

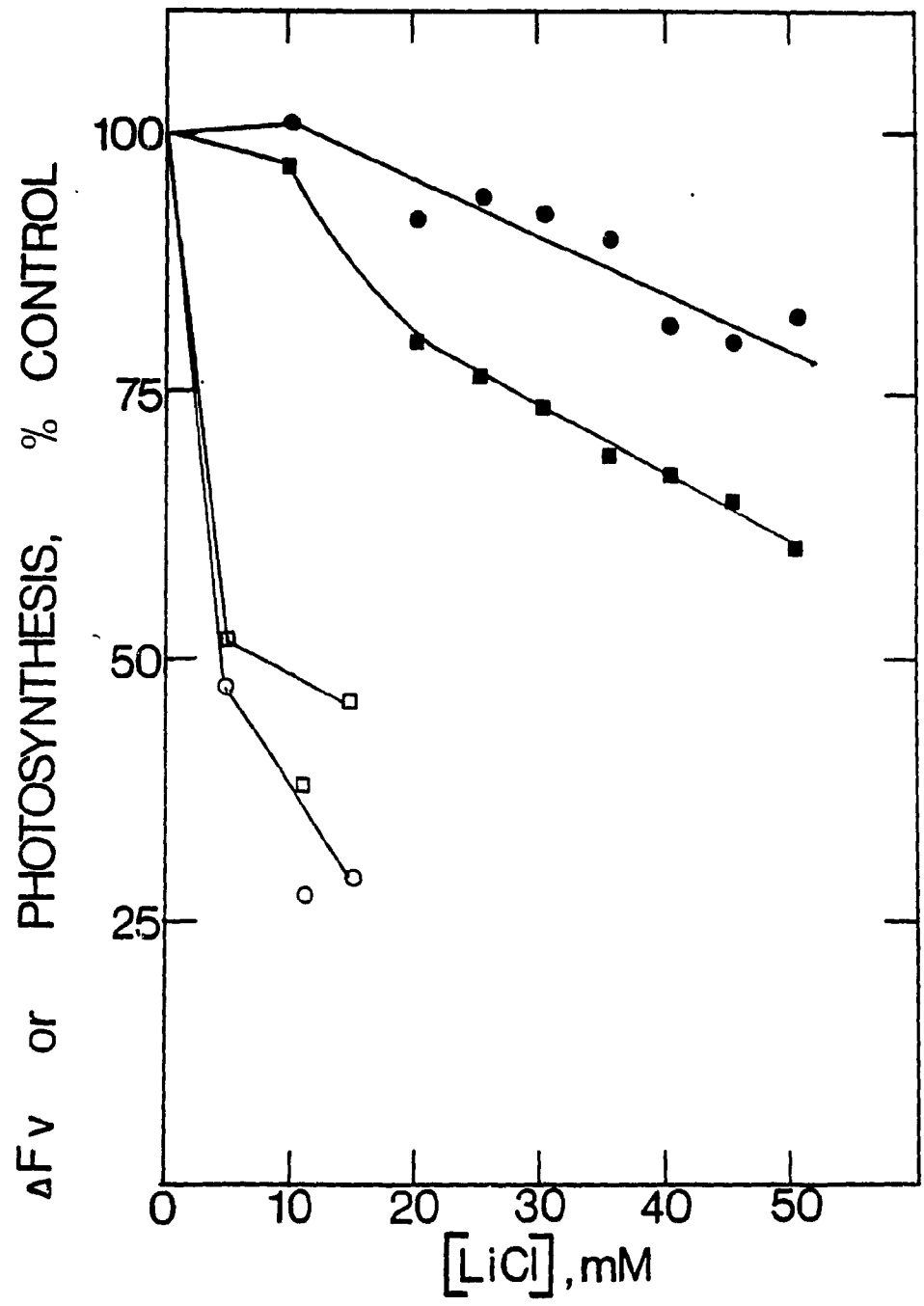
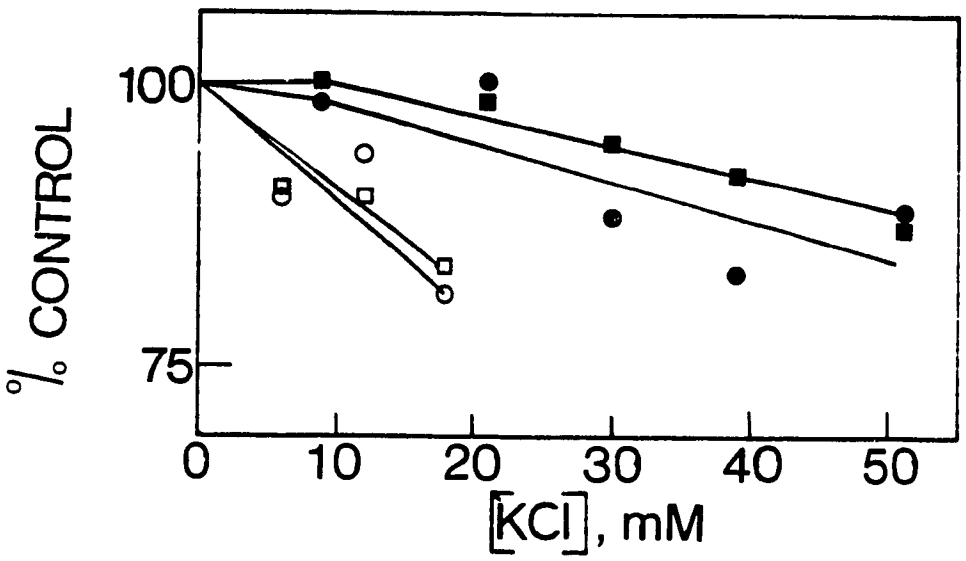

ΔFv or PHOTOSYNTHESIS, % CONTROL
100
75
50
25
0
10
20
30
40
50
[LiCl], mM

FIGURE 15

Effect of [KCL] on standing culture and air-grown cell photosynthesis and fluorescence yield as a percentage of the control. The extent of Chl *a* fluorescence quenching (■,■) and the rate of photosynthetic O_2 evolution (o,o) by the standing culture (■,o) and air-grown (■,o) cells were obtained following the addition of 20 μM DIC. The experimental conditions are similar to those for figure 14.


100
75
% CONTROL
0
10
20
30
40
50
[KCl], mM

standing culture cell photosynthesis and fluorescence quenching. However, this phenomena is distinct from the effect of LiCl on Na^+ - dependent HCO_3^- transport, since the same effect could be observed at high [KCl] (Fig. 15). Consequently, the LiCl effect above 15 - 20 mM was not specific whereas concentrations below 15 mM were specific for HCO_3^- transport. The differential inhibition of [KCl] (Fig. 15) and [LiCl] (Fig. 14) on Na^+ - stimulated photosynthesis and fluorescence quenching by the air - grown cells further suggests that LiCl inhibition was specific for Na^+ - dependent HCO_3^- transport. The $K_I(Li^+)$ for Na^+ stimulated photosynthesis was 1.9 $\pm$ 1.4 mM at 25 µM DIC (Espie et al. 1988b). Studies with KCl indicated that it was the cation Li^+ and not Cl^- that was responsible for the observed inhibition (Fig. 14 & 15; Espie & Canvin 1987; Espie et al. 1988b).

Increasing the extracellular [Li^+] inhibited Chl a fluorescence quenching to a larger degree than photosynthesis in standing culture cells. The observed inhibition of fluorescence quenching was not due to a drastic reduction in the internal inorganic carbon pool size. Studies carried out by other workers (Miller & Colman 1980a; Kaplan et al. 1980; Badger and Andrew 1982) have shown that the rate of photosynthesis is dependent on the intracellular DIC concentration (Fig. 8). Thus, the internal DIC pool size should have been inhibited to the same extent as that of photosynthesis. Direct measurement of intracellular pool

showed the same degree of inhibition (Table 1) as photosynthesis which further supports the above conclusion. The higher degree of inhibition on fluorescence quenching could be due to a direct effect of Li^+ on fluorescence yield.

REVERSIBILITY OF Li^+ INHIBITION

Inhibition of Na^+ - dependent HCO_3^- transport in air - grown cells by low [Li^+] could be reversed by increasing the extracellular Na^+ concentration (Fig. 13). This observation is consistent with previous studies (Espie et al. 1988b). However, in the presence of high [Li^+] (20 mM) the inhibition was not completely reversible by increasing the extracellular [Na^+]. This may be because Na^+ like other cations could cause an osmotic effect on the cells at high concentrations. Maximum fluorescence quenching and high rates of photosynthesis were obtained in the presence of Li^+ if CA was added to the cell suspension. Carbonic anhydrase catalyses the dehydration of HCO_3^- to CO_2. This relieves the rate limitation imposed on the CO_2 transport system at low [DIC] by the slow dehydration of HCO_3^-. Thus high rates of CO_2 uptake could occur in the presence of CA. Lithium inhibition could also be overcome by high [DIC]. This effect was presumably due to the greater supply of CO_2. This shows that Li^+ blocked the cells ability to form a pool through Na^+ - dependent HCO_3^- transport but had little effect on the cell metabolism. The effects of CA on photosynthesis and fluorescence yield in the presence of Li^+

are consistent with the presence of a second pathway for DIC uptake, seperate from Na^+ - dependent HCO_3^- uptake.

EFFECT OF LITHIUM ON PHOTOSYNTHETIC $K_{0.5}$ (DIC)

In the presence of Na^+ (3 mM) the rate of photosynthesis by air - grown cells at low [DIC] was much greater than the theoretical rate of photosynthesis that could be supported solely by CO_2 transport (Fig. 16). This indicated Na^+ - dependent HCO_3^- uptake from the medium. But, in the presence of 20 mM Li^+ the observed rate of photosynthesis paralleled the rate of CO_2 production in the medium with increasing [DIC]. The apparent photosynthetic affinity for extracellular DIC, which reflects the ability of the cells to transport and accumulate DIC within the cell (Kaplan et al. 1980) was decreased by lithium from 55 µM to greater than 200 µM DIC and only at very high [DIC] (10 - 20 mM) did photosynthesis approach the Vmax obtained in the presence of Na^+ alone (data not shown).

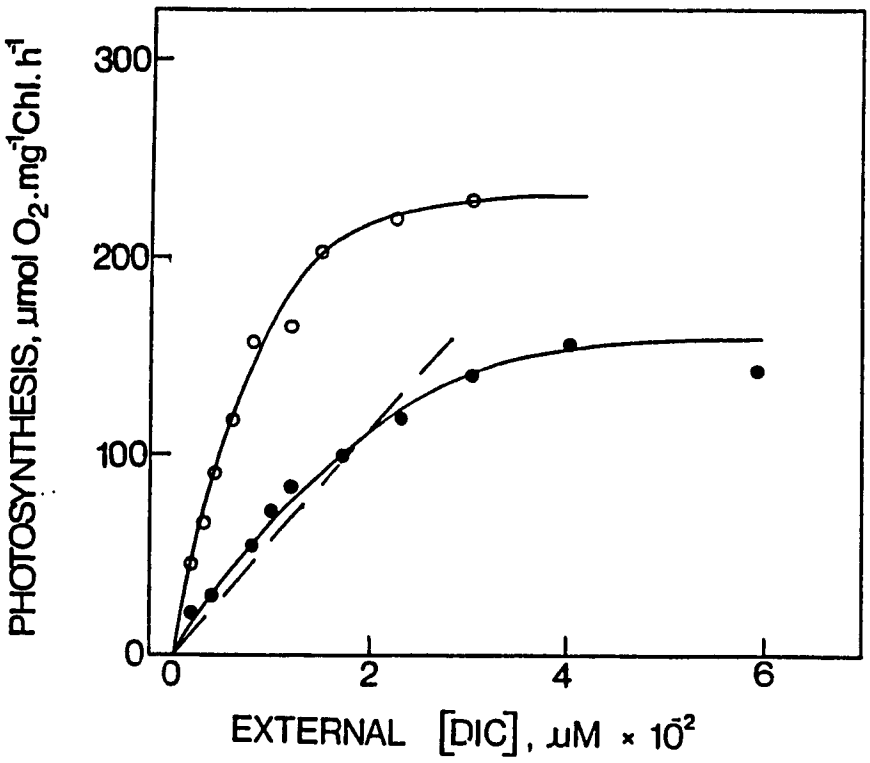
In contrast to air - grown cells, standing culture cells photosynthesis occurred at a rapid rate at low DIC concentration in the absence of Na^+. The presence of Li^+ (20 mM) did not have any effect on photosynthesis with increasing [DIC] (Fig. 17). The $K_{0.5}$ (DIC) determined by the double reciprocal plot method in the absence and presence of Li^+ was in the range of 2.5 - 8 µM DIC.

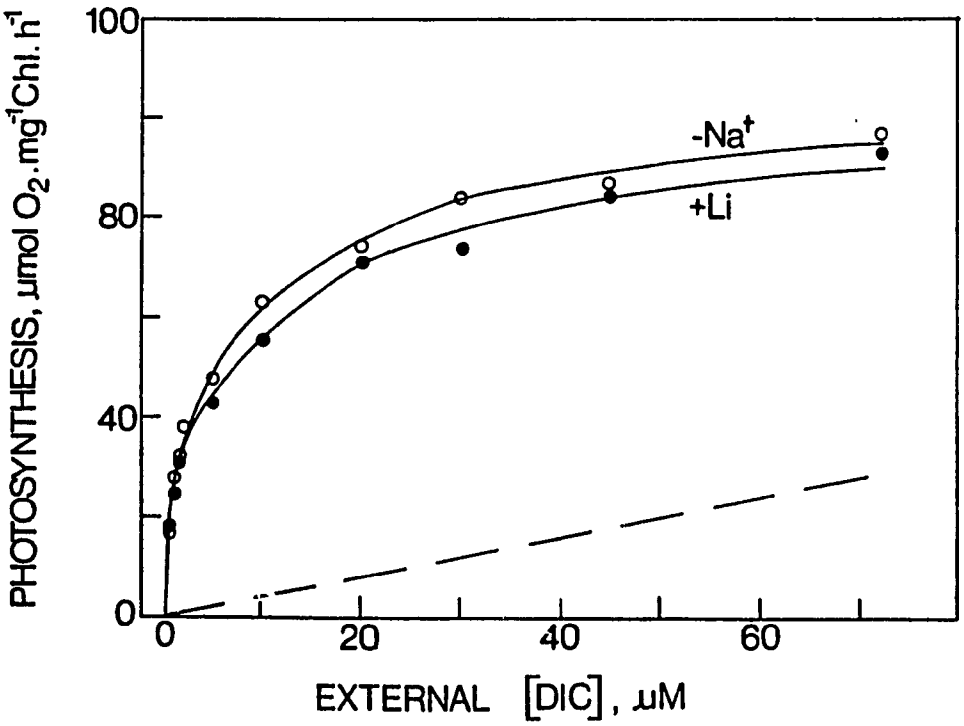
FIGURE 16

DIC response curve for air-grown cell photosynthesis ± 20 mM Li^+. Rate of photosynthetic O_2 evolution as a function of extracellular [DIC] was measured in the presence of 3 mM Na^+ and in the absence (o) and presence (o) of 20 mM LiCl. The experiment was conducted at 30°C and pH 8. The broken line (---) indicates the maximum rate of photosynthesis that could be sustained by the CO_2 supply rate at various [DIC]. The [Chl *a*] was in the range of 4 - 4.5 $\mu g.mL^{-1}$.

FIGURE 17

DIC response curve for standing culture cell photosynthesis ± 20 mM Li^+. Rate of photosynthetic O_2 evolution with respect to the total extracellular [DIC] in the medium was measured in the absence (o) and presence (o) of 20 mM LiCl. The experiment was conducted under the similar conditions as for figure 14 but in the absence of any added Na^+.


PHOTOSYNTHESIS, μmol O_2.mg^{-1}Chl.h^{-1}
300
200
100
0
0
2
4
6
EXTERNAL [DIC], μM × 10^2


PHOTOSYNTHESIS, μmol O_2.mg^{-1}Chl.h^{-1}
100
80
40
0
0
20
40
60
EXTERNAL [DIC], μM
-Na^+
+Li

EFFECT OF LITHIUM ON DIC ACCUMULATION

Measurement of Chl *a* fluorescence yield and photosynthesis at alkaline pH gives an indirect measure of the cells ability to transport and accumulate HCO_3^-. Therefore, silicone fluid centrifugation experiments were performed to directly measure internal DIC accumulation in the presence and absence of Li^+. The results obtained (Table 1) were consistent with those obtained from photosynthesis and fluorescence measurements. Lithium at a concentration of 5 mM drastically reduced (80 %) Na^+ - dependent HCO_3^- accumulation. This inhibition was reversed by increasing the extracellular [Na^+] (Table 1) In standing culture cells, lithium at a concentration of 20 mM only inhibited DIC accumulation by 11%. A similar degree of inhibition of standing culture cell DIC accumulation was also observed with 20 mM KCl (Table 1). These results parallel previous observation of fluorescence quenching and photosynthesis measurenment (Fig. 14). The initial rate of DIC uptake (10 s) by standing culture cells $\pm$ 20 mM Li^+ was similar. Thus, it is clear from the collective results that Li^+ specifically inhibits Na^+ - dependent HCO_3^- transport, but has little effect on Na^+ - independent HCO_3^- transport and accumulation.

TABLE 1

Effect of Li^+ on intracellular DIC accumulation

ADDITIONS	DIC ACCUMULATION (%)
Air grown cells	
a) - NaCl	100
b) +3 mM NaCl	314
c) b + 5 mM Li^+	141
d) c + 30 mM Na^+	388
e) b + 20 mM Li^+	82
f) e + 50 mM Na^+	85
Standing culture cells	
a) - NaCl	100
b) a + 20 mM Li^+	89
c) b + 30 mM Na^+	88
d) a + 20 mM K^+	91
e) a + 3 mM Na^+	119
f) e + 20 mM Li^+	103

35 µM $DI^{14}C$ was supplied to the cells (9 -15 µg $Chl.mL^{-1}$) and the amount of intracellular DIC accumulated was determined by the silicone fluid centrifugation technique following 60 s incubation. The experiment was conducted at pH 8 and 30°C. Light for the cells was provided at 350 µmol $photon.m^{-2}.s^{-1}$.

EFFECT OF MONENSIN ON HCO_3^- TRANSPORT BY THE STANDING CULTURE CELLS

Monensin is a carboxylic polyether antibiotic which collapses the Na^+ gradient between the cells and the medium by forming stable complexes with Na^+ and transporting it across biological membrane (Pressman 1976). When the sodium gradient is collapsed by monensin the membrane potential is not effected (Sandeaux et al. 1978) because it catalyses the electro neutral exchange of Na^+ for H^+ (Sandeaux et al. 1982). We examined the effect of monensin upon standing culture cells photosynthesis and fluorescence yield. At 20 μM DIC, a rapid quenching of Chl a fluorescence was observed in standing culture cells in the absence of Na^+ (Fig. 18). Simultaneous measurements of photosynthesis revealed that the rate was several fold greater than the HCO_3^- dehydration rate. These results indicate HCO_3^- uptake from the medium. Addition of monensin at a concentration as high as 400 μM did not reduce the observed level of photosynthesis nor significantly alter the pattern of fluorescence quenching (Fig. 18). Investigations of the effect of monensin on photosynthesis with increasing [DIC] did not show any inhibition (Fig. 19). The maximum rate of photosynthesis in the absence or presence of monensin was in the range of 85 - 100 μmol $O_2.mg^{-1}Chl.h^{-1}$ and the $K_{0.5}$ (DIC) determined by the double reciprocal plot was in the range of 1.5 - 7 μM. The results obtained by monitoring quenching of Chl a and photosynthesis strongly suggest that a

FIGURE 18

Effect of monensin on Na^+ - independent photosynthesis and fluorescence yield by the standing culture cells. Time course tracings of Chl *a* fluorescence yield and photosynthetic O_2 evolution were obtained in the absence (a) and presence of 200 (b) or 400 (c) μM monensin. The reaction was started by the addition of 20 μM $KHCO_3$ (↓) to cells (5 $\mu g.mL^{-1}$) at the CO_2 compensation point. The experiment was conducted in the absence of any added Na^+ at pH 8.02 and 30°C. The broken line (---), indicates the maximum rate of CO_2 production in the medium. The results of several runs have been overlaid to facilitate comparison.

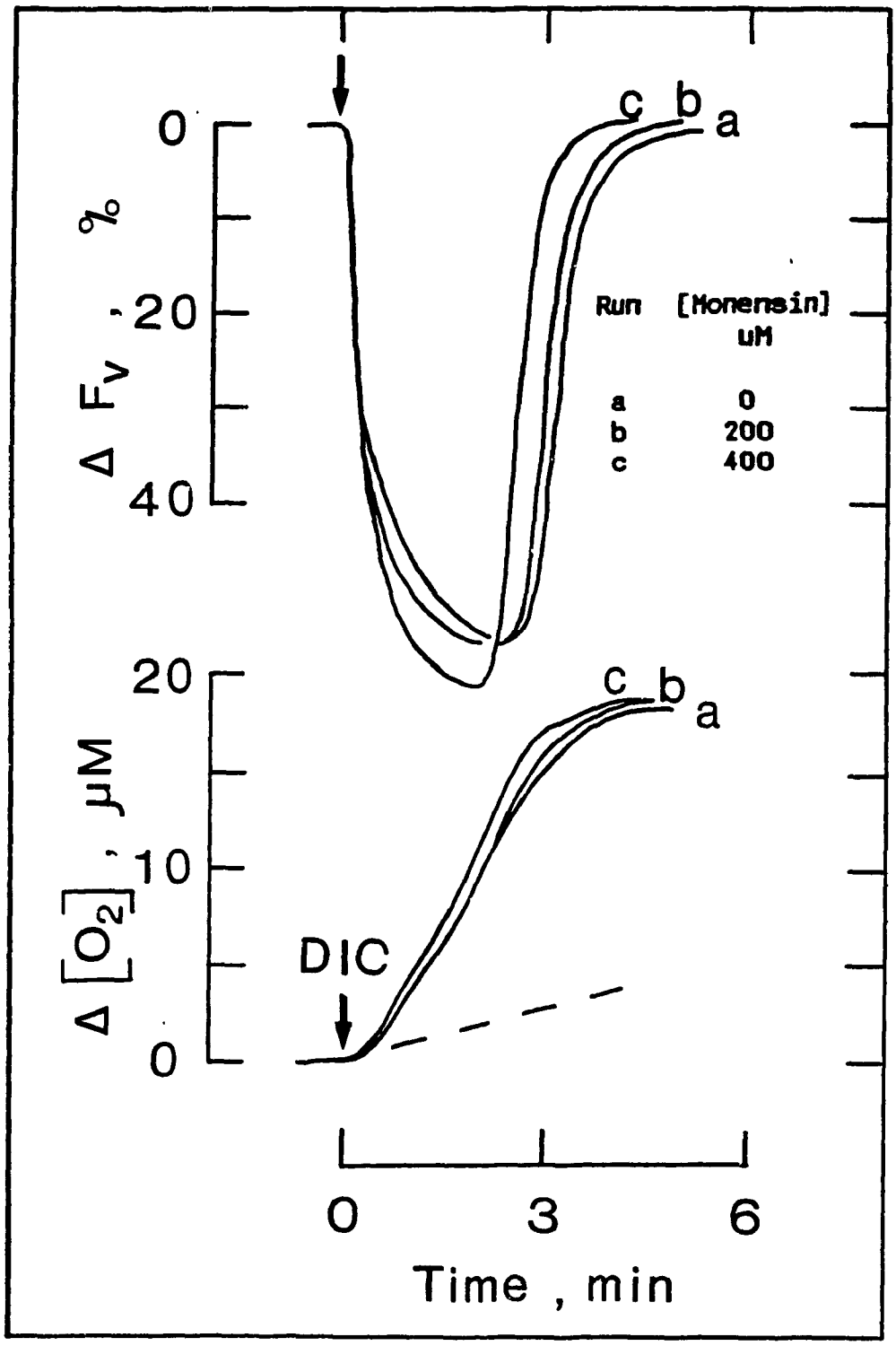
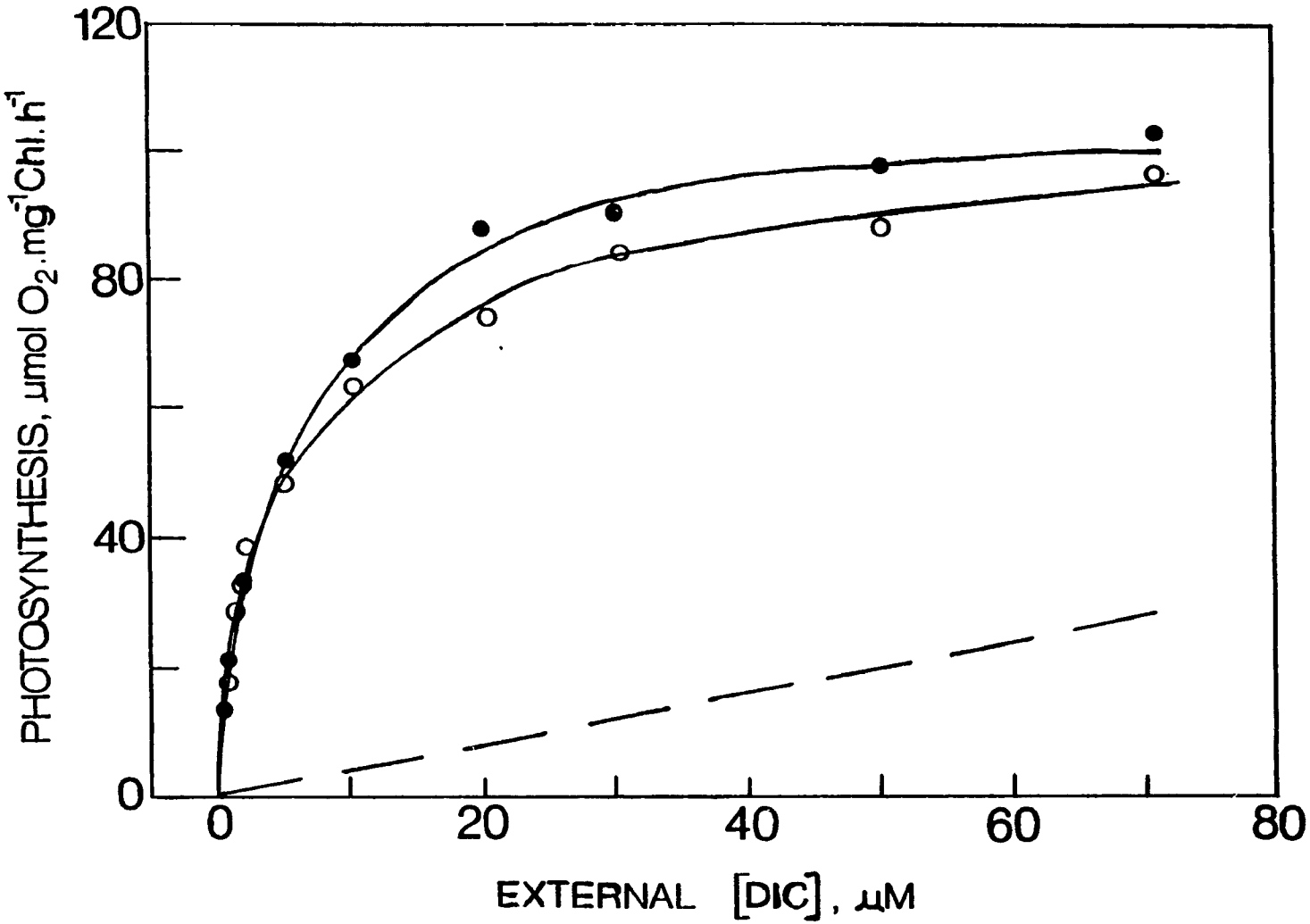

0
20
40
Δ Fv , %
c b a
Run [Monensin] uM
a 0
b 200
c 400
20
10
0
Δ [O2] , µM
c b a
DIC
0
3
6
Time , min

FIGURE 19

DIC response curve for standing culture cell photosynthesis in the presence and absence of monensin. Rate of photosynthesis as a function of extracellular [DIC] was measured in the presence (o) and absence (o) of 50 μM monensin. The experimental conditions were similar to those for figure 18.


120
80
40
0
0
20
40
60
80
PHOTOSYNTHESIS, μmol O_2.mg^{-1}Chl.h^{-1}
EXTERNAL [DIC], μM

Na^+ gradient is not required for HCO_3^- transport in standing culture cells (gradient $[Na^+]o > [Na^+]i$). This was further confirmed by silicone fluid centrifugation experiments. Neither the time course of intracellular DIC accumulation nor the rate of carbon fixation was affected by the presence of monensin (data not shown). These results firmly confirm that monensin has no effect on HCO_3^- transport in standing culture cells. Even if the HCO_3^- transport system in the standing culture cells is a high affinity transporter dependent on the contaminant Na^+ (30 - 40 µM) these results eliminate the possibility that a Na^+ - gradient plays a role in transport.

The effect of monensin on photosynthesis and fluorescence quenching of standing culture cells in the presence of 25 mM Na^+ was also investigated (Fig. 20 & 21). Studies conducted with Streptococcus faecalis indicated that addition of monensin at an extracellular $[Na^+]$ of 25 mM resulted in the increase of intracellular Na^+ concentration from 1.5 mM to 26.5 mM (Delort et al. 1989). In Synechococcus addition of 20 µM DIC at 25 mM Na^+ and pH 8 stimulated the rate of photosynthesis by 1.5 -fold (Fig 20 tracing E & F) as observed previously (Fig. 4). In the presence of 50 µM monensin fluorescence quenching was reduced by 50% (Fig. 20 tracing C) and photosynthesis by 60 % (Fig. 20 tracing G). However, this inhibition did not persist after about 14 - 15 minutes incubation with monensin. The pattern of fluorescence quenching and the measured rate of photosynthesis after 15 min

FIGURE 20

Effect of monensin on standing culture cell photosynthesis (lower panels) and fluorescence quenching (upper panels) in the presence of 25 mM Na^+. Shown in a sequence are tracings obtained in the absence of Na^+ (A,E), presence of 25 mM Na^+ (B,F) and in the presence of Na^+ & 50 µM monensin (C,D,G,H). C & G represents immediate effect of monensin and tracings G & H were obtained following 15 min incubation of the cells with monensin. The rate of photosynthesis in µmol $O_2.mg^{-1}Chl.h^{-1}$ is given beside the O_2 evolution tracings. Fluorescence quenching and photosynthesis was initiated by the addition (↓) of 20 µM DIC at pH 8 and 30°C.

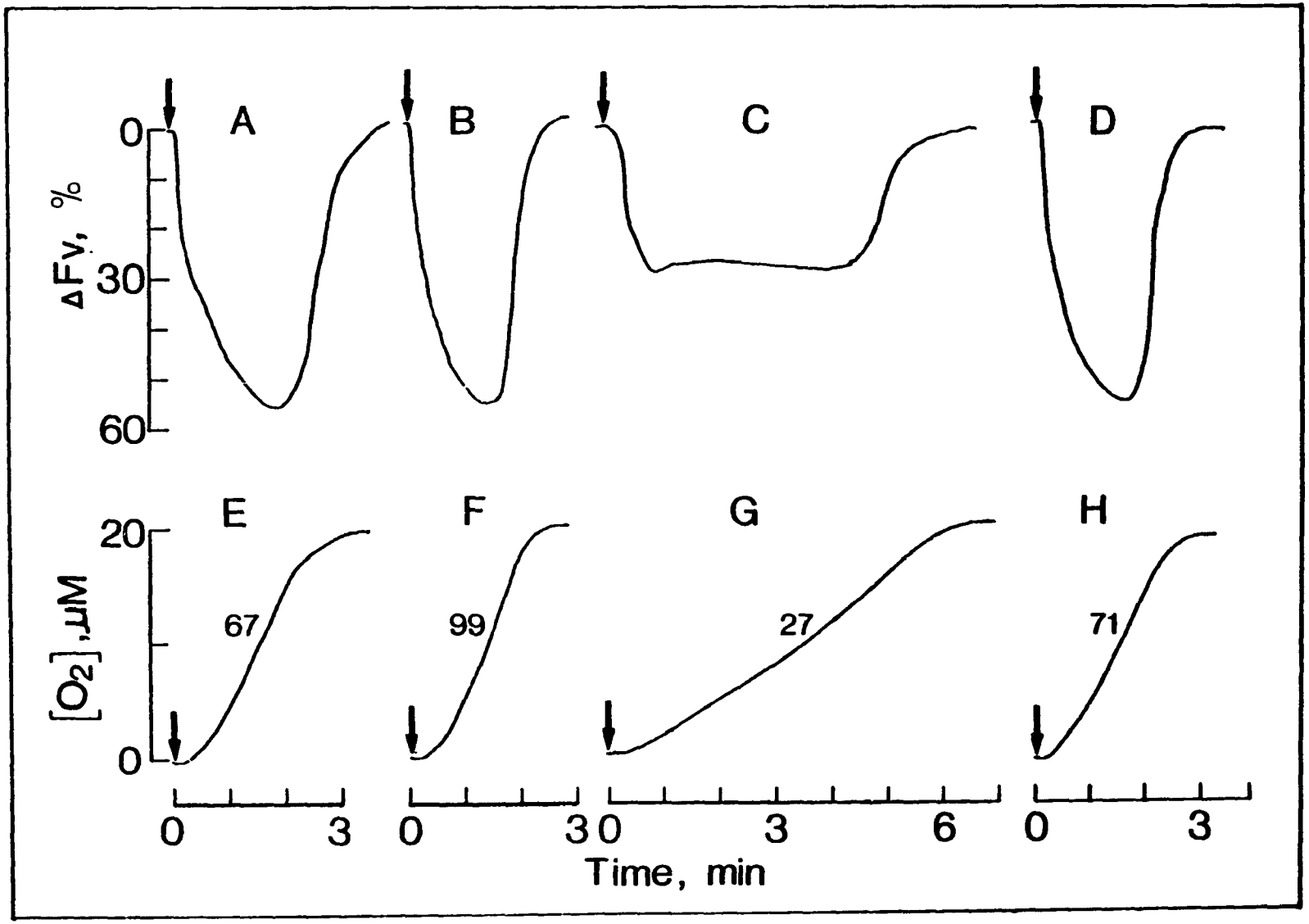
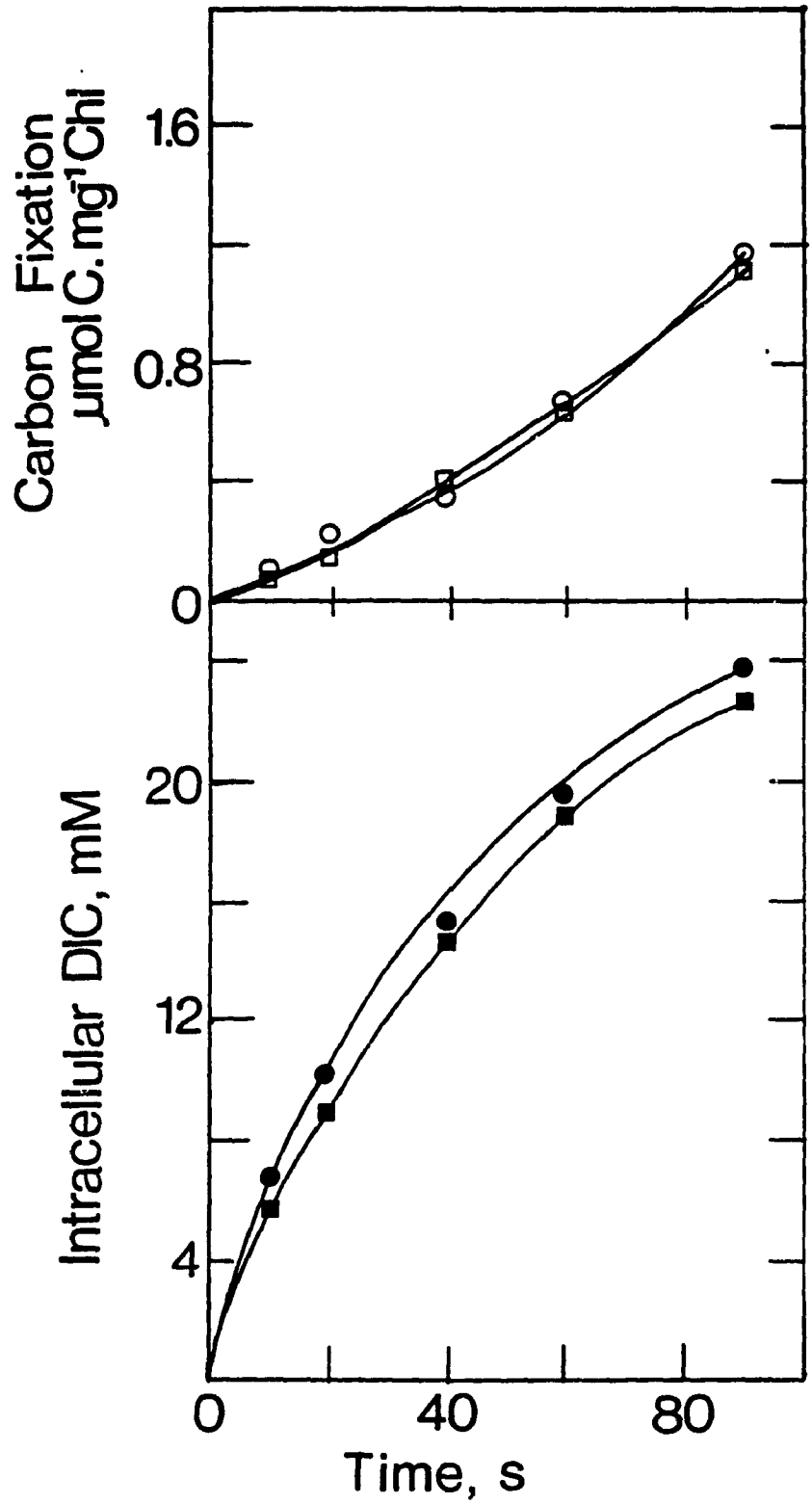

A
B
C
D
0
30
60
ΔFv, %
E
F
G
H
20
0
[O2], μM
67
99
27
71
0
3
0
3
0
3
6
0
3
Time, min

FIGURE 21

Effect of monensin on intracellular DIC accumulation by the standing culture cells in the presence of Na^+. Silicone fluid time course measurement of inorganic carbon accumulation (lower panel) & C fixation (upper panel) was obtained in the absence (o,o) and presence (■,■) of 50 µM monensin. The cells were incubated with monensin for 15 min before intracellular DIC measurements were made. These experiments were performed in the presence of 25 mM Na^+ at pH 8 and 28 - 30°C. The initial inorganic carbon conc. was 37 µM and the [Chl] was 6.5 - 7 $\mu g.mL^{-1}$. The specific activity of ^{14}C in the tube was 40.8 $\mu Ci.\mu mol^{-1}$.


Carbon Fixation
μmol C. mg⁻¹ Chl
1.6
0.8
0
Intracellular DIC, mM
20
12
4
0
40
80
Time, s

incubation (Fig. 20) indicates a lack of inhibition of either HCO_3^- transport or CO_2 fixation. Thus monensin results only in a transient inhibition which was reversed by the naturally occuring metabolic processes of the cell.

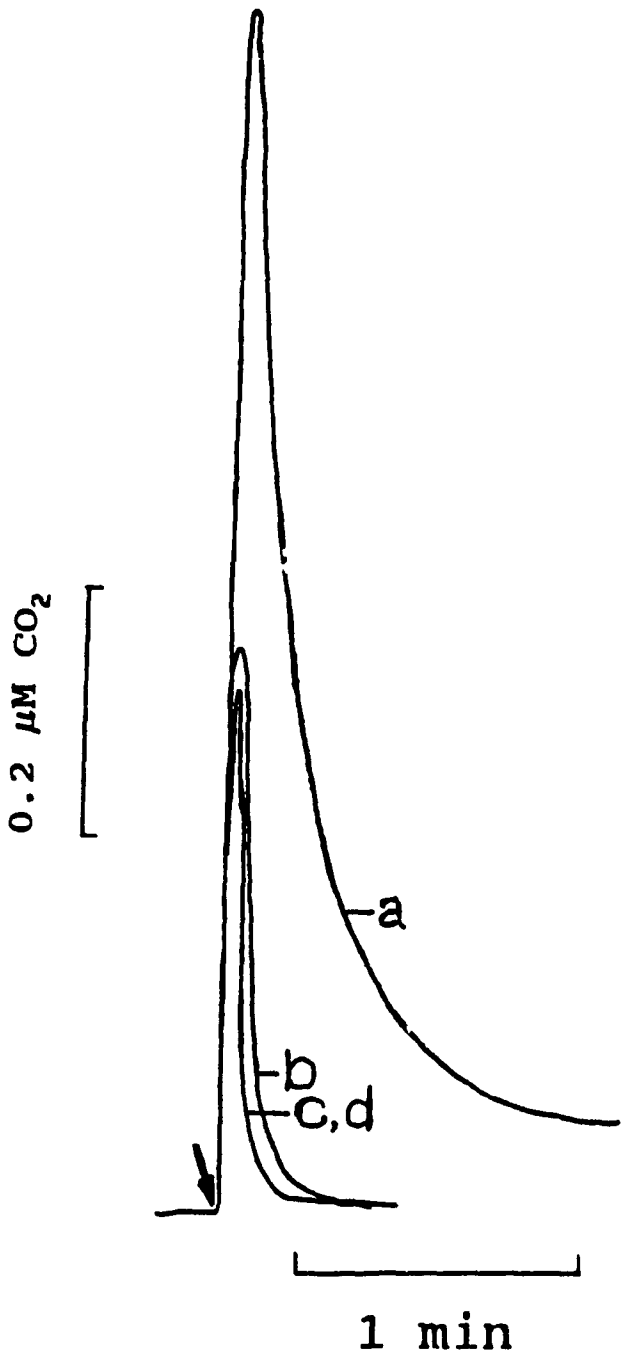
The effect of monensin on standing culture cells in the presence of Na^+ was further investigated using the silicone fluid centrifugation technique (Fig. 21). Time course measurements indicated the formation of a large intracellular DIC pool and a high rate of C fixation in the presence of 25 mM Na^+. Only 1/8 of the observed rate of C fixation could be accounted for solely by CO_2 uptake. DIC accumulation and C fixation in the presence of monensin and 25 mM Na^+ paralleled that observed in the presence of Na^+ alone indicating no effect on HCO_3^- transport.

EFFECT OF MONENSIN ON CO_2 TRANSPORT IN THE STANDING CULTURE CELLS

The effect of monensin on CO_2 transport by standing culture cells was also investigated. CO_2 transport activity was monitored using mass spectrometry to follow CO_2 disappearance from the medium. The transport assay was initiated by adding "pure" CO_2 to the mass spectrometer reaction cuvette which contained cells and BTP buffer (pH 8). The CO_2 for these pulsing experiments was prepared as described in materials and method. Following CO_2 addition to a darkened cell suspension, the CO_2 concentration initially

FIGURE 22

Effect of monensin on CO_2 transport by the standing culture cells. The ability of the cells to transport CO_2 was tested by pulsing small aliquots of acidified H_2O saturated at 0°C with 5% CO_2 to the cells (8 µg Chl.mL^{-1}) in the absence of Na^+ (b), presence of 25 mM Na^+ (c) and in the presence of Na^+ & 50 µM monensin (d). The control (a), obtained by pulsing CO_2 to darkened cells indicates the uncatalysed time course of CO_2 disappearance (conversion to HCO_3^-). Tracing d was obtained following 15 min incubation of the cells with monensin. The experiment was conducted in 25 mM BTP/HCl (pH 8) buffer at 30°C and light was provided at 200 µmol photon.m^{-2}.s^{-1}.


0.2 μM CO_2
a
b
c,d
1 min

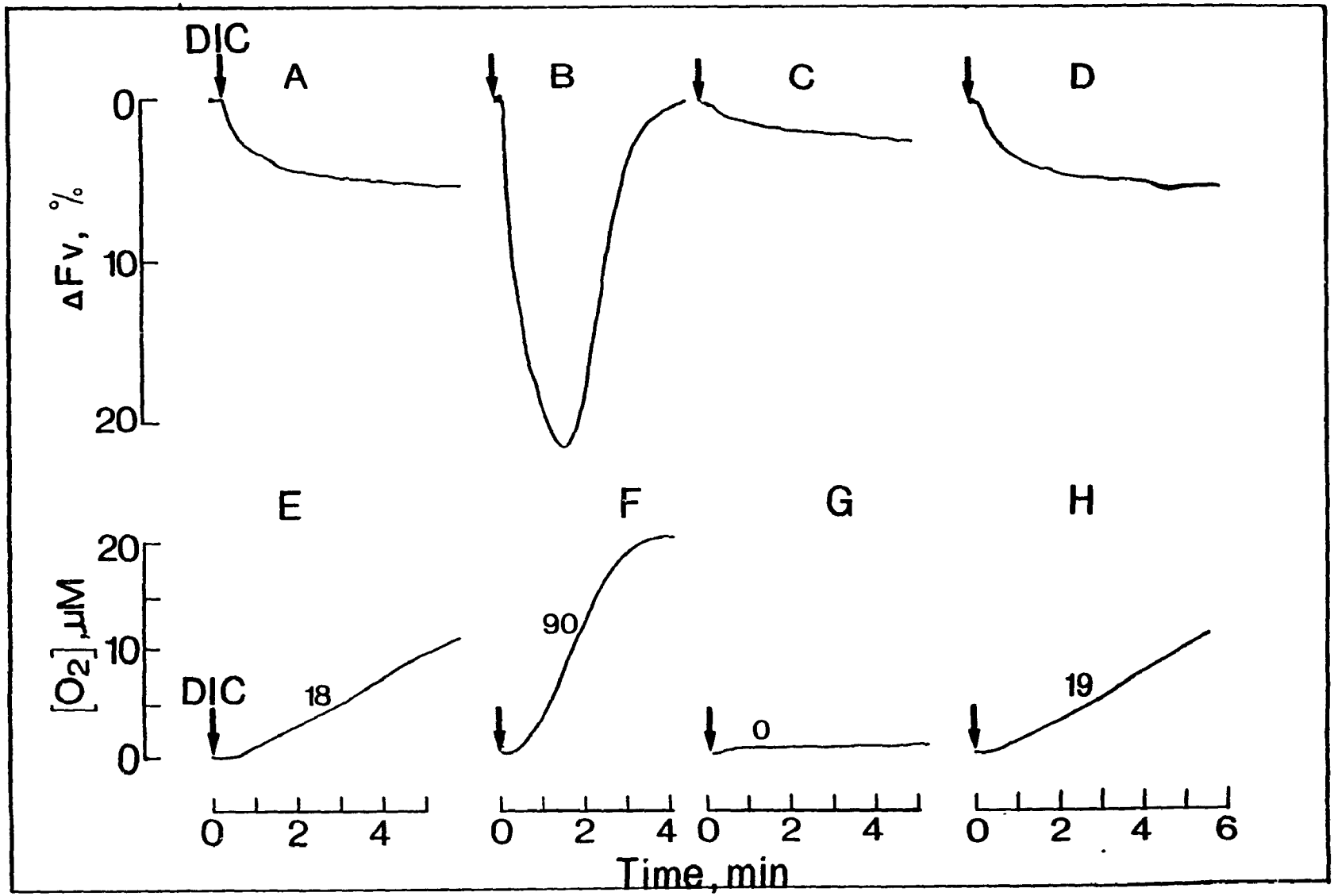
rose to a high level and then gradually declined to a level near the starting level (Fig. 22). The decline in CO_2 concentration in this case was due to it's non-enzymatic conversion to HCO_3^- during the period in which chemical equilibrium between CO_2 and HCO_3^- was being established. A similar time course was observed when CO_2 was added to buffer alone (not shown). Since CO_2 and HCO_3^- transport are light dependent, this curve served as a null control. When the same amount of "pure" CO_2 was added to illuminated cells, a much lower $[CO_2]$ appeared in the medium compared to the control and the $[CO_2]$ rapidly declined to near zero, indicating the presence of a light dependent CO_2 uptake mechanism. The presence of 25 mM Na^+ slightly stimulated CO_2 uptake (Fig. 22). The results obtained following incubation of the cells for 15 min with monensin were similar to that observed in the absence (Fig. 22 tracings c & d). This shows that CO_2 uptake is not dependent upon a Na^+ gradient.

EFFECT OF MONENSIN ON Na^+ - DEPENDENT HCO_3^- TRANSPORT

Figure 23 illustrates the effect of monensin upon Na^+ - dependent fluorescence quenching and photosynthesis by air - grown cells. As observed previously (Fig. 3) very little Chl a fluorescence quenching or photosynthesis was observed in the absence of Na^+ (Fig. 23, tracing A & E) when 20 µM DIC was added to the cell suspension (pH 8). This indicated the lack of significant HCO_3^- transport. Addition of Na^+ greatly

FIGURE 23

Monensin inhibition of Na^+ - stimulated photosynthesis (lower panels) and fluorescence quenching (upper panel) in air- grown cells. Shown in a consecutive time sequence are tracings obtained in the absence of Na^+ (A,E), presence of 25 mM Na^+ (B,F) and in the presence of Na^+ and 50 µM monensin (C,D,G,H). C & G show the immediate effect of monensin and tracings G & H were obtained following 15 min exposure to monensin. Fluorescence quenching and photosynthesis were initiated by the addition (↓) of 20 µM DIC at pH 8 and 30°C.


DIC
A
B
C
D
0
10
20
ΔFv, %
E
F
G
H
20
10
0
[O2] μM
DIC
18
90
0
19
0 2 4
0 2 4
0 2 4
0 2 4 6
Time, min

enhanced the HCO_3^- transport as monitored by Chl a fluorescence yield and photosynthesis (Fig. 23, tracing B & F). Unlike standing culture cells, monensin completely inhibited photosynthesis and fluorescence quenching (Fig. 23, tracing C & G; Fig. 24) in the air - grown cells. This inhibition was instantaneous. However after 15 min photosynthesis and fluorescence quenching returned to the level observed before the addition of Na^+ (Fig. 23, D & H). But the Na^+ - stimulated fluorescence quenching or photosynthesis was not recovered by the incubation, indicating inhibition of Na^+ - dependent HCO_3^- uptake. At a higher pH (9.5), addition of 100 μM monensin completely inhibited the Na^+ - stimulated HCO_3^- uptake. The requirement for higher [monensin] at alkaline pH may suggest that the protonated form of the compound was the inhibitory species (pKa = 6.65). Like that observed for standing culture cells, monensin did not effect CO_2 transport or the photosynthetic carbon metabolism, since, the addition of DIC in the presence of monensin and CA resulted in substantial fluorescence quenching and optimum rate of photosynthesis (Fig. 24 & 25).

Addition of monensin to cells once the intracellular DIC pool had formed (monitored by fluorescence quenching; Fig. 25) resulted in the immediate destruction of the internal DIC pool. This suggests that a Na^+ - gradient was not only required for HCO_3^- uptake but also to maintain the intracellular pool formed through HCO_3^- transport.

FIGURE 24

Reversal of monensin inhibition in the air-grown cells by CA. Shown are overlaid tracings of Chl *a* fluorescence yield and (upper panel) and photosynthetic O_2 evolution (lower panel) obtained in the presence of (a) 25 mM Na^+, (b) 25 mM Na^+ & 50 µM monensin and (c) 25 mM Na^+, 50 µM monensin and & 25 $\mu g.mL^{-1}$ CA following the addition (↓) of 20 µM DIC. The cells (7 $\mu g\ Chl.mL^{-1}$) were incubated for 15 min with monensin before the addition of CA. The experiment was conducted at pH 8 and light for photosynthesis was provided at 100 $\mu mol\ photon.m^{-2}.s^{-1}$.

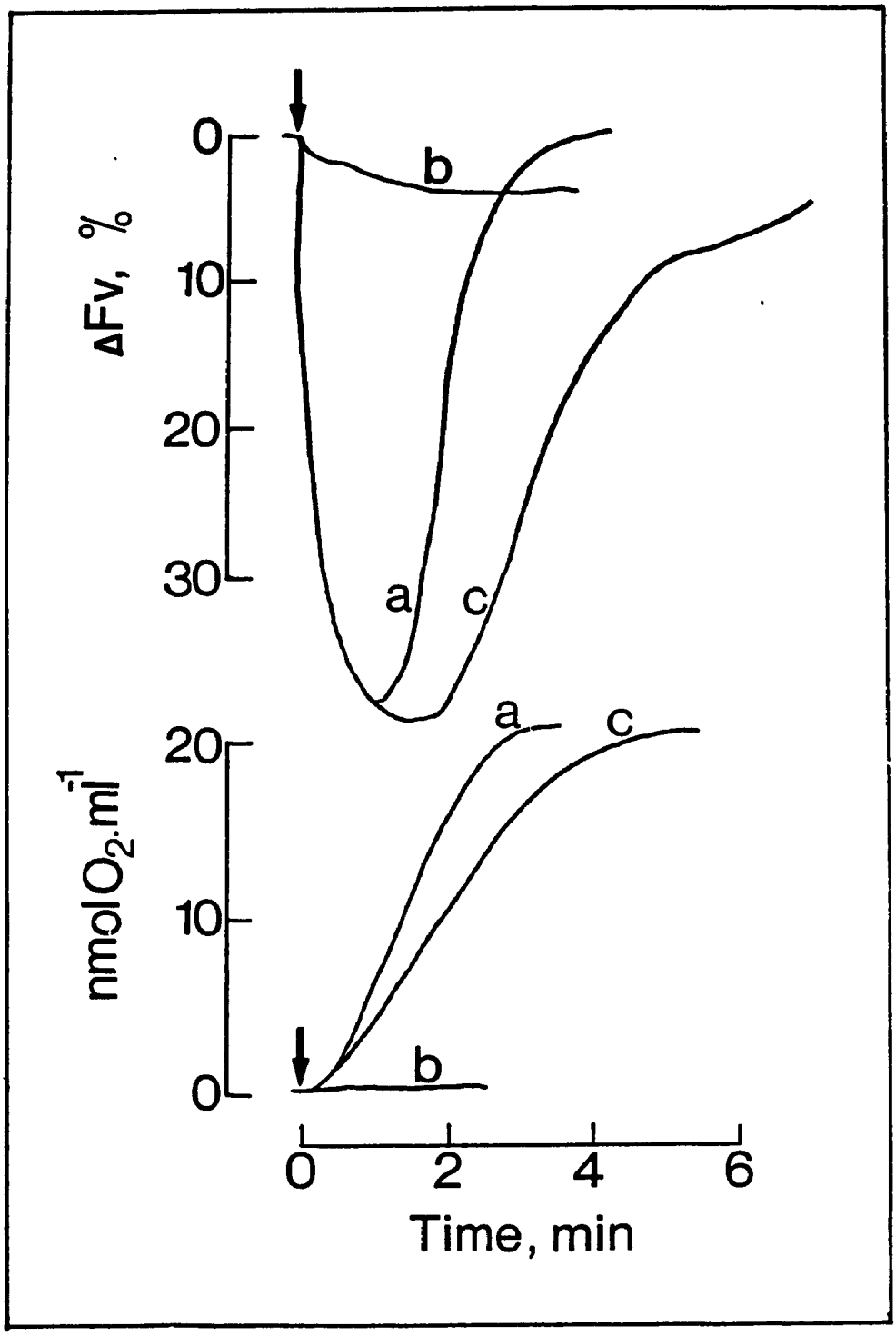

0
10
20
30
ΔFv, %
b
a
c
20
10
0
nmolO2.ml-1
a
c
b
0
2
4
6
Time, min

FIGURE 25

Effect of monensin on a preformed DIC pool in air-grown cells. Shown are the simultaneous time course measurements of Chl *a* fluorescence yield (upper panel) and photosynthetic O_2 evolution (lower panel). Tracings a, was obtained in the presence of only 25 mM Na^+. Addition of monensin at the indicated time resulted in the immediate recovery of fluorescence yield to a near maximum level and inhibition of photosynthetic O_2 evolution (b). This inhibition was partially recoverable by the addition of CA (c). The experimental condition was similar to that described in figure 24. Tracing a,b and c have been overlaid for comparison.

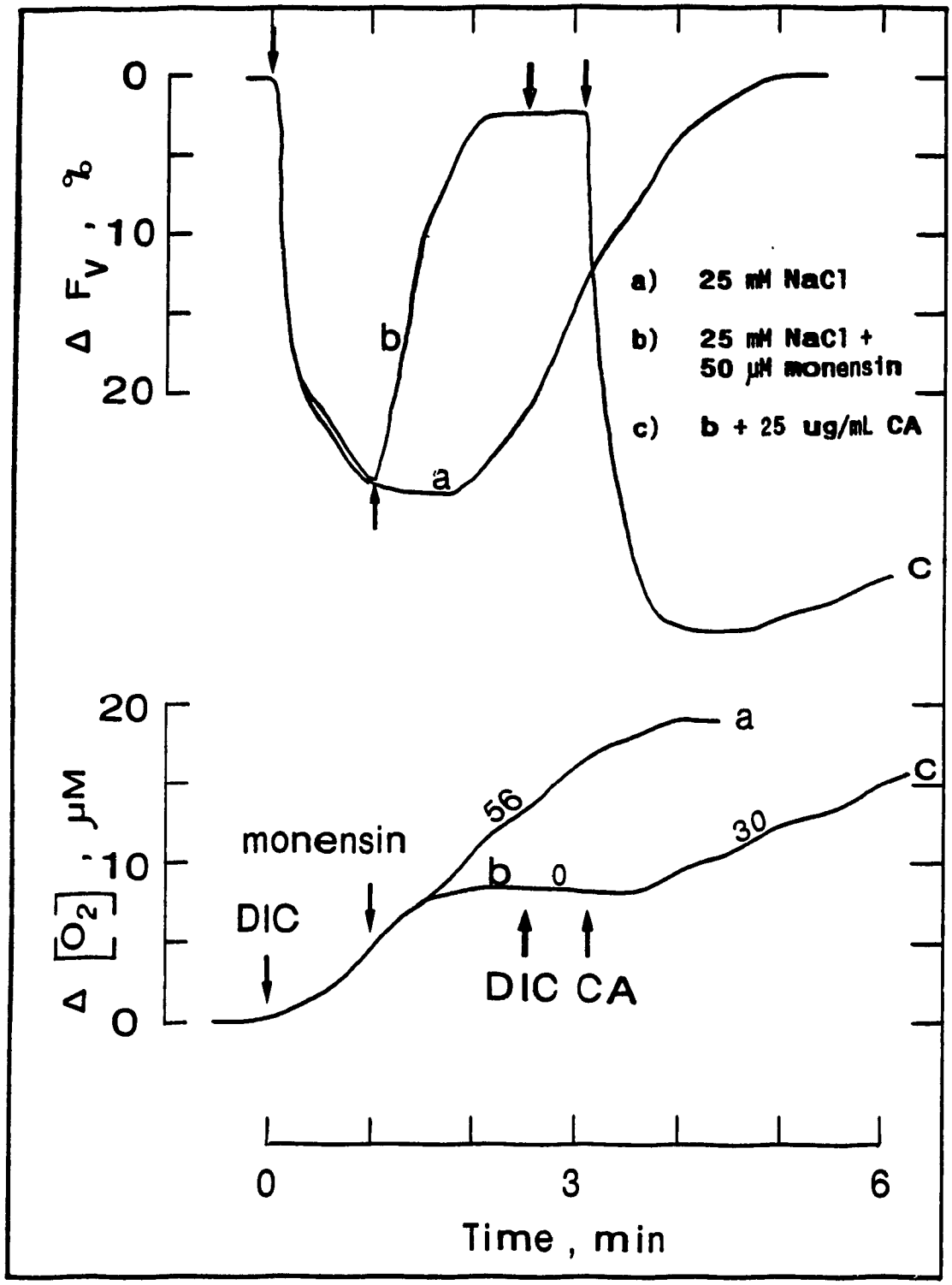
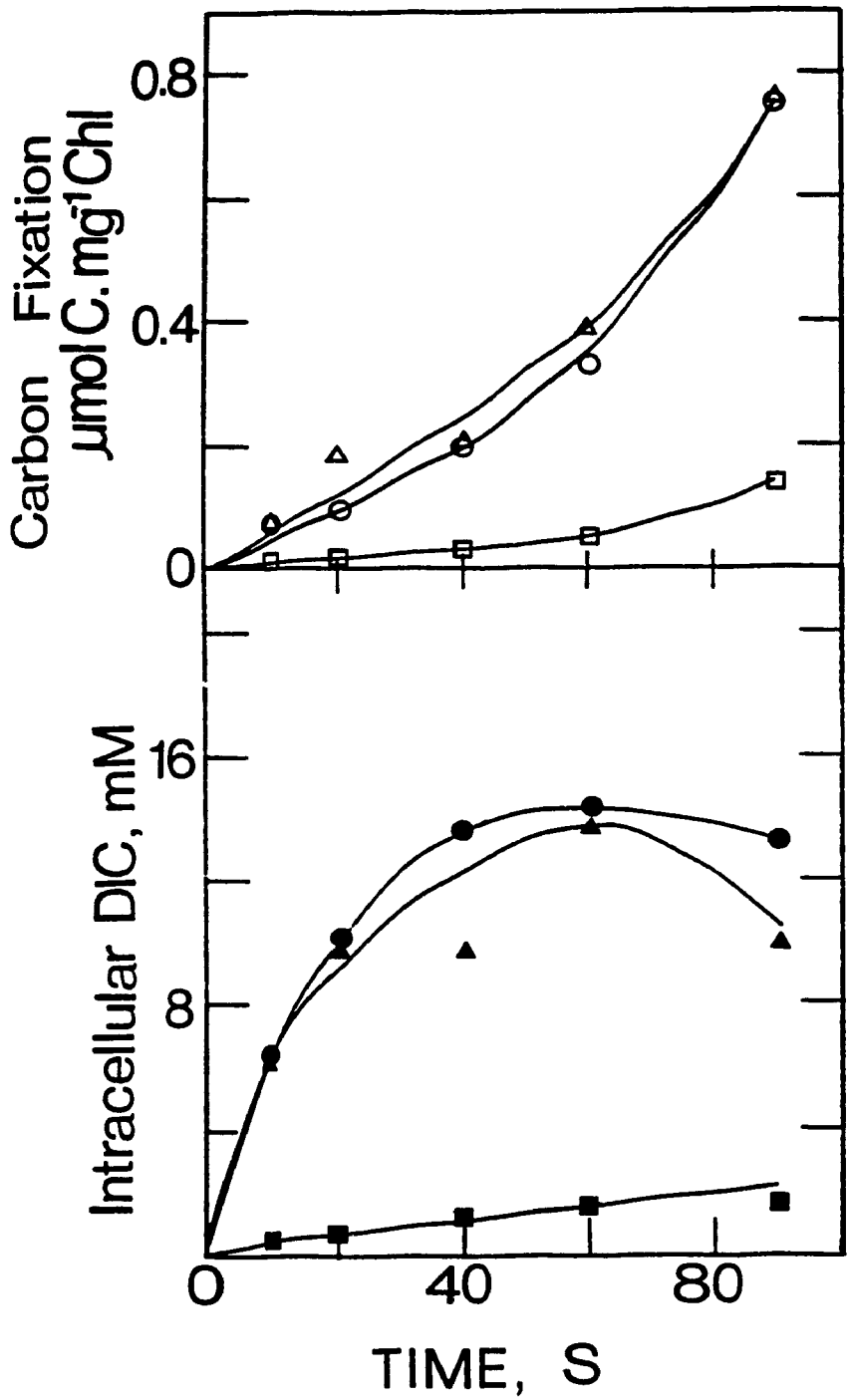

0
10
20
Δ F_v , %
a)
25 mM NaCl
b)
25 mM NaCl +
50 µM monensin
c)
b + 25 ug/mL CA
a
b
c
20
10
0
Δ [O_2] , µM
monensin
DIC
DIC CA
56
30
0
a
b
c
0
3
6
Time , min

FIGURE 26

Monensin inhibition of Na^+ - stimulated intracellular DIC accumulation in air-grown cells. Shown are time courses of intracellular DIC accumulation (o,▪,▲) and ^{14}C fixation (o,▪,▲) obtained in the presence of 25 mM Na^+ (o,o), 25 mM Na^+ & 50 µM monensin (▪,▪) and 25 mM Na^+, 50 µM monensin and 25 $\mu g.mL^{-1}$ CA (▲,▲). The initial [DIC] was 32 µM and the [Chl *a*] was in the range of 12 - 14 $\mu g.mL^{-1}$. The experiment was conducted at pH 8 and illumination for the cells was provided at 200 - 400 µmol photon.$m^{-2}.s^{-1}$.


Carbon Fixation
μmol C. mg⁻¹ Chl
0.8
0.4
0
Intracellular DIC, mM
16
8
0
40
80
TIME, S

We also examined the effect of monensin upon Na^+ - dependent HCO_3^- by direct measurement of the time course of inorganic carbon pool formation and C fixation (Fig. 26). Under our experimental conditions and at an external [DIC] of 35 - 40 µM, maximum intracellular DIC accumulation occurred within 60 - 90 seconds in the presence of 25 mM Na^+ (Fig. 7). In the presence of monensin measurement of DIC accumulation within this 90 seconds time span indicated 85% reduction in the DIC pool and a drastic reduction in the level of C fixation (Fig. 26). The inhibition of C fixation in the presence of monensin is due to a smaller intracellular pool of DIC and was not due to impaired cell metabolism since the maximum accumulation of DIC and C fixation occurred in the presence of CA (30 $\mu g.mL^{-1}$) through the CO_2 transport system (Fig. 26).

EFFECT OF AMILORIDE ON FLUORESCENCE YIELD AND PHOTOSYNTHESIS IN THE STANDING CULTURE AND AIR - GROWN CELLS

In order to further distinguish between the two modes of HCO_3^- uptake in Synechococcus UTEX 625 we examined the effect of amiloride on fluorescence yield and photosynthesis. These experiments were conducted at low [DIC] and at an alkaline pH 8 where HCO_3^- is the predominant species taken up by this organism. Amiloride is a pyrazinoyl guanidine, bearing amino groups on the third and fifth position and a chloro group on the sixth position of the pyrazine ring (Kleyman & Cargoe

1988). In it's protonated form (pKa = 8.8 in H_2O; Kleyman and Cragoe 1988), amiloride is known to inhibit Na^+/H^+ antiporters, Na^+ channels and Na^+ - cotransport (at different concentrations) in many eucaryotic systems (Kaplan et al 1989; Krulwich 1983). Figure 27 illustrates the effect of amiloride on air - grown cells. Photosynthesis was initiated by the addition of 20 μM DIC at pH 8. In the presence of 3 mM Na^+ (Fig. 27), 60 - 65% of the observed rate of photosynthesis in air - grown cells is due to Na^+ - dependent HCO_3^- uptake. Addition of amiloride drastically reduced the level of Na^+ dependent HCO_3^- uptake. Increasing the extracellular [Na^+] in the presence of amiloride reversed the effect on fluorescence quenching and photosynthesis. Previous studies have shown that CO_2 transport is not stimulated by millimolar [Na^+] (Espie et al. 1988). Thus the observed recovery of quenching and photosynthesis was through the Na^+ - dependent HCO_3^- uptake mechanism. Formation of a large intracellular DIC pool (monitored by fluorescence quenching) and the observation of maximum rate of photosynthesis at 20 μM DIC, in the presence of CA indicates lack of inhibition of CO_2 transport or cell metabolism by amiloride. Thus the observed reduction in level of photosynthesis and fluorescence quenching in the presence of amiloride (Fig. 27) was mainly due to inhibition of Na^+ - dependent HCO_3^- transport.

Figure 28 illustates the differential effect of [amiloride] on standing culture and air - grown cell

FIGURE 27

Reversal of amiloride inhibition of air-grown cell fluorescence quenching and photosynthesis. Shown are the tracings of Chl *a* fluorescence yield and photosynthetic O_2 evolution obtained (a) in the presence of 3 mM Na^+ (b) a + 500 µM amiloride (c) b + 25 mM Na+ (d) c + 25 µg/mL CA. The reaction was started by the addition (↓) of 20 µM $KHCO_3$. The rate of photosynthesis in µmol $O_2.mg^{-1}Chl.h^{-1}$ is given beside each curve. The measurements were made at 30°C (pH 8.04) and light was provided at 100 µmol photon.$m^{-2}.s^{-1}$.

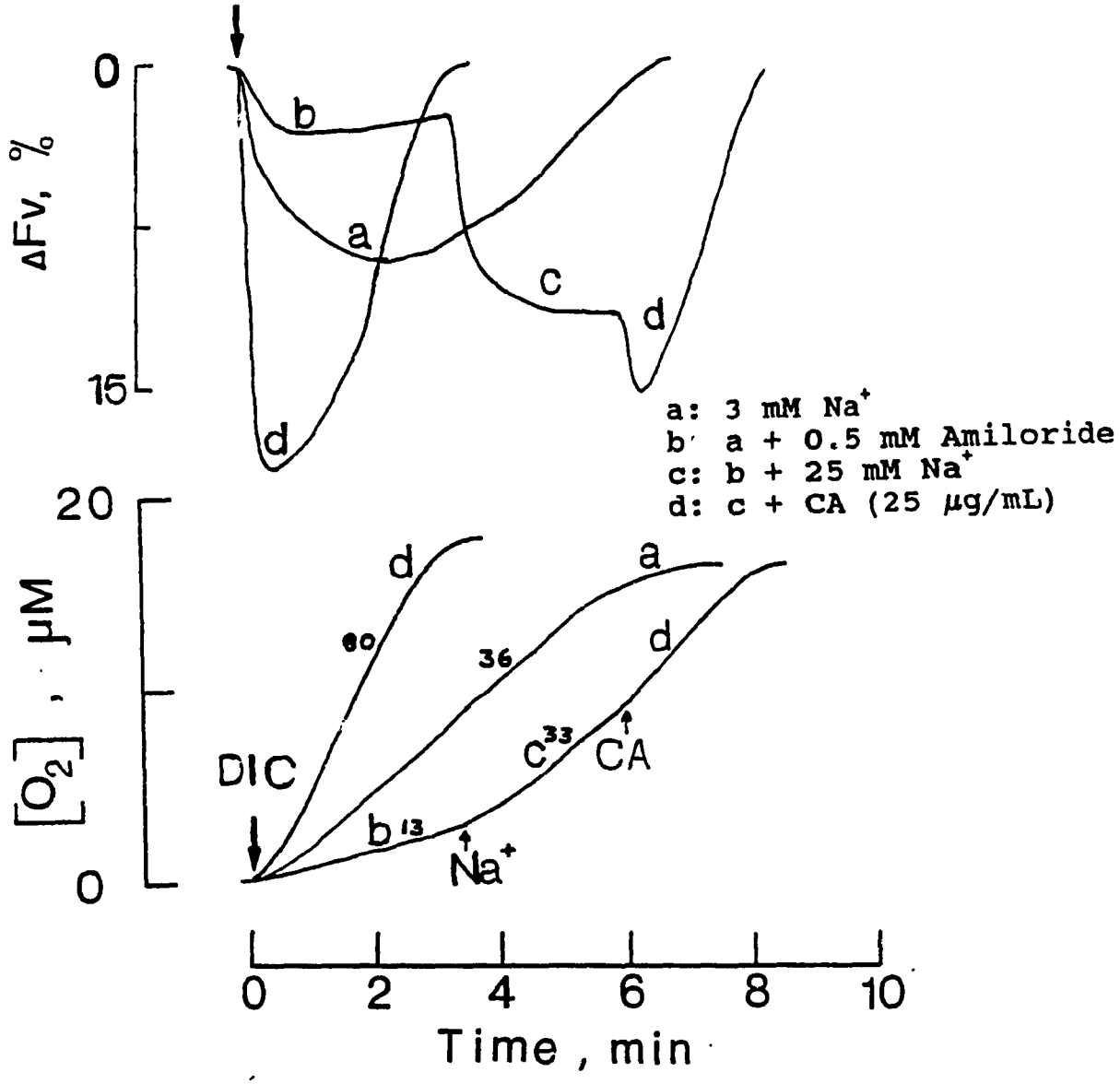
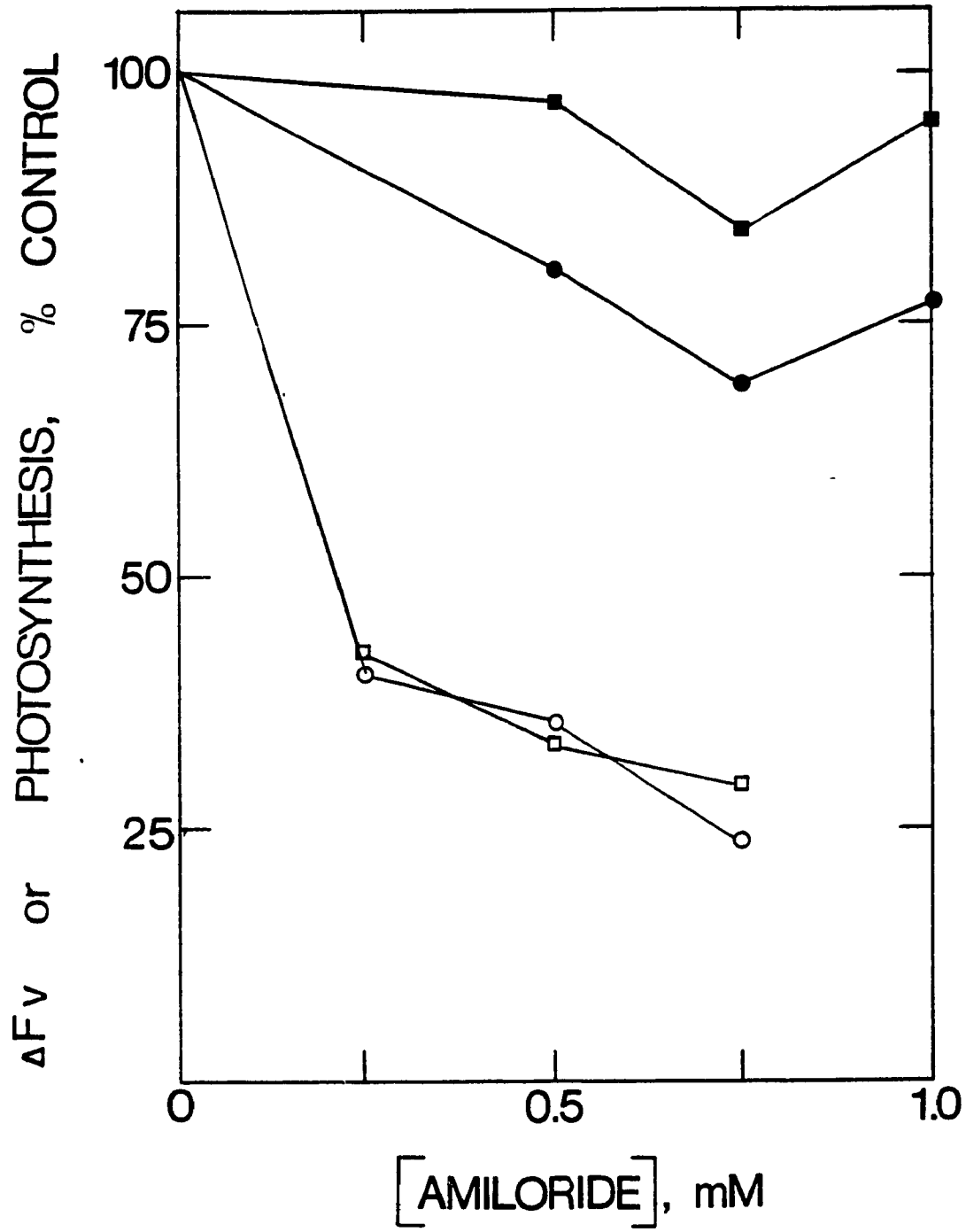

0
15
ΔFv, %
b
a
c
d
d
a: 3 mM Na+
b: a + 0.5 mM Amiloride
c: b + 25 mM Na+
d: c + CA (25 μg/mL)
20
0
[O2], μM
d
a
d
80
36
c33
CA
DIC
b13
Na+
0
2
4
6
8
10
Time, min

FIGURE 28

Effect of [amiloride] on air-grown and standing culture cell photosynthesis and Chl a fluorescence yield. The extent of Chl a fluorescence quenching (■,■) and the rate of photosynthesis (o,o) for the standing culture (o,■) and air-grown culture (■,o) cells were obtained following the addition of 20 µM DIC to cell suspension at pH 8 and 30°C. The experiment was conducted in the presence of 3 mM Na^+ for the air-grown cells (to stimulate Na^+ - dependent HCO_3^- uptake) and in the absence of any added Na^+ for the standing culture cells. The control rate of photosynthesis and fluorescence quenching was 35 µmol $O_2.mg^{-1}Chl.h^{-1}$ and 12% F_v for the air-grown cells and 98 µmol $O_2.mg^{-1}Chl.h^{-1}$ and 57% F_v for the standing culture cells.


ΔFv or PHOTOSYNTHESIS, % CONTROL
100
75
50
25
0
0.5
1.0
[AMILORIDE], mM

photosynthesis and fluorescence quenching. Amiloride at a concentration of 750 μM drastically reduced the rate of photosynthesis and the extent of fluorescence quenching by 75%. Compared to air - grown cells, standing culture cells were less sensitive to amiloride. At a similar concentration of 0.75 - 1 mM fluorescence quenching was only inhibited by 6 - 15 % and photosynthesis by 23 - 30 % (Fig. 28). Addition of Na^+ in the mM range reversed this inhibition in standing culture cells.

Effect of amiloride on the photosynthetic affinity for DIC was also investigated. Amiloride increased the photosynthetic $K_{0.5}$ (DIC) in air - grown cells (Fig. 29). The maximum rate of photosynthesis by the air - grown cells could only be achieved in the presence of amiloride by increasing the [DIC] to about 10 mM. In standing culture cells however, amiloride had no effect on the $K_{0.5}$ (DIC) (Fig. 30). Studies conducted by Kaplan et al., (1989) showed no inhibition of amiloride on the photosynthetic $K_{0.5}$ (DIC) for Synechococcus PCC7942 grown in cultures bubbled with 1 % CO_2 bubbling. This must be because these cells lack the HCO_3^- transport systems (Badger & Gallagher, 1987; Miller & Canvin, 1987).

It is important to note that all experiments performed in the presence of amiloride were short term experiments (10 - 20 minutes). Longer exposure of the cells to amiloride resulted in a higher degree of inhibition of fluorescence quenching and photosynthesis. These effects of amiloride were not reversible

FIGURE 29

DIC response curve for air-grown cell photosynthesis, $\pm$ 0.5 mM amiloride. Rate of photosynthetic O_2 evolution as a function of extracellular [DIC] was measured in the presence of 3 mM Na^+ and in the absence (o) & presence (o) of 0.5 mM amiloride. The experiment was conducted at 30°C and pH 8. The [Chl *a*] was in the range of 4.5 - 6 $\mu g.mL^{-1}$. The solid line indicates the maximum rate of photosynthesis that could be sustained by CO_2.

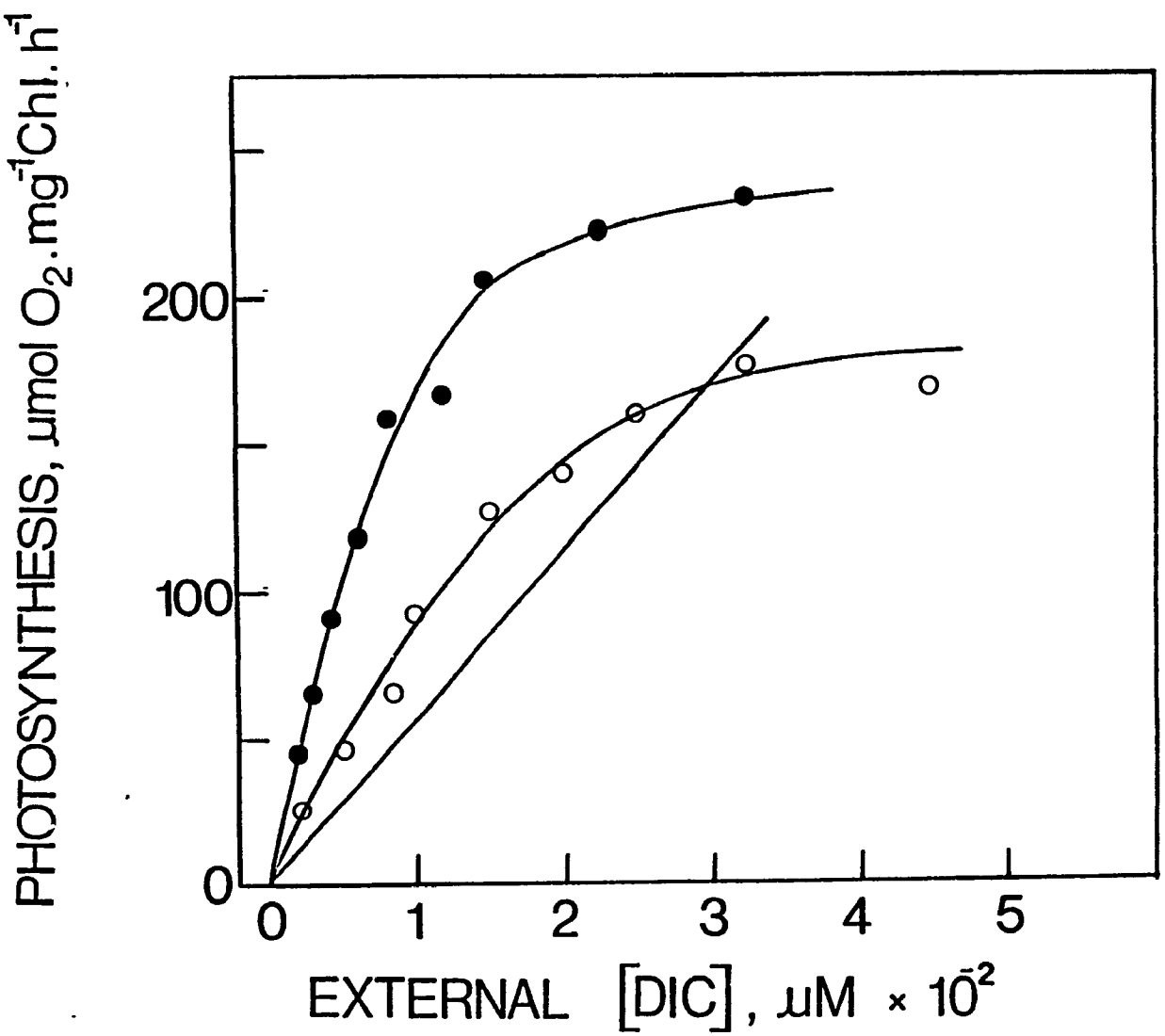
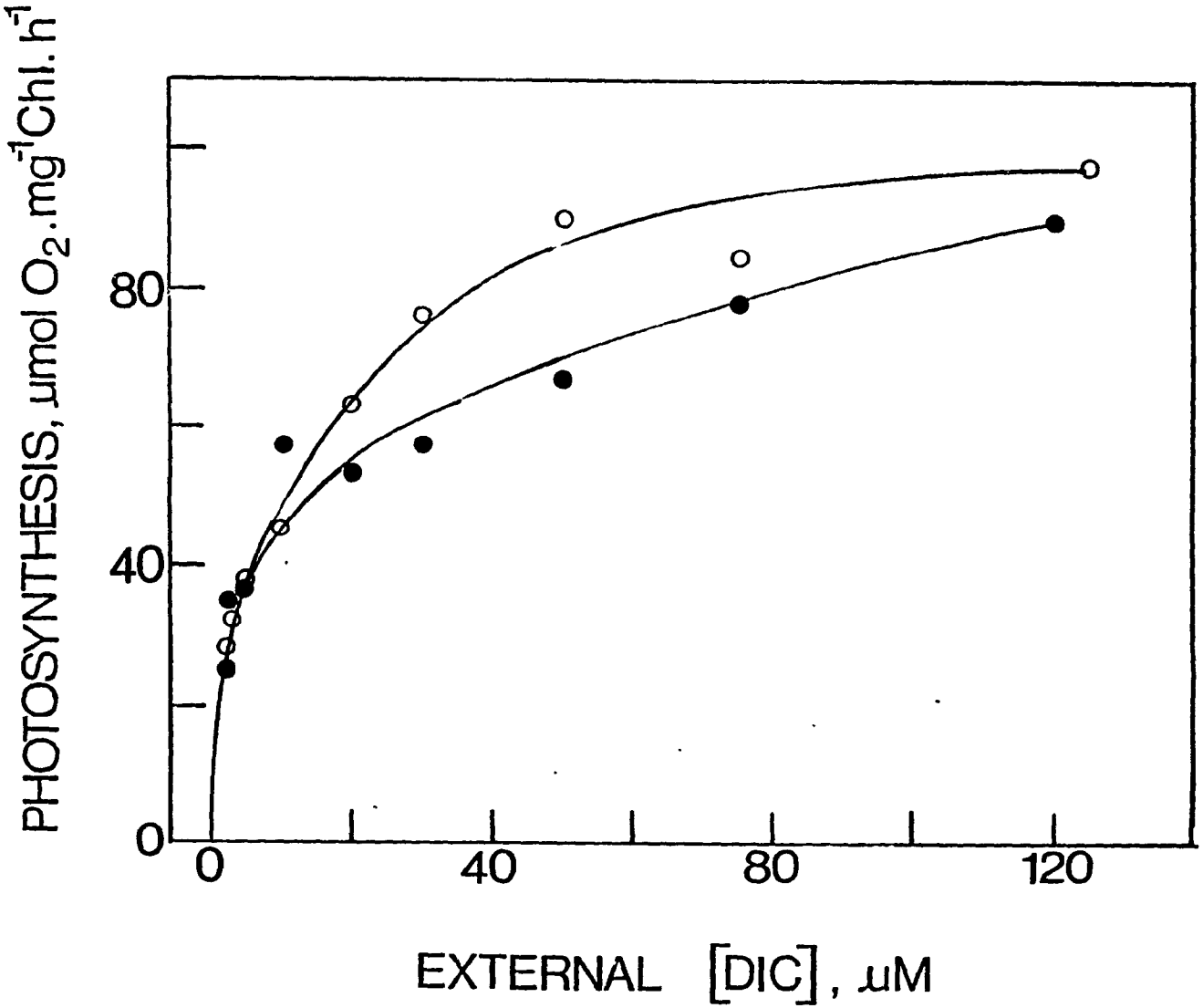

PHOTOSYNTHESIS, μmol O_2.mg^{-1}Chl.h^{-1}
200
100
0
0
1
2
3
4
5
EXTERNAL [DIC], μM × 10^2

FIGURE 30

DIC response curve for standing culture cell photosynthesis, $\pm$ 0.5 mM amiloride. Rate of photosynthetic O_2 evolution with respect to the total extracellular [DIC] in the medium was measured in the presence (o) and absence (o) 0.5 mM amiloride. The [Chl *a*] was in the range of 8.9 - 9.4 $\mu g.mL^{-1}$. The experiment was conducted under the similar conditions as in figure 29 but in the absence of any added Na^+.


PHOTOSYNTHESIS, μmol O_2.mg^{-1}Chl.h^{-1}
0
40
80
0
40
80
120
EXTERNAL [DIC], μM

by CA or high [DIC] indicating inhibition of cell metabolism. In eucaryotic systems amiloride is known to accumulate within cells and inhibit protein kinase through competition with ATP for an ATP binding site (Kleyman & Cragoe 1988).

[EZA] DEPENDENT INHIBITION OF PHOTOSYNTHESIS AND FLUORESCENCE YIELD IN STANDING CULTURE AND AIR - GROWN CELLS

Previous studies have raised the possibility of the involvement of a CA - like moiety in inorganic carbon transport in cyanobacteria (Volokita et al. 1984; Abe et al. 1987; Price & Badger 1989). This idea has found experimental support in the observation that various inhibitors of carbonic anhydrase are also effective against DIC transport. These inhibitors include H_2S (Espie et al. 1989), COS (Miller et al. 1989) and EZA (Price & Badger 1989,. Ethoxyzolamide is a sulfanoamide which is known to inhibit CA activity by binding to the Zn at the active site (Coleman 1976). Price & Badger found that EZA effectively inhibited active CO_2 transport in Synechococcus PCC7942 at around 300 µM. Recent work in our lab has confirmed this finding for Synechococcus UTEX 625 (Espie et al. unpublished). It was also found in Synechococcus PCC7942 EZA inhibited HCO_3^- transport to the same degree as it inhibited CO_2 transport (Price & Badger, 1989).

In order to further distinguish the modes of HCO_3^- transport in the two cell types of Synechococcus UTEX 625, we examined the effect of EZA. Figure 31 shows the effect of

different [EZA] on photosynthesis in standing culture and air - grown cells. Photosynthesis was initiated by the addition 20 μM DIC at pH 8. For the air - grown cells, experiments were conducted in the presence of 10 mM Na^+ for the occurrence of HCO_3^- transport. The standing culture cells were more sensitive to EZA than air-grown cells. At an [EZA] of 40 μM photosynthesis in standing culture cells was reduced by 75% and at 80 μM reduced by 85% relative to the control. At similar [EZA], photosynthesis in air -grown cells was only inhibited by 12 and 25%. The K_I(EZA) for inhibition of photosynthesis in standing culture cells was about 8 μM (Fig. 31). Simultaneous measurements of internal DIC pool formation as monitored by Chl a fluorescence yield indicated similar degree of inhibition as that observed for photosynthesis (Fig. 32). The solvent, DMSO in which EZA was dissolved, at the concentration used had little effect on fluorescence quenching and photosynthesis (not shown). The observed EZA inhibition on standing culture cells is not due to inhibition of cell metabolism because EZA inhibition was reversed by the addition of Na^+ (Fig. 33). Thus, the observed inhibition of photosynthesis in standing culture cells could be due either to the inhibition of DIC uptake or due to inhibition of intracellular CA. Studies by Price & Badger (1989) with intact Synechococcus PCC7942 strains have clearly shown that at the concentration used EZA has little effect on intracellular CA. It was found that the rate of photosynthesis as a function of

FIGURE 31

Effect of [EZA] on standing culture and air-grown culture photosynthesis. The rate of photosynthesis was measured following 20 µM DIC addition at pH 8.05 and 30°C. The experiment was conducted in the presence of 10 mM Na^+ for air - grown cells and in the absence of any added Na^+ for the standing culture cells. The control rate of photosynthesis for the air-grown cell and standing culture cell was 59 µmol $O_2.mg^{-1}Chl.h^{-1}$ and 69 µmol $O_2.mg^{-1}Chl.h^{-1}$ respectively.

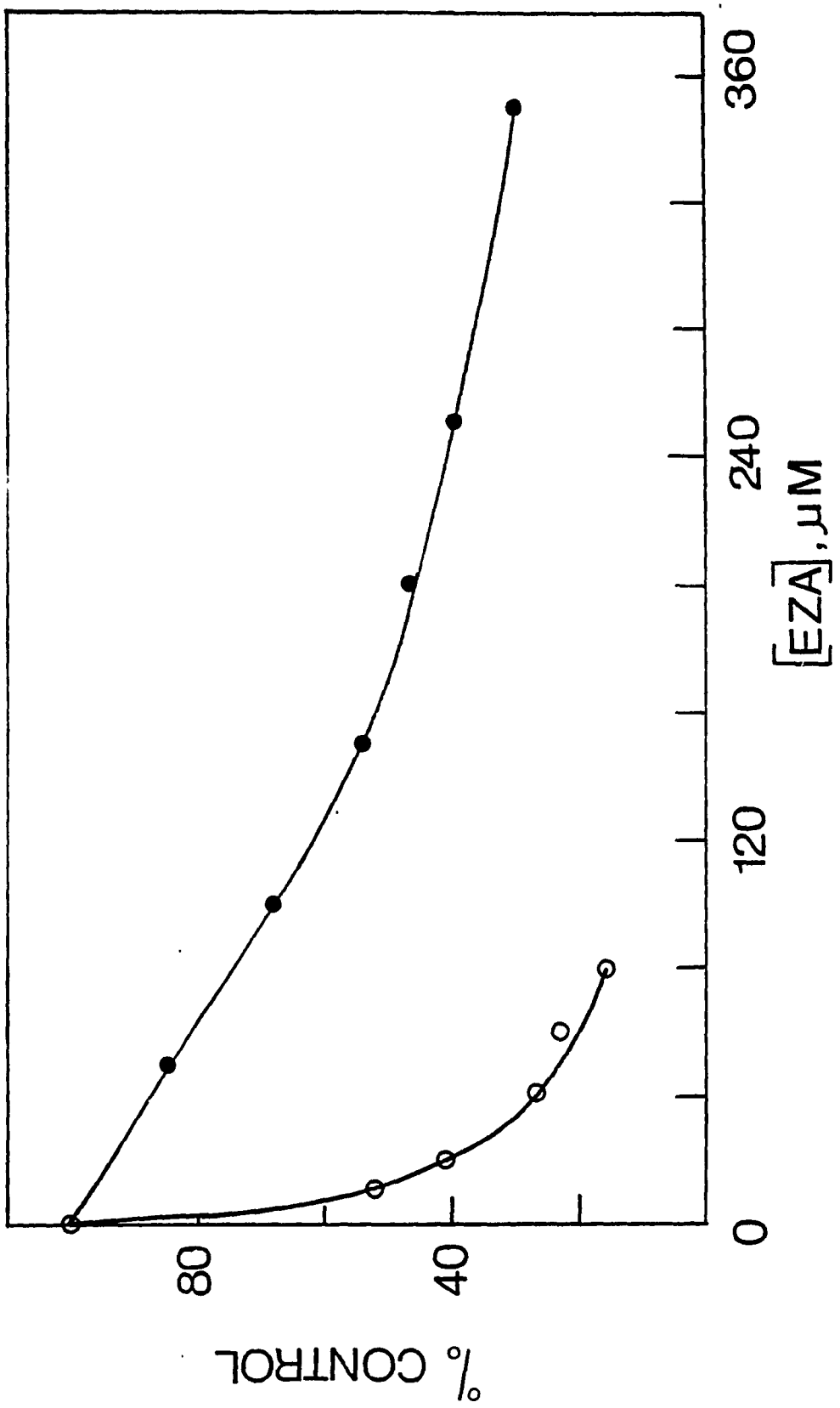
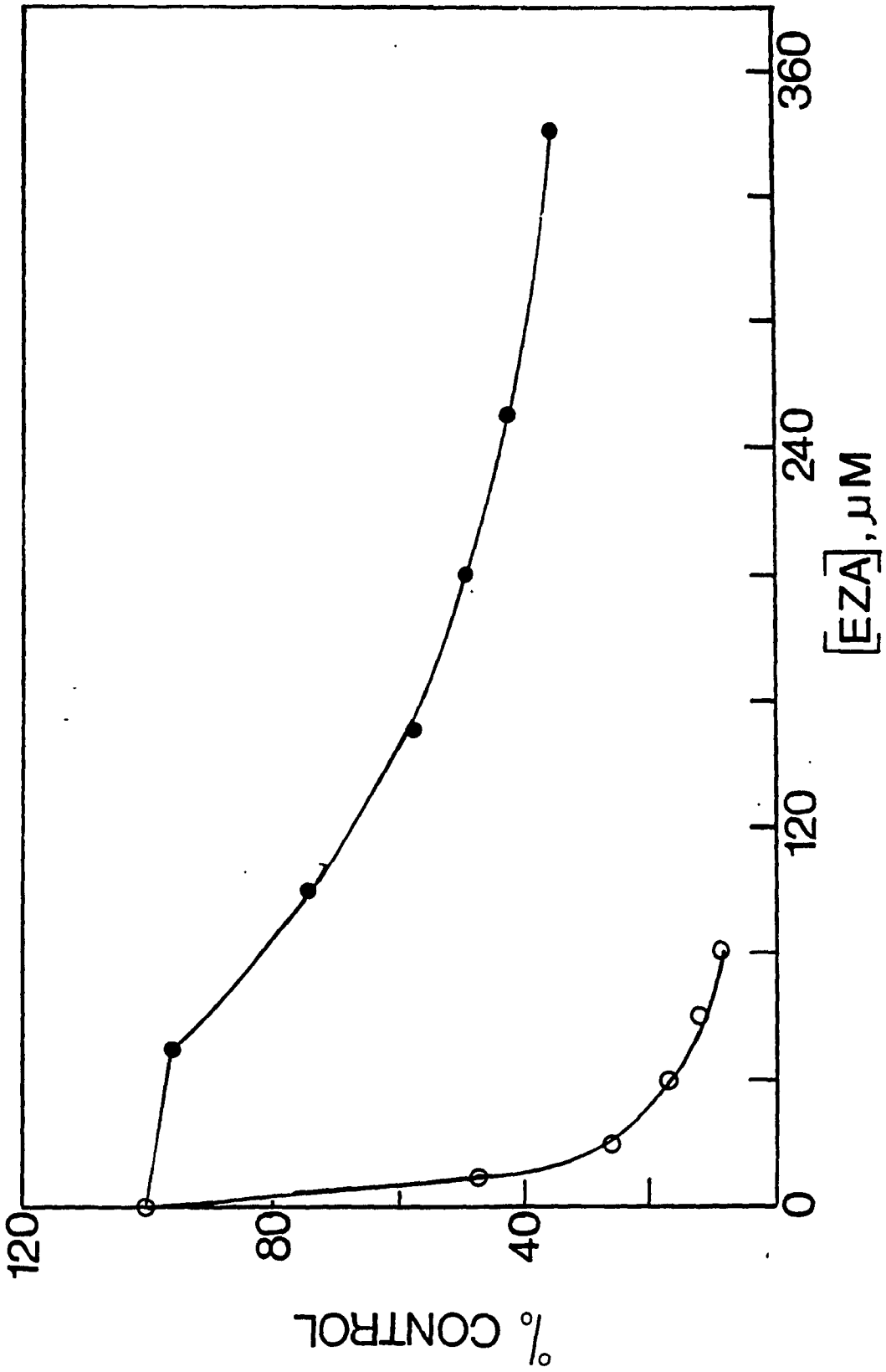

% CONTROL
80
40
0
120
240
360
[EZA], μM

FIGURE 32

Ethoxyzolamide response curve for air-grown and standing culture cell fluorescence yield. The experimental conditions were similar to those for figure 31. Control extent of fluorescence quenching was 21% F_v for the air-grown culture cells and 57% F_v for the standing culture cells.


% CONTROL
120
80
40
0
120
240
360
[EZA], μM

the internal [DIC] was unaffected by the EZA. If the intracellular dehydration of HCO_3^- to CO_2 had been effected, a much larger internal DIC pool would be necessary to supply CO_2 to Rubisco for fixation in the absence of catalysis. Consistent with the evidence of Price & Badger, (1989) our fluorescence quenching studies (Fig. 32 & 33) indicated that EZA caused a reduction in the magnitude of internal DIC pool and provided evidence that DIC transport was inhibited rather than intracellular CA. This latter conclusion is supported by the finding in standing culture cells that Na^+ overcomes the inhibitory effect EZA on photosynthesis and fluorescence quenching. There is no known role for Na^+ in the catalytic mechanism of CA. Collectively, these data support the idea that EZA inhibits the DIC uptake mechanism.

At low [EZA], the EZA inhibition of DIC uptake in standing culture cell is mainly due to the reduction in Na^+ - independent HCO_3^- uptake rather than CO_2 transport. At pH 8 and 20 μM DIC, CO_2 transport could only account for 15% of the total observed rate of photosynthesis. Whereas the observed inhibition of photosynthesis was 85% at 80 μM [EZA]. Recent studies using cells which only transport CO_2 have shown that 80 μM EZA only brings about a 25% reduction in CO_2 transport activity (G.Espie et al. unpublished). Although EZA appears to inhibit both CO_2 and HCO_3^- transport, the concentration requirement is very different. Estimates of K_i(EZA) for CO_2 transport are around 150 μM compare to 8 μM for Na^+ -

dependent HCO_3^- transport.

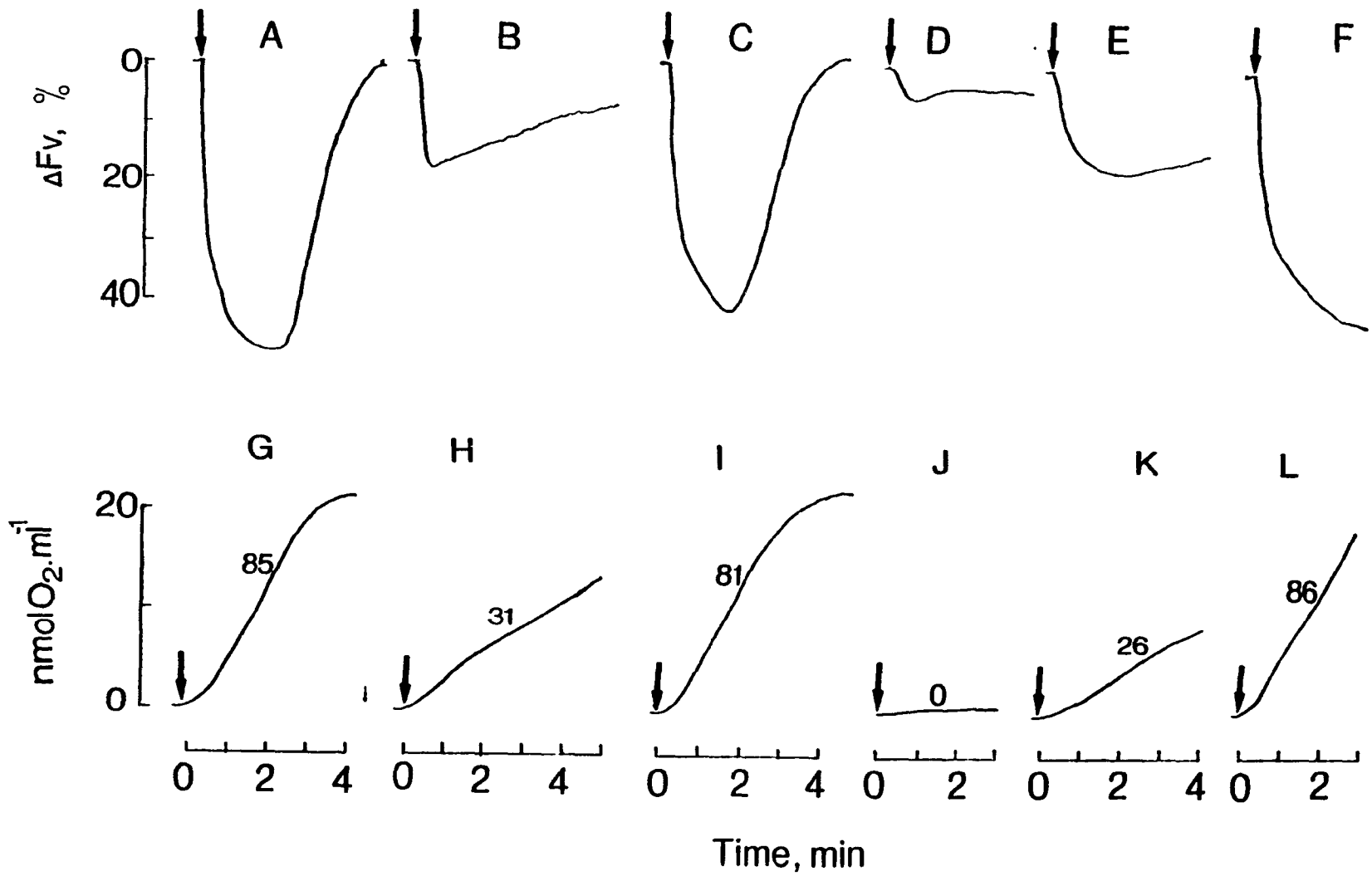
REVERSIBILITY OF EZA INHIBITION IN THE STANDING CULTURE CELLS

It has been clearly established that standing culture cells have a Na^+ - independent HCO_3^- uptake mechanism. Ethoxyzolamide at a concentration of 50 μM drastically reduced the Na^+ - independent HCO_3^- transport (as monitored from Chl *a* fluorescence yield and photosynthesis) at 20 μM DIC and pH 8 (Fig. 33 B & H). Addition of 25 mM Na^+ in the presence of EZA recovered photosynthesis to a rate several fold greater than the rate of CO_2 supply to the cells (Fig. 33, tracing I). Simultaneous measurements of fluorescence quenching showed large intracellular pool formation. Under the experimental condition used CO_2 supply to the cell was limiting and only 1/7 of the observed rate of photosynthesis could be supported solely by CO_2 uptake. Therefore, the observed recovery was due to Na^+ - dependent HCO_3^- uptake. Thus, using EZA we have unmasked the ability of standing culture cells to take up HCO_3^- through an Na^+ -dependent mechanism. The Na^+ - dependent HCO_3^- is normally obscured by the Na^+ - independent uptake mechanism.

The millimolar requirement for [Na^+] in standing culture cell photosynthesis and fluorescence quenching was reminiscent of the Na^+ - dependent HCO_3^- uptake present in the air - grown cells. In order to further verify that this Na^+ - dependent HCO_3^- uptake mechanism was similar to that found in air - grown

FIGURE 33

Demonstration of the presence of the Na^+ - dependent and the Na^+ - independent HCO_3^- transport system in standing culture cells. Shown in a time sequence are Chl *a* fluorescence yield (upper panels) and photosynthetic O_2 evolution (lower panels) tracings obtained a) in the absence of Na^+ (A,G) b) a + 40 μM EZA (B,H), c) b + 25 mM Na^+ (C,I), d) c + 50 μM monensin (D,E,F,J,K,L). Tracings E,F,K & L were obtained after 15 min. incubation of the cells with monensin. All tracings except F & L were obtained following 20 μM DIC addition (↓). Tracing F & L were obtained following 1 mM DIC addition. The [Chl *a*] of the cell suspension was 7.2 $\mu g.mL^{-1}$.


A
B
C
D
E
F
0
20
40
ΔFv, %
G
H
I
J
K
L
20
0
nmolO2.ml-1
85
31
81
0
26
86
0 2 4
0 2 4
0 2 4
0 2
0 2 4
0 2
Time, min

cells, we examined the effect of monensin following the inhibition of most of the Na^+ - independent HCO_3^- transport. Addition of 50 μM monensin completely inhibited Na^+ - stimulated photosynthesis and fluorescence quenching (Fig. 33, tracings D & J). Incubating the cells for 15 minutes partially recovered the level of photosynthesis and fluorescence quenching to the level observed before addition of Na^+ (Fig. 33 compare E & K with B & H). Monensin has no inhibitory effect on CO_2 transport (Fig. 22). Therefore, the observed reduction in DIC uptake following the addition of monensin (as monitored from fluorescence quenching and photosynthesis) is due to inhibition of Na^+ - dependent HCO_3^- transport. Monensin inhibition was recoverable by increasing the [DIC]. Thus the standing culture cells of Synechococcus UTEX 625 have active Na^+ - independent and Na^+ - dependent HCO_3^- transport systems as well as active CO_2 transport systems.

DISCUSSION

Cyanobacteria are classified, as alkalotolerant organisms (Krulwich & Guffanti 1989). They preferentially grow and photosynthesis at alkaline pH between 8 - 10 (Miller & Colman 1980b). At this pH and at low [DIC], HCO_3^- is the predominant species of inorganic carbon (pKa = 6.3) available in the aquatic environment. Cyanobacteria grown under these conditions are known to have mechanism for the efficient transport of HCO_3^- (Badger & Gallagher 1987; Kaplan et al. 1980; Miller & Canvin 1985; Miller & Colman 1980; Price & Badger 1989) which permits their adaptation to the surrounding conditions. Consistent with previous findings, standing culture and air-grown cells of Synechococcus UTEX 625 also possess efficient HCO_3^- transport mechanisms. However, many lines of evidence presented in this investigation suggest the presence of two distinct HCO_3^- uptake mechanism in Synechococcus UTEX 625.

Na^+ - INDEPENDENT HCO_3^- TRANSPORT

Previous studies with air-grown cells of Synechococcus UTEX 625 (Espie & Canvin 1987; Espie et al. 1988b; Miller et al. 1984; Miller & Canvin 1985) and Anabaena variabilis (Kaplan et al. 1984; Reinhold et al. 1984) have demonstrated a requirement for extracellular sodium in the HCO_3^- transport processes. For Synechococcus at pH 8 about 25 mM was found to be optimum at low (20 µM) [DIC]. In contrast, standing culture

cells did not require extracellular Na^+ for HCO_3^- uptake. Various methods have been used to demonstrate the presence of Na^+ - independent HCO_3^- transport in standing culture cells. First, the rate of photosynthesis by the standing culture cells in the absence of sodium was not limited by the rate of HCO_3^- dehydration in a closed system (Fig.4 & 6; Espie & Canvin 1987) at alkaline pH. The actual rate of photosynthesis was many fold greater than the uncatalysed rate of HCO_3^- conversion to CO_2. If the cells had the ability to produce extracellular CA (which would catalyse the convertion of HCO_3^- to CO_2), then the high rate of photosynthesis could be accounted for solely by CO_2 uptake. But, electrometric assay, isotopic disequilibrium experiments (Espie & Canvin 1987) and mass spectrometer studies (Fig.9 & 22) showed no extracellular CA present in standing culture cells. Thus, HCO_3^- has to be directly taken up from the extracellular medium to obtain rates of photosynthesis above the slow uncatalysed rate of HCO_3^- conversion to CO_2.

The occurrence of Chl *a* fluorescence quenching following the addition of DIC to cyanobacterial cell suspensions has been shown to be strongly correlated with intracellular DIC accumulation (CO_2 and/or HCO_3^-) and not due simply to carbon fixation (Miller *et al*. 1988b). Using this method to monitor intracellular DIC, substantial quenching of Chl *a* fluorescence was observed when low concentrations of DIC were added to standing culture cells in the absence of Na^+ (Fig. 4). We interpret this result to indicate that standing culture cells

accumulate a large pool of internal DIC in the absence of Na^+. In contrast, in air-grown cells little quenching of Chl *a* fluorescence occurred in the absence of Na^+ and the rate of photosynthesis was dramatically depressed (Fig. 3a). The larger degree of fluorescence quenching observed for the standing culture cells compared to air-grown cells in the absence of Na^+, suggests that a larger DIC pool was formed through the accumulation of HCO_3^-. The formation of this large intracellular DIC pool, in turn, could support a high rate of photosynthesis above the CO_2 supply rate.

Cells grown in standing cultures possess an extremely high photosynthetic affinity for DIC in the absence of Na^+ ($K_{0.5}$ (DIC) = 10 μM or $K_{0.5}$ (CO_2) = 0.15 μM; Espie & Canvin 1987) at pH 8. In contrast, the primary carboxylating enzyme Ribulose bisphosphate carboxylase in cyanobacteria (Kaplan *et al*. 1980) has a K_m (CO_2) of 200 - 300 μM, regardless of cellular growth conditions (Andrew & Abel 1981; Badger 1980; Kaplan *et al*. 1980). Thus, the apparent photosynthetic affinity for DIC $K_{0.5}$ (CO_2) is some 3 orders of magnitude lower than the K_m (CO_2) of Rubisco. If Rubisco kinetics alone determined the overall photosynthetic affinity of the cells for DIC a $K_{0.5}$ (CO_2): Km (CO_2) ratio of one would be expected. Clearly, some mechanism must exist prior to the carboxylation reaction which is capable of enhancing cellular affinity for CO_2. The observed dependence of photosynthesis on the size of the internal DIC pool (Fig. 8) suggests the high

photosynthetic affinity is due to the ability to form a large intracellular DIC pool. The fact that $K_{0.5}$ (DIC) remains fairly constant as the pH of the medium increases (Espie & Canvin 1987) further suggests that HCO_3^- uptake is involved in intracellular accumulation of DIC (Miller 1985). If the cells were transporting CO_2 alone then $K_{0.5}$ (CO_2) should remain constant with increasing pH (Miller 1985). However, $K_{0.5}$ (CO_2) was observed to decrease by several orders of magnitude when the pH was increased from 8 to 9.5 (Espie & Canvin 1987).

Measurement of photosynthetic O_2 evolution and chlorophyll a fluorescence yield only provides an indirect measure of the cells ability to transport DIC. Thus, direct measurements were made using the silicone fluid filtering centrifugation technique. DIC depletion of the medium and intracellular accumulation by the standing culture cells in the absence of Na^+ occurred much faster than the CO_2 supply rate (Fig.7d). Simultaneous direct measurements of carbon fixation also indicated dependence on HCO_3^- transport (Fig.7b).

Sodium - independent HCO_3^- transport in standing culture cells was also apparent from the mass spectrometer studies. The rapid rise in [$^{13}CO_2$] above the equilibrium level, when the lights were switched off following a brief period of illumination (Fig. 9) did not require extracellular Na^+. The observed rise could only be accounted for by intracellular DIC accumulation through active $H^{13}CO_3$ transport followed by

intracellular dehydration of $H^{13}CO_3$ to $^{13}CO_2$ which then leaks back into the medium along the concentration gradient when the lights are switched off. This phenomenon could not have been a result of respiratory CO_2 release as labelled $DI^{13}C$ was used. Inhibition of HCO_3^- accumulation and loss of intracellular pool in the absence of light suggest the requirement of energy from the photosynthetic electron transport system for DIC transport processes.

Na^+ - DEPENDENT HCO_3^- TRANSPORT

In contrast to standing culture cells, millimolar concentrations of extracellular Na^+ were essential for intracellular accumulation of HCO_3^- in air-grown cells (Fig. 3, 5 & 7; Espie et al. 1988b; Miller et al. 1984; Miller & Canvin 1985). In the absence of Na^+ and under circumstances where there was limited availability of CO_2, and HCO_3^- was the predominant dissolved inorganic carbon species, fluorescence quenching data suggested very little intracellular accumulation of DIC and the rate of photosynthesis paralleled the HCO_3^- dehydration rate (Fig. 3a). Mass spectrometer studies (Espie et al. 1988b) have indicated that this DIC accumulation and photosynthesis could be fully accounted for by CO_2 uptake alone. Thus, unlike standing culture cells, in air-grown cells HCO_3^- accumulation is severely inhibited in the absence of Na^+. Intracellular accumulation of DIC and high rates of photosynthesis could be obtained in the absence of

Na^+ through the CO_2 transport system by several means. These include: a) decreasing the pH; b) increasing the [DIC] in the medium or c) by the addition of carbonic anhydrase, to catalyse the HCO_3^- dehydration to CO_2. All of these treatments essentially increase the rate of CO_2 supply to the cells and thus CO_2 transport can support high rates of photosynthesis.

The initial rate and extent of fluorescence quenching by the air-grown cells increased with increasing [Na^+] (millimolar) at pH 8 following the addition of 20 μM DIC (Fig. 3). This suggests that the rate of HCO_3^- transport and the magnitude of the internal pool formed in air-grown cells is dependent on extracellular [Na^+] concentration. This is further supported by the observation of the increase in rate of photosynthesis (above the rate of CO_2 supply) with increasing [Na^+] concentration (Fig. 3). The requirement of Na^+ for HCO_3^- transport is also reflected in the low photosynthetic affinity for DIC in the absence of Na^+. In the absence of Na^+ the rate of photosynthesis paralleled the theoretical rate of CO_2 production in the medium (Fig. 5). The $K_{0.5}$ (DIC) in the absence of Na^+ was similar to high [DIC] grown cells (Badger & Gallagher 1987; Miller & Canvin 1987). These high [DIC] grown cells have no capacity for HCO_3^- uptake but have a marked ability for CO_2 transport (Badger & Gallagher 1987; Miller & Canvin 1987; Price & Badger 1989). The low photosynthetic affinity for DIC in the absence of Na^+ indicates the reduced ability to concentrate CO_2 at the site of Rubisco.

This was due to the very low potential for HCO_3^- transport. Whereas in the presence of 25 mM Na^+ the $K_{0.5}$ (DIC) was reduced by 5 -fold because of their ability to transport HCO_3^-.

The requirement of Na^+ in HCO_3^- transport by the air-grown cells was also confirmed by direct measurments of DIC accumulation. In the absence of sodium there was low level of intracellular DIC accumulation and the rate of carbon fixation paralleled the rate of HCO_3^- dehydration. The intracellular accumulation of DIC and carbon fixation which occurred in the absence of Na^+ is likely due mainly to CO_2 uptake as the rate of photosynthesis paralleled the CO_2 supply. In the presence of 25 mM Na^+ external DIC was depleted faster than the rate of CO_2 production. Only 1/7 of the observed rate of carbon fixation could be accounted for by CO_2 transport reflecting the large contribution of HCO_3^- to the formation of intracellular DIC pool. The requirement of Na^+ in the HCO_3^- transport system is highly specific. This requirement for Na^+ cannot be met by substituting other cations such as K^+, Mg^{2+}, Li^+ etc. (Kaplan et al. 1984; Miller et al. 1984).

ACTIVE HCO_3^- TRANSPORT

The air-grown cells (in the presence of 25 mM Na+) and standing culture cells were able to concentrate inorganic carbon to a level 1000 times greater than in the external medium. The internal pH has been reported to be around 7.6 - 8 at an external pH of 8 (Coleman & Colman 1981; Kaplan et al.

1980; Miller & Colman 1980b). Since the pH in both the compartments are almost similar the CO_2:HCO_3^- ratio would also be same. Thus, the Na^+ - independent HCO_3^- uptake by the standing culture cells and the Na^+ - dependent HCO_3^- transport in air-grown cells must be considered as occurring against approximately a 1000 -fold concentration gradient.

Bicarbonate could be distributed passively according to the electrochemical gradient across the cell membrane and a 1000 -fold HCO_3^- accumulation could be achieved if the membrane potential is about 180 mV positive inside (Miller & Colman 1980a). But so far only negative membrane potentials have been measured in cyanobacteria and other prokaryotic organisms (Kaplan et al. 1980; Miller & Colman 1980a). The observed HCO_3^- accumulation ratio could also be achieved by the passive distribution of CO_2 across the plasma membrane along the pH gradient if the intracellular pH was more alkaline (pH 10.7) than the external pH (8) (Kaplan et al. 1980; Miller et al. 1988a). But the intracellular pH has been found to be similar to that of the medium at an external pH of 8. Thus, it is evident from these studies that the Na^+ - independent and Na^+ - dependent HCO_3^- transport is an active process occurring against a electrochemical gradient in the cyanobacteria Synechococcus UTEX 625.

ACTIVE CO_2 TRANSPORT

The presence of a CO_2 uptake mechanism in standing culture cells is evident from the mass spectrometer studies. Upon illumination of the cells, in the presence of DIC, the external [CO_2] rapidly dropped to a level near zero (Fig. 9). This draw down in external [CO_2] from equilibrium could only be accounted for by the selective uptake of CO_2 from the external medium. This was also evident from the CO_2 pulsing experiment. The CO_2 in the medium rapidly disappeared when aliquots of CO_2 saturated water was pulsed to illuminated standing culture cells compared to the dark control (Fig. 22). This further indicates the ability of the standing culture cells to take up CO_2 directly from the medium apart from HCO_3^- transport capabilities. The inhibition of CO_2 uptake in the dark (Fig. 9 & 22) and the out burst of accumulated CO_2 to the external medium when the lights are switched off (Fig. 9) suggest the requirement of metabolic energy from the light driven electron transport system for CO_2 transport. Similar mass spectrometer studies (Espie *et al.* 1988a; Miller *et al.* 1988a) and CO_2 pulsing experiments (Miller *et al.* 1988a) have shown the presence of an efficient CO_2 transport system in the air-grown cells.

The uptake of CO_2 occurred against a large concentration gradient. In the absence of Na^+, HCO_3^- transport is inhibited in air-grown cells. Thus, all of the DIC accumulation that occurred in the air-grown cells in the absence of Na^+ was

predominantly due to CO_2 uptake (Fig. 7). The accumulated intracellular [CO_2] far exceeded the extracellular concentration. At an external [DIC] of 28 μM the internal concentration was 4.1 mM. Assuming the CO_2 and HCO_3^- are at equilibrium in both compartments then the uptake of CO_2 occurred against approximately 145 -fold concentration gradient. This, however, is an under estimate since the external [CO_2] is very much lower than the chemical equilibrium at the [DIC] used (Espie et al. 1988a; Miller et al. 1988a). Similarly, CO_2 uptake in the standing culture cells also occurred against a large concentration gradient. Studies indicated rapid depletion of the external [CO_2] to 0.2 μM following illumination of the cells (Fig. 9). Direct measurement of accumulated intracellular [DIC] concentration at 60 s time point was found to be around 15 - 20 mM (Fig. 7 & 21). This indicates occurrence of CO_2 uptake against approximately 1360 -fold gradient. This again is an under estimate because the external [DIC] was 37 - 40 μM for the silicone fluid experiment and was 65 μM for the mass spectrometer experiment. Thus, the actual CO_2 uptake in standing culture cells occurred against a larger concentration gradient. The CO_2 uptake could be driven passively long the pH gradient and the observed accumulation gradient could achieved if the intracellular pH was more alkaline than the external medium. But at an external pH of 8 the intracellular pH has been found to be similar to that of the medium indicating the

CO_2 uptake is an active process.

The characteristics of the CO_2 transport system in the standing culture cells are very similar to that reported for the air-grown cells of Synechococcus UTEX 625 (Espie et al. 1988a; Miller et al. 1988a,b). Like that observed for the air-grown cells (Miller et al. 1988b), monensin did not have any effect on the CO_2 transport in standing culture cells (Fig. 22). This indicates that a Na^+ - gradient is not required for CO_2 uptake. Lithium did not inhibit CO_2 transport in standing culture cells, like the situation observed for air-grown cells (Espie et al. 1988b). This is evident from the normal level of Na^+ - independent DIC accumulation by the standing culture in the presence of Li^+ (Table 1), where part of the observed accumulation is due to CO_2 transport. EZA at a similar concentration inhibited the CO_2 uptake in air-grown and standing culture cells without much effect on the cell metabolism (Espie & Tyrrell, unpublished). Other studies (Espie, unpublished) indicated that H_2S inhibited CO_2 transport in standing culture and air-grown cells to a similar degree. Based on these characteristics it seems reasonable to assume that the same CO_2 transport system is present in both cell types.

DIFFERENTIATION BETWEEN Na^+ - DEPENDENT AND Na^+ - INDEPENDENT HCO_3^- TRANSPORT

This study has established the presence of two modes of

HCO_3^- uptake in Synechococcus UTEX 625 with respect to the requirement for Na^+. A clear distinction between these two modes of HCO_3^- uptake was essential in order to avoid confusion, since the same inorganic carbon species was transported in both cases. Monensin which was found to inhibit Na^+ - gradient dependent serotonin uptake by human blood platelets (Feinstein et al. 1977), selectively inhibited Na^+ - dependent HCO_3^- transport in air-grown cells. Monensin, however, only had a modest effect on cell metabolism or on Na^+ -independent HCO_3^- uptake. When 25 mM Na^+ was present to stimulate Na^+ - dependent HCO_3^- transport in air-grown cells, addition of monensin drastically reduced the level of Chl a fluorescence quenching and the rate of photosynthesis (Fig. 23 - 25). This reduction was due to inhibition of Na^+ -dependent HCO_3^- uptake and not due to reduction in CO_2 transport. This conclusion is based on the following observation. Firstly, the experiments were conducted at pH 8 and at a low [DIC] of 20 μM where HCO_3^- uptake accounts for 6/7 of the observed rate of photosynthesis. Secondly, addition of monensin to air-grown cells in the absence of Na^+ did not cause any reduction in the level of fluorescence quenching nor photosynthesis. Under these circumstances CO_2 is the predominant DIC species transported by the air-grown cells. Thirdly, monensin inhibition of Na^+ - stimulated photosynthesis fluorescence quenching and intracellular DIC accumulation (dependent on Na^+ - dependent HCO_3^- uptake) was recoverable to a near maximum

level by the addition of CA (Fig.24 - 26). Carbonic anhydrase catalyses the dehydration of HCO_3^-, thereby relieving the rate limitation of CO_2 supply to the cells. Thus, increasing the availability of CO_2 to the cells reversed the monensin inhibition. These observations clearly indicate that the maintenance of a Na^+ - gradient facilitates HCO_3^- transport in air-grown cells but was not required for CO_2 uptake.

In contrast, monensin did not have any effect on the extent of fluorescence quenching or the rate of photosynthesis supported by Na^+ - independent HCO_3^- transport (Fig. 18). To avoid ambiguity as to the form of DIC species transported (CO_2 or HCO_3^-), low [DIC] was used and the experiment was carried out at pH 8. The apparent photosynthetic affinity for extracellular inorganic carbon in standing culture cells was also not effected by monensin. These data suggest that monensin had no effect on DIC (CO_2) concentrating mechanism, which was based upon the operation of a Na^+ - independent HCO_3^- transport system. This was confirmed by measuring intracellular DIC accumulation and carbon fixation supported by Na^+ - independent HCO_3^- transport in the presence of monensin (Fig. 21).

Monensin is a carboxylic polyether ionophore (Pressman, 1983) which collapses the gradient by exchanging Na^+ for H^+ with a stoichiometry of 1:1 (Sandeaux et al. 1982). Thus, the immediate inhibition of DIC accumulation and carbon fixation following the addition of monensin in the presence of 25 mM

Na^+ (Fig. 20) could have been due to immediate alkalization of the cytoplasm. With time the cells may be able to regulate and bring the intracellular pH to a normal level through H^+ extrusion. This possibility must be examined by directly measuring the intracellular pH following monensin addition. In Streptococcus faecalis the intracellular pH changed by 0.2 pH units following the collapse of the Na^+ gradient at an extracellular [Na^+] of 25 mM (Delort et al. 1989). The recovery of carbon fixation in Synechococcus to near maximum levels following 10 - 15 min incubation suggests that the monensin had no effect on the light dependent electron transport system and ATP production.

Lithium, like monensin, selectively inhibited Na^+ - dependent HCO_3^- transport in intact air-grown cells with a very modest effect on Na^+ - independent HCO_3^- transport. The sodium stimulated fluorescence quenching and photosynthesis in cells grown on air was drastically reduced by lithium (Fig 13 & 14). The reduction in fluorescence quenching and photosynthesis brought about by Li^+, paralleled the decrease in intracellular DIC accumulation (Table 1). These findings for air-grown cells of Synechococcus UTEX 625 are consistent with those of Espie et al. (1988b) and Espie & Canvin (1987). The marked reduction in intracellular pool formation is due to inhibition of Na^+ - dependent HCO_3^- transport because in mass spectrometer experiment which allows continuous monitoring of CO_2, lithium was shown to have very little effect on CO_2 uptake

(Espie et al. 1988b). Complete recovery of fluorescence quenching and photosynthesis following the addition of CA, which permits a continuous supply of CO_2 to the cells in the presence of Li^+ (Fig. 13) further supports the above statement. The lithium inhibition of Na^+ - dependent HCO_3^- transport was reversible by increasing the extracellular [Na^+].

Lithium which inhibited sodium stimulated HCO_3^- uptake at Li^+:Na^+ ratio of 4:1 (fig. 14; Espie et al. 1988b) had a very slight effect on the Na^+ - independent rate of photosynthesis in standing culture cells (supported mainly by Na^+ - independent HCO_3^- transport) at Li^+:Na^+ (contaminant sodium in the buffer) ratio around 1500. An identical degree of inhibition was observed on standing culture cells at similar K^+:Na^+ ratio (Fig. 15). The decrease in intracellular [DIC] in the presence of Li^+ or K^+ paralleled the degree of inhibition observed on photosynthesis. This suggests that the observed inhibition of standing culture cells could have been due to an osmotic effect. The photosynthetic $K_{0.5}$ for (DIC) in the absence and presence of 20 mM Li^+ was similar in the standing culture cells. This further confirms the above conclusion.

Amiloride a pyrazinoylguanidine, has been used extensively in eucaryotic systems to inhibit Na^+ co-transport, Na^+/H^+ antiporters and Na^+ channels (Krulwich, 1983). Experimental results in this study showed that amiloride differentially inhibited the two modes of HCO_3^- transport (Fig. 28). Amiloride at a concentration of 500 µM, completely

inhibited Na^+ (3 mM) stimulated fluorescence quenching and the rate of photosynthesis was reduced to a level similar to that of the HCO_3^- dehydration rate (Fig. 27 & 28). The reduction in the intracellular DIC pool (monitored by Chl a fluorescence quenching) was not due to inhibition of CO_2 transport nor carbon fixation because near maximum rates of photosynthesis were observed when intracellular DIC accumulation was supported by CA - mediated CO_2 transport (Fig. 27). Thus, the observed reduction in photosynthesis and fluorescence quenching was due to inhibition of Na^+ - dependent HCO_3^- transport.

Amiloride at a concentration which completely inhibited Na^+ - dependent HCO_3^- transport only reduced the Na^+ - independent rate of photosynthesis and fluorescence quenching in the standing culture cells by 10 - 20% relative to control (in the absence of any added Na^+, Fig. 28). Only at high [DIC] there was observable inhibition of photosynthesis by amiloride (Fig. 30). But, the rate of photosynthesis was still several folds greater than the rate of CO_2 production in the medium indicating ongoing HCO_3^- transport. At higher [DIC], the build up of OH^- internally and the possible inhibition of Na^+/H^+ antiporter may have been responsible for the reduction in photosynthesis. Na^+/H^+ antiporter activity has been reported in Synechococcus grown under sodium stress (Blumwald et al. 1984) and in cultures bubbled with 1% CO_2 (Kaplan et al. 1989). The ability of the standing culture and air-grown cells to grow at

an alkaline pH (fig. 10 & 11) further suggests the possible presence of Na^+/H^+ exchanger to aid in intracellular pH regulation. Thus, the slight inhibition of Na^+ - independent HCO_3^- transport could have been due to the inhibition of the Na^+/H^+ antiporter. In this case, the antiporter would take up H^+ from the surrounding environment in exchange for intracellular Na^+. Operation of the antiporter in this direction would be driven by the electrochemical gradient for protons.

Another means of distinguishing between the two modes of HCO_3^- transport is by selectively inhibiting Na^+ - independent HCO_3^- uptake. Ethoxyzolamide selectively inhibited Na^+ - independent HCO_3^- uptake with very moderate effect on Na^+ - dependent HCO_3^- transport. At pH 8 and low [DIC], fluorescence quenching and photosynthesis in standing culture cells (Fig. 31 - 33) was predominantly supported by Na^+ - independent HCO_3^- transport. But when the standing culture cells were treated with 40 μM EZA, the Na^+ - independent rate of photosynthesis and fluorescence quenching was drastically reduced indicating inhibition of Na^+ - independent HCO_3^- transport. The CO_2 transport system of standing culture cells was also inhibited by EZA but only at a much higher [EZA] (Espie & Tyrrell unpublished). Similar concentration of EZA only had a very slight inhibitory effect on Na^+ - dependent HCO_3^- uptake as monitored by the Chl *a* fluorescence quenching technique and photosynthesis (Fig.31 & 32). The observed reduction in Na^+ -

dependent photosynthesis and fluorescence quenching in air-grown cells was due to inhibition of CO_2 uptake and probably not due to a reduction in Na^+ - dependent HCO_3^- transport (Espie & Tyrrell unpublished). Even though the observed high rate of photosynthesis under the circumstances was mainly due to Na^+ - dependent HCO_3^- uptake, the leakage of CO_2 from the cytoplasm and the lack of CO_2 scavenging mechanism would result in reduced DIC accumulation and lead to the observed inhibition. This conclusion should be confirmed by measuring the $[CO_2]$ in the medium following the treatment of the cells with EZA.

SODIUM REQUIREMENT FOR HCO_3^- TRANSPORT

Sodium has been shown to be required for many cyanobacterial functions (Miller et al. 1984; Blumwald et al. 1984). The millimolar requirement of Na^+ for HCO_3^- transport (Espie et al. 1988b; Kaplan et al. 1984; Miller et al. 1984; Reinhold et al. 1984) and the micromolar requirement for CO_2 uptake clearly distinguishes the two sites of action of Na^+ in the DIC transport system. There are 3 possible mechanism by which Na^+ may stimulate HCO_3^- transport (Kaplan et al. 1984; Reinhold et al. 1984): a) sodium could act as an allosteric effector changing the binding parameters of the transporter for HCO_3^-. In this model, transport of sodium across the membrane is not required; b) Na^+ may be required for the regulation of the intracellular pH through a Na^+/H^+ antiporter

or Na^+/OH^- symport which helps in the elimination of the OH^- built up during the intracellular dehydration of HCO_3^-; c) direct involvement of Na^+ in the HCO_3^- transport through a Na^+/HCO_3^- symport energised by a Na^+ ATPase or an electrogenic Na^+/H^+ antiporter. Enhancement of the initial rate of HCO_3^- transport monitored from fluorescence quenching data (Fig. 3) suggest a direct involvement of Na^+ in HCO_3^- transport by the air-grown cells. Inhibition of Na^+.- dependent HCO_3^- uptake by monensin (Fig. 23 - 26) indicates the requirement for a Na^+ - gradient for HCO_3^- uptake. The results of the study with monensin are inconsistent with the involvement of a HCO_3^- translocatory ATPase. If there was a primary HCO_3^- uptake mechanism and Na^+ only was involved in the regulation of the intracellular pH through a Na^+/H^+ antiporter in air-grown cells then the $\Delta \mu H^+$ created by the build up of OH^- should only rate limit HCO_3^- efflux. But the enhancement of initial rate of HCO_3^- uptake by Na^+ and complete inhibition of this stimulation by monensin supports the direct involvement of Na^+ in HCO_3^- uptake.

Sodium accelerated the rate of intracellular DIC pool utilization which was built up predominantly by HCO_3^- accumulation (Fig. 3) also suggests the additional involvement of sodium in the elimination of the OH^- built up during intracellular HCO_3^- dehydration. Studies have indicated the presence of Na^+/H^+ antiporter in Synechococcus cells grown under sodium stress (Blumwald et al. 1984). Further studies

should be performed in order to conclude if Na^+/HCO_3^- symport is energised by an electrogenic Na^+/H^+ antiporter as in E.coli, where Na^+/solute symport is coupled to Na^+/H^+ antiporter (Shiota et al. 1984), or if the Na^+/HCO_3^- uptake is energised by Na^+ ATPase.

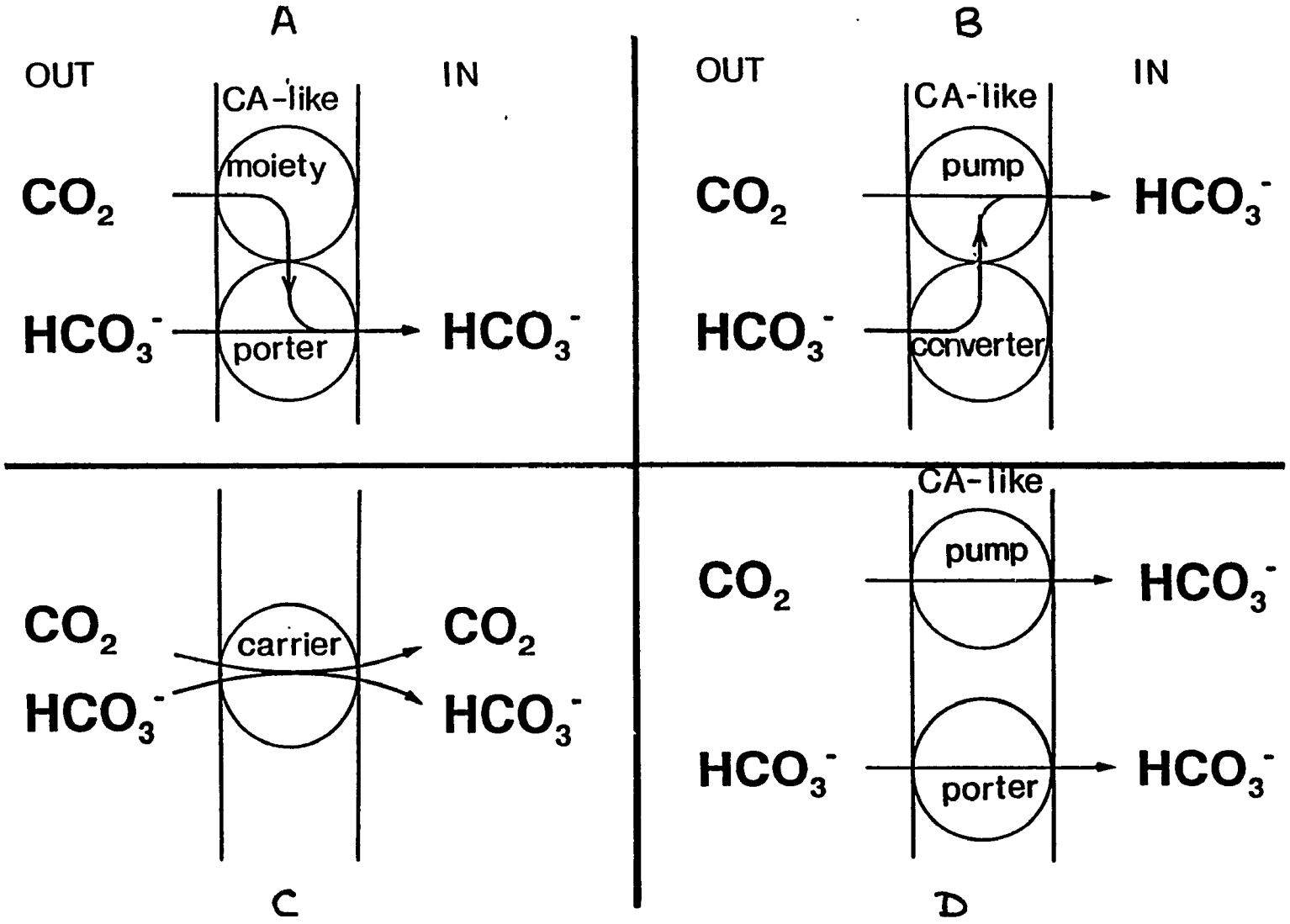
MODEL FOR DIC TRANSPORT

Different mechanistic models have been put forward for DIC transport in cyanobacteria (Fig. 34). Volokita et al. 1984 proposed that Anabaena variabilis M3 grown in cultures bubbled with 0.03% CO_2 in air has a primary HCO_3^- transport system (Fig. 34A). In addition, they postulated that there is associated with the primary porter, a CA - like " front end" mechanism which converts CO_2 to HCO_3^- within the membrane for use by the primary HCO_3^- porter. This model was based mainly on the observation that EZA selectively inhibited CO_2 transport and HCO_3^- was the DIC species which arrived on the cytoplasmic side of the membrane, regardless of the inorganic carbon species supplied to the organism.

Price & Badger (1989) also suggested the involvement of CA - like activity for DIC transport in Synechococcus PCC7942 cells grown on 30 ppm CO_2 in air. However, it was proposed that these cells had a central CO_2 transporter (Fig. 34b). The central porter takes up CO_2 but converts it to HCO_3^- through a CA - like step during translocation across the membrane. Thus, HCO_3^- arrived in the cytoplasm. Attached to this central

FIGURE 34

DIC transport models proposed for cyanobacteria. a) A primary HCO_3^- transporter with a CA - like activity (Volokita *et al.* 1984). b) A primary CO_2 transporter with a CA - like front end (Price & Badger 1989). c) A common porter for the transport of CO_2 and HCO_3^- d) Two distinct transport system for CO_2 and HCO_3^- transport (Espie *et al.* 1989).


A
OUT
IN
CA-like
moiety
CO_2
HCO_3^-
porter
HCO_3^-
B
OUT
IN
CA-like
pump
CO_2
HCO_3^-
converter
HCO_3^-
CO_2
carrier
CO_2
HCO_3^-
HCO_3^-
C
CA-like
pump
CO_2
HCO_3^-
HCO_3^-
porter
HCO_3^-
D

pump is an additional CA - like front end which converts HCO_3^- from the external medium to CO_2 for transport by the CO_2 pump. The CA - like moiety is not directly involved in transport and it's activity is cryptic (no extracelleular products). This proposal was based on the observation that a) HCO_3^- transport being induced only in low DIC grown cells while CO_2 transport is expressed constitutively in all cell type; b) inhibition of both, CO_2 and HCO_3^- transport systems by EZA or COS (Badger & Price 1990); c) HCO_3^- being the only species delivered to the cytoplasm regardless of the inorganic carbon species supplied to the cells.

As in Anabaena variabilis in air-grown cells of Synechococcus UTEX 625, CO_2 transport was selectively inhibited by EZA with very moderate effect on Na^+ - dependent HCO_3^- transport (Espie & Tyrrell, unpublished). CO_2 transport in air-grown cells of Synechococcus UTEX 625 was also selectively inhibited by the use of H_2S (Espie et al. 1989) or COS (Miller et al. 1989). However it was also observed that CO_2 transport could occur normally in air-grown cells under conditions where the HCO_3^- transport was inhibited by Li^+ or by omitting sodium (Espie et al. 1988). Thus, it appeared that CO_2 uptake was not obligatorily dependent upon a HCO_3^- transport system nor was the HCO_3^- uptake dependent upon a CO_2 transport. Based on these observation two separate, specific and independent transporters for the uptake of CO_2 and HCO_3^- was proposed by Espie et al. (1989; Fig. 34D).

The conflict in the results obtained for air-grown cells of Synechococcus UTEX 625 (Espie et al. 1989) and for the 30 ppm CO_2 in air-grown Synechococcus PCC7942 (Price & Badger 1989) could be due the presence of Na^+ - independent HCO_3^- transport in the 30 ppm CO_2 grown cells. Synechococcus UTEX 625 grown on 30 ppm CO_2 (Crotty & Espie, unpublished) possessed Na^+ - independent HCO_3^- transport system. This observation supports the above statement. The results presented here for the Na^+ - independent HCO_3^- transport system is in agreement with Price & Badger (1989) studies with 30 ppm CO_2 grown cells while those obtained for Na^+ - dependent HCO_3^- are consistent with the work of Espie et al. 1989. So far, the Na^+ - independent HCO_3^- transport system has not been considered by any of the current models.

The Na^+ - dependent HCO_3^- uptake mechanism present in the air-grown cells is distinctly different from the Na^+ - independent transport present in the standing culture cells. Monensin (Fig.24 - 26), Li^+ (Fig. 13 -15) and amiloride (Fig. 27 - 29) specifically inhibited Na^+ stimulated HCO_3^- transport in air-grown cells without any effect on CO_2 transport system. This observations further supports the Espie et al. (1989) model for the presence of two different porters for the transport of HCO_3^- and CO_2 in air-grown cells. Furthermore, the fact that CO_2 transport is constitutive and the HCO_3^- transport is induced eliminates the possibility of a CO_2 uptake mechanism dependent on a primary HCO_3^- transporter as proposed

by Volokita et al. (1984).

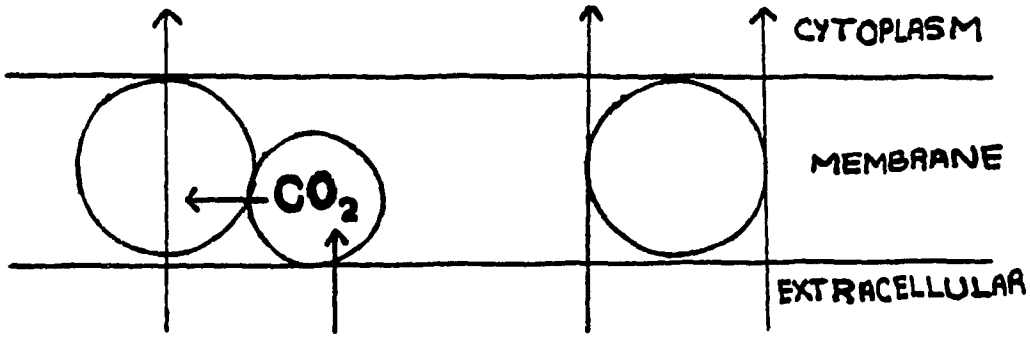
The Na^+ - independent HCO_3^- uptake was inhibited by low [EZA] (Fig. 31 - 33). The inhibition of Na^+ - independent HCO_3^- transport by EZA suggests the involvement of CA or CA - like activity within this transporter. So far, extracellular CA has not been detected in standing culture cells (Fig. 9 & 22; Espie & Canvin 1987) as judged by three different methods. Thus it seems that the CA - like activity is associated with the interior of the cytoplasm or the transporter itself. The Na^+ - independent HCO_3^- uptake, unlike the Na^+ - dependent HCO_3^- transport system did not require extracellular sodium nor a Na^+ - gradient for HCO_3^- transport. Li+ and amiloride which inhibited Na^+ - dependent HCO_3^- transport had no effect on Na^+ - independent HCO_3^- transport. These results strongly suggest the presence of a distinctly different HCO_3^- transporter in the standing culture cells.

Figure 35 illustrates the 2 possible mechanisms for Na^+ - independent HCO_3^- uptake in standing culture cells. One possibility (Fig. 35A) is that Na^+ - independent HCO_3^- uptake is dependent upon the control of CO_2 transport to transfer DIC across the membrane. In this model, we suggest that a CA - like front end attached to the primary transporter is involved in the conversion of HCO_3^- to CO_2. The CO_2 generated by the front end is subsequently transported through the primary porter. This model is supported by the observation of: a) CO_2 transport was not affected by Li^+, monensin or amiloride like

FIGURE 35

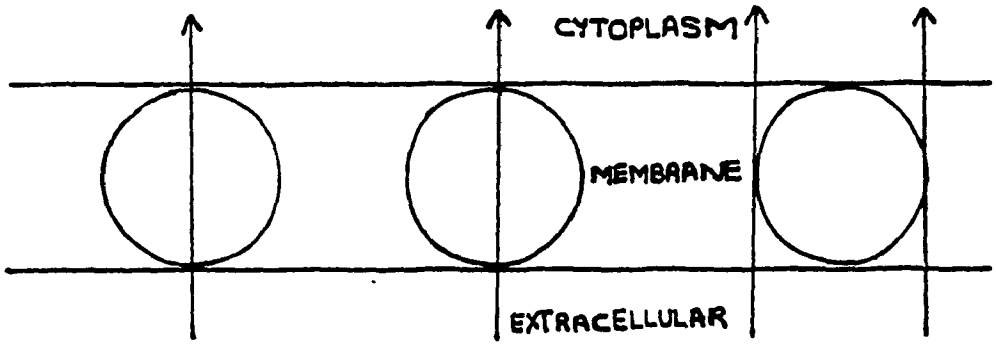
Mechanisms of DIC uptake in standing culture cells of Synechococcus UTEX 625. Na^+ - independent HCO_3^- uptake could occur through 2 possible means, A primary CO_2 transporter with a CA - like front end (A) or by a primary HCO_3^- transporter independent of CO_2 transport (B). Also illustrated is a separate Na^+ - dependent HCO_3^- porter similar to that present in air-grown cells.

MODEL A



CO_2 HCO_3^- Na^+ HCO_3^-

MODEL B



CO_2 HCO_3^- Na^+ HCO_3^-

that observed for Na^+ - dependent HCO_3^- transport; b) CO_2 transport was expressed constitutively in Synechococcus UTEX 625 grown on plates, standing, air-grown and high CO_2 cultures whereas Na^+ - independent HCO_3^- transport was only expressed when the cells were grown in standing cultures or 30 ppm grown cells. This suggest that the Na^+ - independent HCO_3^- transport may be dependent on a primary CO_2 transporter as described in figure 35A. However the effect of CO_2 transporter inhibitors, COS and H_2S have not been tested on Na^+ - independent HCO_3^- transport. Secondly, experiments have not been performed to see if there is competition between HCO_3^- (Na^+ - independent) and CO_2 for uptake as would be predicted for the coupled system shown in Fig. 35A. If there is no competitive inhibition for uptake between the species and if COS and H_2S have no effect on Na^+ - independent HCO_3^- transport, then the Na^+ - independent HCO_3^- transporter could be a separate entity from that of CO_2 (Fig. 35B). The primary HCO_3^- uptake could be a H^+/HCO_3^- symport energised by a proton gradient or it could be energised primarily by an ATPase.

Direct measurements of intracellular DIC accumulation using silicone fluid centrifugation technique indicated that addition of 25 mM Na^+ enhanced DIC accumulation (Fig.7D) and carbon fixation (Fig.7B) in standing culture cells. The larger intracellular pool of DIC in the presence of Na^+ was also evident from the mass spectrometer experiment (Fig. 9). This stimulation was not due to increased CO_2 uptake because the

experiment was conducted at low [DIC] where the CO_2 concentration was limited. Secondly, mass spectrometer studies indicated that CO_2 depletion from the medium by the standing culture cells in the presence and absence of Na^+ was similar at low [DIC] (Fig. 9). Thus, the observed accumulation was due to greater accumulation of HCO_3^-. Studies with the inhibitors suggested that the residual Na^+ - dependent HCO_3^- uptake in standing culture cells is similar to that present in air-grown cells. Like that observed for air-grown cells EZA did not show any inhibition of Na^+ - dependent HCO_3^- transport in standing culture cells. Addition of Na^+ following the inhibition of Na^+ - independent HCO_3^- transport with EZA recovered fluorescence quenching and photosynthesis (Fig. 33). This recovery was due to Na^+ - dependent HCO_3^- uptake as the rate of photosynthesis was greater than the rate of HCO_3^- dehydration. Subsequent additions of monensin inhibited this Na^+ - dependent HCO_3^- transport like that observed for the air-grown cells. Lithium also inhibited Na^+ dependent photosynthesis and HCO_3^- accumulation (Table 1; Espie & Canvin 1987) in standing culture cells. The photosynthetic $K_{0.5}$ (Na^+) for standing culture and air-grown cells was found to be similar (Espie & Canvin 1987). These results suggest that the standing culture cells have residual Na^+ - dependent HCO_3^- transport similar to that present in air-grown cells (Fig. 35).

It is clear that at low [CO_2] and the presence of HCO_3^- in the medium induces HCO_3^- uptake in cyanobacteria (Badger &

Gallagher 1987; Kaplan et al. 1980; Price & Badger 1989). Transfering the standing culture cells to air-grown cultures resulted in the total loss of Na^+ - independent HCO_3^- transport and increase in level of Na^+ - dependent HCO_3^- transport (Fig. 12). This suggest that very low [DIC] and subsequently the low growth rate of the organism may induce Na^+ - independent HCO_3^- transport. However, on some occassions the air-grown cells expressed a large component of the Na^+ - independent HCO_3^- uptake. The reason for this variability is not properly understood.

In conclusion, this study indicates the presence of two inducible HCO_3^- transport system with respect to the requirenment of extracellular Na^+. Monensin, Li^+ and amiloride selectively inhibits Na^+ - dependent HCO_3^- uptake without any apparent effect on the Na^+ - independent HCO_3^- transport. The Na^+ - independent HCO_3^- transport could be selectively inhibited at low [EZA]. This suggests the involvement of a CA - like moiety for Na^+ - independent HCO_3^- uptake. Unlike the HCO_3^- uptake mechanism, the CO_2 transport system is present in all cell types of Synechococcus UTEX 625 regardless of the growth condition.

REFERENCES

Abe, T., Tsuzuki, M.,Miyachi, S. 1987. Transport and fixation of inorganic carbon during photosynthesis in cells of Anabaena grown under ordinary air III. Some characteristics of bicarbonate transport system in cells grown under ordinary air. Plant Cell Physiol. 28: 867 - 874

Aizawa, K., Miyachi, S. 1986. Carbonic anhydrase and CO_2 concentrating mechanism in microalgae and cyanobacteria. FEMS Micobiol.Rev.39: 215 - 233

Allen, M.M. 1968. Simple conditions for the growth of unicellular blue-green algae on plates. J.Phycol. 4: 1 - 4

Andrew, T.J., Abel, K.M. 1981. Kinetics and subunit interaction of Ribulose bisphosphate carboxylase - oxygenase from the cyanobacterium Synechococcus sp. J.Biol.Chem. 256: 8445 - 8451

Badger, M. R. 1980. Kinetic properties of ribulose 1, 5-bisphosphate carboxylase/oxygenase from Anabaena variabilis. Arch. Biochem.Biophys. 231: 233 - 242

Badger, M. R. 1987. The CO_2 - concentrating mechanism in aquatic phototrophs. The Biochem. Plants. 10: 219 - 274

Badger, M. R., Andrew, T. J.1982. Photosynthesis and inorganic

carbon usage by the marine cyanobacterium, Synechococcus sp. Plant Physiol. 70: 517 - 523

Badger, M.R., Basset, M., Comin, H.N. 1985. A model for HCO_3^- accumulation and photosynthesis in the cyanobacterium Synechococcus sp. Plant Physiol. 77: 465 - 471

Badger, M.R., Gallagher, A. 1987. Adaptation of photosynthetic CO_2 and HCO_3^- accumulation by the cyanobacterium Synechococcus PCC6301 to growth at different inorganic carbon concentrations. Aust.J.Plant Physiol. 14: 189 - 201

Badger,M.R., kaplan,A., Berry, J.A. 1978. A mechanism for concentrating CO_2 in Chlamydomonas reinhardtii and Anabaena variabilis and it's role in photosynthetic CO_2 fixation. Carnegie Inst. Wash. year Book. 77: 251 - 261

Badger, M.R., Price, D.G. 1990. Carbon oxysulfide is an inhibitor of both CO_2 and HCO_3^- uptake in the cyanobacterium Synechococcus PCC7942. Plant Physiol. 94: 35 - 39

Birmingham, B.C., Colman, B. 1979. Measurement of carbon dioxide compensation points of fresh water algae. plant Physiol. 64: 892 - 895

Blumwald, E., Wolosyn, J.M., Packer, L. 1984. Na^+/H^+ exchange

in the cyanobacterium Synechococcus 6311. Biochem. Biophys. Res. Commun.122: 452 - 459

Buch, K. 1960. Dissoziation der kohlensaure, Gleichgenichte und Puffe systeme. In: WRuhland, ed, Handbuch Pflanzenphysiologie, Vol.1 Springer - Verlag, Berlin. pp 1 - 11

Coleman, J.R. 1975. Chemical reaction of sulfanamides with carbonic anhydrase. Ann. Rev. Pharmacol. 15: 221 - 242

Coleman, J.R., Colman, B. 1981. Inorganic carbon accumulation and photosynthesis in a blue - green alga as a function of external pH. Plant Physiol. 67: 917 - 921

Delieu, T., Walker, D.A. 1972. An improved cathode for the measurement of photosynthetic oxygen evolution by isolated chloroplasts. New Phytol. 71: 201 - 225

Delort, A., Dauphin, G., Guyot, D., Jeminet, G. 1989. Study by NMR of the mode of action of monensin on Streptococcus faecalis de-energised and energised cells. Biochem.Biophys.Acta. 1013: 11 20

Espie, G.S., Canvin, D.T. 1987. evidence for Na^+ - independent HCO_3^- uptake by the cyanobacterium Synechococcus leopoliensis

Plant Physiol. 84: 125 - 130

Espie, G. S., Gehl, K.A., Owttrim, G. W., Colman, B. 1984. Inorganic carbon utilization by cyanobacteria. In: Advances in photosynthetic research. Proc. of the Int. Congress on photosynthesis, Brussels, Belgium, Ed. by C.Sybesma. Vol. 3 Martinus Nijhoff/ Dr. W.Junk Publishers, The Hague. pp. 457 - 460

Espie, G.S., Miller, A.G., Birch, D.G., Canvin, D.T. 1988a. Simultaneous transport of CO_2 and HCO_3^- by the cyanobacterium Synechococcus UTEX 625. Plant Physiol. 87: 551 - 554

Espie, G.S., Miller, A.G., Canvin, D.T. 1988b. Characterization of the Na+ - requirement in cyanobacterial photosynthesis. Plant Physiol. 88: 757 - 763

Espie, G.S., Miller, A.G., Canvin, D.T., 1989. selective and reversible inhibition of active CO_2 transport by hydrogen sulfide in a cyanobacterium. Plant Physiol. 91: 387 - 394

Feinstein, M.B., Henderson, E.G., Shaafi, R.I. 1977. The effect of alterations of transmembrane sodium ion and potassium ion gradients by ionophores in human blood platelets. Biochem.Biophys.Acta. 468: 284

Kaplan, A., Badger, M.R., Berry, J.A. 1980. Photosynthesis and the intracellular inorganic carbon pool in the blue-green alga Anabaena variabilis: response to external CO_2 concentration. Planta 149: 219 - 226

Kaplan, A., Scherer, S., Lerner, M. 1989. Nature of the light-induced H^+ efflux and Na^+ uptake in cyanobacteria. Plant Physiol. 89: 1220 - 1225

Kaplan, A., Volokita, M., Zenvirth, D., Reinhold, L. 1984. An essential role for sodium in the bicarbonate transporting system of the cyanobacterium Anabaena variabilis. FEBS Lett. 176: 166 - 168

Kleyman, T.R., Cragoe, E.J. 1988. Amiloride and it's analogs as tools in the study of ion transport. J.Membrane Biol. 105: 1 - 21

Krulwich, T.A. 1983. Na^+/H^+ antiporters. Biochem.Biophys.Acta. 726: 245 - 264

Krulwich, T.A., Guffani, A.A. 1989. Alkalophilic bacteria. Ann.Rev.Microbiol. 43: 435 - 463

Mackinney, G. 1941. Absorption of light by chloroophyll solutions. J.Biol.Chem. 140: 315 - 322

Mayo, W.P., Elrifi, I.R., Turpin, D.H. 1989. The relationship between ribulose bisphosphate concentration, dissolved inorganic carbon (DIC) transport and DIC - limited photosynthesis in the cyanobacterium Synechococcus leopoliensis grown at different concentrations of inorganic carbon. Plant Physiol. 90: 720 - 722

Mayo, W.P., Williams, T.G., Birch, D.G., Turpin, D.H. 1986. Photosynthetic adaptation by Synechococcus leopoliensis in response to exogenous dissolved inorganic carbon. Plant Physiol. 80: 1038 - 1040

Miller, A.G. 1985. Study of inorganic carbon transport: The kinetic reaction approach. In: Inorganic carbon uptake by aquatic photosynthetic organisms. Ed. W.J.Lucas and J.A. Berry. Ammerican society of plant physiologists. Rockville, MD: pp. 17 - 37

Miller, A.G., Canvin, D.T. 1985. Distinction between HCO_3^- and CO_2 dependent photosynthesis in the cyanobacterium Synechococcus leopoliensis based on the selective response of the HCO_3^- transport to Na^+. FEBS. 187: 29 - 32

Miller, A.G., Canvin,D.T. 1987. The quenching of chlorophyll a fluorescence as a consequence of the transport of inorganic carbon by the cyanobacterium Synechococcus UTEX 625. Biochem.

Biophys. Acta. 894: 407 - 413

Miller, A.G., Canvin, D.T.1987b. Na^+ - stimulation of photosynthesis in the cyanobacterium Synechococcus UTEX 625 grown on high levels of inorganic carbon. Plant Physiol. 84: 118 - 124

Miller, A.G., Colman, B. 1980a. Active transport and accumulation bicarbonate by a unicellular cyanobacterium. J.Bacteriol. 143: 1253 - 1259

Miller, A.G., Colman, B. 1980b. Evidence for HCO_3^- transport by the blue-green alga (cyanobacterium) Coccochloris penıocystis. Plant Physiol. 65: 397 - 402

Miller, A.G., Espie, G.S., Canvin, D.T. 1988a. Active transport of CO_2 by the cyanobacterium Synechococcus UTEX 625. Plant Physiol. 86: 677 - 683

Miller, A.G., Espie, G.S., Canvin, D.T. 1988b. Chlorophyll a fluorescence yield as a monitor of both active CO_2 and HCO_3^- transport by the cyanobacterium Synechococcus UTEX 625. Plant Physiol. 86: 655 - 658

Miller A.G., Espie, G.S., Canvin, D.T. 1989. Use of carbon oxysulfide, a structural analog of CO_2, to study active

transport in the cyanobacterium Synechococcus UTEX 625. Plant Physiol. 90: 1221 - 1231

Miller, A.G., Espie, G.S., Canvin, D.T. 1991. The effect of inorganic carbon and oxygen upon fluorescence in the cyanobacterium Synechococcus UTEX 625. Can. J. Bot. (in press)

Miller,A.G., Turpin, D.H., Canvin, D.T. 1984. Na^+ requirement for growth, photosynthesis and pH regulation in the alkalotolerant cyanobacterium Synechococcus leopoliensis. J. Bacteriol. 159: 100 - 106

Pressman, B.C. 1976. Biological application of ionophore. Ann. Rev. Biochem. 45: 501 - 530

Price ,G.D., Badger, M.R. 1989. Ethoxyzolamide inhibition of CO_2 uptake in the cyanobacterium Synechococcus PCC7942 without apparent inhibition of internal carbonic anhydrase activity. Plant Physiol. 89: 37 - 43

Reinhold, L., Volokita, M., Zenvirth, D., Kaplan, A. 1984. Is HCO_3^- transport in Anabaena a Na^+ symport? Plant Physiol. 76: 1090 - 1092

Sandeaux, R., Sandeaux, J., Gavach, C., Brun, B. 1982. Transport of Na^+ by monensin across bimolecular lipid

membranes. Biochem.Biophys.Acta. 684: 127 - 132

Sandeaux, R., Seta, P., Jeminet, G., Alleaume, M., Gavach, C. 1978. The influence of pH on the conductance of lipid bimolecular membranes in relation to the alkaline ion transport induced by carboxylic carriers Grisorixin, Alborixin and monensin. Biochem.Biophys.Acta. 51: 499 - 508

Shelp B.J., Canvin, D.T. 1984. Evidence for bicarbonate accumulation by Anacystis nidulans. Can.J.Bot. 62: 1398 - 1403

Shiota, S., Yazyu, H., Tsuchiy, T. 1984. Escherichia coli mutants with altered cation recognition by the melibiose carrier. J.Bacteriol. 160: 445 - 447

Shiraiwa, Y., Miyachi, S. 1985. Role of carbonic anhydrase in the photosynthesis of blue-green alga (cyanobacterium) Anabaena variabilis ATCC29413. Plant cell Physiol. 26: 109 - 116

Truesdale, A., Downing, B. 1955. The solubility of oxygen in pure water and sea water. J. Appl. Chem. (London) 5; 53 - 63

Tu, C.,Spiller, H., Wynns, G.C., Silverman, D.N. 1987. Carbonic anhydrase and the uptake of inorganic carbon by

Synechococcus sp. (UTEX 2390). Plant Physiol. 85: 72 - 77

Turpin, D.H., Miller, A.G., Parslow, J.S., Elrifi, I.R., Canvin, D.T. 1985. Predicting the kinetics of dissolved inorganic carbon limited growth from the short-term kineics of photosynthesis in Synechococcus leopoliensis (Cyanophyta). J. Phycol. 21:409 - 418

Volokita, M., Zenvirth, D., Kaplan, A., Reinhold, L. 1984. Nature of the inorganic carbon species actively taken up by the cyanobacterium Anabaena variabilis Plant Physiol. 76: 599 - 602

Yokota, A., Kitaoka, S. 1985. Correct pK value for dissociation constant of carbonic acid lower the reported Km values of Ribulose bisphosphate carboxylase to half. Presentation of a nomograph and an equation for determining the pK values. Biochem.Biophys.Res.Commun. 131: 1075 - 1079