The University of Manitoba

STUDIES ON THE PHYSIOLOGY AND BIOCHEMISTRY OF *CHLAMYDOMONAS SEGNIS* IN SYNCHRONOUS CULTURE

by

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To my Mother and Father,

without whom I would

never have known

The autumn leaves
Fluttering, leaves
Behind sad memories
Of summer's death
Of webs we weave
Entangled in the
Spokes of life
A state of constant
Temporariness
Never ending
Nor beginning
The wheel takes
Another turn
Bringing forth,
The autumn leaves.

Tan Cheng Keat

ABSTRACT

The growth and division of the Chlorophyte, *Chlamydomonas segnis*, were synchronized by an alternating light and dark regime of 12 hours light (11,000 lux) and 12 hours dark under a number of CO_2 concentrations. Two and four daughter cells were produced in air (0.03% CO_2) and 5% CO_2 respectively. In the latter, the 4 daughter cells formed a tetrad stage during the dark period and were not released until exposed to a light interval of at least one hour. The zoospore release in light was enhanced when the cultures were bubbled with carbon dioxide-free air. Regardless of CO_2 concentration, the first mitotic division occurred at approximately the 12th hour of illumination. The second mitotic division was delayed for 4 hours in cells dividing in 1% or 5% CO_2 as compared to those in 0.1% CO_2 cultures, which also produced 4 daughter cells. Provision of 5% CO_2 resulted in the production of zoospores with higher cell mass, RNA, carbohydrate and chlorophyll content than their counterparts formed in cultures supplied with air only.

The cell cycle of *Chlamydomonas segnis* was also followed in continuous light to avoid the inhibitory effect

of darkness on zoospore release. Using zoospores with high initial mass at the start of the cycle in cultures aerated with 5% CO_2, the cells spent a relatively short time in the G_1 (0.38) and a long time in S-phase (0.62). On the other hand, zoospores having low initial mass, exhibited an extended G_1-phase (0.7) and a short S-phase (0.3) in cultures supplied with air.

Photosynthesis, p-benzoquinone Hill reaction as well as the activities of carbonic anhydrase, ribulose diphosphate and phosphoenolpyruvate carboxylases were determined during the interphase in air and 5% CO_2 cultures. The incorporation of $^{14}CO_2$ into the organic acid, amino-acid and sugar fractions was also followed in both cultures. Photosynthetic capacity, photosystem II and carbonic anhydrase activity declined at the end of G_1 in air cultures. Although the G_1 the 5% CO_2 was terminated at the same time as in air cultures by the onset of the S-phase (9th hour of illumination), the period of active photo-synthesis extended into the S-phase for 4 hours.

The relatively high and very low activity of carbonic anhydrase in air and 5% CO_2 cultures, respectively suggested that this enzyme was involved in the regulation of photosynthesis during the growth phase of the normal cell cycle in *Chlamydomonas segnis* aerated with air. During this cycle, the growth phase of *Chlamydomonas segnis* was marked by substantial carbonic anhydrase activity and

photosynthetic capacity. The drop in photosystem II appeared to terminate the growth phase. During the growth phase a period (0 - 4 hours light) could be distinguished in which RNA, amino-acids, and intermediates of the reductive pentose phosphate cycle accumulated. A single step increase in ribulose diphosphate carboxylase activity occurred after the termination of G_1, but prior to the onset of the S-phase. The latter was characterized by less incorporation of $^{14}CO_2$ into amino-acids. This was associated with the accumulation of isocitrate lactone probably via the phosphoenolpyruvate carboxylase reaction. From this observation, it was speculated that the accumulation of isocitrate or its precursors or products may have inhibited photosystem II.

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	(Ethylenedinitrilo) tetraacetic Acid
MES	2-(N-morpholino) ethanesulfonic Acid
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
PEP	Phosphoenolpyruvate
POPOP	1,4-Bis (2-5) phenyloxazoylbenzene
PPO	2,5-Diphenyloxazole
RNA	Ribonucleic Acid
Tris	Tris(hydroxymethyl) aminomethane

INTRODUCTION

It is known that growth of autotrophic green algae is dependent on light, temperature, and available carbon dioxide. Until recently, it has been the custom to grow autotrophic green algae in relatively high concentrations of CO_2 (1 - 5% v/v) to prevent limitations of growth, although 0.5% CO_2 seems to be more than adequate (Steemann-Nielsen, 1955; Myers, 1962). However, concentrations of CO_2 above 0.5% by volume have been used during synchronous cultures of unicellular green algae of the *Chlorella* type (Kuhl and Lorenzen, 1964; Bishop and Senger, 1971; Surzycki, 1971). Such synchronous cultures raise a problem about the relation of their cell cycle to those of most other organisms (Mitchison, 1971). This is because all *Chlorella* systems show an increase not of a factor of two (i.e., one normal cell cycle with one mitotic division) but of four to sixteen which makes it difficult to draw useful comparison between this system and other cycles.

It was thought that the failure to produce the normal cell cycle in unicellular green algae might be due to the use of relatively high CO_2 concentration (1 - 5% by volume), resulting in enhanced growth and subsequent production of more than two daughter cells during the cell cycle. There-

fore, it was felt necessary to conduct some studies on the effects of CO_2 concentrations on the synchrony of growth and cell division in *Chlamydomonas segnis* to see whether low CO_2 concentration (0.03% found in air) may be sufficient to produce a two-fold increase in growth and, hence, one cell division. The results embodied in the present investigations demonstrated that aeration of the cultures with air during synchronization by light and dark regime gave rise to a normal cell cycle of a factor of two. On the other hand, higher concentrations of CO_2 (0.1 - 5% by volume) enhanced growth and increased the number of daughter cells into four. The effects of high CO_2 concentration on the zoospore release, chemical composition of the cell and its capacity to photosynthesize have also been shown. Since the ultimate scope of using synchronous cultures is to characterize the various developmental stages during the cell cycle (Tamiya, 1964 and 1966; Pirson and Lorenzen, 1966; Senger and Bishop, 1969; Mitchison, 1969 and 1971; John *et al.*, 1973) an attempt has been made to follow some of the physiological and biochemical events that may regulate growth during interphase in *Chlamydomonas segnis* grown in air or 5% CO_2.

LITERATURE REVIEW

I. EFFECTS OF CARBON DIOXIDE ON SOME PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS IN ALGAE

Besides its role as a source of carbon for autotrophic growth of algae, carbon dioxide has been shown to influence various physiological and biochemical processes. The effects of carbon dioxide on algal growth and metabolism are summarized in the following.

A. pH of the Nutrient Media

Changes in the pH may affect the performance of the growing algae. It has been shown that the aeration of algal media causes a decrease in the pH of the media (Steemann-Nielsen, 1955; Galloway and Krauss, 1961; Sorokin, 1971). The rate of decrease is enhanced by increasing the carbon dioxide concentration of the aerating gas (Galloway and Krauss, 1961). In addition, the light-induced increase in pH of the medium depended on the availability and concentration of carbon dioxide (Neumann and Levine, 1971; Atkins and Graham, 1971). This increase was greater in cells previously cultured in air than in 5% CO_2 (Graham *et al.*, 1971).

B. Growth

It has been the bone of contention as to the effective concentration of carbon dioxide required to support growth of autotrophic cultures of algae. Most investigators use 1 - 5% CO_2, although 0.5% CO_2 is more than adequate (Myers, 1962). Steemann Nielsen (1955), using very dilute suspension of *Chlorella pyrenoidosa*, showed that the rate of growth was independent of the CO_2 concentration from 0.15% down to 0.01 - 0.03%. He and Willemoes (1966) further observed in *Chlorella vulgaris* that there was no difference in the growth rate, size of autospores and number of autospores formed within the CO_2 range of 0.03% to 12%. However, in *Chlorella pyrenoidosa*, the initial rate in air was greater than those grown in 5% or 12% CO_2, the same growth rate being obtained after 24 hours adaptation. The size of the autospores produced at 5% and 12% CO_2 were the same, but smaller than that produced in air grown cells; the number of autospores produced being greater in 5 and 12% CO_2 (n=16) than air grown cells (n=8). Cooksey (1971) reported that the growth rate of *Chlorella pyrenoidosa* was the same in air and 5% CO_2 while in *Chlamydomonas reinhardii*, the rate of growth was faster in CO_2-enriched air, the doubling time being reduced by a factor of two. Using synchronous cultures of *Chlamydomonas segnis*, Badour *et al.* (1971a, 1973)

observed no difference in the number of zoospores produced at 0.1% and 5% CO_2 (n=4) while in air grown cells the number was reduced (n=2). The body length of daughter cells was essentially the same in all concentrations of carbon dioxide (Badour *et al.*, 1973). The toxic effect of carbon dioxide of more than 1% CO_2 on plants has been reported (Rabinowitch, 1951; Steemann Nielsen, 1955). However, recently it was observed in the unicellular hot spring acidophillic alga *Cyanidium caldarium* that during the 25 days of growth, the growth rate in 100% CO_2 was very much greater than that grown in air, although initially (3 days adaptation), the former growth rate was less than the latter (Seckbach and Baker, 1970).

C. Synchronization of Algae

Senger (1961, 1962) using 1.5% CO_2 showed that CO_2 enrichment is an essential requirement for obtaining complete synchrony of autotrophic cultures of *Chlorella*. He showed that cells of *Chlorella pyrenoidosa* could not be synchronized in continuous light by 16+CO_2:12-CO_2 hour changes. The deleterious effect of comparatively low concentrations of CO_2 have been detected in synchronous cultures of *Chlorella fusca* (Soeder *et al.*, 1964). However, synchronization of *Euglena* could be achieved in air using the 12:12 hour light-dark regime (Edmunds,

1965; Codd and Merrett, 1971) and group synchrony in air for the green alga *Chlorella pyrenoidosa* (Wanka, 1959). Recently, it was reported that synchronization of *Chlamydomonas reinhardii* Dangeard WT^+ was achieved in air (Cechacek and Hillova, 1970). Badour *et al.* (1971a, 1973) observed in *Chlamydomonas segnis* that synchronization could be achieved in air, 0.1% and 5% CO_2 using the 12:12 hour light-dark regime. The synchronizing effect of carbon dioxide, using air:50% CO_2 changes, on cell division in the protozoan *Tetrahymena* has been demonstrated (Hjelm, 1971).

D. Cell Division and Release of Daughter Cells

Sorokin (1962a) reported a total inhibition of cell division in the high temperature strain of *Chlorella sorokiana* in the dark by as little as 1% CO_2 when the cells were suspended in distilled water. This inhibitory effect of carbon dioxide was counteracted by resuspension of the cells in bicarbonate buffer (Sorokin, 1962b, 1964). A permanent delay of 2 hours in the liberation of autospores by high CO_2 tension was detected in some strains of *Chlorella* suspended in nutrient media (Soeder *et al.*, 1964, 1966). However, Senger (1962) could not find this effect. The delay in autospore release at high CO_2 tension was enhanced by light and was counteracted by low CO_2 concentration (Soeder *et al.*,

1966). Badour and Waygood (1971a) observed a 4 hour delay in the generation time of *Chlamydomonas segnis* (previously referred to as *Gloeomonas*) when the cells were grown in 5% CO_2 as compared to 0.1% CO_2.

E. Cell Composition

During the synchronous growth of *Chlamydomonas segnis*, Badour and Waygood (1971a) found that the formation of protein and carbohydrate was greater with 5% CO_2 than with 0.1% CO_2. Formation and accumulation of starch and sugars in tomato leaves was enhanced when the concentration of CO_2 provided during growth was increased from 0.035% to 0.15% (Madsen, 1968, 1971). Similar findings were shown in *Chlorella pyrenoidosa* (Steemann Nielsen and Willemoes, 1966) when the cells were provided with 5% CO_2 instead of air during growth. Increases in CO_2 concentration from 0% to 0.03% to 3% resulted in dramatic increases in both the total level of protein and the incorporation of radioactive leucine into protein in the etiolated coleoptiles of *Avena sativa* (Bown and Lampman, 1972). Total pigment in the blue-green alga *Anacystis nidulans* constituted 20.5% of the dry weight when grown in 1% CO_2 while with air grown cells it was only 11.1% of the dry weight (Eley, 1971). Ullrich (1972) showed that labelling of polyphosphates by ^{32}P was inhibited by the presence of CO_2-enriched air

in *Ankistrodesmus braunii*, while ^{32}P labelling in organic phosphates was enhanced. The reverse effect was observed in oxygen (CO_2-free air). Labelling of nucleic acids was indifferent to both oxygen and CO_2.

F. Dark Respiration and Related Processes

Respiration rates of *Chlorella pyrenoidosa* and *Chlorella vulgaris* cultured in air were found to be twice as high in CO_2-bicarbonate buffer containing 0.04 mM/l free CO_2 than at 1.68 and 3.96 mM/l of free CO_2 (Steemann Nielsen and Willemoes, 1966). In cells grown in 5% CO_2 no conclusive results were obtained. However, Soeder *et al.* (1964) observed that respiration in *Chlorella* sp. was inhibited by high CO_2 tension. The suppression of respiration by high CO_2 in numerous higher plants has been shown (Kidd, 1915; Thomas, 1925). In *Ricinis* sp. mitochondria oxidation of succinate to fumarate was retarded by CO_2-bicarbonate mixtures containing more than 10% CO_2 (Bonner, 1950; Bendall *et al.*, 1958). Recently, Shipway and Bramlage (1973) showed that in mitochondrial preparations of *Malus pumila* malate oxidation was stimulated, while oxidations of citrate, α-ketoglutarate, fumarate, pyruvate, succinate and NADH were suppressed at CO_2-bicarbonate mixtures containing 18% CO_2 as compared to 3% CO_2. In addition, high concentration of CO_2 was found to inhibit NAD-cytochrome

c-reductase system, cytochrome c-oxidase system and oxidative phosphorylation in many plant species (Bendall *et al.*, 1960; Miller and Evans, 1956; Miller and Hsu, 1965).

G. Photosynthesis and Photosynthetic Reactions

1. Photosynthetic CO_2 Uptake and O_2-evolution

Photosynthesis in *Chlorella* cells was found to increase with increasing concentrations of free CO_2 reaching a saturation point of approximately 0.01 - 0.03% CO_2 (Whittingham, 1952; Steemann Nielsen, 1955; Steemann Nielsen and Jensen, 1958). Further increase in the amount of free CO_2 above 1% resulted in the decrease in photosynthesis (Steemann Nielsen, 1955; Steeman Nielsen and Willemoes, 1966). In addition, the previous history of the cells also determines the ability of the cells to photosynthesize. *Chlorella* cells previously grown in air and allowed to photosynthesize in either high or low CO_2 showed a short induction phase (Briggs and Whittingham, 1952; Graham and Whittingham, 1968; Graham *et al.*, 1971). When grown in 4 or 5% CO_2, the cells showed a short induction phase only when they photosynthesize in buffer of higher CO_2 concentration, and a prolonged induction phase in buffer of low CO_2 concentration. The inhibitory effect of oxygen on photosynthesis in

Chlorella, *Chlamydomonas* and *Euglena* was shown to be partially overcome by increasing the HCO_3^- or CO_2 concentrations (Bowes and Berry, 1971; Fock *et al.*, 1971; Ellyard and Pietro, 1969).

2. Hill-reaction

The dependency on CO_2 or HCO_3^- for the Hill-reaction has been reported by a number of investigators (Warburg and Krippahl, 1960a; Stern and Vennesland, 1960; Abeles *et al.*, 1961; Stemler and Govinjee, 1973). Heise and Gaffron (1963) stated that this dependency should be viewed with caution as CO_2 has a general catalytic effect.

3. Related Processes

Badour (1959, 1961) found that aeration with 1.5% CO_2 in air enhanced the uptake of phosphate in continuously illuminated cultures of *Chlorella vulgaris* as compared to aeration with CO_2-free air. Cultures maintained for 4 - 12 hours in light and bubbled with CO_2-free air, accumulated polyphosphates but not if CO_2 was provided. Recently, it has been shown that non-cyclic photophosphorylation is dependent on the presence of CO_2 in *Ankistrodesmus* and *Chlorella* species, while cyclic photophosphorylation is relatively impartial to the presence or absence of CO_2 (Gimmler *et al.*, 1971; Glagoleva *et al.*, 1972; Ullrich, 1972; Klob *et al.*, 1973).

In short term experiments with *Chlorella fusca*, ATP level was shown to increase to a maximum within 20 seconds of deprival of CO_2 (Lewenstein and Bachofen, 1972). In several oscillations, the ATP level dropped back to the original level in approximately 3 minutes reflecting the simultaneous action of the different control systems for ATP production, consumption and translocation reactions located in the different compartments of the eucaryote cell. A recent report showed that in *Chlorella pyrenoidosa* the fluorescence yield is strongly affected by CO_2 concentration; the yield being approximately two-fold higher in the presence of CO_2 than in its absence (Slovacek and Bannister, 1973).

H. Other Physiological and Biochemical Responses

1. Photosynthetic Pathways

Bassham and Calvin and their colleagues (1960) have shown that in *Chlorella* species grown and allowed to photosynthesize in relatively high CO_2 concentrations (1 - 5%), the major pathway of photosynthesis occurs via the reductive pentose phosphate cycle. Several observers (Rabson *et al.*, 1962; Pritchard *et al.*, 1962, 1963; Whittingham and Pritchard, 1963; Ahmed and Ries, 1969) have shown that the photosynthetic intermediary products at low

CO_2 concentrations were quantitatively different from that observed at high CO_2 concentration. Graham and Whittingham (1968) observed that in *Chlorella pyrenoidosa* grown in 5% CO_2, the pathway of $^{14}CO_2$ incorporation occurs via β-carboxylation when photosynthesis was carried out in comparatively low CO_2 concentration but via the Calvin-cycle when allowed to photosynthesize in high CO_2 concentration. In air grown cells, the pathway was of the Calvin type irrespective of the CO_2 concentrations used during the photosynthetic experiments.

2. Glycollate Production and Excretion

Algal cells previously grown in air were unable to excrete glycollate, while those grown in high CO_2 (greater than air) exhibited glycollate excretion (Pritchard *et al.*, 1962; Whittingham and Pritchard, 1963; Miller *et al.*1963; Warburg and Krippahl, 1960b). Glycollate formation and excretion were induced at relatively low CO_2 concentration and reached saturation at levels of 0.2% CO_2 in the gaseous phase. Further increase in CO_2 concentration resulted in a dramatic decline in glycollate production (Whittingham and Pritchard, 1963; Bowes and Berry, 1971). Increased light intensity resulted in an increase in glycollate production (Tolbert and Zill, 1956; Whittingham and Pritchard, 1963; Merrett

and Lord, 1973). However, it has been reported that in *Chlamydomonas segnis* and *Chlorella fusca*, glycollate excretion was not observed under conditions known to favour glycollate biosynthesis and excretion (Badour and Waygood, 1971a; Goulding *et al.*, 1969).

3. Excretion of Ketomalonic Acid Semialdehyde

Badour and Waygood (1971a) observed in *Chlamydomonas segnis* that excretion of ketomalonic acid semialdehyde was enhanced in cells grown in 5% CO_2 as compared to cells grown in 0.1% CO_2. The excretion occurred at high light intensity and was limited to the phases of cellular division and zoospores release.

I. Enzymes

1. Catalase

Activity of catalase in microbodies was found to be totally absent in acetate bleached cells and during the process of greening of *Euglena* when the medium was aerated with CO_2-free air instead of air (Brody and White, 1972).

2. Nitrate Reductase

The activity of nitrate reductase in *Chlamydomonas reinhardi* in autotrophic cultures was shown to decline when the cultures were aerated with

CO_2-free air instead of air supplemented with 0.5% CO_2 (Thacker and Syrett, 1972). Similar findings were shown for the higher plant *Perilla* when CO_2 was deprived (Kannangara and Woolhouse, 1967).

3. Isocitrate Lyase

The presence of isocitrate lyase activity in *Chlamydomonas segnis* grown in 5% CO_2 was demonstrated by Badour and Waygood (1971b), whereas in 0.1% CO_2 isocitrate lyase was totally absent.

4. Glycollate Dehydrogenase

The specific activity of glycollate dehydrogenase was found to be regulated by the CO_2 concentration in *Chlamydomonas reinhardtii* Dangeard (-) (No. 90) and *Euglena gracilis* Z Klebs (Nelson and Tolbert, 1969; Codd *et al.*, 1969). Cells grown in high CO_2 (1 - 5% by vol.) exhibited repressed enzyme activity, while growth in air derepressed the enzyme. In *Chlorella fusca* (previously *Chlorella pyrenoidosa* 211/8P) however, the activity was relatively unaffected by the CO_2 concentration provided during growth (Codd *et al.*, 1969). Cooksey (1971) observed in *Chlamydomonas reinhardtii* 137c mt+ that the enzyme was synthesized initially in 5% CO_2 in air at similar rate to that in air only. The enzyme disappeared from the cell only under nitrogen limitation. Lord and Merrett (1970a) showed that the

derepression of glycollate dehydrogenase in *Chlamydomonas mundana* previously grown in acetate, by transference to inorganic media occurs at equal rates irrespective of the CO_2 concentration (5% or air) used in the aerating gas.

5. Calvin Cycle and β-Carboxylation Enzymes

RuDP-carboxylase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1, 6-biphosphatase, aldolase, phosphoriboisomerase, ribulose-5-phosphate kinase and PEP-carboxylase of air and 5% CO_2 grown cells of *Chlorella*, and during the period of adaptation to low CO_2 showed little difference in the activities of these enzymes or in the amount of Fraction I protein (Reed and Graham, 1968).

6. Carbonic Anhydrase

Growth in high CO_2 concentrations in C_3-plants and in the algae *Chlorella*, *Chlamydomonas* and *Euglena* resulted in the repression of carbonic anhydrase activity, while growth in air derepress the activity (Graham *et al.*, 1971; Nelson *et al.*, 1969; Cervigni *et al.*, 1971). In C_4-plants, the activity of carbonic anhydrase was enhanced when higher concentrations of CO_2 than air were provided during growth (Cervigni *et al.*, 1971).

MATERIALS AND METHODS

The Alga

Chlamydomonas segnis Ettl (Ettl, 1965; Badour *et al.*, 1973) previously referred to as *Gloeomonas* sp. (Badour and Waygood, 1971a) was used as the experimental organism. Stock cultures of this alga were maintained on agar slants composed of the mineral nutrient medium with 2% agar, at 25° and in dim light.

The Nutrient Medium

The mineral nutrient medium of Kuhl and Lorenzen (1964), after being enriched with phosphate, was used for the cultivation of *Chlamydomonas segnis*. It consists of the following.

		mg/l	moles/l
1.	KNO_3	1010.0	1.0×10^{-2}
2.	$MgSO_4.7H_2O$	246.5	1.0×10^{-3}
3.	$CaCl_2$	11.1	1.0×10^{-4}
4.	$NaH_2PO_4.1H_2O$	517.0	3.7×10^{-3}
5.	K_2HPO_4	780.0	4.5×10^{-3}
6.	$FeSO_4.7H_2O$	3.8	1.4×10^{-5}
7.	EDTA (Trisodium)	9.3	2.5×10^{-5}
8.	H_3BO_3	0.093	1.3×10^{-6}

9.	$ZnSO_4.7H_2O$	0.100	3.5×10^{-7}
10.	$MnSO_4.4H_2O$	0.020	1.2×10^{-7}
11.	$CuSO_4$	0.005	4.5×10^{-8}
12.	$CoCl_2.6H_2O$	0.008	3.4×10^{-8}
13.	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.006	4.9×10^{-9}

The pH of the nutrient medium after autoclaving was 6.9.

Aeration of the Cultures

The aeration of the cultures with appropriate volumes of CO_2 in air or air only was maintained by passing compressed air and CO_2 through a flowmeter (Matheson). The outflowing gas mixture or air only (1000 cc/min) was then passed through 1% $CuSO_4$ solution, sterile cotton filter, sterile distilled water and through a sterile four-arm manifold connected to low-form Fernbach culture flasks. CO_2-free air was obtained by passing the outflowing air through 40% KOH twice and 10% $Ba(OH)_2$.

Preparation of Synchronous Cultures

For the synchronization of the algal cultures, the method of Pirson and Lorenzen (1966) was used. An inoculum was aseptically introduced from the slants into 100-150 mls each of the sterilized nutrient medium in 250 ml Erlenmyer flasks. The flasks were placed in a controlled environment chamber at 25°. Illumination from the top of the chamber by 'cool white' fluorescent lamps provided an intensity of 11,000 lux at approximately 3.0 cm from the bottom of the

flasks. The inocula were then allowed to grow and multiply for 3 days in continuous light and bubbled with the appropriate volumes of CO_2. From these precultures, the algal suspensions were diluted with fresh medium to a cell concentration of 1×10^6 cells per ml. One thousand mls of each of the diluted algal suspension were transferred into 2500 ml low-form Fernbach culture flasks. The cultures were then subjected to alternating periods of 12 hrs light and 12 hrs darkness combined with dilution of the algal suspension to a constant cell number of 10^6 cells per ml at the end of the dark period. After 3 - 4 cycles of 12:12 light-dark regimen followed by dilution, synchronized cultures were obtained for the experiments.

Cell Number

Cell counts were obtained using an Haemocytometer after fixing the cell in iodine solution.

Body Length and Flagellum Length

After fixing the cells in 0.1% OsO_4, the body and flagellum length were measured using a calibrated occular micrometer.

Chlorophyll

Total chlorophyll per ml algal suspension was determined according to the method of Holden (1965). To the pellet obtained by centrifugation of 10 mls of algal

suspension were added 10 mg $MgCO_3$ and 8 ml of 95% methanol. After heating the mixture in a water-bath at 70°C for 15 minutes, followed by centrifugation, the supernatant was cooled, made up to 10 ml with 95% methanol and measured at 650 nm and 665 nm against methanol using a Zeiss spectrophotometer. Total chlorophyll per ml algal suspension was determined from the following equation.

$$\text{Total Chlorophyll (mg/l)} = 25.5 \times OD_{650} + 4 \times OD_{665}$$

Carbohydrate

Total carbohydrate was determined by the anthrone method (Roe, 1955). To 1 ml of cell suspension, twice washed with distilled water, was added 4 ml of anthrone reagent. 1 ml of algal suspension and 4 ml of 75% H_2SO_4 served as a blank. After heating at 100° in a water-bath for 15 minutes, the blue-green color was measured against the blank at 620 nm using a Zeiss spectrophotometer. The total carbohydrates were expressed as micrograms glucose from a standard calibration curve made for glucose.

Protein

To 2 ml of twice washed with distilled water algal suspension was added 2 ml of 2 N NaOH and heated for 30 minutes in a water-bath at 100°. 0.5 ml of the cooled extract was used for the colorimetric determination of protein according to Lowry *et al.* (1951). Total protein in

micrograms per ml of algal suspension was calculated from a graph prepared for Bovine serum albumin.

Dry Weight

50 ml of the algal suspension were washed by centrifugation, concentrated to about 2 - 5 ml , quantitatively transferred to an aluminum dish of known weight and then dried overnight at 100°.

Extraction and Determination of Nucleic Acids

150 ml of algal suspension were washed with distilled water. The resultant pellet after centrifugation was extracted twice with 7.0 ml of 0.2 N perchloric acid in 50% ethanol at room temperature for 30 minutes each. The residue was extracted three times for 10 minutes each at 70° with 6.0 ml of ethanol:ether (3:1 v/v) mixture and washed with 6.0 ml of absolute ethanol. The resultant residue was finally extracted with 5.0 ml of 0.5 N perchloric acid at 45° for 6 hours, thereafter centrifuged and the resulting supernatant was used to determine total nucleic acids and DNA according to Senger (1965) and Burton (1956). For the determination of total nucleic acids, 3.0 ml of the final extract was placed in a cuvette with a 1 cm light path and read in the Zeiss spectrophotometer at the indicated wavelengths.

$$\text{Total Nucleic Acids} = OD_{268.5} - OD_{320}$$

For DNA determination, 2.0 mls of Diphenylamine Acetaldehyde reagent were added to 1.0 ml of the final extract and incubated for 18 hours at 30°. The resultant blue color was read at 595 nm and 650 mn.

$$\text{Total DNA} = OD_{595} - OD_{650}$$

RNA was obtained by subtracting total DNA from Total Nucleic Acids. The data were expressed as micrograms per ml algal suspension using the calibration curve of Senger (1965).

Determination of Photosynthetic O_2 Evolution by Warburg Apparatus

80 mls of algal suspension were washed with distilled water, and then rewashed in Warburg No:9 buffer (Stauffer, 1957) which consisted of a mixture of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ (15:85 v/v, respectively), pH 9.1. After concentrating the algal suspension to a total volume of 8 mls, 2.0 mls each were used to measure the oxygen evolution at 25° with a Gilson Differential Respirometer using a light intensity of approximately 12,000 lux. Two flasks were kept in the light and one in the dark (wrapped with aluminum foil). After equilibration for 15 minutes, readings were taken every 10 minutes for 30 minutes. Oxygen evolved was linear with time and the respiratory oxygen uptake was negligible or absent in this buffer.

Determination of Photosynthetic O_2 Evolution, Respiration and Benzoquinone Hill Reaction by Clark Oxygen Electrode

100 mls of algal cultures were centrifuged and washed once with distilled water. After being rewashed and resuspended in a total volume of 10 mls of 0.05 M phosphate buffer, pH 7.0, the oxygen evolved and uptake were measured with a Clark Oxygen Electrode. Temperature was maintained at 25° by a cooling jacket surrounding the reaction vessel. A convex cone of light from a Koehler illuminator (Baker's, London, England) provided approximately 12,000 lux at the surface of the reaction vessel.

(i) Photosynthetic O_2 Evolution and Respiration

The reaction vessel contained 4 mls of the cell suspension and 10 µl of $NaHCO_3$ (final concentration 5×10^{-3} M). After equilibration at room light, the illuminator was switched on and photosynthetic oxygen evolution was measured for 3 minutes. This was followed by 3 minutes of darkness to measure respiration. Photosynthetic evolution of oxygen was corrected for the respiratory uptake.

(ii) Benzoquinone Hill Reaction

The reaction vessel contained 4 mls algal suspension 10 µl $NaHCO_3$ (final concentration 5 mM) and 10 µl of resublimated benzoquinone (2 mg). After 3 minutes equilibration in darkness, the algal cells were illuminated and oxygen evolved was measured for 3

minutes. This was followed by 3 minutes of darkness to measure oxygen uptake. No respiratory uptake of oxygen was noticed either during the equilibration period or during the period subsequent to the measurement of oxygen evolved in the light.

The oxygen exchange was linear with time and calculations were based on the solubility of oxygen in water (Hodgman *et al.*, 1962, 1963).

Photosynthetic $NaH^{14}CO_3$ Fixation and Extraction of Radioactivity

After washing the algal suspension, the cells were resuspended in 0.05 M phosphate buffer, pH 7.0 and made to a 10 times concentrated suspension. 2.0 mls of the latter suspension was pipetted into the main compartment of the manometer flasks, and equilibrated for 15 minutes on the Gilson Differential Respirometer at 25° and 12,000 lux with a constant shaking rate of 100 oscillation per minute. 10 µCi of $NaH^{14}CO_3$ (s.a. 59 mCi/mM, Amersham/Searle, England) were added to the contents of each flask. After incubation for different time period, the cells were killed by the addition of 0.5 ml of glacial acetic acid and quantitatively transferred to 15 ml glass centrifuge tube. The cells were dried by aeration and then frozen for later analysis.

The frozen samples were extracted once with 5 mls portions of 80%, 50%, 20% of ethanol and twice with water at

75° for 20 minutes each. A glass marble placed on top of each test tube prevented excessive evaporation. After each alcoholic-water extraction, the samples were centrifuged at 10,000 rpm for 10 minutes, and the residue was quantitatively transferred to a scintillation vial. The supernatants from each extraction were pooled together and reduced in volume by aeration at 40° and the lipid substances were removed from this fraction by extracting 4 times with an equal volume of chloroform. The chloroform-soluble fraction was washed twice with 1 ml aliquots of distilled water, and was pooled together with the residue fraction, dried down and resuspended in 0.5 ml of 20% ethanol. Their radioactivity was determined.

The ethanol-water soluble fractions (free from lipids) were pooled, dried down by aeration at 40° and redissolved in 1.0 ml of distilled water. 0.2 ml aliquot was taken and determined for radioactivity. The rest of the ethanol-water fraction was then fractionated by ion exchange chromatography.

Ion Exchange Chromatography of Ethanol-Water Soluble Fraction

This fraction was separated into the following three main fractions as described by Canvin and Beevers (1961), and Cossins and Beevers (1963).

(i) Basic compounds, largely amino-acids;

(ii) Acidic compounds, mainly organic acids and sugar phosphates;

(iii) Neutral compounds, mainly sugars.

The following two types of resins (Bio-Rad Labs.; Richmond, California) were employed:

(i) Cation exchange resin, Dowex 50W-8 (hydrogen form, 200 - 400 mesh)

(ii) Anion exchange resin, Ag 1-x10 (chloride form, 200 - 400 mesh)

Both types of resins were prepared in bulk according to Atkins and Canvin (1971). Each resin was then slurried into a large column plugged at the base with glass wool. The cation exchange resin, Dowex 50W-8 (H^+; 200 - 400 mesh) was treated with 10 ml of 2 N HCl per 5 ml resin and then washed with distilled water until the effluent was close to neutral. The anion exchange resin, Ag 1-x10 (Cl^-; 200 - 400 mesh) was converted to the formate form by treatment with 1 M sodium formate until the effluent gave a negative test for chlorides. The resin was washed with 0.1 N formic acid (50 ml/5 ml resin), followed by distilled water until the effluent was close to neutral.

6 x 1 cm ion exchange columns were prepared by pouring a slurry of the above resins into 13 x 1 cm glass columns plugged at the base with glass wool. The columns were rinsed with 2 bed volumes of distilled water before use.

Fractionation of the Ethanol-water Soluble Extract

The ethanol-water soluble extract was loaded on to the Dowex 50W-X8-H^+ mini columns and flushed with 80 mls of distilled water. The effluent was concentrated and designated as the organic acid-sugar fraction. The amino-acids were then eluted with 80 mls of 2 N ammonium hydroxide followed by 20 mls of 3 N ammonium hydroxide. The effluent was evaporated to dryness.

The organic acid-sugar fraction was quantitatively transferred to the Ag 1-X10-$HCOO^-$ column and rinsed with 100 mls of distilled water. The effluent containing mainly sugars was collected and dried down. The organic acids and sugar phosphates retained by this column were eluted with 80 mls of 4 N formic acid, followed by 20 mls of 6 N formic acid. The effluent was dried down.

All three main fractions were redissolved in 1.0 ml distilled water for radioactivity determination and thin-layer chromatography.

Measurement of Radioactivity

All samples were determined for radioactivity by means of the Picker Nuclear Liquimat Scintillation Counter. Samples were corrected for quenching by using a quench curve prepared with picric acid and ^{14}C-toluene.

Aliquots of the aqueous samples obtained were placed in scintillation vials and made up to 0.5 ml with 20%

ethanol. 10 mls of scintillation cocktail (0.03 g POPOP, 7.0 g PPO and 100 g napthalene dissolved in a total volume of 1 litre of p-dioxane) was added to each sample and measured for radioactivity.

Thin-layer Chromatography (TLC)

The cellulose powder MN300 (Machery, Nagel and Co.) was prepared as described by Cook and Bieleski (1969). 15 gm of washed, dried cellulose was mixed with 90 ml water and homogenized at 20,000 rpm for 1 minute, left 30 seconds and homogenized again for 1 minute. The slurry was then allowed to sit for 1½ minutes before spreading with a Desaga spreader (Desaga, Heidelberg, West Germany). 20 x 20 cm plates of 250 microns thickness of cellulose were prepared and allowed to age for one week after spreading to ensure stability. Where possible, an aliquot containing approximately 5,000 - 10,000 dpm was spotted in a 2.5 cm band, 2.0 cm from the edges of the plate. To ensure uniformity of run, plates were run in sets of five as they were made, the first direction run being against the direction of spreading. Prior to thin-layer chromatography in the second direction, the bands on the plate were condensed to spots by eluting them 6 times with 1% acetic acid for the amino-acid and organic acid plates. The sugar plates were eluted with water. At least 4 sets of plates were chromatographed for each sample, two of which were

determined for radioactivity, while the radioactive spots were eluted from the other two plates and cochromatographed with the authentic cold chemicals using the same solvent systems. A plate consisting of cold authentic compounds only was always run together with the sample plates. The plates were run for 15 cm from the origin in each direction. To aid drying and evaporation of the solvents, the plates were dried in an oven at 40° for 20 minutes after each direction run.

Separation of Amino-acid and Organic Acid Fractions

Two dimensional TLC was used to separate the various products using the solvent system described by Bieleski and Young (1963). The first direction run was achieved in 100 ml of n-propanol:ammonium hydroxide:water (6:3:1 v/v). The second solvent system consisted of 140 ml of n-propyl acetate:formic acid:water (11:5:3 v/v). After drying, the approximate glycollic acid area in the organic acid plates were sprayed with 0.1 M Na_2CO_3 solution in 50% ethanol to prevent sublimation.

Separation of Sugars

The sugars were separated by two dimensional TLC in 100 ml of n-propanol:water:n-propyl acetate:acetic acid: pyridine (120:60:20:4:1 v/v) in the first direction and 140 ml of n-butanol:acetic acid:water (12:5:3 v/v) for the second direction as described by Cook and Bieleski

(1969).

Detection of Compounds and Measurement of Radioactivity

Radioautograms were made by exposing the dried plates to "Kodak Medical X-ray Film, No-screen" from Eastman Company, Rochester, New York. After an appropriate period (1 to 2 weeks) of exposure, the films were developed to locate the radioactive spots. The spots were scraped off as a roll and quantitatively transferred to scintillation vials for radioactivity determination or to a pasteur pipette plugged at the tip with cotton wool for elution and subsequent cochromatography. Radioactivity determination was carried out as previously described. After radio-autographic exposure, 1 sample plate, cochromatographed plates and the reference plates were sprayed with the appropriate reagents (Smith, 1960; Mezzetti *et al.*, 1972).

(i) Amino-acids were detected by a solution of 0.5% ninhydrin in acetone;

(ii) Organic acids by bromocresol green (0.4% solution in 90% ethanol) or aniline-xylose (1 ml aniline, 1 g xylose in 100 ml ethanol);

(iii) Sugar phosphates by ammonium molybdate (25 ml 4% ammonium molybdate, 5 ml 60% perchloric acid 10 ml 1 N HCl and 60 ml water);

(iv) Sugars by a solution of nathoresorcinol (20 mg napthoresorcinol, 10 ml ethanol and 0.5 ml

concentrated H_2SO_4).

Identification of Isocitrate Lactone

The radioactive spot thought to be isocitrate lactone was eluted and converted to isocitric acid by alkali hydrolysis and then to glyoxylate and succinate by isocitrate lyase. Isocitrate lactone (approximately 20,000 cpm) was hydrolyzed to isocitric acid in the presence of 0.2 ml 1 N NaOH in a boiling bath for 15 minutes (Deutsch and Phillips, 1957). The sample was cooled, adjusted to pH 7.2 and reduced in volume. To this sample was added in a total volume of 3.0 ml the following in μmoles according to Foo *et al.* (1971): MES buffer, pH 7.2, 100; $MgCl_2$, 7.5; glutathione (reduced), 2; DL-isocitrate (trisodium salt), 2 and 0.2 ml of isocitrate lyase (EC 4.1.3.1) purified from *Chlamydomonas segnis* by Mr. S.K. Foo. After 90 minutes incubation at 30°, the glyoxylate produced from the authentic isocitrate and radioactive sample was determined as the 2,4-dinitrophenylhydrazone derivatives. The latter was extracted from the aqueous phase by ethyl acetate. The resulting yellowish extract was then rinsed twice with distilled water, reduced in volume and chromatographed on a cellulose thin layer plate using tertiary amyl alcohol:ethanol:water (5:1:4 v/v) as solvent. After exposure to X-ray film, the plate was sprayed with an ethanolic NaOH solution to produce the characteristic brick-

red color of glyoxylate dinitrophenylhyrazone derivative. The location of the two colored spots of glyoxylate perfectly superimposed the locations of the radioactivity on the radioautogram. A control run using boiled enzyme did not result in gyloxylate formation nor radioactivity in the radioautogram. This, together with the fact that the product formed after alkali hydrolysis of the suspected radioactive isocitrate lactone was identical to the position of authentic isocitrate, provided evidence that the unknown acid was probably isocitrate lactone.

Enzyme Preparation

After washing 500 ml of algal suspension with distilled water, the cell pellet was resuspended in 10 ml of 0.05 M Tris-HCl buffer, pH 8.0 containing 1 mM EDTA and 5 mM 2-mercaptoethanol. The latter suspension was sonicated in an ice bucket and then centrifuged. To the resulting supernatant, crystalline ammonium sulphate was added to give a 60% saturation and the mixture was allowed to precipitate overnight. After centrifugation, the residue was redissolved in a total volume of 5 ml of the same buffer but without EDTA. The supernatant obtained after centrifugation was used as the crude enzyme. All the above procedures were performed at 5° and the centrifugation was carried out at 17,500 rpm for 30 minutes. Protein was determined by the method of Lowry *et al.* (1951).

Enzyme Assay

Ribulose 1,5-diphosphate carboxylase (EC4.1.1.f) was assayed according to the method of Paulsen and Lane (1966). The incubation mixture contained in μmoles: RuDP (tetrasodium salt), 0.35; $NaH^{14}CO_3$, 25 (known dpm, >5 x 10^6 dpm); glutathione (reduced), 3; EDTA, 0.03; $MgCl_2$, 5; Tris-HCl, pH 7.8, 100; and crude enzyme (100 μg of protein) in a total volume of 0.5 ml. The reaction mixture was preincubated at 30° for 10 minutes. The reaction was initiated by the addition of RuDP. Control samples were without RuDP. The reaction was terminated after 15 minutes by the addition of 1 ml of 2 N HCl. The sample was then centrifuged and 0.5 ml of the supernatant was taken into a scintillation vial, dried down and redissolved in 0.5 ml distilled water for determination of radioactivity as previously described.

Phosphoenolpyruvate carboxylase (EC4.1.1.31) The assay procedures and incubation mixture were essentially the same as that used in the assay of ribulose diphosphate carboxylase. Instead of RuDP, the following were added in μmoles: PEP (trisodium salt), 2; NADH, 0.8; and malate dehydrogenase (EC1.1.1.37), 1 i.u. and crude enzyme (400 μg of protein).

For both RuDP and PEP carboxylases, the activities were found to be dependent on protein concentration and linear with time for at least 30 minutes (Figure 1).

0-representable, 1-representable), then Q is strongly representable (resp. 0-representable, 1-representable).

Proof. Using the same arguments given in the proof of Theorem 1.17 and by Remark 2.

Corollary 1.19. Let P be a representable poset. Then

(i) For $a_1, a_2, \ldots, a_n, b_1, b_2, \ldots, b_m \in P, n, m \geq 1$, $\bigcup_{i=1}^{n}[a_i) \cup \bigcup_{j=1}^{n}(b_j]$ is strongly representable. In particular, if $a \leq b$ in P, $[a,b]$ is strongly representable.

(ii) For any non-empty subset A of P, $\bigcap_{a\in A}(a]$ is 1-representable and $\bigcap_{a\in A}[a)$ is 0-representable.

(iii) Every maximal chain in P is representable.

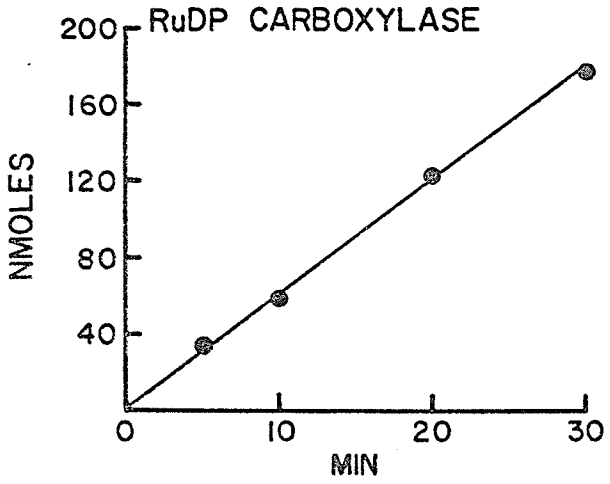
Proof. (i) By Theorem 1.17 and Remark 7.

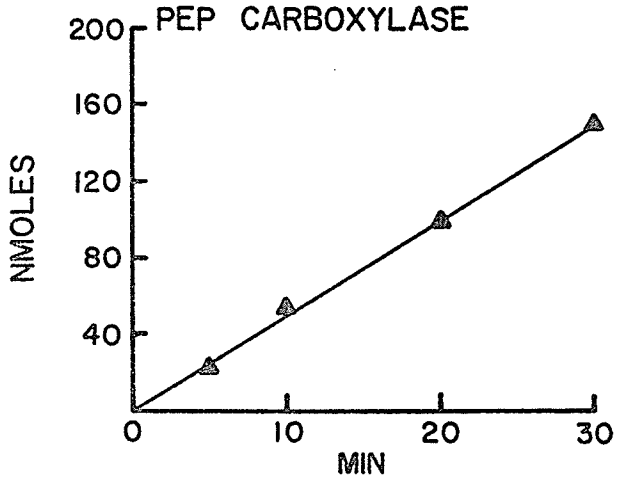
(ii) Let $Q = \bigcap_{a\in A}(a]$. Then there is a family $\{P_i\}_{i\in\Delta}$ of finite posets such that P^* is a subposet of $\prod_{i\in\Delta}P_i$, of the form $\bigcap_{k\in F}[\bigcup_{l\in T_k}A_{kl}]$. So $\bigcap_{k\in F}[\bigcup_{l\in T_k}A_{kl}] \cap \bigcap_{a\in A}(a]$ is order isomorphic to one of $\{Q, Q\cup\{0\}\}$. By Theorem 1.1 and Lemma 1.5, $\bigcap_{k\in F}[\bigcup_{l\in T_k}A_{kl}] \cap \bigcap_{a\in A}(a]$ is strongly representable. Hence by Remark 2, Q is 1-representable in either case. Dually, we can show that $\bigcap_{a\in A}[a)$ is 0-representable.

(iii) Suppose C is a maximal chain in P. Then it is obvious that

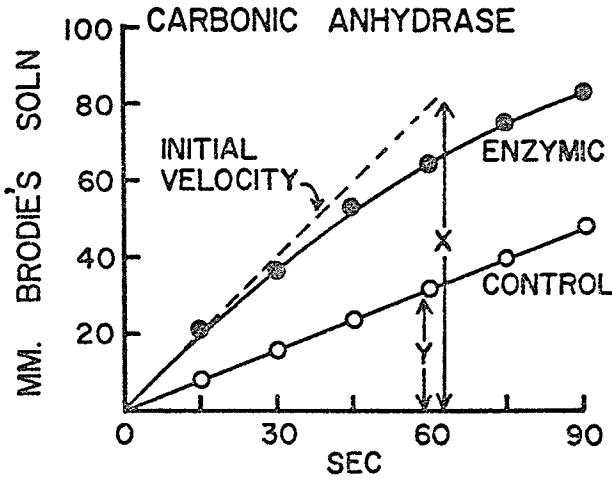
$$C = \bigcap_{q\in C}\left([q) \cup (q]\right).$$

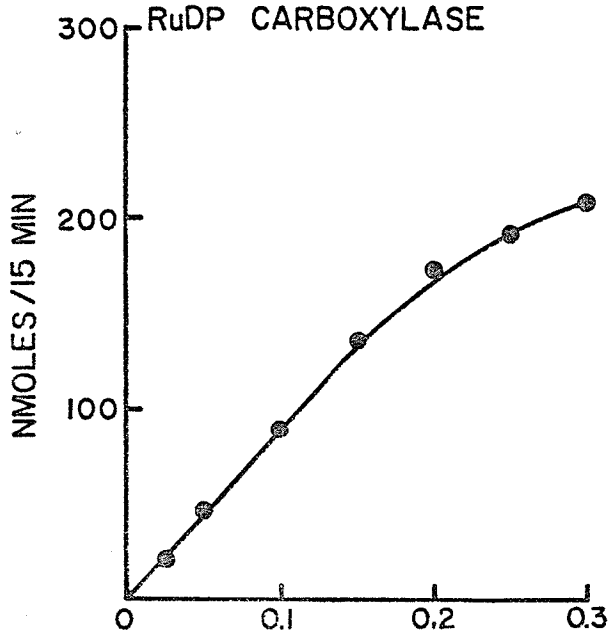
Thus, by Theorem 1.17, C is representable.

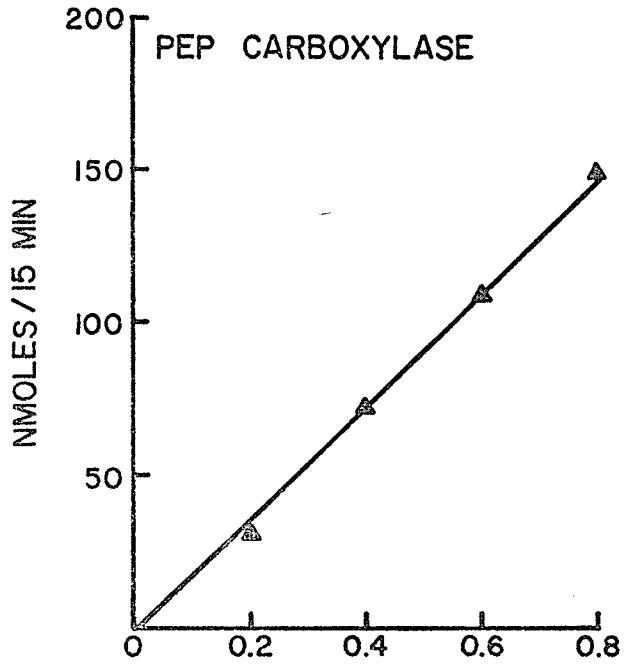




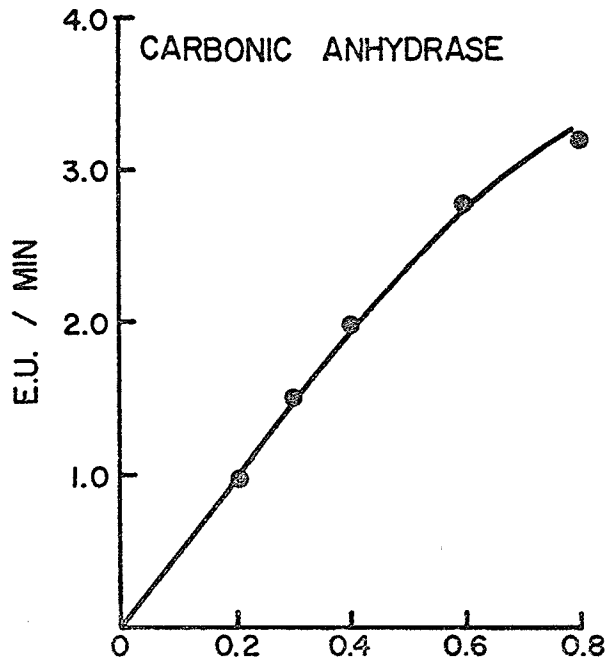
INCUBATION TIME







PROTEIN (MG)



Carbonic anhydrase (EC4.2.1.1) was assayed manometrically at 15° according to Waygood (1955). The main compartment of the Warburg flask contained 1.0 ml of phosphate buffer (0.1 M Na_2HPO_4: 0.1 M KH_2PO_4, 3:2 v/v), pH 7.0; crude enzyme (300 µg of protein) and distilled water to make up the volume to 1.7 mls. The side-arm contained 0.5 ml of 0.1 M $NaHCO_3$. After equilibration for 10 minutes, the reaction was started by tipping the $NaHCO_3$ into the main compartment. The reaction was followed for 1½ minutes. The control consisted of boiled enzyme. The activity was found to be dependent on protein concentration and the initial rate was used in the determination of enzyme activity (Figure 1). The activity was expressed as Enzyme Units (E.U.) where

$$E.U. = \frac{(x - y)}{y}$$

x = initial velocity of enzymic reaction

y = initial velocity of control

Partial Purification of Crude Enzyme

After 5 days growth, the cells were harvested and the crude enzyme prepared as previously described. A known volume of the crude enzyme preparation was applied to a Sephadex G-100 (coarse) column (2.5 cm x 16.5 cm) previously equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 containing

1 mM EDTA and 5 mM 2-mercaptoethanol. The protein was eluted with the same buffer at a flow rate of 0.6 ml per minute. 70 tubes were collected, each containing 3.5 mls of the eluate. RuDP-carboxylase, PEP-carboxylase and carbonic anhydrase were determined in the fractions collected using the assay systems described before.

RESULTS

I. CHARACTERIZATION OF ZOOSPORES PRODUCED IN SYNCHRONOUS CULTURES BUBBLED WITH AIR OR 5% CO_2-IN AIR

A. Number of Zoospores Produced

When *Chlamydomonas segnis* was grown synchronously in cultures bubbled with air using the programmed light-dark regime (12:12 hours, LD change) combined with dilution of algal suspension at the end of each dark period to 10^6 cells per ml, each mother cell produced two zoospores (Figure 2). This was indicated by the doubling in the cell number at the 20th hour during the dark period. Figure 3a shows the complete formation of 2 daughter cells (diads) at the 13th hour but the zoospores were not yet released. The onset of zoospore release occurred usually at the 14 - 16th hour of the cell cycle. The two-fold increase in cell volume* to

* This was computed by assuming that a zoospore which was 10 μ in length should have a volume of $1000\mu^3$. In order to produce two identical daughter cells, it should undergo a two-fold increase in volume, i.e., to acquire a volume of $2000\mu^3$. This volume corresponds approximately to an increase of 26% in the cell length at the end of the growth period. To produce four daughter cells, the zoospore should quadruplicate its volume and correspondingly should show 60% increase in length (Badour *et al.*, 1973).

Figure 2. Synchronization of *Chlamydomonas segnis* using the light-dark regime (12:12 hour, LD change) followed by dilution in either air (o,●) or 5% CO_2 (Δ,▲).

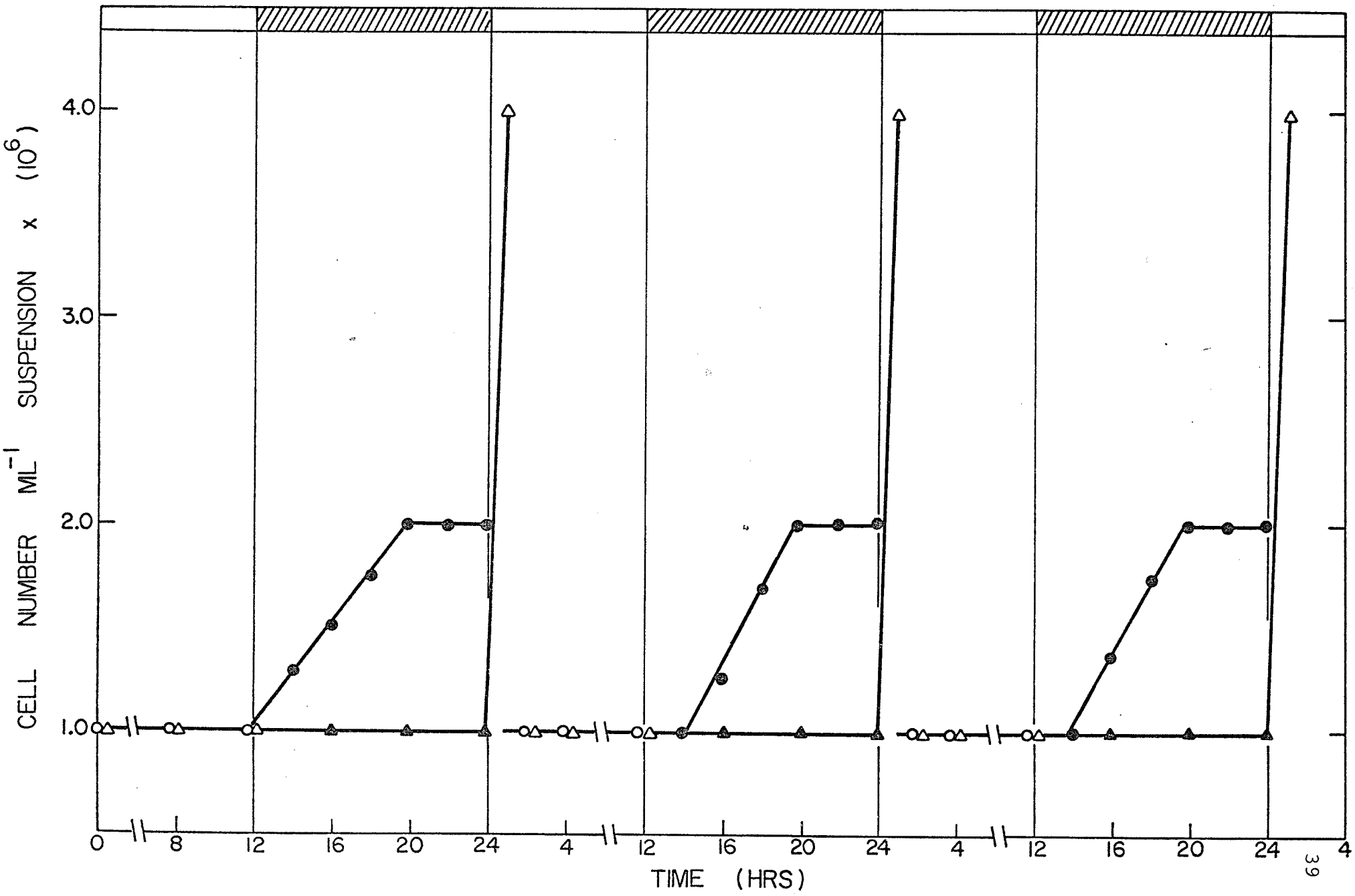
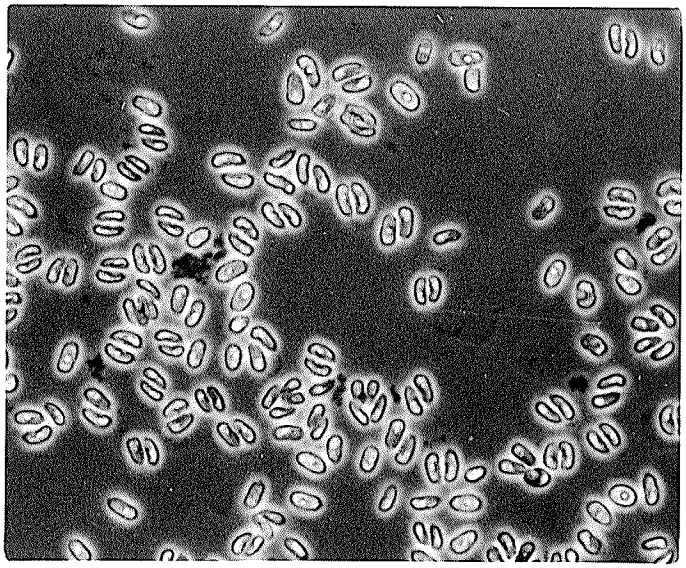
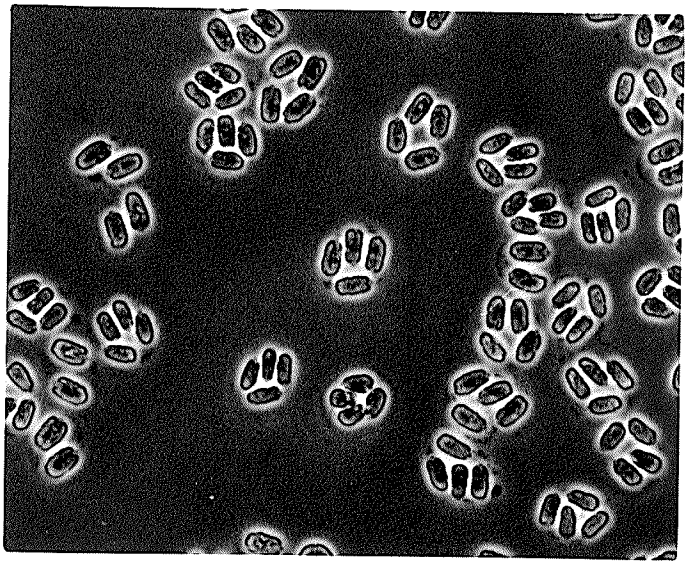

CELL NUMBER ML^{-1} SUSPENSION x (10^6)
4.0
3.0
2.0
1.0
0
8
12
16
20
24
4
12
16
20
24
4
12
16
20
24
4
TIME (HRS)

Figure 3. Completed formation but unreleased zoospores in the dark in (a) air, or (b) 5% CO_2.



(a)

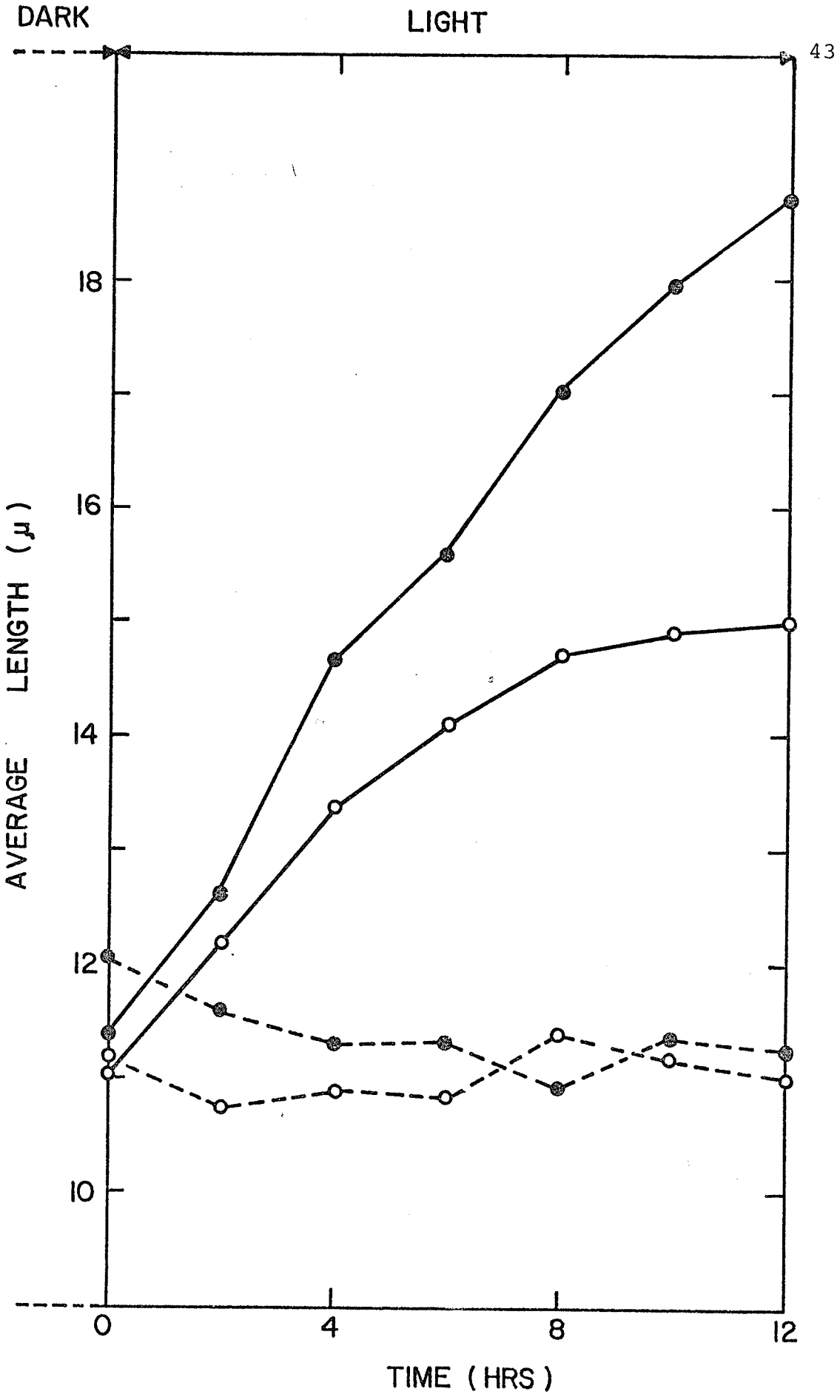


(b)

20μ

Figure 4. Body and flagellum lengths of cells grown in air or 5% CO_2.

o Air
● 5% CO_2
—— Body length
-- Flagellum length


DARK
LIGHT
18
16
14
12
10
AVERAGE LENGTH (μ)
0
4
8
12
TIME (HRS)

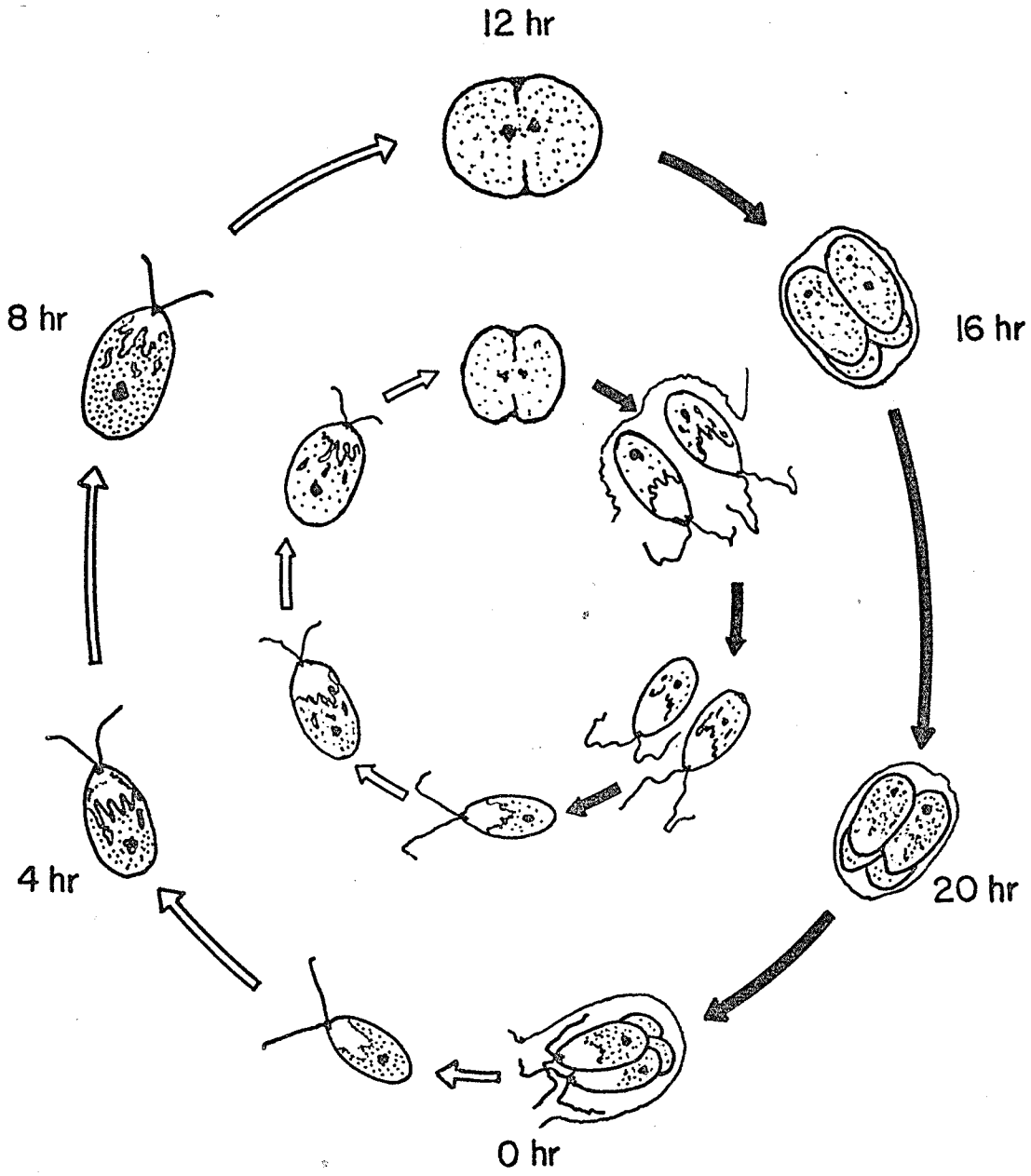
produce 2 daughter cells was approximately indicated by the increase in the average body length from 11μ to 15μ during the 12 hour light period and was not associated with any significant changes in flagellum length (Figure 4).

On the other hand, cells growing and dividing in cultures maintained under the same culture condition but gassed with 5% CO_2 in air (v/v) instead of air, quadruplicated their volume during the 12 hour light period showing 65% increase in the average cell length (Figure 4). This increase should account for the formation of 4 daughter cells in 5% CO_2 cultures. Microscopic examination of the algal suspension during the dark period revealed (Figure 3b) that the formation of 4 daughter cells in these cultures was achieved at the 18th hour, but the formed zoospores were not released. Subsequently, no increase in the cell number could be shown throughout the following 6 hours of darkness (Figure 2). The zoospores remained within the hyaline mother cell wall assuming a tetrad-stage. Each tetrad was counted as one cell and, hence, the unchanged cell number observed during the 24 hours of the cell cycle. In order to maintain the synchronous growth under the light-dark regime, the algal suspension was routinely diluted at the end of the dark period by adding 3 volumes of the fresh medium to one volume of

Figure 5. Diagrammatic representation of the cell cycle of *Chlamydomonas segnis* during the light-dark cycle.

Inner circle represents cultures grown in air.

Outer circle represents cultures grown in 5% CO_2.


12 hr
16 hr
20 hr
0 hr
4 hr
8 hr

algal suspension. When this diluted suspension of tetrads was exposed to light in the following light-dark cycle, the release of zoospores was accomplished within the hour of illumination giving rise to the initial cell number of 10^6 cells per ml. A diagrammatic representation of the life cycle in air or 5% CO_2 during the light-dark regime is given in Figure 5.

B. Initiation of Zoospore Release

The observed inhibitory effect of high CO_2 tension (5% CO_2 in air) on zoospore release in *Chlamydomonas segnis* during darkness and the release of zoospore on subsequent illumination has also been shown in other species of *Chlamydomonas* (Mihara and Hase, 1971; Kates and Jones, 1964) and in *Chlorella fusca* - 8P (John *et al.*, 1973). The latter authors interpreted the effect of light in terms of initiating the growth of the autospores within the mother cell wall. The autospore enlargement would exert pressure on the mother cell wall leading to its rupture and, hence, the release of autospores. In *Chlamydomonas segnis*, however, the release of zoospores could be initiated when tetrads at the 20th hour of the cell cycle were either illuminated or bubbled with CO_2-free air for 2 hours (Table 1). This may imply that the initiation of zoospore release by light was due to the provision of

Table I. Effect of provision of light and CO_2-free air for 2 hours (20 - 22nd hour of cell cycle) on the release of zoospores during the dark period in cultures bubbled with 5% CO_2 in air.

Treatment	Increase in Cell Number (% Initial Cell Number, Time:0)
Control	100
Light	212
CO_2-free air	148
Light + CO_2-free air	400

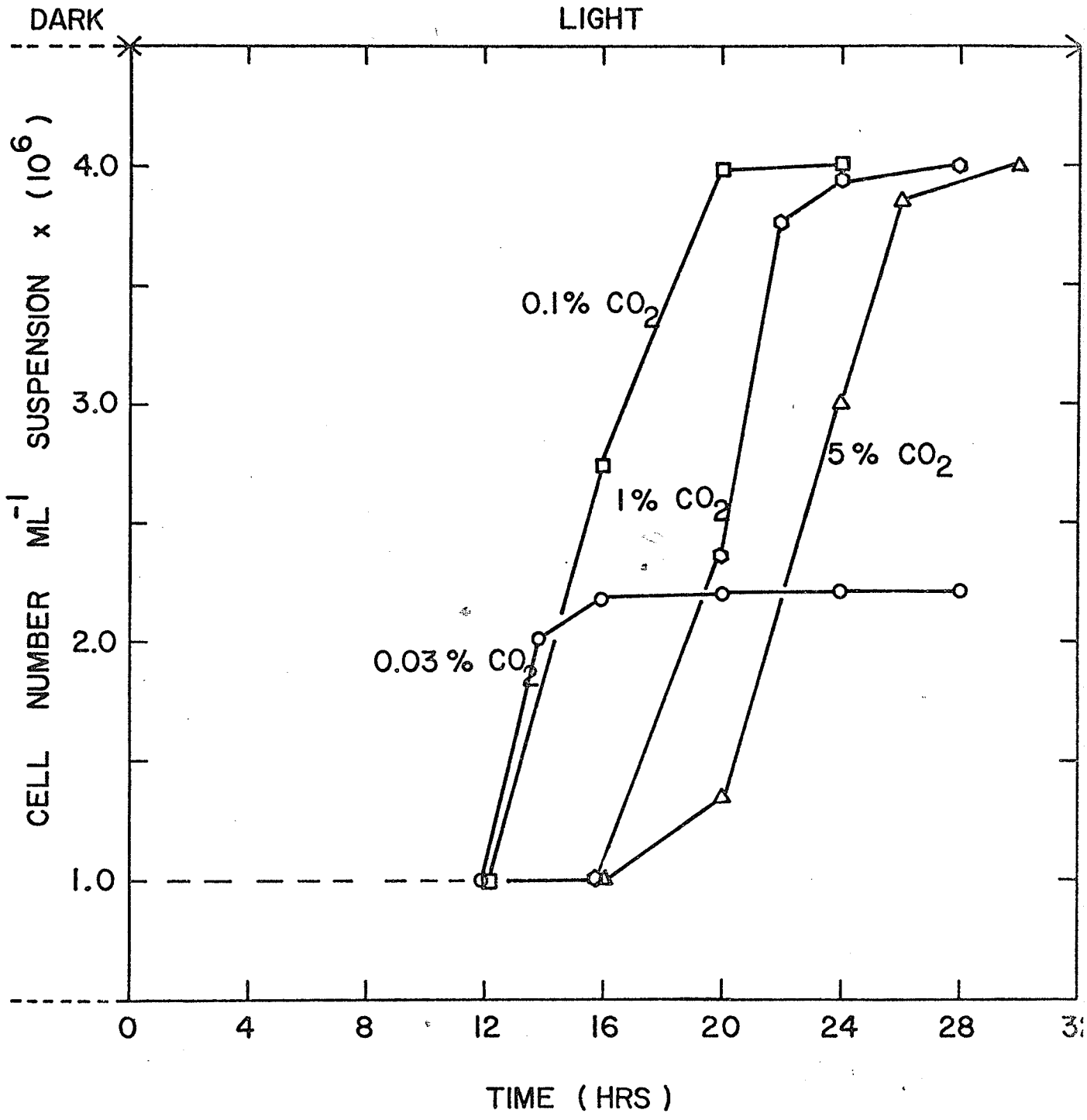
energy which enabled the zoospores to penetrate the mother cell envelope. This energy was unavailable in the dark under high CO_2 tension (5% CO_2 in air). Removal of the latter by bubbling with CO_2-free air for 2 hours would provide the zoospores with the suitable environment to generate the energy required for their release. The cumulative effect of light and aeration with CO_2-free air was manifested by the enhancement of zoospore release and its completion within 2 hours (20 - 22nd hour of the cell cycle). Under such conditions, where CO_2 was almost lacking, most of the energy trapped by the daughter cells would be available for the release of zoospores. The initiation of autospore release by light in *Chlorella pyrenoidosa* during the dark period of the cell cycle was also reported by Lorenzen and Schleif (1966).

It is obvious, therefore, that darkness generally exerts an inhibitory effect on the release of autospores and zoospores. This inhibitory effect is profound in cultures maintained at high CO_2 tension and can be overcome by exposure to light.

C. Timing of the Onset and Completion of Zoospore Release as Influenced by CO_2 Concentration

In this experiment, zoospores of *Chlamydomonas segnis* were produced in darkness from synchronous

Figure 6. Timing of the onset and completion of zoospore release as influenced by CO_2 concentration.


DARK
LIGHT
CELL NUMBER ML^{-1} SUSPENSION x (10^6)
4.0
3.0
2.0
1.0
0.1% CO_2
1% CO_2
5% CO_2
0.03% CO_2
0
4
8
12
16
20
24
28
TIME (HRS)

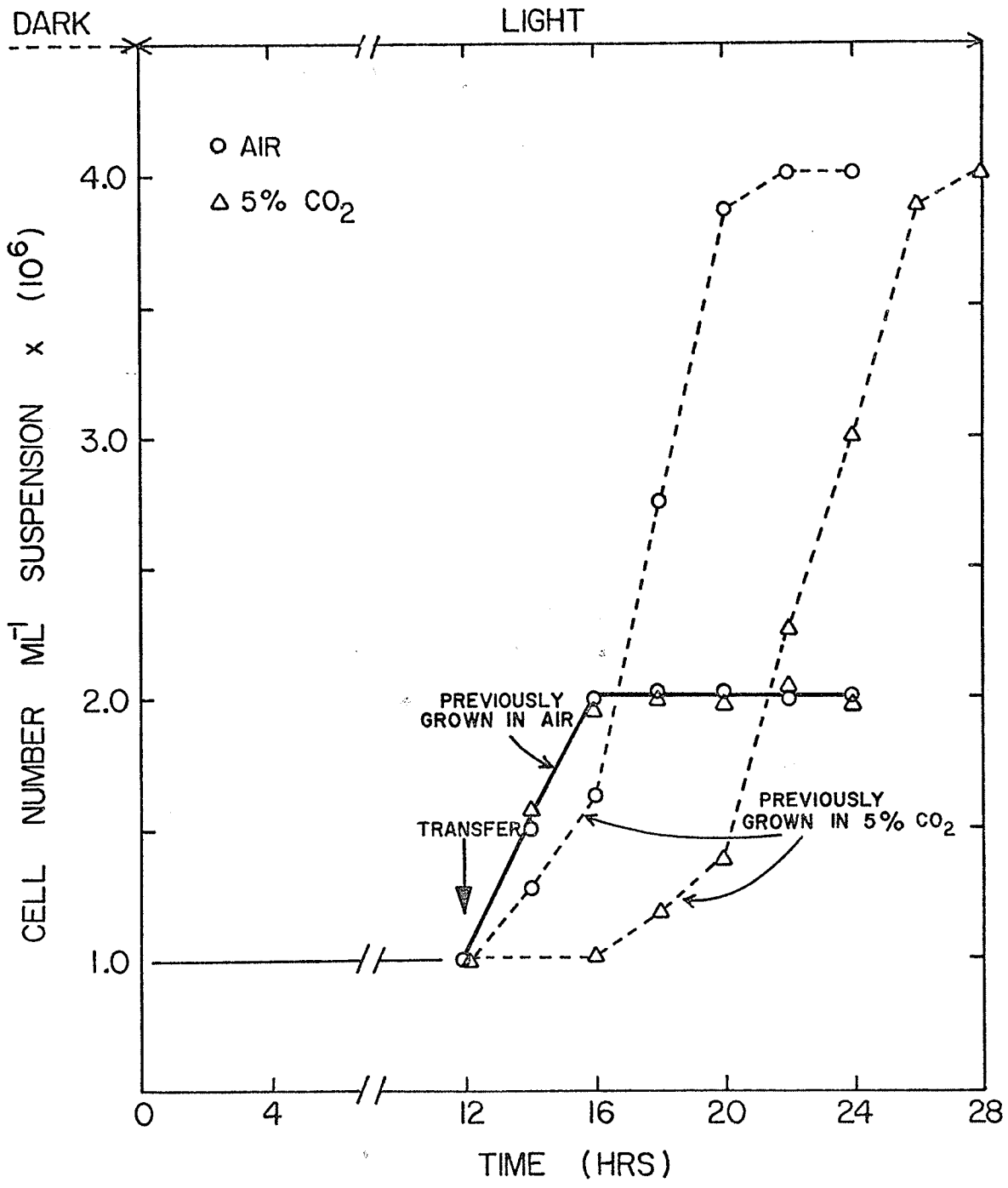
cultures gassed with various CO_2 concentrations, namely 0.03% CO_2 (air), 0.1% CO_2, 1% CO_2 and 5% CO_2 in air (v/v). After dilution of the cell suspension to a constant number of 10^6 cells per ml, the zoospores from each culture were left to grow and develop in their respective CO_2 concentrations. Figure 6 showed that a delay of 4 hours in the timing of the onset of zoospore release in 1.0% and 5% CO_2 cultures as compared to cultures bubbled with either air or 0.1% CO_2 in air. This delay appeared to be attributed to delayed mitotic division and the subsequent formation of tetrads rather than to a delay in the release of zoospores. Obviously, the latter should commence in light even in 5% CO_2 (compare Table 1) if the two mitotic divisions necessary for the formation of tetrads had been achieved earlier. Since the incipient cell division or the onset of cytokinesis (Pirson and Lorenzen, 1966; Senger and Bishop, 1969) occurred in all cultures at the same time at approximately the 12th hour of illumination, the delay in the onset of zoospore release in light in 1% and 5% CO_2 cultures was probably due to delay in the second mitotic division. The latter appeared to be retarded for 4 hours as compared to the situation in cultures provided with 0.1% CO_2 in air.

When cells grown in air cultures were supplied at the 12th hour of the cell cycle with 5% CO_2 in air

(Figure 7), the timing of the onset of zoospore release did not change. This was evident because the cells at that time of the cell cycle had already accomplished one mitotic division and production of the 2 daughter cells characteristic of these cultures. On the other hand, when the cells grown in 5% CO_2 were bubbled with air at the 12th hour, the onset of zoospore release occurred without delay and resulted in the four-fold increase in cell number at the 20th hour. Obviously, this increase would have not occurred unless the daughter cells from the first mitosis had entered the second mitotic division and completed it in some cells of the population. The onset of zoospore release, in cultures left in 5% CO_2, was evidenced at the 20th hour of the cell cycle, although the cells had their first mitotic division ready by the 12 - 14th hour. This again may show that the second mitotic division, a prerequisite for the formation of tetrads and subsequent zoospore release was halted for 8 hours in cultures supplemented with 5% CO_2 in air. The latter seemed not to influence the first mitotic division which occurred around the 12th hour of the cell cycle.

The period of time elapsed between the onset and completion of zoospore release known as the "division time" in the literature increased with increasing the CO_2 concentration provided to the cultures (Figure 6).

Figure 7. Effect of air (o) or 5% CO_2 (Δ) on the onset of zoospore release in cells previously grown in 5% CO_2 or air, respectively.

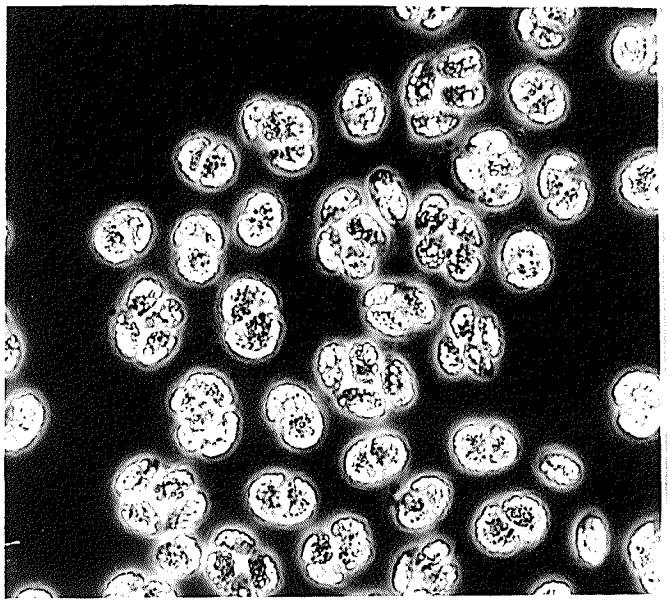

DARK
LIGHT
AIR
5% CO_2
4.0
3.0
2.0
1.0
CELL NUMBER ML^{-1} SUSPENSION x (10^6)
PREVIOUSLY GROWN IN AIR
TRANSFER
PREVIOUSLY GROWN IN 5% CO_2
0
4
12
16
20
24
28
TIME (HRS)

It was found that 8, 12 and 14 hours were required in light for the completion of zoospore release (i.e., four-fold increase in cell number) in 0.1%, 1% and 5% CO_2 cultures respectively. This observation, coupled with the fact that 4 zoospores were produced in each of these cultures, provided indirect evidence that relatively high CO_2 concentration (1% and 5% CO_2 in air) acted to delay the second mitotic division. The photomicrograph of *Chlamydomonas segnis* during the phase of second mitosis in light and in cultures aerated with 5% CO_2 in air (Figure 8) showed clearly that about 50% of the cells were still in the diad stage indicating the delayed second mitosis.

D. DNA, RNA, Protein, Carbohydrate and Chlorophyll Contents of Zoospores Produced in Darkness in Either Air or 5% CO_2 Cultures

The data presented in Table II were obtained from zoospores (air cultures) and tetrads (unreleased zoospores in 5% CO_2 cultures) at the end of the 12 hour dark period. Although the DNA content of zoospores from both cultures was almost the same, the RNA content was three times greater in zoospores produced in 5% CO_2 than in air cultures. This resulted in a higher RNA/DNA ratio of 8.1 for zoospores formed in 5% CO_2 as compared to 3.2 for those produced in air. The former zoospores

Figure 8. Delay of second mitotic division in *Chlamydomonas segnis* cultured in 5% CO_2.



20μ

Table II. DNA, RNA, protein, carbohydrate and chlorophyll content of zoospores produced in darkness in either air or 5% CO_2 cultures.

Cell Composition	µg per 10^6 zoospores	
	Air	5% CO_2
DNA	0.28	0.32
RNA	0.90	2.60
Protein	18.00	40.00
Carbohydrate	4.00	10.00
Chlorophyll	1.20	1.70
Dry weight	26.90	69.90

with higher level of RNA contained almost twice as much protein and carbohydrate as their counterparts in air cultures which had a lower RNA content. Furthermore, a 40% increase in chlorophyll content was shown in zoospores obtained from 5% CO_2 cultures as compared to those from air cultures. The greater accumulation of RNA, protein, carbohydrate and chlorophyll in zoospores produced in 5% CO_2 was indicated by the increased dry weight of the zoospores. This was also reflected in the increased width of the zoospore cell as shown in Figure 3b, but not in the cell length which remained almost unchanged (11.0 - 11.4μ).

These results showed that aeration with 5% CO_2 favoured the accumulation of RNA, protein, carbohydrate and chlorophyll but not DNA. Obviously, the accumulation of such cell materials took place in the cytoplasm and cytoplasmic inclusions (chloroplasts, etc.).

The question whether the increase in cell material in zoospores produced in 5% CO_2 cultures were equally distributed among the individual zoospore population as a result of equal cytoplasmic division could not be answered satisfactorily because of the lack of cytological evidence. However, the number of daughter cells produced by such a population after growth in cultures with limited CO_2 concentration (air) may be taken as a

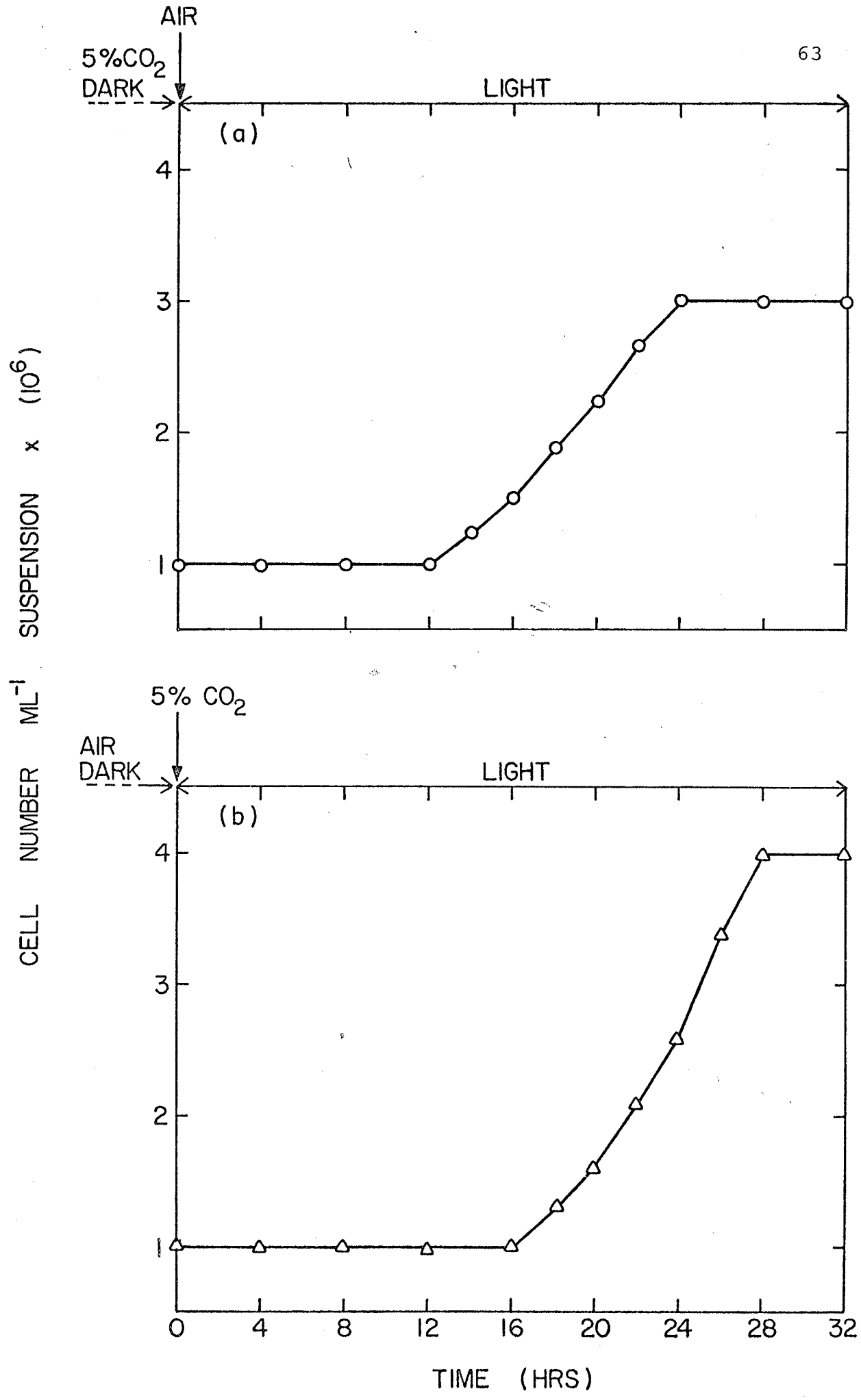
criterion for equal cell mass per zoospore. As shown in Figure 9a, when zoospores produced in darkness in 5% CO_2 cultures were bubbled with air throughout the cell cycle, growth and cell division resulted in three-fold increase in the cell number. The concept of the critical cell mass (Mitchison, 1971) suggests that there may be a critical mass at which cells initiate DNA synthesis and, hence, the subsequent mitotic division(s). If only one doubling of mass occurred in cultures bubbled with air and the initial high cell mass was equally distributed among the individual zoospores (from 5% CO_2), then either a two-fold or four-fold increase in the cell number would have been obtained. The three-fold increase in cell number implied that the zoospore population from 5% CO_2 cultures was heterogeneous. Half of the zoospores were higher in cell mass than the others. During their growth in air, they behaved differently so that one-half grew and subsequently underwent only one mitotic division whereas the other half divided twice and, hence, the three-fold increase in cell number.

Zoospores produced in air cultures showing low initial cell mass responded equally to the high CO_2 concentration of 5% CO_2 in air as shown in Figure 9b. This culture gave rise to four-fold increase in the cell number at the 28th hour indicating that all the zoospores grew and quadruplicated their mass in preparation for two

Figure 9. Onset of zoospore release and increase in cell number as affected by provision of air or 5% CO_2 during the cell cycle to zoospores produced in the dark in 5% CO_2 or air, respectively.

(a) Zoospores produced in the dark in 5% CO_2 and subsequently cultured in air.

(b) Zoospores produced in the dark in air and subsequently cultured in 5% CO_2.


AIR
5%CO2
DARK
LIGHT
(a)
4
3
2
1
SUSPENSION x (10^6)
5% CO2
AIR
DARK
LIGHT
(b)
CELL NUMBER ML^-1
0
4
8
12
16
20
24
28
32
TIME (HRS)

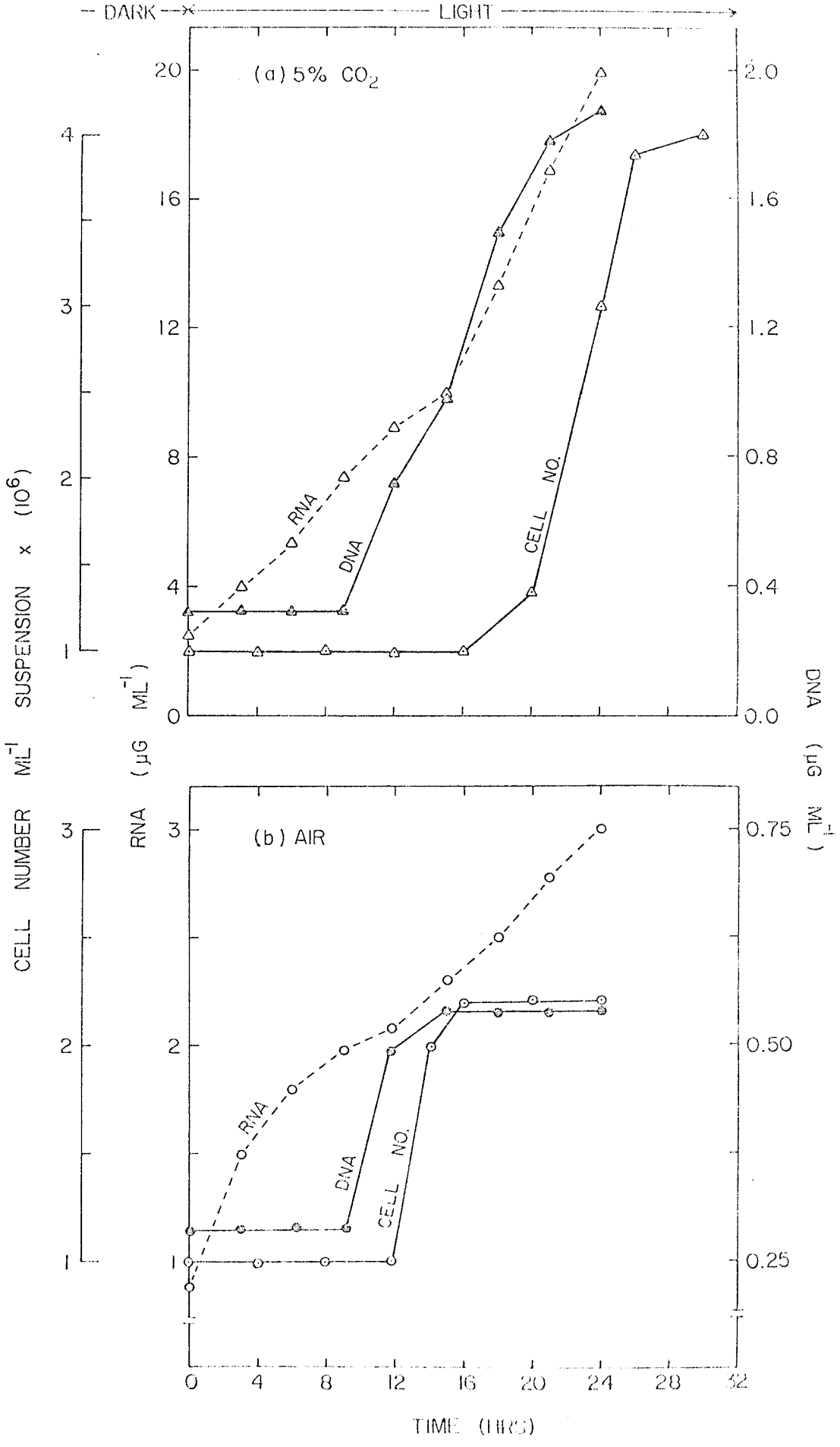
mitotic divisions. Therefore, the zoospores produced in darkness in air cultures represented a relatively homogeneous population.

E. Characterization of the Cell Cycle in 5% CO_2 and Air Cultures

Using zoospores with high and low initial mass, i.e., produced in darkness in 5% CO_2 and air cultures respectively, the cell cycle in their respective CO_2 concentration was characterized under continuous illumination (Figure 10). Since the length of one cell cycle is determined from the onset of illumination till the time of attainment of 50% of the final percent increase in cell number (Schmidt, 1969), the cell cycle length could not be calculated in cultures subjected to 12 hour light followed by 12 hour darkness (Figure 11). This is because in the dark period a delay or total inhibition of zoospore release occurred.

As shown in Figure 10, the total cell cycle lasted 24 hours in 5% CO_2 cultures and 13 hours in air cultures. Although both cultures commenced their S-phase at the 9th hour (end of G_1-phase), the difference could be observed in the length of G_1 and S-phases when expressed as a fraction of one cell cycle. Thus, in air cultures the G_1 and S period occupied approximately 0.7 and 0.3 of the cell cycle, whereas a shorter G_1 (0.38) and longer

Figure 10. Periodic increases in RNA, DNA and cell number of *Chlamydomonas segnis* cultured (a) in 5% CO_2, or (b) in air in continuous light.

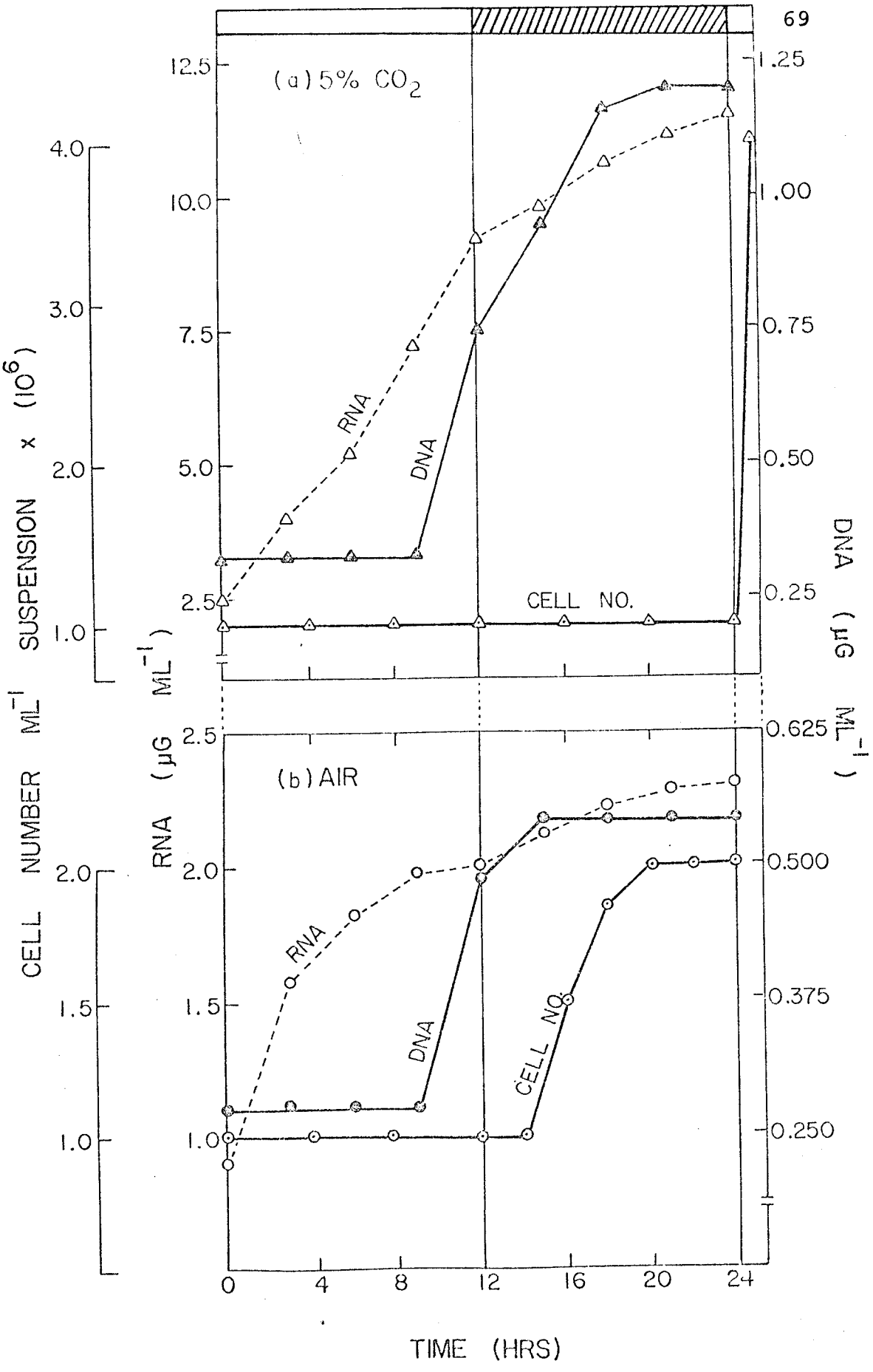

DARK
LIGHT
(a) 5% CO_2
(b) AIR
RNA
DNA
CELL NO.
CELL NUMBER ML^{-1} SUSPENSION x (10^6)
RNA (μG ML^{-1})
DNA (μG ML^{-1})
TIME (HRS)
0
4
8
12
16
20
24
28
32
0.0
0.4
0.8
1.2
1.6
2.0
0.25
0.50
0.75

S(0.62) phases were observed in 5% CO_2 cultures. This observation may lead to the conclusion that high CO_2 tension (i.e., greater than air) would result in a relatively short G_1 phase and subsequently a long S-phase.

Comparing the accumulation of RNA in continuous light and in light-dark cultures (Figures 10 and 11) it was clear that regardless of the CO_2 concentration provided to the cultures, the imposition of darkness at the 12th hour resulted in a lower rate of RNA accumulation. DNA synthesis was stimulated in light in 5% CO_2 but not in air cultures. Such stimulation increased the DNA content of the zoospore (0.47µg/10^6 cells) as compared to its dark counterpart (0.32µg/10^6 cells) or to those formed in air (0.27µg/10^6 cells). It appeared, therefore, that 5% CO_2 in air coupled with light enhanced the accumulation of DNA. The latter, however, did not trigger a third mitosis to produce 8 zoospores instead of 4.

The results obtained from these experiments provide evidence that aeration of cultures of *Chlamydomonas segnis* with 5% CO_2 in air exerted an inhibitory effect on zoospore release in darkness. This was overcome by exposing the culture to a light interval. Growth in 5% CO_2 resulted in the production of heterogeneous zoospore population with an average cell mass higher

Figure 11. Periodic increases in RNA, DNA and cell number in *Chlamydomonas segnis* cultured (a) in 5% CO_2, or (b) in air during one light-dark (12:12 hour LD) cycle.


(a) 5% CO_2
RNA
DNA
CELL NO.
(b) AIR
RNA
DNA
CELL NO.
CELL NUMBER ML^{-1} SUSPENSION x (10^6)
RNA (µG ML^{-1})
DNA (µG ML^{-1})
TIME (HRS)
0
4
8
12
16
20
24

than that produced in air cultures. The cell cycle was characterized by a shorter G_1 and a longer S-phase in 5% CO_2 than in air cultures. The former culture favoured the accumulation of DNA in light.

II. LEVELS OF PHOTOSYNTHESIS, CARBONIC ANHYDRASE, RIBULOSE-1,5-DIPHOSPHATE AND PHOSPHOENOLPYRUVATE CARBOXYLASES ACTIVITIES DURING THE CELL CYCLE OF *CHLAMYDOMONAS SEGNIS* IN AIR OR 5% CO_2

Unless indicated, in this and the following experiments, the zoospores produced from cultures obtained by the alternating light-dark regime were left to grow and complete their life cycle under continuous illumination in their respective CO_2 concentration, i.e., air or 5% CO_2 in air (v/v). Continuous light was employed to avoid any effects that may occur by the imposed long dark period (Mitchison, 1971; John *et al.*, 1973). The results were expressed per ml algal suspension (Duynstee and Schmidt, 1967; Walther and Edmunds, 1973) and on protein basis (Bishop and Senger, 1971). However, the latter which paralleled the increases in dry weight (Figure 12) when used as the basis, might lead to erroneous or misleading interpretation of the results because of the differential rates in protein synthesis and other physiological or biochemical parameters (Duynstee and Schmidt, 1967).

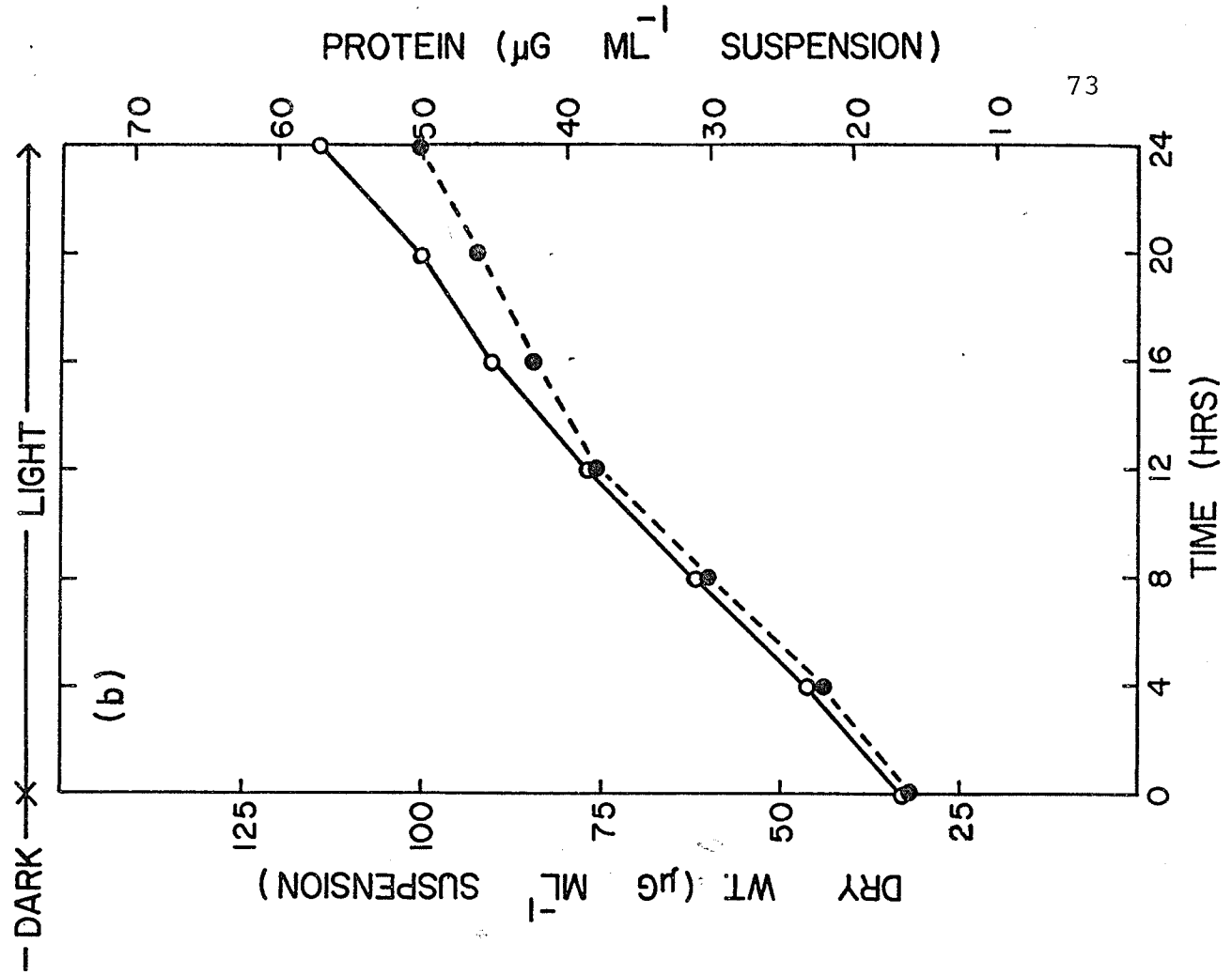
A. Oxygen Evolution and the p-Benzoquinone Hill Reaction

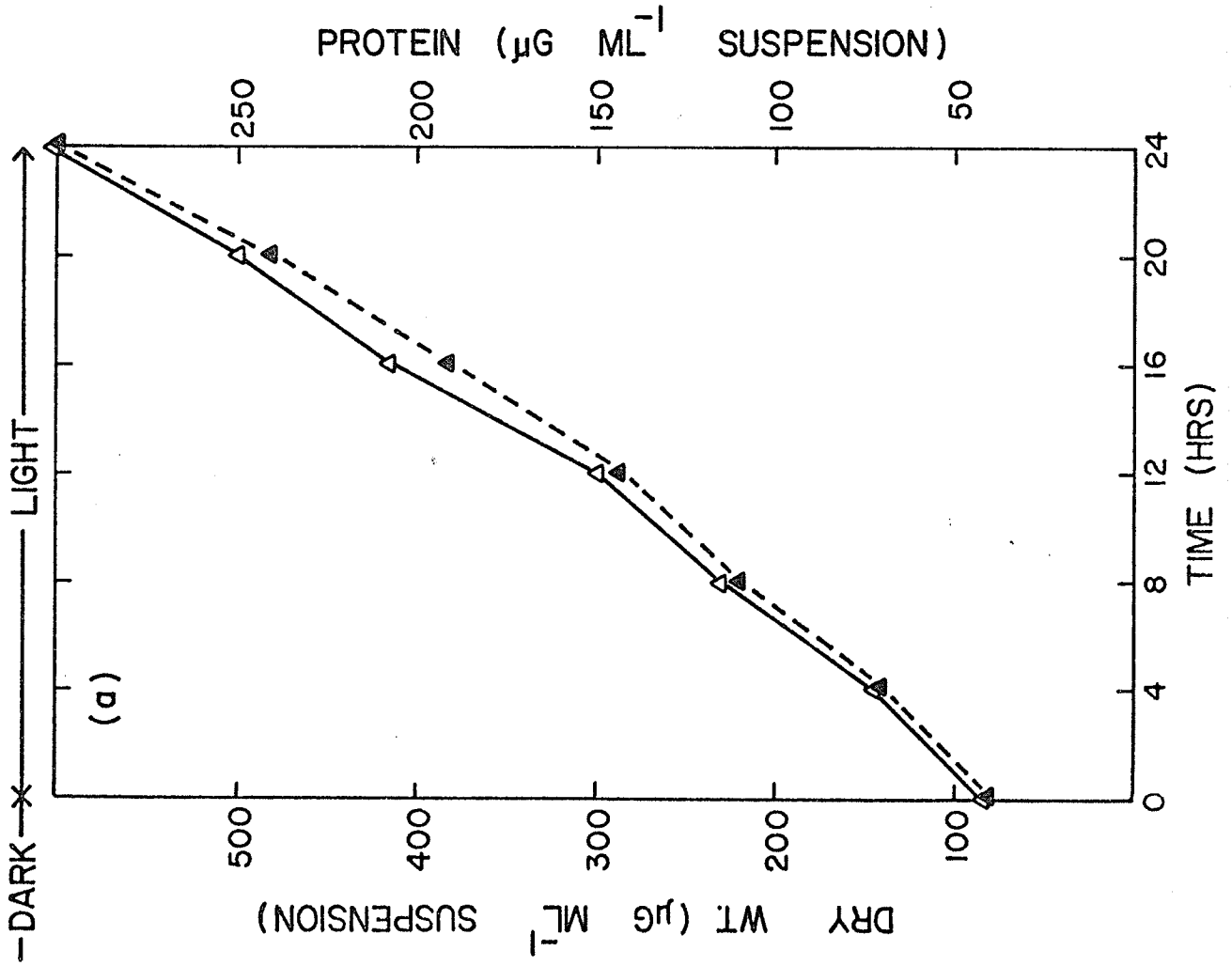
The photosynthetic capacity of *Chlamydomonas segnis* (per ml algal suspension) measured manometrically or by the oxygen electrode during the life cycle in cultures bubbled with air or 5% CO_2 in air under continuous

Figure 12. Periodic increases in dry weight and protein during the cell cycle in air or 5% CO_2 cultures.

(a) 5% CO_2 Culture
Δ——Δ Dry weight
▲---▲ Protein

(b) Air Culture
o——o Dry weight
●---● Protein


PROTEIN (μG ML⁻¹ SUSPENSION)
70
60
50
40
30
20
10
0
4
8
12
16
20
24
TIME (HRS)
DARK
LIGHT
(b)
125
100
75
50
25
DRY WT. (μG ML⁻¹ SUSPENSION)


PROTEIN (μG ML⁻¹ SUSPENSION)
250
200
150
100
50
0
4
8
12
16
20
24
TIME (HRS)
DARK
LIGHT
(a)
500
400
300
200
100
DRY WT. (μG ML⁻¹ SUSPENSION)

illumination is shown in Figures 13 and 14. Regardless of the differences in the amount of oxygen evolved between air and 5% CO_2 grown cells (compare Figure 16), the photosynthesis increased in both cultures during the growth phase and declined during maturity and division. This was followed by an increase in the photosynthetic capacity during and after the release of zoospores. This pattern is similar to that reported for various unicellular green algae growing synchronously in cultures subjected to light-dark periods (Gerhardt, 1964; Kates and Jones, 1966; Bishop and Senger, 1971) or to continuous dim light (Walther and Edmunds, 1973). However, it was obvious that zoospores growing in 5% CO_2 attained the peak of oxygen evolution 4 hours later than those growing in air. Consequently, the decline of oxygen evolved commenced 4 hours earlier in air than in 5% CO_2 cultures. Actually, this decline in photosynthesis represents a drop in the efficiency of photosystem II as indicated by the decline of p-benzoquinone Hill reaction (Senger and Bishop, 1967; Senger, 1970) as shown in Figure 15. The drop in photosystem II activity was greater (40%) in cells grown in air rather than in 5% CO_2 (15 - 20%). This observation may suggest that cells grown at high CO_2 were relatively more capable of maintaining the activity of photosystem II.

Figure 13. Photosynthetic oxygen evolution in Warburg No: 9 buffer (pH 9.1) of *Chlamydomonas segnis* grown in either air or 5% CO_2 in synchronous cultures and continuous light.

o Air grown
Δ 5% CO_2 grown

——— Nmoles/min/ml algal suspension
---- Nmoles/min/mg protein

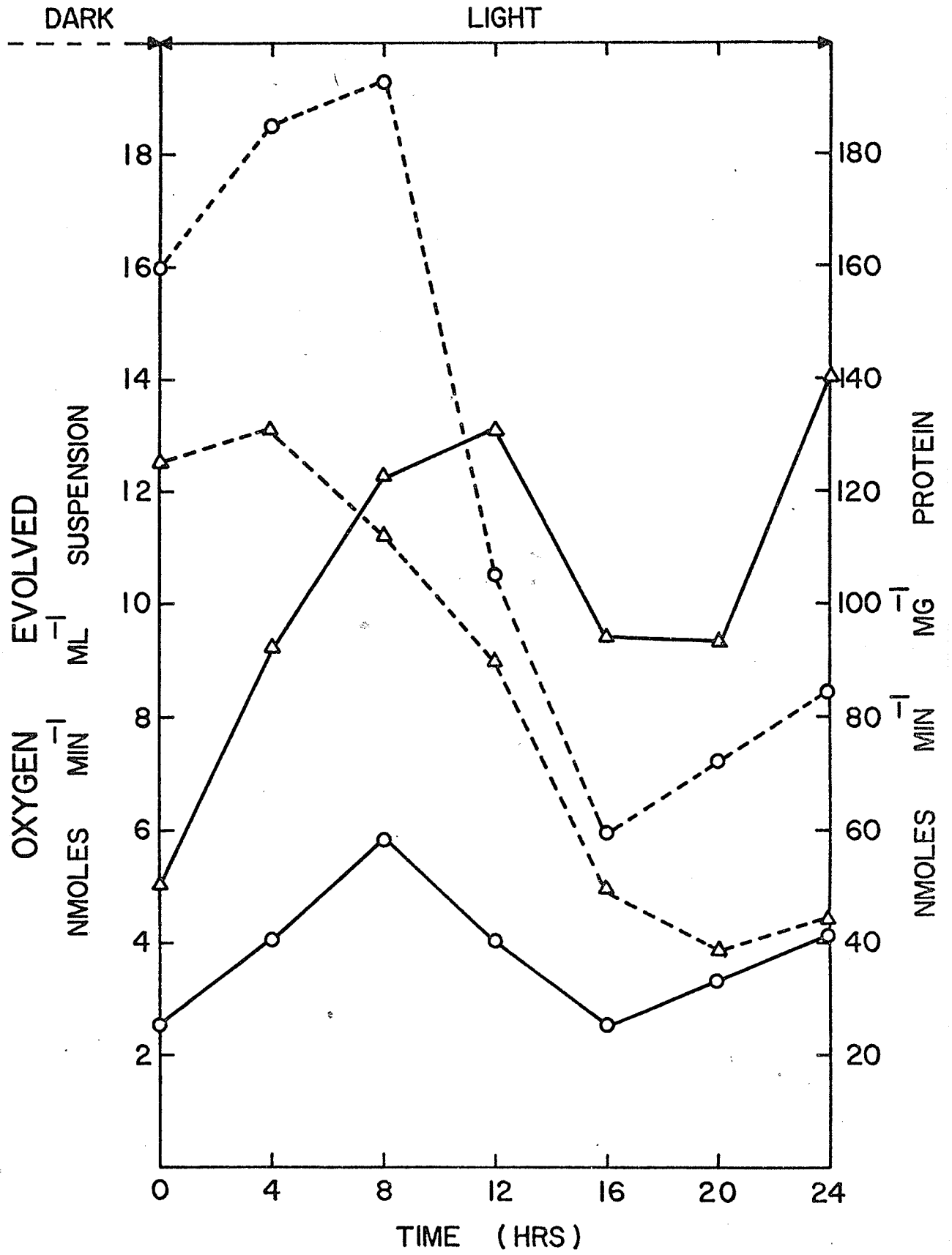

DARK
LIGHT
OXYGEN EVOLVED
NMOLES MIN⁻¹ ML⁻¹ SUSPENSION
NMOLES MIN⁻¹ MG⁻¹ PROTEIN
18
16
14
12
10
8
6
4
2
180
160
140
120
100
80
60
40
20
0
4
8
12
16
20
24
TIME (HRS)

Figure 14. Photosynthetic oxygen evolution of *Chlamydomonas segnis* grown either in air or 5% CO_2 in synchronous cultures and continuous light measured in phosphate buffer (pH 7.0) with the oxygen electrode.

o Air grown
Δ 5% CO_2 grown

——— Nmoles/min/ml algal suspension
---- Nmoles/min/mg protein

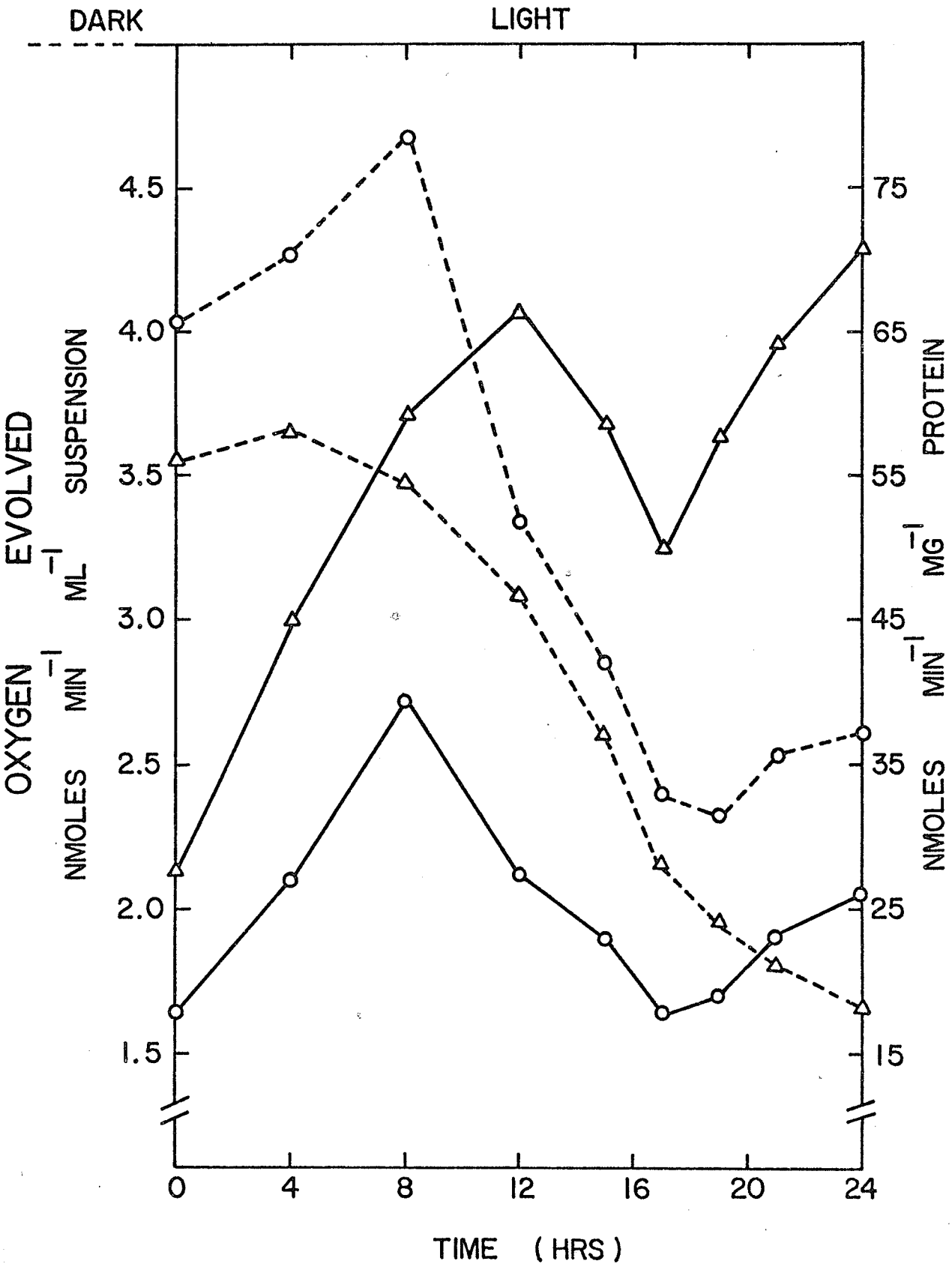

DARK
LIGHT
OXYGEN EVOLVED
NMOLES MIN⁻¹ ML⁻¹ SUSPENSION
NMOLES MIN⁻¹ MG⁻¹ PROTEIN
4.5
4.0
3.5
3.0
2.5
2.0
1.5
75
65
55
45
35
25
15
0
4
8
12
16
20
24
TIME (HRS)

Figure 15. Benzoquinone Hill reaction of *Chlamydomonas segnis* grown in either air or 5% CO_2 in synchronous cultures and continuous light measured in phosphate buffer (pH 7.0) with the oxygen electrode.

o Air grown
Δ 5% CO_2 grown

——— Nmoles/min/ml algal suspension
---- Nmoles/min/mg protein

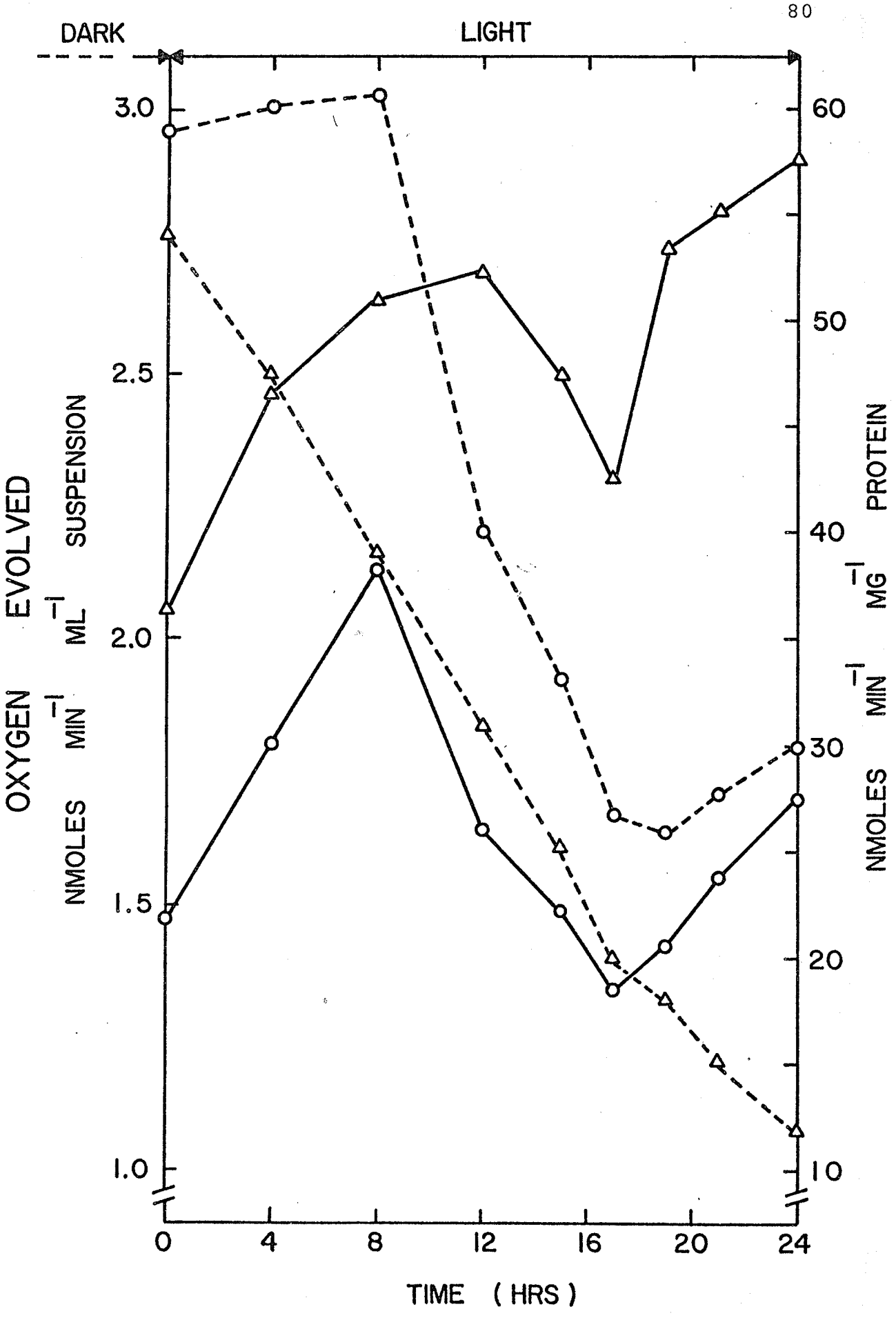

DARK
LIGHT
3.0
2.5
2.0
1.5
1.0
60
50
40
30
20
10
0
4
8
12
16
20
24
TIME (HRS)
OXYGEN EVOLVED
NMOLES MIN⁻¹ ML⁻¹ SUSPENSION
NMOLES MIN⁻¹ MG⁻¹ PROTEIN

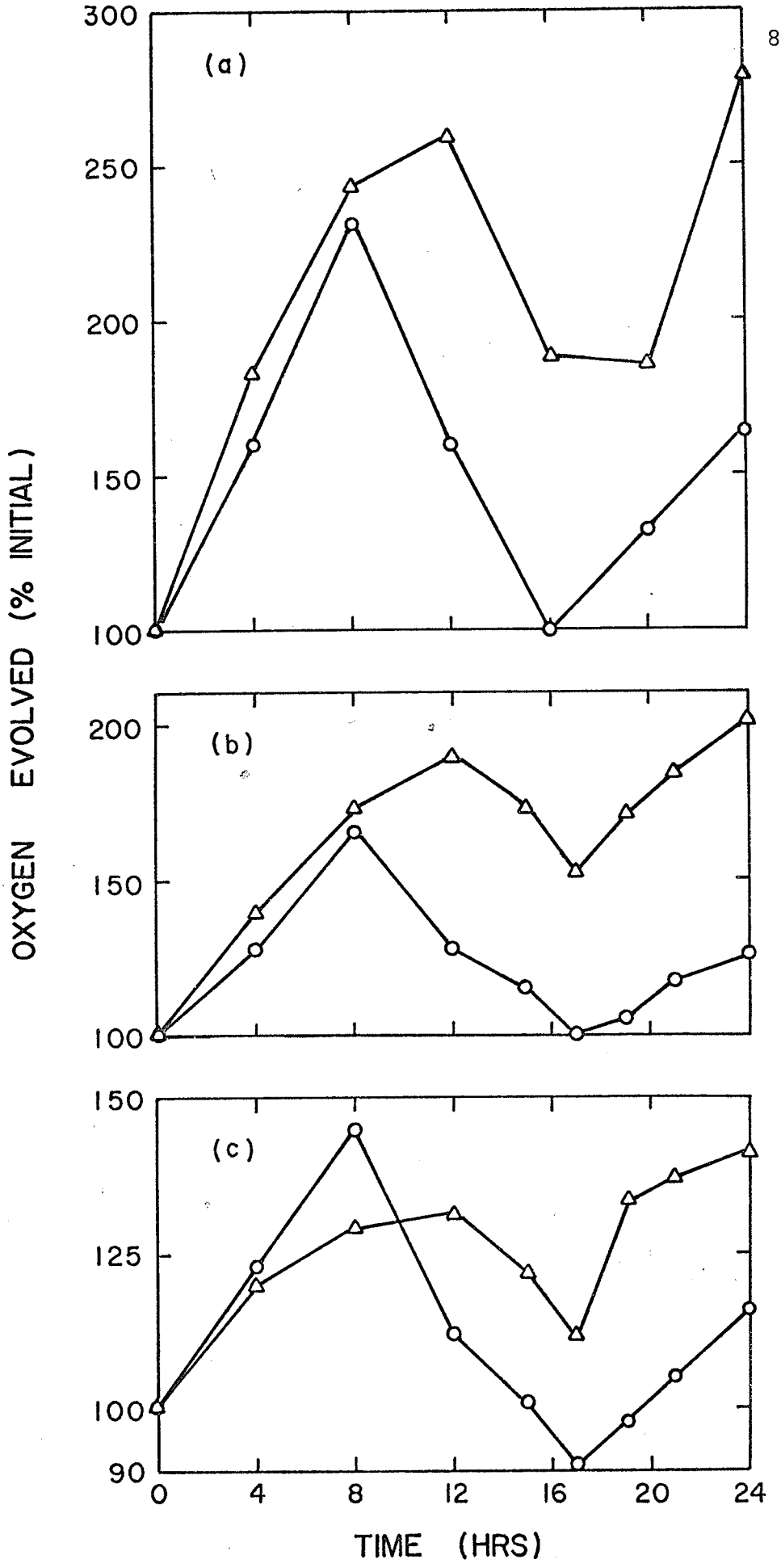
Figure 16. Oxygen evolution expressed as percentage of initial (time:0).

(a) Oxygen evolution in Warburg No. 9 buffer plotted from Figure 13.

(b) Oxygen evolution in phosphate buffer plotted from Figure 14.

(c) Benzoquinone Hill reaction plotted from Figure 15.

o——o Air grown
Δ——Δ 5% CO_2 grown


(a)
(b)
(c)
OXYGEN EVOLVED (% INITIAL)
TIME (HRS)
300
250
200
150
100
200
150
100
150
125
100
90
0
4
8
12
16
20
24

B. Carbonic Anhydrase Activity

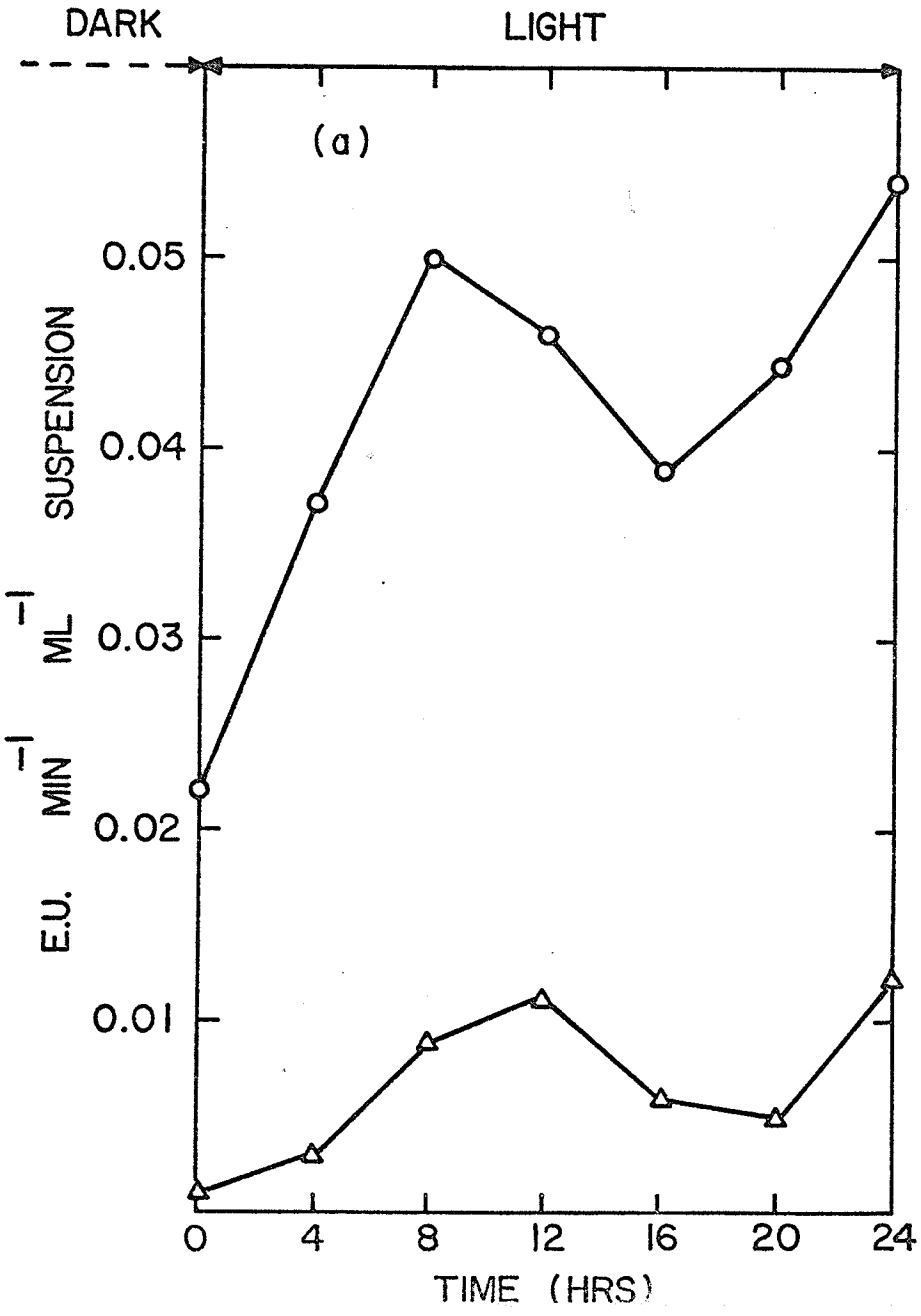
As shown in Figure 17, carbonic anhydrase activity was 4 - 25 times higher in zoospores produced and developed in air than in 5% CO_2 cultures. In some experiments, the carbonic anhydrase activity could not be detected in 5% CO_2 grown cells. These results are in agreement with the various reports (Reed and Graham, 1968; Nelson *et al.*, 1969; Graham *et al.*, 1971) that high CO_2 tension represses the synthesis of carbonic anhydrase. The levels of the enzyme activity per ml algal suspension during the cell cycle formed a pattern characteristic of a peak enzyme. Thus, the activity increased during growth and reached the peak at the 8th and 12th hour in air and 5% CO_2 cultures respectively. This was followed by a decline in enzyme activity during cell division and zoospore release. The changes in the levels of the enzyme activity corresponded with the observed variations in the photosynthetic oxygen evolution (Figures 13 and 14) and the p-benzoquinone Hill reaction (Figure 15). Such a correlation may suggest a regulatory relationship between carbonic anhydrase and photosystem II activities.

C. Ribulose-1,5-Diphosphate Carboxylase Activity

In air and 5% CO_2 cultures, the RuDP-carboxylase activity (Figure 18) showed a single step increase

Figure 17. Carbonic anhydrase activity in synchronous cultures of *Chlamydomonas segnis* grown in either air (o) or 5% CO_2 (Δ) in continuous light.

The activity was expressed
(a) per ml suspension, or
(b) per mg protein


DARK
LIGHT
(a)
0.05
0.04
0.03
0.02
0.01
E.U. MIN⁻¹ ML⁻¹ SUSPENSION
0
4
8
12
16
20
24
TIME (HRS)

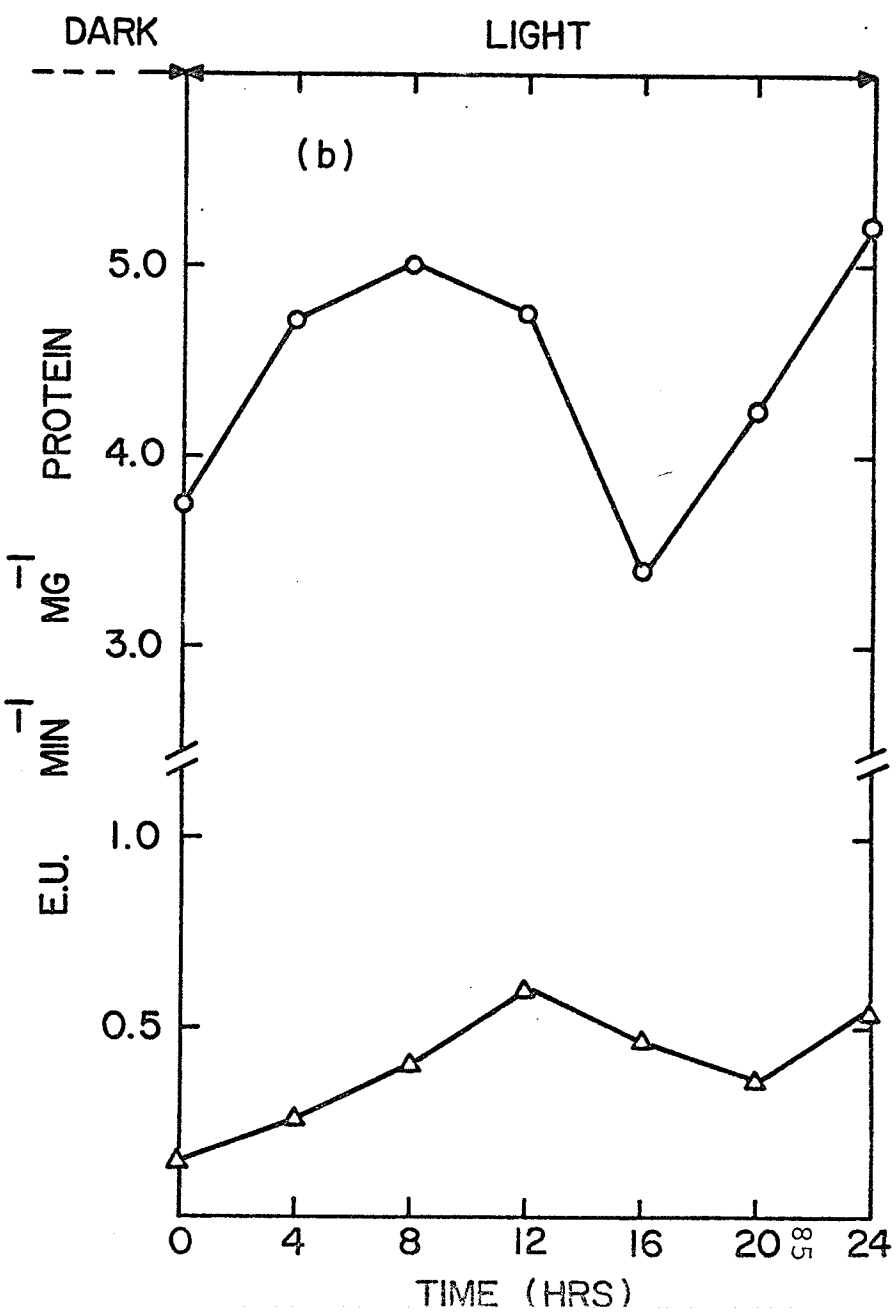
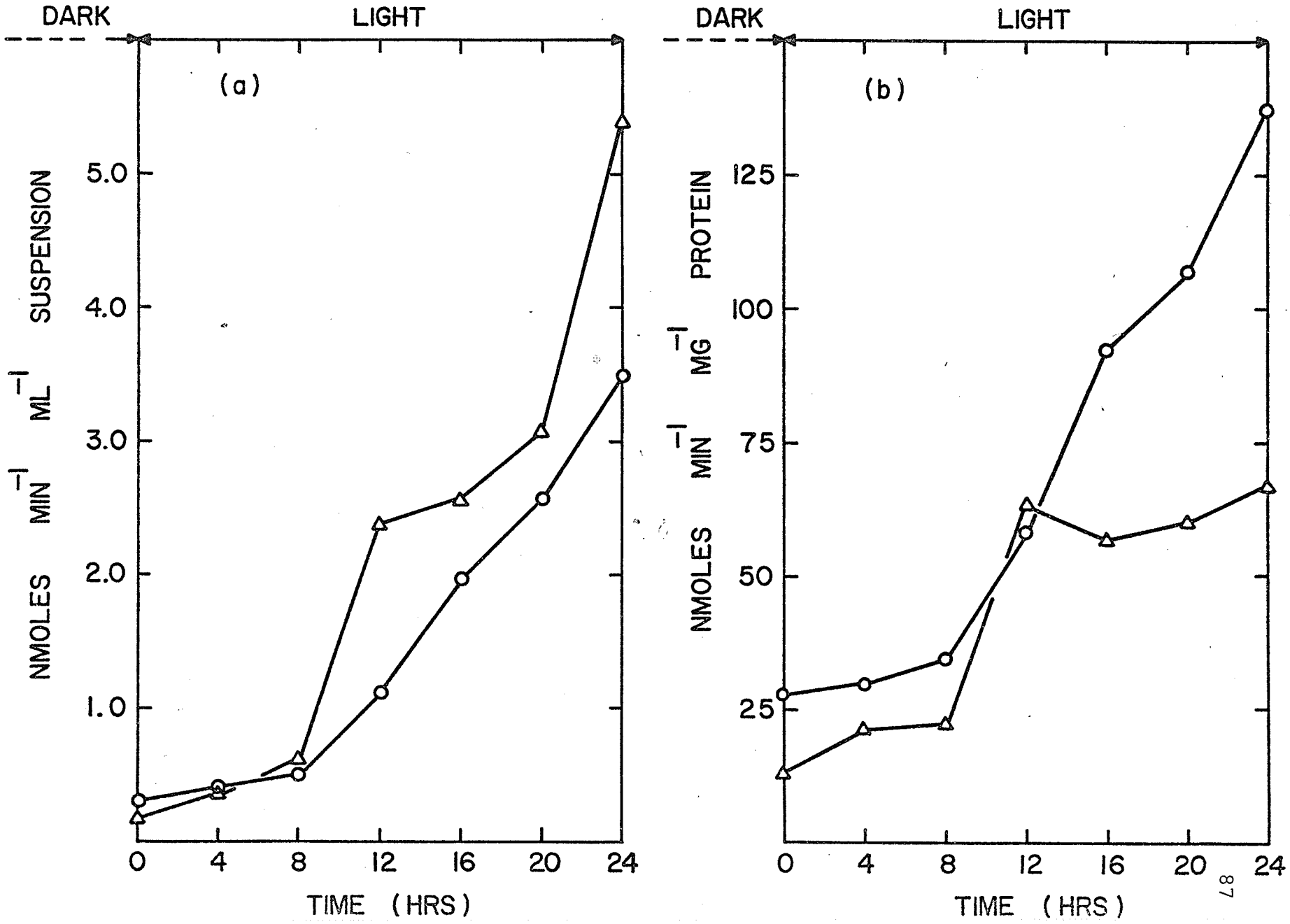

DARK
LIGHT
(b)
5.0
4.0
3.0
1.0
0.5
E.U. MIN⁻¹ MG⁻¹ PROTEIN
0
4
8
12
16
20
24
TIME (HRS)

Figure 18. Ribulose-1,5-diphosphate carboxylase activity in synchronous cultures of *Chlamydomonas segnis* grown in air (o) or 5% CO_2 (Δ) in continuous light.

The activity was expressed (a) per ml suspension or (b) per mg protein.


DARK
LIGHT
(a)
NMOLES MIN⁻¹ ML⁻¹ SUSPENSION
5.0
4.0
3.0
2.0
1.0
0
4
8
12
16
20
24
TIME (HRS)
DARK
LIGHT
(b)
NMOLES MIN⁻¹ MG⁻¹ PROTEIN
125
100
75
50
25
0
4
8
12
16
20
24
TIME (HRS)

commencing at the 8th hour just prior to DNA synthesis (compare Figure 10). Within 4 hours (8th - 12th hour), the enzyme activity was doubled in air grown cells and quadruplicated in 5% CO_2 cultures in accordance with the number of zoospores that will be produced, 2 and 4 respectively. The presence of the enzyme under different growth conditions indicated that it is constitutive, while the single step increase may represent a direct relationship between gene transcription and expression (Molloy and Schmidt, 1970; Mitchison, 1969). This single step increase and continued synthesis in the presence of light may be due to a de novo synthesis or activation (light induced or removal of co-repressors) or both (Molloy and Schmidt, 1970; Walker, 1973). The effect of CO_2 concentration on the enzyme activity was manifested by the higher specific activity in zoospores grown and produced in air than in 5% CO_2 (Figure 18b). It seems that the lower substrate level (0.03%) in air provided during growth is compensated by a higher specific activity of RuDP-carboxylase as suggested by Graham and Whittingham (1968).

D. Phosphoenolpyruvate Carboxylase Activity

PEP-carboxylase activity was either very low or undetectable in crude extracts prepared from cells grown in air. On the other hand, 5% CO_2 grown cells showed

substantial enzyme activity which was 40 - 80 times greater than that determined in air grown cells during the cell cycle (Figure 19a). This represented 10 - 25 times increase in the specific activity when compared to air grown cells (Figure 19b).

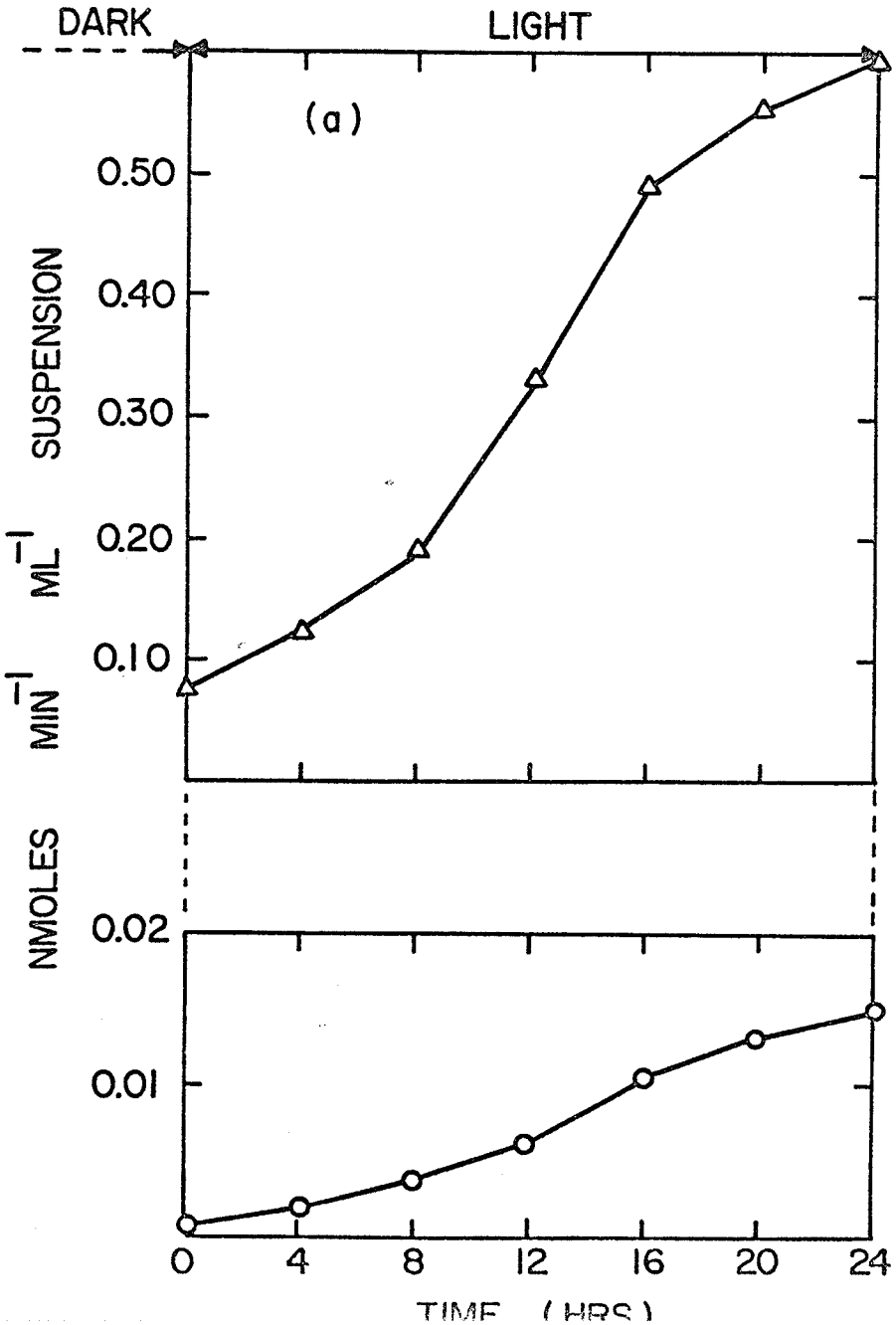
The variations in the levels of RuDP- and PEP-carboxylases during the cell cycle of *Chlamydomonas segnis* in air or 5% CO_2 cultures did not parallel the characteristic changes in photosynthetic O_2-evolution and the p-benzoquinone Hill reaction (Figures 13, 14 and 15).

E. Detection of Endogenous Inhibitor(s) and Repression of Enzyme(s)

The low enzyme activity measured in crude extracts prepared from cells grown in either air or 5% CO_2 may be attributed to the presence of endogenous inhibitor(s) rather than repression of enzyme synthesis. In order to test for inhibitors, the crude enzyme preparation with high activities were mixed with those showing low enzyme activities. A decrease or increase in the specific activity of the combined extracts as compared to the expected activity obtained by addition of activities determined separately would indicate the presence of inhibitor(s) or activator(s) respectively. Table III showed that neither soluble endogenous

Figure 19. Phosphoenolpyruvate carboxylase activity in synchronous cultures of *Chlamydomonas segnis* grown either in air (o) or 5% CO_2 (Δ) in continuous light.

The activity was expressed (a) per ml suspension, or (b) per mg protein.


DARK
LIGHT
(a)
0.50
0.40
0.30
0.20
0.10
SUSPENSION
ML⁻¹
MIN⁻¹
NMOLES
0.02
0.01
0
4
8
12
16
20
24
TIME (HRS)

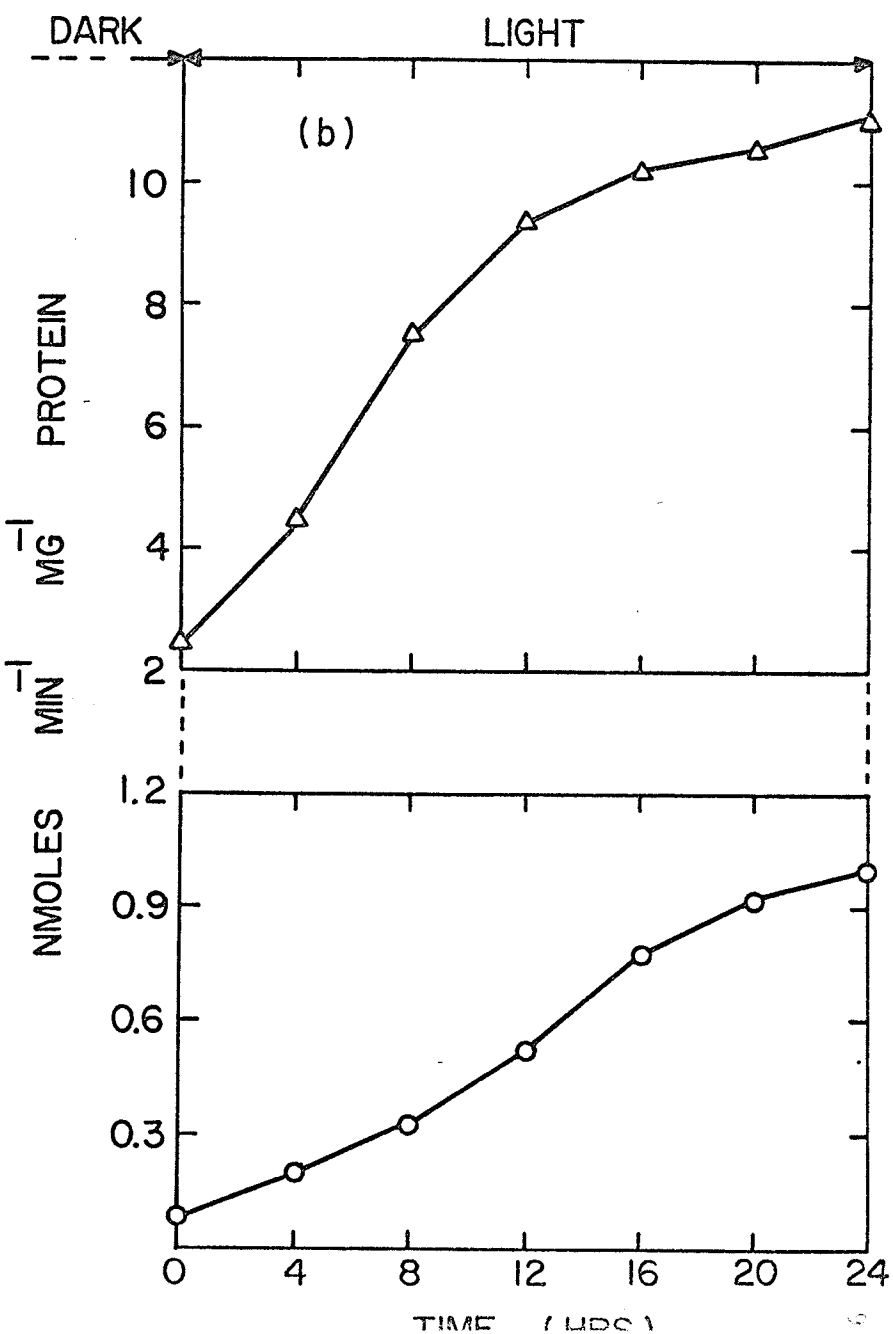

DARK
LIGHT
(b)
10
8
6
4
2
PROTEIN
MG⁻¹
MIN⁻¹
NMOLES
1.2
0.9
0.6
0.3
0
4
8
12
16
20
24
TIME (HRS)

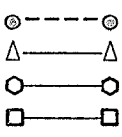
Table III. Absence of endogenous inhibitors of RuDP-carboxylase, PEP-carboxylase and carbonic anhydrase in crude extracts of *Chlamydomonas segnis* during synchronous growth in either air or 5% CO_2.

	Ru-DP-carboxylase (nmoles/min/mg protein)		%	PEP-carboxylase (nmoles/min/mg protein)		%	Carbonic Anydrase (E.U./min/mg protein)		%
Air grown, 6th hour	40.0	68.0	100.0	0.0	0.70	100.0	4.5	4.0	100.0
Air grown, 16th hour	96.0			1.4			3.5		
Combined		66.0	97.0		0.70	100.0		4.1	102.5
5% CO_2, 6th hour	32.3	47.3	100.0	8.0	9.10	100.0	0.0		
5% CO_2, 16th hour	62.3			10.2					
Combined		48.7	103.0		8.90	97.8			
Air, 6th hour							4.5	2.25	100.0
5% CO_2, 6th hour							0.0		
Combined								2.25	100.0
Air, 16th hour	96.0	79.1	100.0						
5% CO_2, 16th hour	62.3								
Combined		75.0	94.5						
Air, 6th hour				0.0	5.1	100.0			
5% CO_2, 16th hour				10.2					
Combined					5.0	98.0			

inhibitors nor activators were present in the crude extracts.

The presence of tightly bound inhibitors may be revealed by passing the crude extracts with low enzyme activity through Sephadex G-100. Increases in the specific activity of the enzyme in the eluates may provide some evidence of such inhibitors. Figures 20 and 21 showed that in air as well as 5% CO_2 grown cells the protein emerged as one major peak containing RuDP- and PEP-carboxylases. Whereas carbonic anhydrase activity was evident in the preparation from air culture, it was completely absent in the preparation from 5% CO_2 grown cells, even in fractions collected before and after the first protein fraction. The data in Tables IV and V showed that the relatively lower activity of RuDP-carboxylase in both air and 5% CO_2 grown cells during this early phase of growth was not due to the presence of inhibitors but rather the synthesis may be repressed. On the other hand, the increase in specific activity and purification of PEP-carboxylase in both air and 5% CO_2 grown cells was indicative of the presence of a tightly bound inhibitor(s). The higher increase in the purification of PEP-carboxylase in air grown cells as compared to 5% CO_2 grown cells showed that a higher amount of inhibitor(s) was produced as a resultant of growth in

Figure 20. Sephadex G-100 column chromatography of crude extracts prepared from cells after 6 hours growth in air and light.



Protein concentration
RuDP-carboxylase activity
PEP-carboxylase activity
Carbonic anhydrase activity

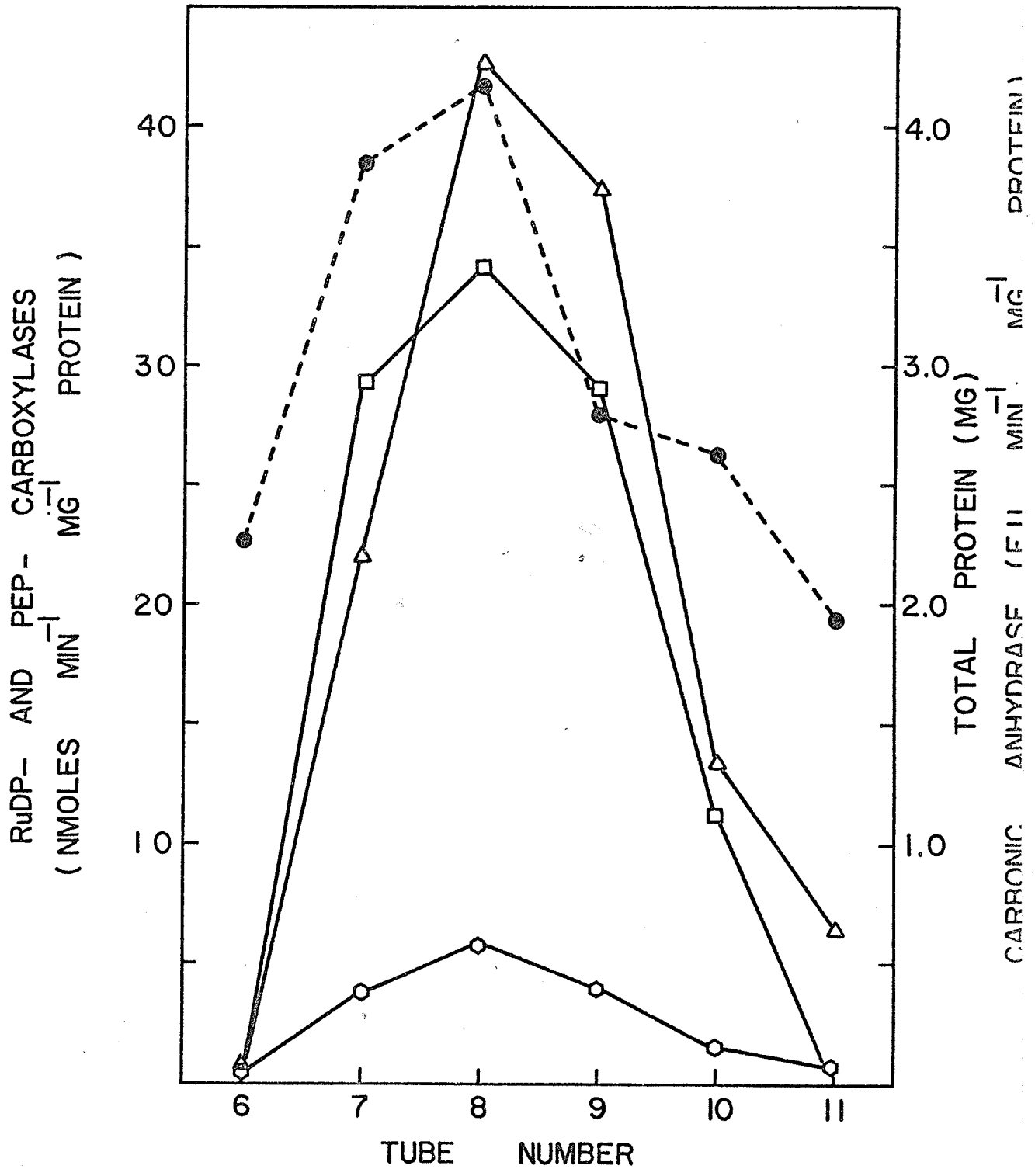

RuDP- AND PEP- CARBOXYLASES
(NMOLES MIN⁻¹ MG⁻¹ PROTEIN)
TOTAL PROTEIN (MG)
CARBONIC ANHYDRASE (E.U. MIN⁻¹ MG⁻¹ PROTEIN)
40
30
20
10
4.0
3.0
2.0
1.0
6
7
8
9
10
11
TUBE NUMBER

Figure 21. Sephadex G-100 column chromatography of crude extracts from cells after 6 hours growth in 5% CO_2 and light.

⊙-----⊙ Protein concentration
Δ——Δ RuDP carboxylase activity
O——O PEP-carboxylase activity

Carbonic anhydrase activity was absent.

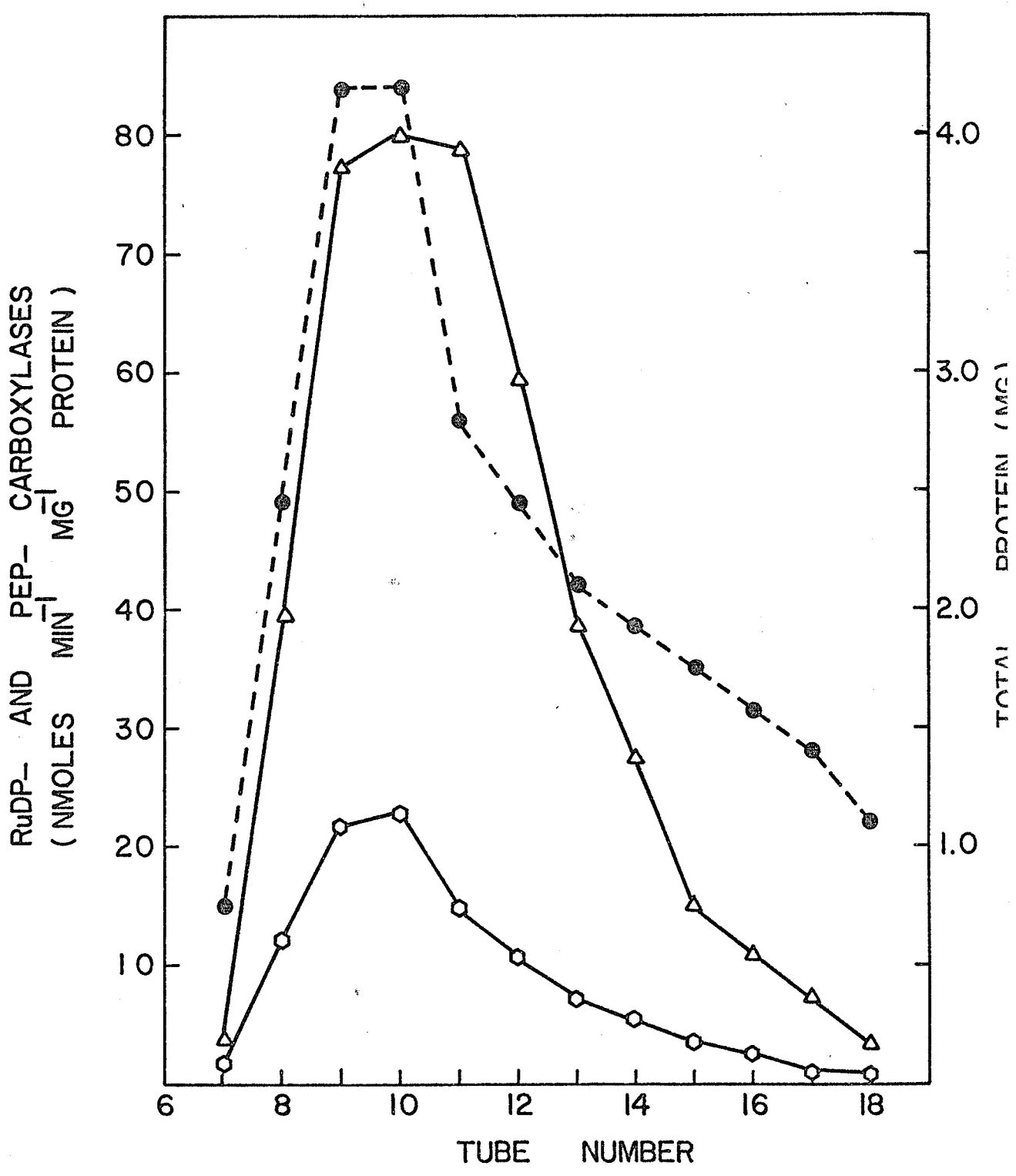

RuDP- AND PEP- CARBOXYLASES
(NMOLES MIN⁻¹ MG⁻¹ PROTEIN)
TOTAL PROTEIN (MG)
80
70
60
50
40
30
20
10
4.0
3.0
2.0
1.0
6
8
10
12
14
16
18
TUBE NUMBER

Table IV. Partial purification of crude extracts of cells grown in air. Data computed from Figure 20.

	Volume in ml	Total Units	Specific Activity	% Recovery	Purification
RuDP Carboxylase	-	(nmoles/min)	(nmoles/min/mg protein)		
Fraction					
0-60% $(NH_4)_2SO_4$	4.0	420.6	20.2	100.0	-
Sephadex G-100	21.0 (6-11)*	421.8	23.8	100.3	1.18
PEP Carboxylase	-	(nmoles/min)	(nmoles/min/mg protein)		
Fraction					
0-60% $(NH_4)_2SO_4$	4.0	4.4	0.2	100.0	-
Sephadex G-100	21.0 (6-11)*	57.1	3.2	1297.7	15.33
Carbonic Anhydrase	-	(E.U./min)	(E.U./min/mg protein)		
Fraction					
0-60% $(NH_4)_2SO_4$	4.0	43.1	2.07	100.0	-
Sephadex G-100	14.0 (7-10)*	36.7	2.72	85.2	1.37

*Denotes tube number

Table V. Partial purification of crude extracts of cells grown in 5% CO_2. Data computed from Figure 21.

	Volume in ml	Total Units	Specific Activity	% Recovery	Purification
RuDP carboxylase		(nmoles/min)	(nmoles/min/mg protein)	-	-
Fraction					
0-60% $(NH_4)_2SO_4$	3.5	1434	51.2	100.0	-
Sephadex G-100	38.5 (7-18)*	1322	49.6	92.2	0.96
PEP carboxylase	-	(nmoles/min)	(nmoles/min/mg protein)		
Fraction					
0-60% $(NH_4)_2SO_4$	3.5	256	8.0	100.0	-
Sephadex G-100	38.5 (7-18)*	317	11.9	123.8	1.48
Carbonic Anhydrase	-	(E.U./min)	(E.U./min/mg protein)	-	-
Fraction					
0-60% $(NH_4)_2SO_4$	3.5	0	0	-	-
Sephadex G-100	38.5 (7-18)*	0	0	-	-

* Denotes tube number

air. The complete absence of carbonic anhydrase activity in preparations from 5% CO_2 grown cells even after gel filtration suggested that the synthesis of this enzyme was repressed by high CO_2 tension. This was substantiated by the results presented in Table VI which showed decreased carbonic anhydrase activity with increased CO_2 concentration provided during growth.

The results obtained thus showed that the autotrophic growth of *Chlamydomonas segnis* in air was associated with relatively high carbonic anhydrase and RuDP-carboxylase activities. PEP-carboxylase activity was relatively low. Growth in 5% CO_2 appeared to be also regulated by carbonic anhydrase although its activity was comparatively low. RuDP-carboxylase also exhibited lesser activity. However, PEP-carboxylase was relatively high. From this comparison, it is clear that cells grown in 5% CO_2 require relatively lower carbonic anhydrase activity than cells grown in air (0.03% CO_2). In other words, cells grown in low CO_2 tension are compensated by a greater carbonic anhydrase activity compared to those grown at higher CO_2 tensions.

Table VI. The influence of CO_2 concentration on the activity of carbonic anhydrase in *Chlamydomonas segnis*.
Activity was determined in crude extracts after 6 hours growth in cells previously synchronized and grown in various concentrations of carbon dioxide.

	E.U./min/ml	E.U./min/mg protein
Air	0.045	4.5
0.1% CO_2	0.020	1.6
1.0% CO_2	0.004	0.4
5.0% CO_2	0.000	0.0

III. REGULATION OF PHOTOSYNTHESIS BY CARBONIC ANHYDRASE DURING THE GROWTH PHASE

If carbonic anhydrase is responsible for the regulation of the photosynthetic capacity, and RuDP- and PEP-carboxylases determine the path of carbon during photosynthesis, then altering the activities of these enzymes by transient changes of CO_2 concentration during growth should result in changes in the rate of the photosynthetic capacity and the path of carbon (see Part IV and V).

A. Enzyme(s) Levels as Influenced by Changes in CO_2 Concentration

Zoospores obtained from air cultures (Time:0) and subjected to 5% CO_2 during growth (Figure 22), acquired a relatively high level of RuDP-carboxylase (1.6 - 2.3 times the activity of the control) although the specific activity remained unchanged. This suggested that the enhanced synthesis of protein in 5% CO_2 might have contributed to the increase in the level of the enzyme expressed per ml algal suspension. PEP-carboxylase was initially undetectable and aeration with 5% CO_2 during growth did not activate the enzyme indicating the inhibitor(s) already present in zoospores secured from air cultures could not be removed by growth at high CO_2 tension. The level and specific activity of carbonic anhydrase were reduced

Figure 22. Effect of 5% CO_2 on RuDP-carboxylase and PEP-carboxylase activities provided during the growth of zoospores produced (Time:0) in air.

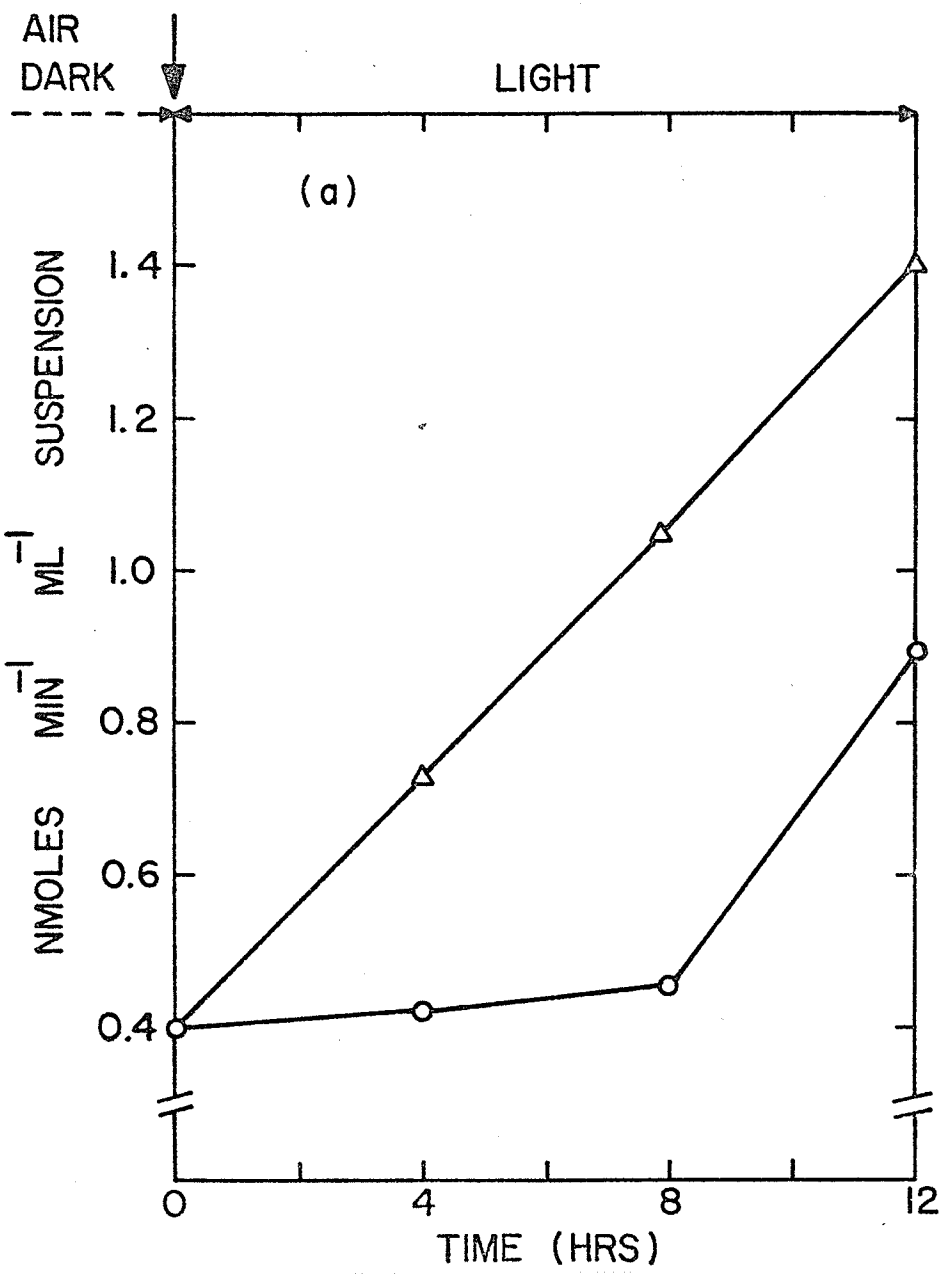
The activities were determined in crude extracts and expressed
(a) per ml suspension, or
(b) per mg protein.

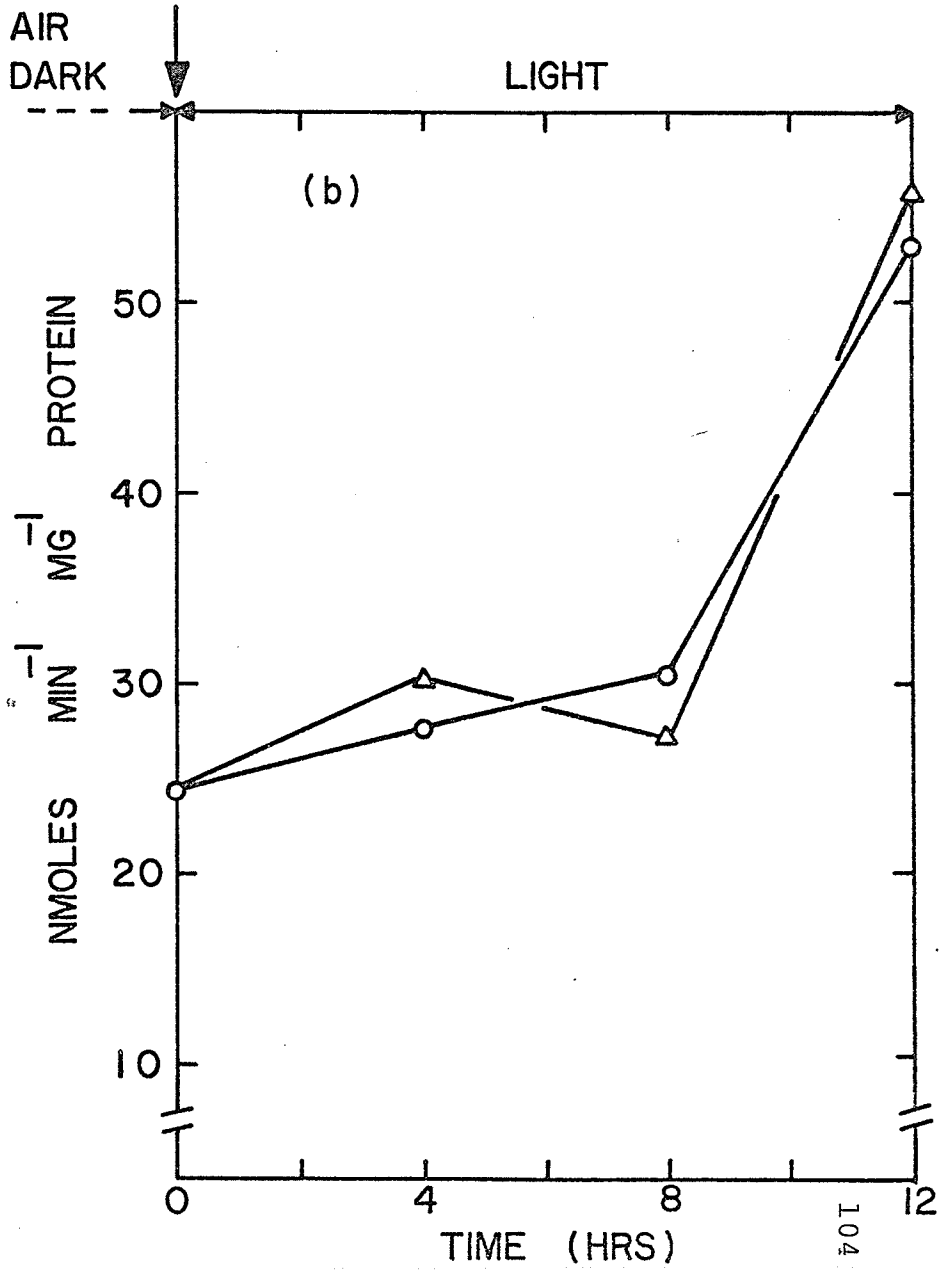
RuDP-carboxylase Activity

o—o—o Air to air
o—Δ—Δ Air to 5% CO_2

PEP-carboxylase Activity

Not detectable


AIR
DARK
LIGHT
(a)
NMOLES MIN⁻¹ ML⁻¹ SUSPENSION
1.4
1.2
1.0
0.8
0.6
0.4
0
4
8
12
TIME (HRS)


AIR
DARK
LIGHT
(b)
NMOLES MIN⁻¹ MG⁻¹ PROTEIN
50
40
30
20
10
0
4
8
12
TIME (HRS)

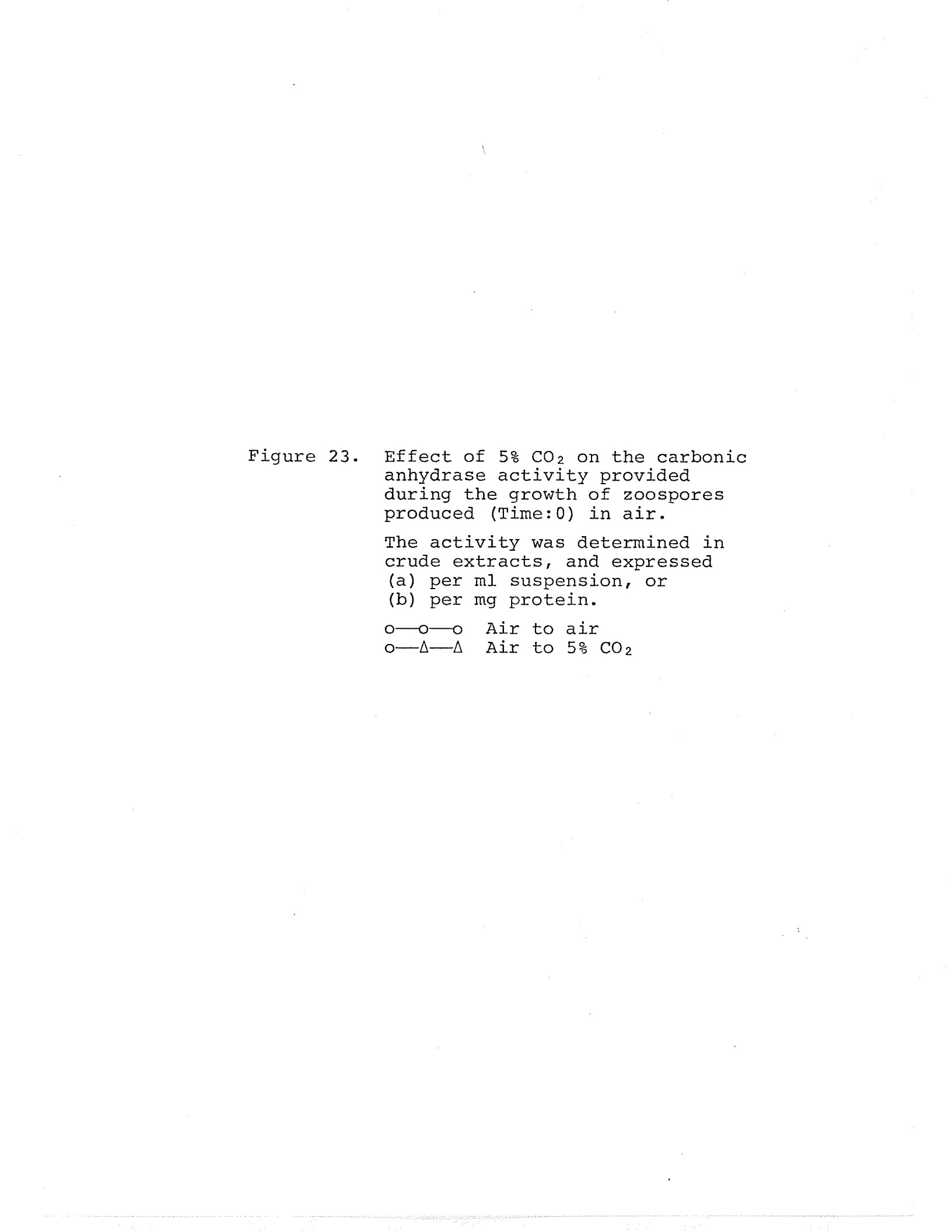
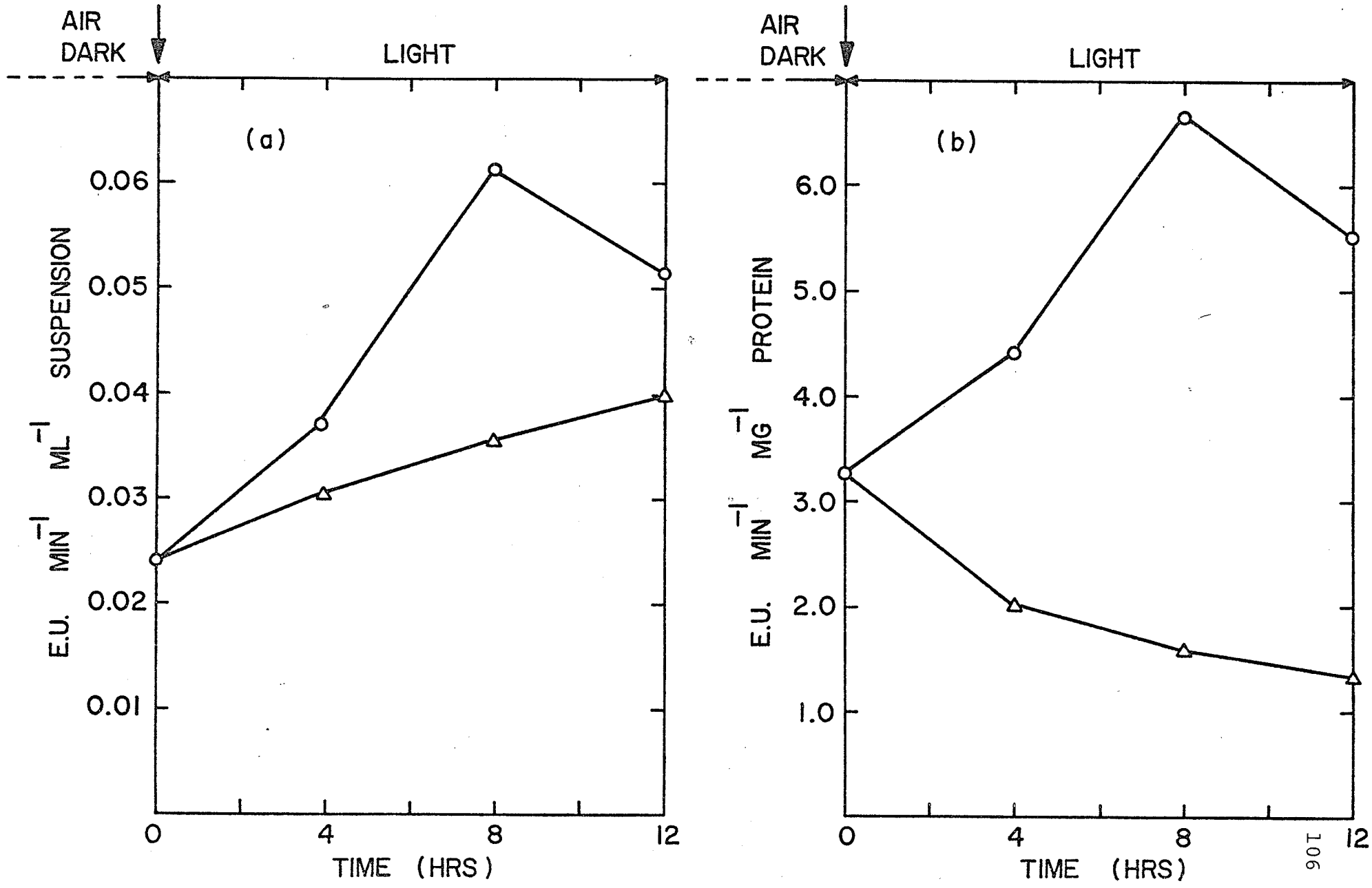


Figure 23. Effect of 5% CO_2 on the carbonic anhydrase activity provided during the growth of zoospores produced (Time:0) in air.

The activity was determined in crude extracts, and expressed
(a) per ml suspension, or
(b) per mg protein.

o—o—o Air to air
o—Δ—Δ Air to 5% CO_2


AIR
DARK
LIGHT
(a)
0.06
0.05
0.04
0.03
0.02
0.01
E.U. MIN^{-1} ML^{-1} SUSPENSION
0
4
8
12
TIME (HRS)
AIR
DARK
LIGHT
(b)
6.0
5.0
4.0
3.0
2.0
1.0
E.U. MIN^{-1} MG^{-1} PROTEIN
0
4
8
12
TIME (HRS)

by 1.2 - 1.8 and 2.2 - 4.1 times, respectively as compared to the control (Figure 23).

When zoospores produced in 5% CO_2 (Time:0) were provided with air during growth and compared with the control left in 5% CO_2, neither the level (per ml) nor the specific activity of RuDP-carboxylase changed (Figure 24). On the other hand, both the level and specific activity of PEP-carboxylase (Figure 24) decreased by 7 - 14 times and 8 - 12 times, respectively. Meanwhile, the activity of carbonic anhydrase (Figure 25) increased 3 - 5 times as indicated by the enzyme level and specific activity.

It is obvious from these results that carbonic anhydrase was the enzyme which responded consistently to the changes in CO_2 concentration. Regardless of the prehistory of the zoospores (i.e. produced in the dark in air or 5% CO_2 cultures), the cells responded characteristically by acquiring the high and low levels of carbonic anhydrase activity when allowed to grow in air and 5% CO_2 respectively. RuDP- and PEP-carboxylases did not respond in a similar manner. The former remained unchanged when cultures were provided with air instead of 5% CO_2 and increased when 5% CO_2 replaced air. PEP-carboxylase decreased in activity on departure from 5% CO_2 to air but did not change when 5% CO_2 was supplied to zoospores produced in air.

Figure 24. Effect of air on the RuDP-carboxylase and PEP-carboxylase activities, provided during the growth of zoospores produced (Time:0) in 5% CO_2.

The activities were determined in crude extracts, and expressed (a) per ml suspension, or (b) per mg protein.

RuDP-carboxylase Activity

Δ—Δ—Δ 5% CO_2 to 5% CO_2
Δ—o—o 5% CO_2 to air

PEP-carboxylase Activity

▲---▲---▲ 5% CO_2 to 5% CO_2
▲---●---● 5% CO_2 to air

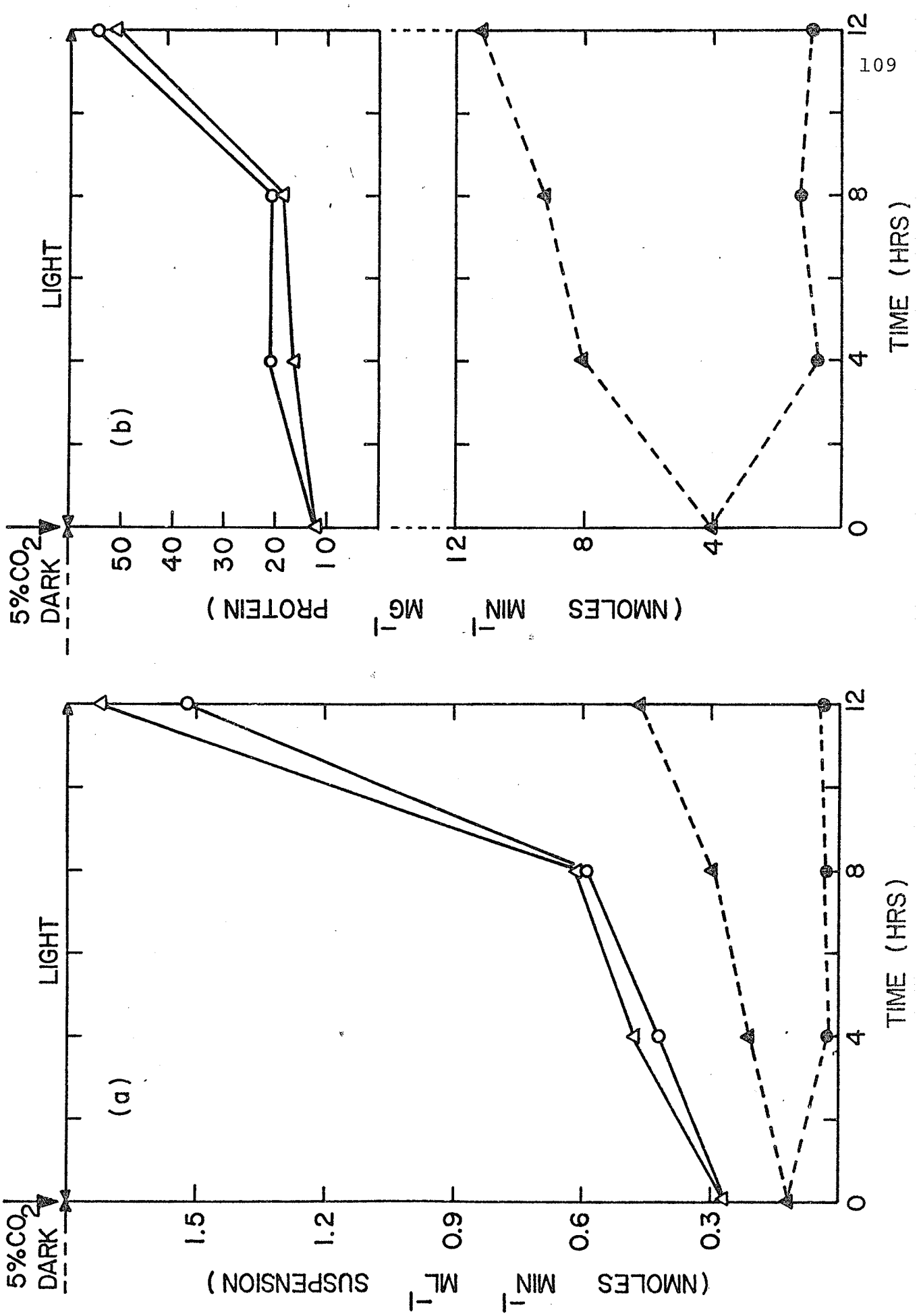
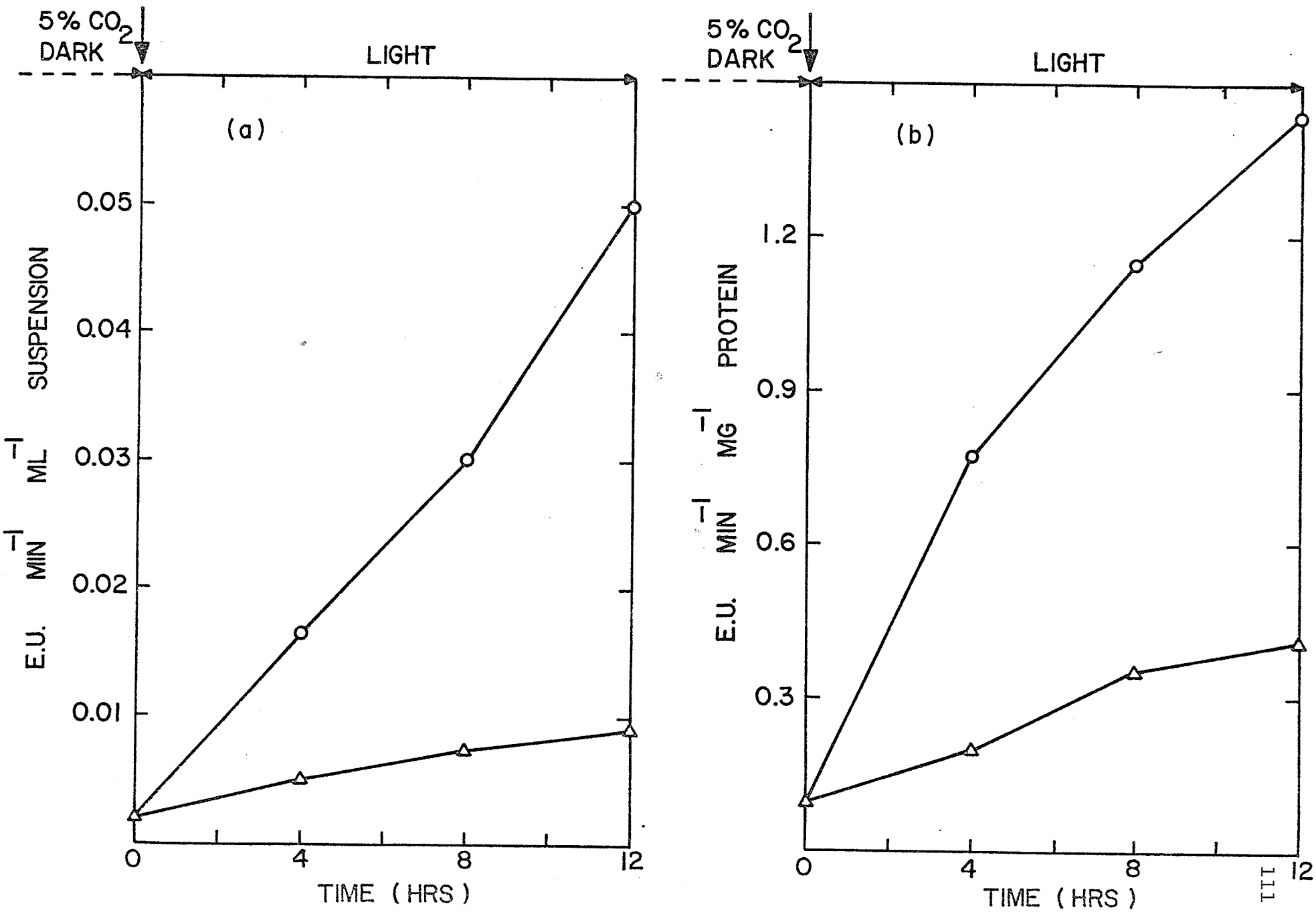

LIGHT
(b)
5%CO2
DARK
50
40
30
20
10
(MG PROTEIN)
12
8
4
(NMOLES MIN-1)
0
4
8
12
TIME (HRS)
LIGHT
(a)
5%CO2
DARK
1.5
1.2
0.9
0.6
0.3
(ML-1 SUSPENSION)
(NMOLES MIN-1)
0
4
8
12
TIME (HRS)

Figure 25. Effect of air on the carbonic anhydrase activity provided during the growth of zoospores produced (Time:0) in 5% CO_2.

The activity was determined in crude extracts and expressed (a) per ml suspension, or (b) per mg protein.

Δ—Δ—Δ 5% CO_2 to 5% CO_2
Δ—o—o 5% CO_2 to air


5% CO_2
DARK
LIGHT
(a)
E.U. MIN^{-1} ML^{-1} SUSPENSION
0.05
0.04
0.03
0.02
0.01
0
4
8
12
TIME (HRS)
5% CO_2
DARK
LIGHT
(b)
E.U. MIN^{-1} MG^{-1} PROTEIN
1.2
0.9
0.6
0.3
0
4
8
12
TIME (HRS)

B. Photosynthetic Capacity and Carbonic Anhydrase Activity

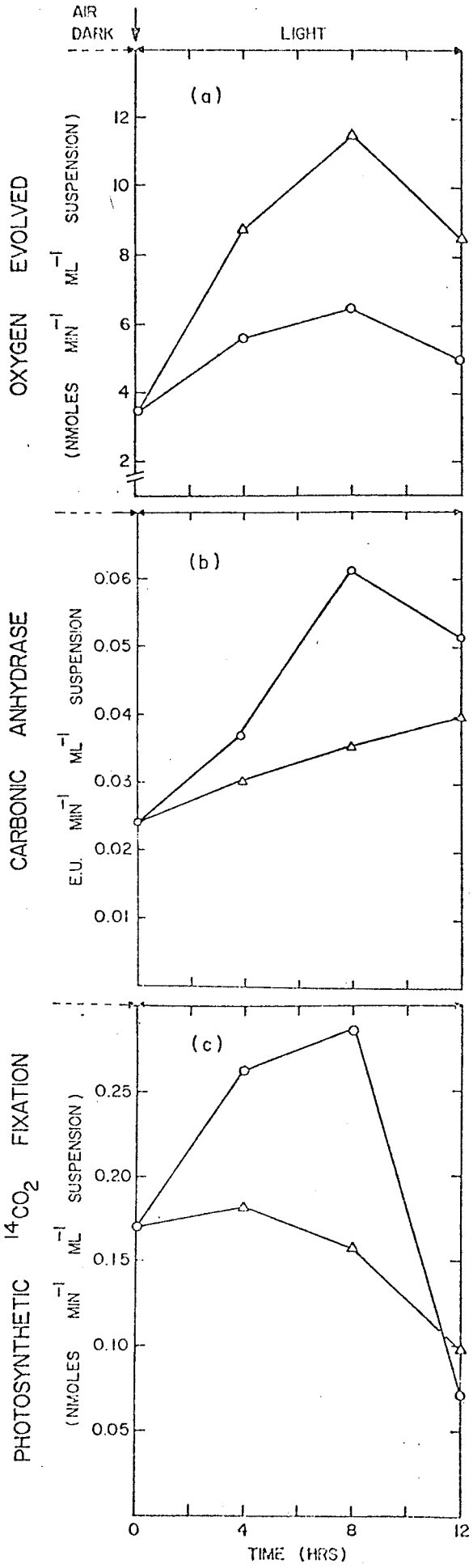
In zoospores produced in air (Time:0 ; homogeneous population), the oxygen evolved (Figure 26a) doubled and quadruplicated when grown either in air or 5% CO_2, respectively in preparation for the production of one or two mitotic divisions (Figure 9). In order to achieve this, air grown cells increased their carbonic anhydrase to account for the provision of the substrate (CO_2 or HCO_3^-) and by this could cope with the low CO_2 in air (0.03%). On the other hand, zoospores grown in 5% CO_2, which showed just a slow increase in carbonic anhydrase activity (Figure 26b), would still require carbonic anhydrase to adjust the amount of substrate presumably essential for just the production of 4 daughter cells. This may suggest that carbonic anhydrase was working in air cultures to drive in the substrate to the sites of reactions, and in 5% CO_2 cultures to protect the cells from the high endogenous substrate concentration that may extend growth, resulting in the production of more than 4 zoospores. The higher concentration of endogenous substrate in cells grown in 5% CO_2 as compared to that of air grown cells could be seen from the $^{14}CO_2$ fixation experiment. 5% CO_2 grown cells, shown to be more photosynthetically active than air grown cells (Figure 26a) fixed less $^{14}CO_2$ than the air

Figure 26. Photosynthetic capacity, carbonic anhydrase activity and photosynthetic $^{14}CO_2$ fixation of zoospores produced (Time:0) in air and during their subsequent growth in air (o), or 5% CO_2 (Δ).

(a) Photosynthetic oxygen evolution in Warburg buffer No. 9.

(b) Carbonic anhydrase activity from Figure 23a.

(c) Photosynthetic $^{14}CO_2$ fixation. 10 μCi $NaH^{14}CO_3$ (59 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$.


AIR
DARK
LIGHT
(a)
OXYGEN EVOLVED
(NMOLES MIN⁻¹ ML⁻¹ SUSPENSION)
12
10
8
6
4
2
(b)
CARBONIC ANHYDRASE
E.U. MIN⁻¹ ML⁻¹ SUSPENSION
0.06
0.05
0.04
0.03
0.02
0.01
(c)
PHOTOSYNTHETIC $^{14}CO_2$ FIXATION
(NMOLES MIN⁻¹ ML⁻¹ SUSPENSION)
0.25
0.20
0.15
0.10
0.05
0
4
8
12
TIME (HRS)

grown cells (Figure 26c). This could be attributed to a dilution effect exerted by the high cold endogenous substrate (CO_2 or HCO_3^-). In other words, cells grown in 5% CO_2 appeared to be saturated by the substrate and if carbonic anhydrase activity increased during growth in such cells, it must have been to control the substrate pool size. Therefore, it could be concluded that carbonic anhydrase regulates photosynthesis resulting in controlled growth. The latter would trigger the S-phase in preparation of cell division.

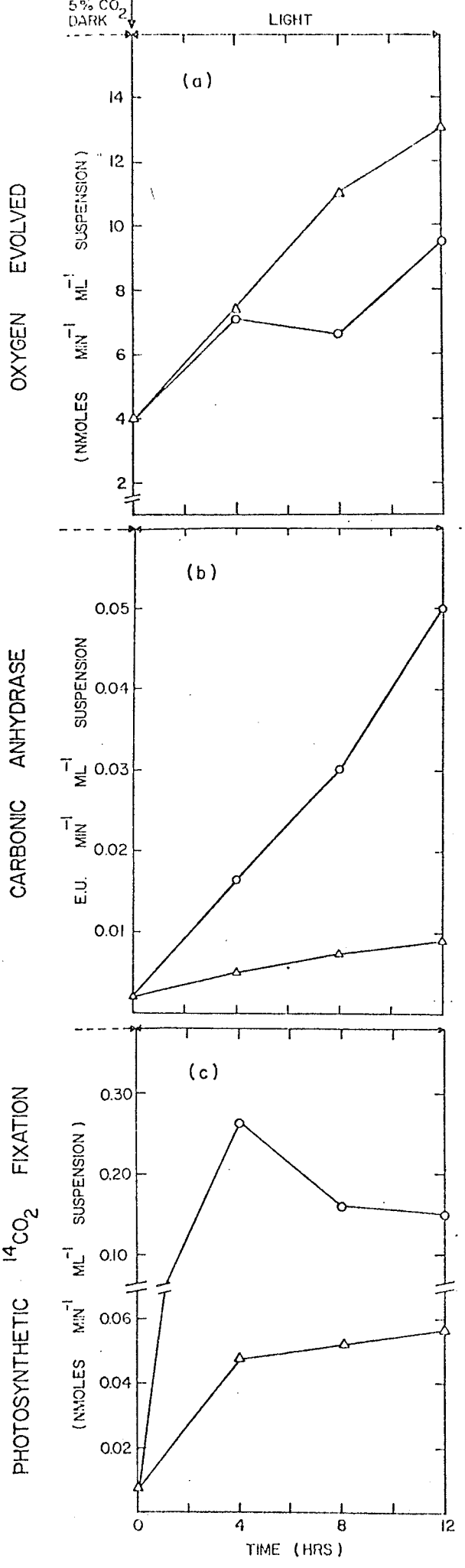
If the zoospores produced in 5% CO_2 (Time:0; heterogeneous population) were used, the photosynthetic O_2-evolution did not double nor quadruplicate (Figure 27a) within the 8 hours growth phase as observed in homogeneous zoospores (Figure 26a). When placed in air, carbonic anhydrase again increased in air to support the photosynthetic activity in air cultures to account for growth in preparation for cell division (Figure 27b). In 5% CO_2 cultures, the slow increase in carbonic anhydrase again supported the requirement for carbonic anhydrase to adjust the endogenous substrate level, and to provide for the growth beyond 8 hours required to produce higher number of zoospores. The results of $^{14}CO_2$ fixation (Figure 27c) could again be attributable to a high endogenous CO_2 or HCO_3^- pool in 5% CO_2 grown cells,thus causing a dilution effect and,hence, lower

Figure 27. Photosynthetic capacity, carbonic anhydrase activity, and photosynthetic $^{14}CO_2$ fixation of zoospores produced (Time:0) in 5% CO_2 and during their subsequent growth in air (o) or 5% CO_2 (Δ).

(a) Photosynthetic oxygen evolution in Warburg buffer No. 9.

(b) Carbonic anhydrase activity from Figure 25a.

(c) Photosynthetic $^{14}CO_2$ fixation. 10 μCi Na $H^{14}CO_3$ (59 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$.


5% CO_2
DARK
LIGHT
(a)
OXYGEN EVOLVED
(NMOLES MIN^{-1} ML^{-1} SUSPENSION)
14
12
10
8
6
4
2
(b)
CARBONIC ANHYDRASE
E.U. MIN^{-1} ML^{-1} SUSPENSION
0.05
0.04
0.03
0.02
0.01
(c)
PHOTOSYNTHETIC $^{14}CO_2$ FIXATION
(NMOLES MIN^{-1} ML^{-1} SUSPENSION)
0.30
0.20
0.10
0.06
0.04
0.02
0
4
8
12
TIME (HRS)

$^{14}CO_2$ fixation.

In addition to the dilution effect, the high specific activities of carbonic anhydrase and RuDP-carboxylase in air grown cells could be responsible for the higher $^{14}CO_2$ fixation which was conducted at very low CO_2 concentration (160 nmoles/2ml).

IV. PRODUCTS OF PHOTOSYNTHETIC $^{14}CO_2$ FIXATION

Previous investigators have shown that the products of $^{14}CO_2$ fixation varied during the cell cycle (Smith *et al.*, 1961; Ahmed and Ries, 1969; Walther and Edmunds, 1973). Using asynchronous cultures, the products of fixation were shown to be dependent on the previous history of the cell as well as the photosynthesizing conditions (Graham and Whittingham 1968; Döhler, 1973; Merrett and Lord, 1973). The products of $NaH^{14}CO_3$ fixation in air and 5% CO_2 grown cells were subsequently investigated during the first 12 hours of illumination to throw some light on the metabolism of *Chlamydomonas segnis* as influenced by CO_2 concentration during growth and the S-phase.

A. Rate of Incorporation of $^{14}CO_2$ as Influenced by Growth in Either Air or 5% CO_2

Figures 28 to 30 are sample results showing that the total incorporation of $^{14}CO_2$ was linear with incubation time whether zoospores or grown cells were used and regardless of the CO_2 concentration used to aerate the cultures. The rate of incorporation into the ethanol-water soluble fraction was linear for about 1 minute after which the rate slowed down. The residue plus the chloroform-soluble fraction exhibited an exponential increase with incubation time. The

Figure 28. Incorporation of $^{14}CO_2$ into the various cell fraction as a function of incubation time. Using

(a) Zoospores produced (Time:0) in air cultures. Data are obtained from Table I in Appendix.

(b) Zoospores produced (Time:0) and grown in air for 8 hours. Data are obtained from Table II in Appendix.

T = Total fixation
E = Ethanol-water soluble fraction
R = Residue and chloroform soluble fraction

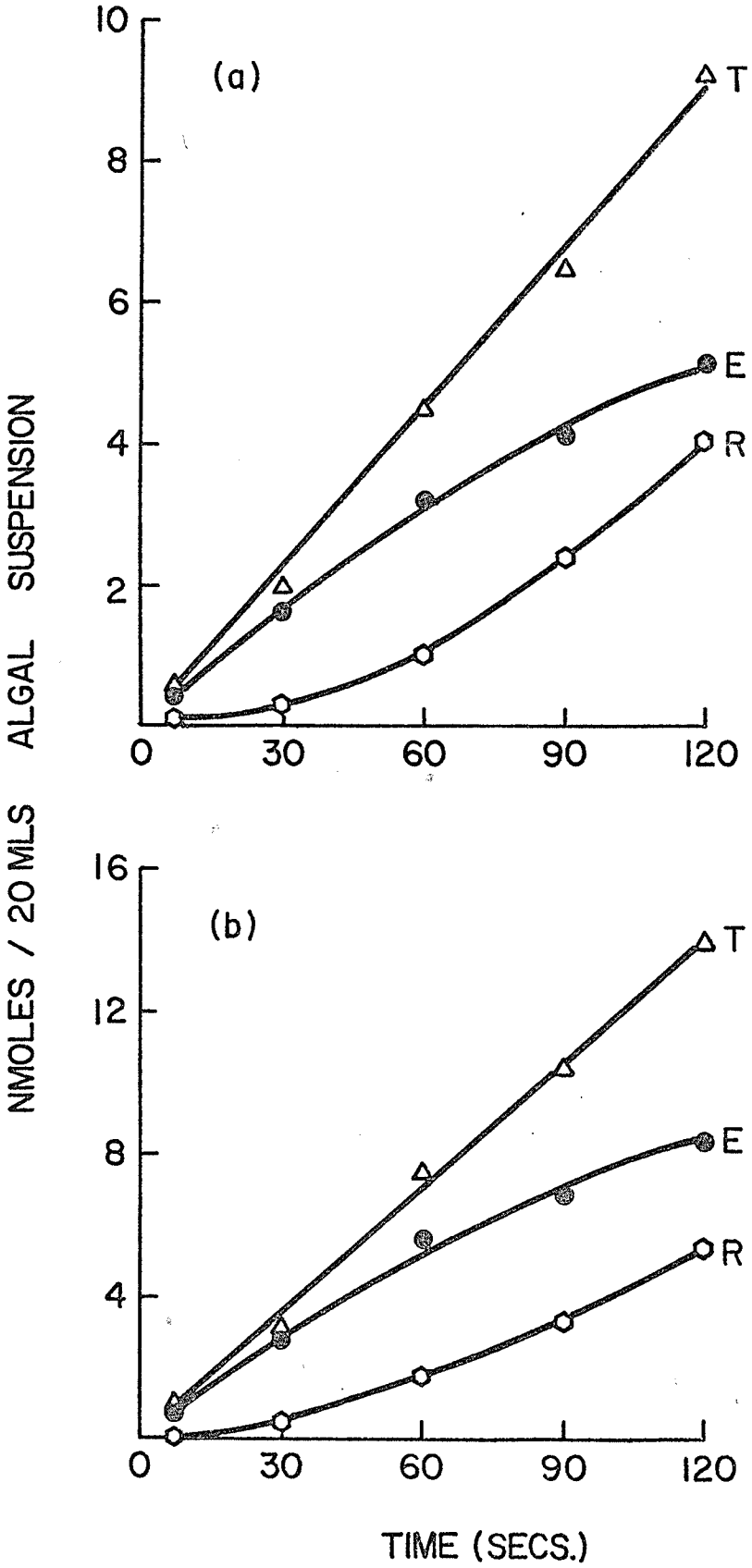

(a)
(b)
T
E
R
NMOLES / 20 MLS ALGAL SUSPENSION
TIME (SECS.)
0
30
60
90
120
2
4
6
8
10
12
16

Figure 29. Incorporation of $^{14}CO_2$ into the various cell fractions as a function of incubation time. Using

(a) Zoospores produced (Time:0) in air but grown in 5% CO_2 for 8 hours. Data are obtained from Table III in Appendix.

(b) Zoospores produced (Time:0) in 5% CO_2 but grown in air for 8 hours. Data are obtained from Table IV in Appendix.

T = Total fixation
E = Ethanol-water soluble fraction
R = Residue + chloroform soluble fractions

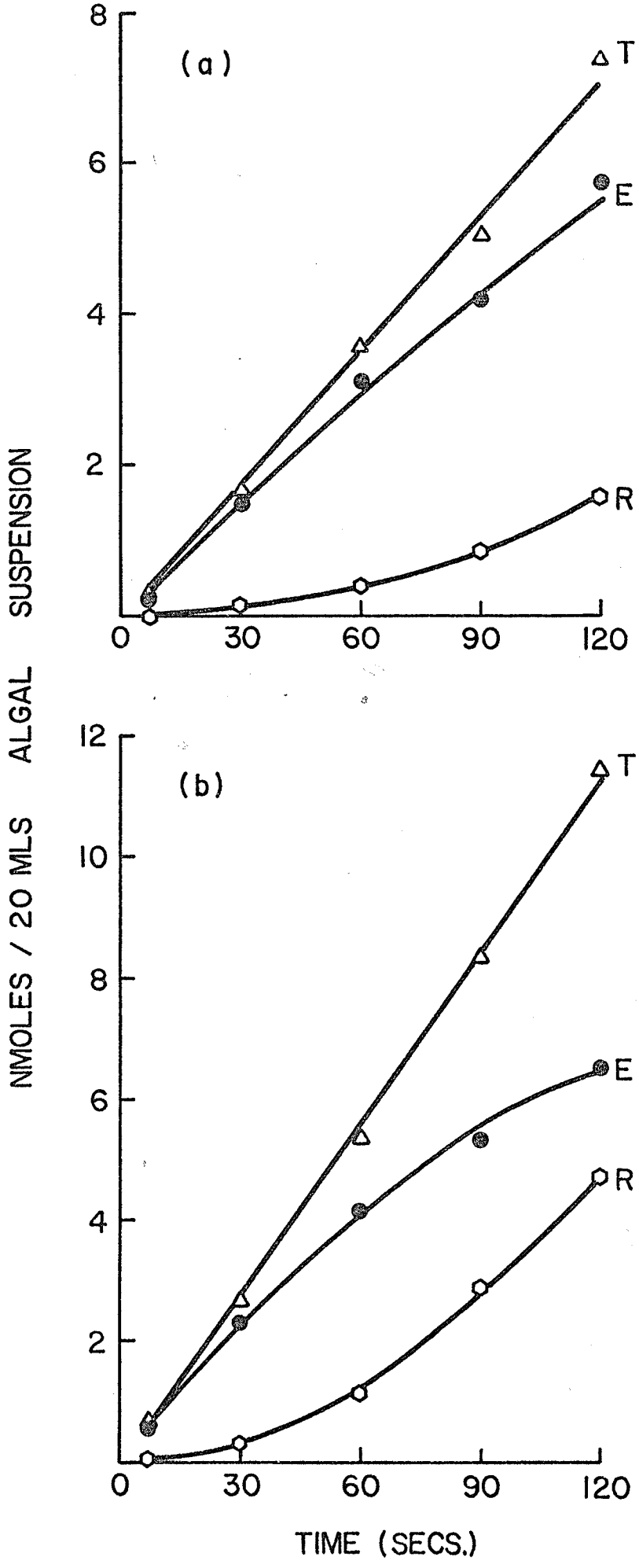
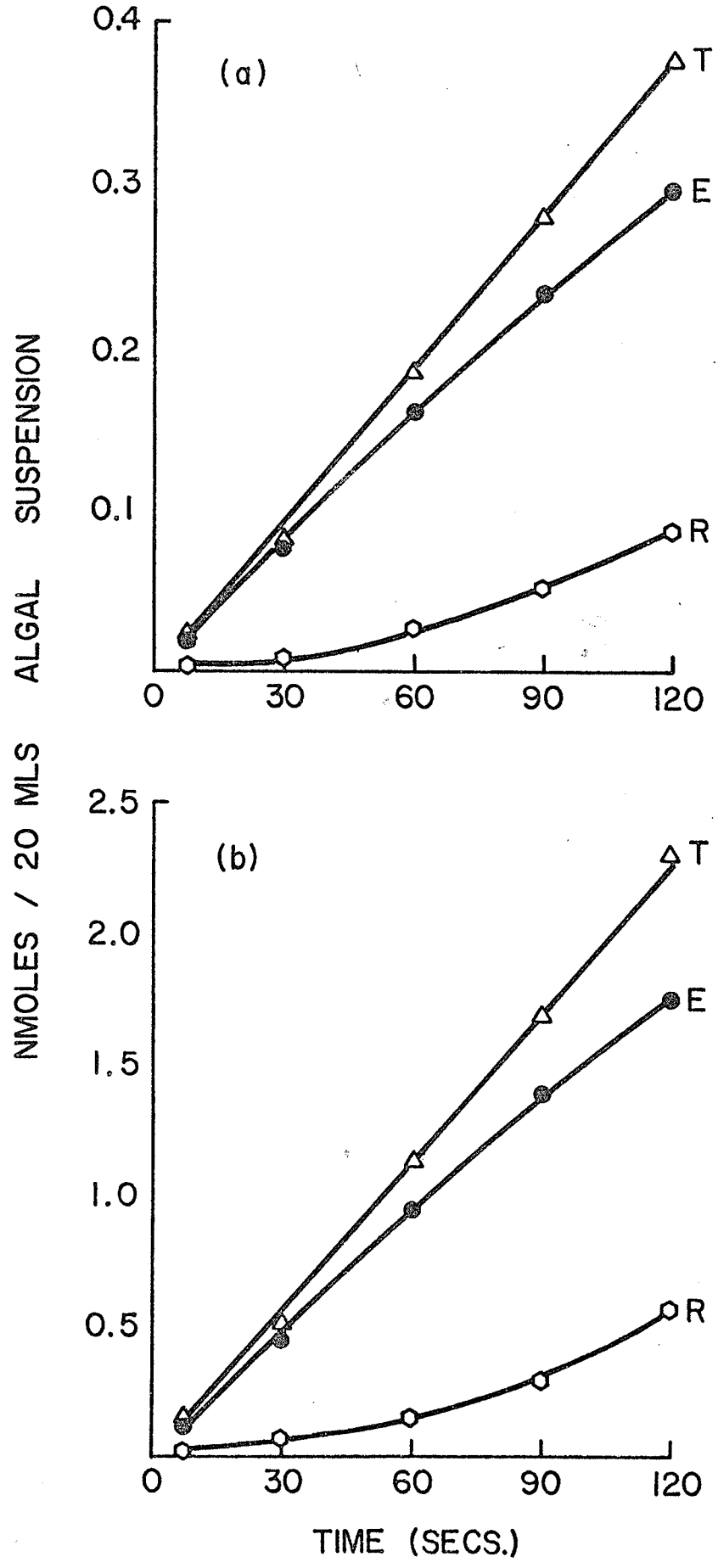

(a)
(b)
T
E
R
NMOLES / 20 MLS ALGAL SUSPENSION
TIME (SECS.)
0
30
60
90
120
2
4
6
8
10
12

Figure 30. Incorporation of $^{14}CO_2$ into the various cell fraction as a function of incubation time using

(a) Zoospores produced (Time:0) in 5% CO_2 cultures. Data are obtained from Table V in Appendix.

(b) Zoospores produced (Time:0) and grown in 5% CO_2 for 8 hours. Data are obtained from Table VI in Appendix.

T = Total fixation
E = Ethanol-water soluble fraction
R = Residue + chloroform soluble fractions


(a)
(b)
T
E
R
0.4
0.3
0.2
0.1
2.5
2.0
1.5
1.0
0.5
0
30
60
90
120
NMOLES / 20 MLS ALGAL SUSPENSION
TIME (SECS.)

amount incorporated at any one time interval into the residue plus chloroform-soluble fraction was lower than the ethanol-water soluble fraction. The rate of flow of ^{14}C into the residue plus chloroform-soluble fraction was faster in cells grown in air (Tables I, II, IV in Appendix) than in 5% CO_2 (Tables III, V and VI in Appendix) regardless of whether in zoospores were produced in air or 5% CO_2. The products of 1 minute fixation of the ethanol-water soluble fraction at different stages of the cell cycle were further investigated.

B. Distribution of ^{14}C in the Ethanol-water Soluble Fraction

Figure 31 summarizes the distribution of ^{14}C into the various fractions after 1 minute fixation during the phases of growth and DNA synthesis.

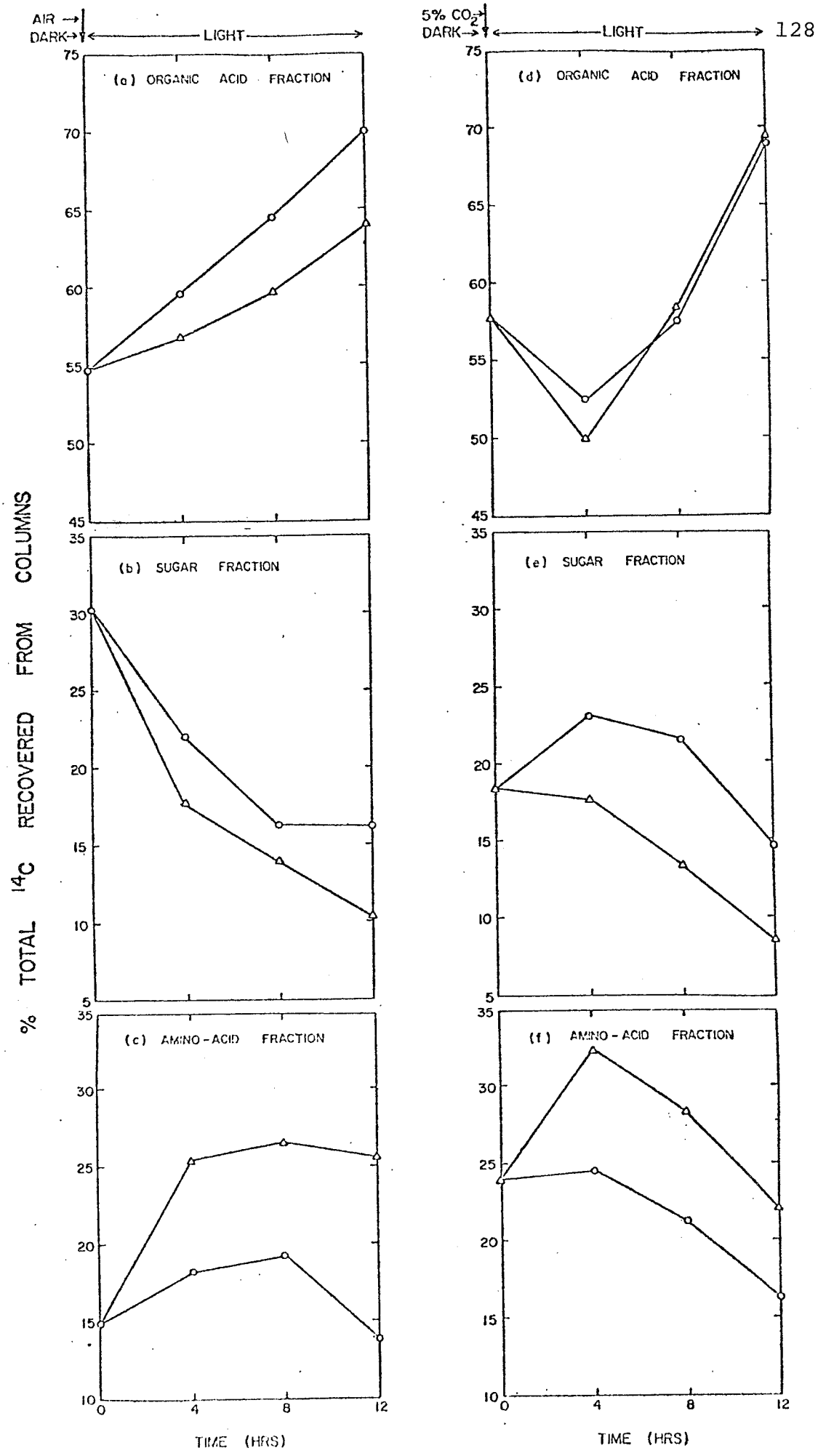
Figure 31 (a, b, and c) showed that in zoospores produced and grown in air, the organic acid fraction exhibited a linear increase. Meanwhile, the sugar fraction showed a continued decrease. The amino-acid fraction increased during the growth phase (0 - 8th hour) and then declined. Provision of 5% CO_2 to these zoospores instead of air did not alter the general pattern observed in zoospores left to grow in air. However, the percentage distribution of ^{14}C between the

Figure 31. Distribution of ^{14}C in the various fractions of the ethanol-water soluble fraction during the phases of growth and DNA synthesis after one minute $^{14}CO_2$ fixation.

a, b, and c: Zoospores produced in air cultures (Time:0) and during their growth in air (o), or 5% CO_2 (Δ)

d, e, and f: Zoospores produced in 5% CO_2 cultures (Time:0) and during their growth in either 5% CO_2 (Δ), or air (o).

Data are obtained from Table VII in Appendix.


AIR →
DARK→
LIGHT
5% CO2→
DARK→
LIGHT
(a) ORGANIC ACID FRACTION
(b) SUGAR FRACTION
(c) AMINO-ACID FRACTION
(d) ORGANIC ACID FRACTION
(e) SUGAR FRACTION
(f) AMINO-ACID FRACTION
% TOTAL 14C RECOVERED FROM COLUMNS
TIME (HRS)
TIME (HRS)

various fractions was affected. Both the sugar and organic acid fractions showed a lower percentage of ^{14}C, while the amino-acid fraction showed a higher percentage when compared to cells growing in air. The bulk of ^{14}C (55 - 70%) was in the organic acid fraction while the rest was distributed between the amino-acid and sugar fractions.

On the other hand, in zoospores produced and grown in 5% CO_2 (Figure 31d, e and f), the incorporation of ^{14}C into the organic acid fraction initially decreased after 4 hours growth and then increased. The sugar fraction showed a continuous decrease while the amino-acid fraction exhibited an increase during the first 4 hours of growth, followed by a decrease. When the zoospores produced in 5% CO_2 (Time:0) were provided with air during the growth instead of 5% CO_2, the pattern of ^{14}C distribution in the various fractions was not altered very much during the 12 hour period. The percentage incorporation of ^{14}C into the organic acid fraction was virtually unaltered. However, incorporation into the amino-acid fraction was greatly reduced with a concomitant increase in the sugar fraction. The bulk of ^{14}C (50 - 70%) was found in the organic acid fraction while the rest was distributed between the amino-acid and the sugar fractions.

Generally speaking, the incorporation of ^{14}C into

the various fractions varied according to whether the zoospores were produced in air or 5% CO_2 and the difference between them can be manifested in their ability to incorporate ^{14}C into amino-acids or sugars during growth as summarized in the following:

(i) Organic acid fraction > sugar fraction > amino-acid fraction in zoospores produced and grown in air.

(ii) Organic acid fraction > amino-acid fraction > sugar fraction in zoospores produced and grown in 5% CO_2.

(iii) Organic acid fraction > amino acid fraction $\geq$ sugar fraction in zoospores produced in either air or 5% CO_2 and then grown in 5% CO_2 or air respectively.

The higher incorporation of amino-acids in cells grown in 5% CO_2 may explain why zoospores produced in 5% CO_2 cultures contain a higher protein content (Table II). As the pool size of the various products becomes saturated, the ability to incorporate ^{14}C into it diminishes. The ability to direct the flow of carbon into any cell constituents would therefore be dependent on the rate at which that particular pool becomes saturated under different growth conditions. This was probably the case when the zoospores rich in protein (i.e., produced in 5% CO_2) were grown in air, their

ability to incorporate ^{14}C into amino-acid declined (Figure 31f) and, hence, the production of zoospores with relatively lower protein content in air cultures (Table II).

C. Distribution of ^{14}C of One Minute Fixation in the Various Products of the Organic Acid, Sugar and Amino-acid Fractions

Chromatography and radioautography of the organic acid, sugar and amino-acid fractions showed no qualitative difference but a quantitative difference was observed during growth and S-phase in air and 5% CO_2 cultures. The results given in Tables VIII to XIII (in the Appendix) are summarized in Figures 32 to 35.

1. Effect of 5% CO_2 Provided During Growth of Zoospores Previously Produced in Air

Figure 32 gives the results of zoospores produced and grown in air. The effect of imposition of 5% CO_2 on the distribution of the products is given in Figure 33. In zoospores produced and grown in air, sugar monophosphates decreased slightly after 4 hours growth, after which the level increased to its original level and remained constant. The imposition of 5% CO_2 decreased the cells' ability to incorporate ^{14}C into the sugar monophosphates which exhibited a declining

Figure 32. Photosynthetic products of one minute $^{14}CO_2$ fixation during growth and S-phases of zoospores produced and grown in air. The data presented are obtained from Tables VIII, IX, and X in Appendix.

Abbreviations	Products
	Organic acid fraction
G3P	Glyceraldehyde-3-phosphate
ISL	Isocitric lactone
MAL	Malic acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
GLY	Glyceraldehyde
POL	Polysaccharides
SUC	Sucrose
	Amino acid Fraction
ALA	Alanine
GLY	Glycine
SER	Serine

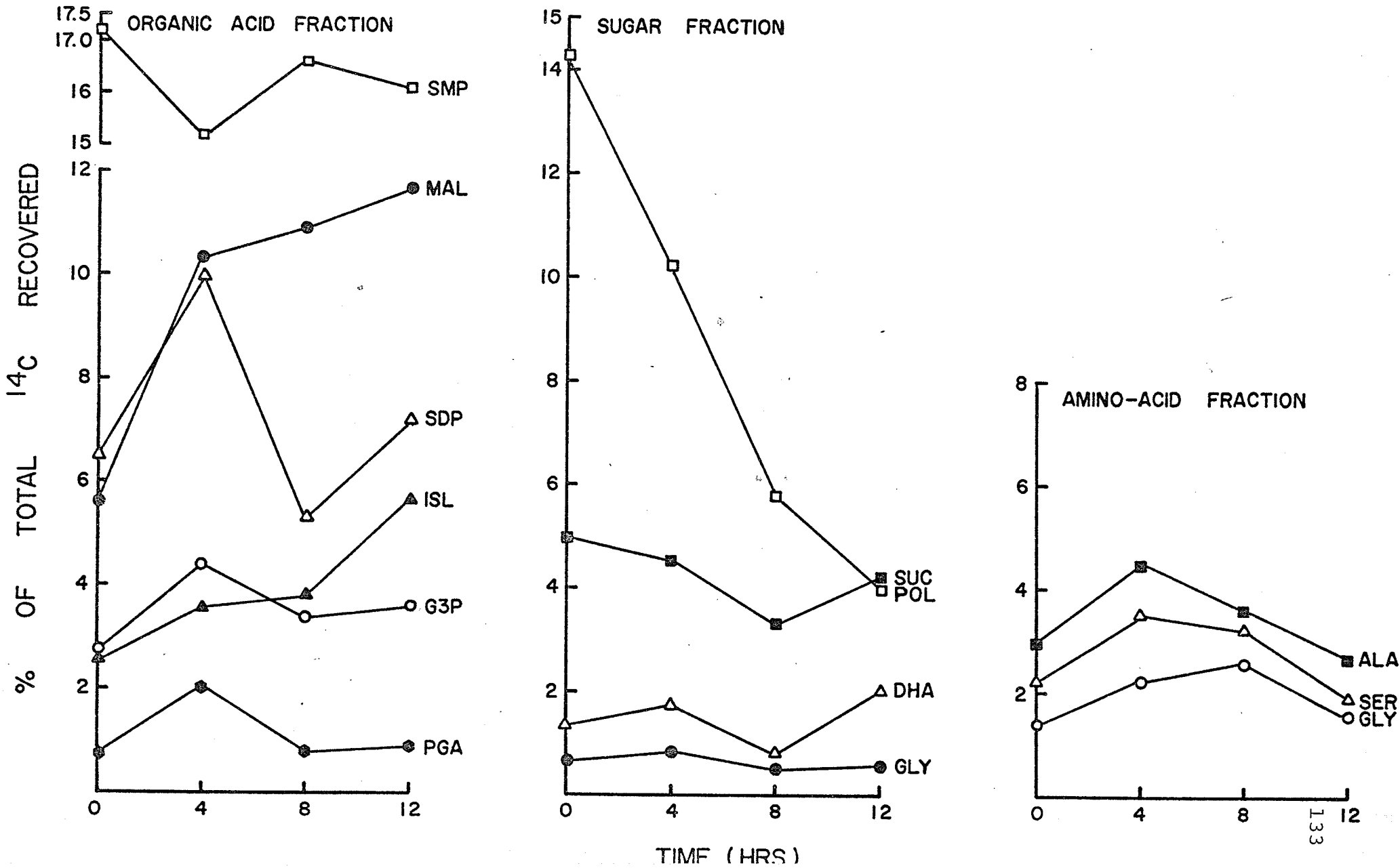
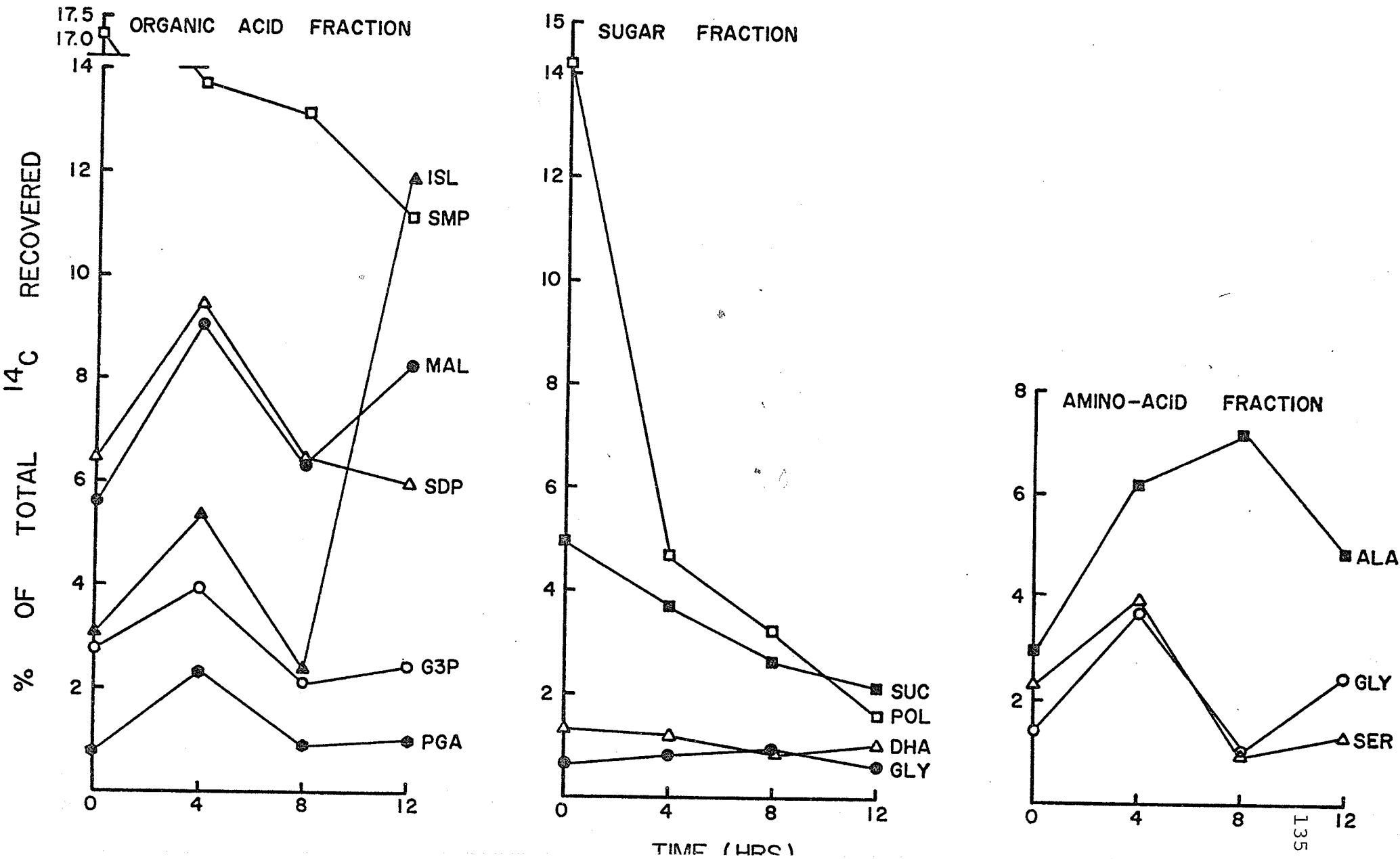

PRODUCTS OF 1 MIN FIXATION
ORGANIC ACID FRACTION
SUGAR FRACTION
AMINO-ACID FRACTION
% OF TOTAL 14C RECOVERED
TIME (HRS)
SMP
MAL
SDP
ISL
G3P
PGA
SUC
POL
DHA
GLY
ALA
SER
GLY
17.5
17.0
16
15
12
10
8
6
4
2
0
4
8
12
14

Figure 33. Photosynthetic products of one minute $^{14}CO_2$ fixation of zoospores produced in air (Time:0) but grown in 5% CO_2 during growth and S-phases.
The data are obtained from Tables VIII, IX and X in Appendix.

Abbreviations	Products
	Organic acid fraction
G3P	Glyceraldehyde-3-Phosphate
ISL	Isocitric lactone
MAL	Malic acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
GLY	Glyceraldehyde
POL	Polysaccharides
SUC	Sucrose
	Amino acid fraction
ALA	Alanine
GLY	Glycine
SER	Serine


PRODUCTS OF 1 MIN FIXATION
ORGANIC ACID FRACTION
SUGAR FRACTION
AMINO-ACID FRACTION
% OF TOTAL ^{14}C RECOVERED
TIME (HRS)
ISL
SMP
MAL
SDP
G3P
PGA
SUC
POL
DHA
GLY
ALA
GLY
SER

trend during the cell development. The amount and trend of sugar diphosphates, glyceraldehyde-3-phosphate and 3-phosphoglycerate remained virtually unaltered as a resultant of increased CO_2 tension during growth. All exhibited an increase after 4 hours growth, declined and then increased slightly from the 8th to the 12th hour. Malate and isocitrate lactone in air grown cells exhibited continued increase during the cell cycle. The amount of malate was very much greater than isocitrate lactone. The imposition of 5% CO_2 during growth, resulted in a decrease in the amount of malate and an increase in the amount of isocitrate lactone. The pattern was also altered. Both malate and isocitrate lactone exhibited an increase after 4 hours growth, declined and then increased between 8th and 12th hour. The amount of malate was higher than isocitrate lactone during growth phase (0 - 8th hour, light), after which isocitrate lactone level surpassed that of malate.

Of the sugar fraction, the major incorporation of ^{14}C occurred in polysaccharides and sucrose, the former being greater than the latter in both air and 5% CO_2 growing cells. In air grown cells, the amount of polysaccharides and sucrose were respectively greater than the polysaccharides and

sucrose in 5% CO_2 cells. Both exhibited a declining trend during cellular development in both types of culture.

Alanine, serine and glycine contributed to the major amino-acids formed. In air grown cells, alanine and serine increased after 4 hours growth and then declined, whereas this decline was not observed for glycine until after the 8th hour, the amount present being alanine > serine > glycine. Provision of 5% CO_2 altered the pattern as well as the amount incorporated into alanine, serine and glycine. Alanine showed an increase during the growth phase (0 - 8th hour) before declining. Both serine and glycine increased at the 4th hour, declined at the 8th and then increased. The amount present being alanine > serine = glycine. The amount of alanine present in air grown cells was less than that present in 5% CO_2 cells. Serine and glycine were almost the same in both type of cultures.

2. Effect of Air Provided During Growth of Zoospores Previously Produced in 5% CO_2

The ^{14}C products from zoospores produced and grown in 5% CO_2 are represented in Figure 34. The products from those zoospores when grown in air are shown in Figure 35. The amount of sugar mono-phosphates in 5% CO_2 grown cells was lower than that

Figure 34. Photosynthetic products of one minute $^{14}CO_2$ fixation during growth and S-phases of zoospores produced and grown in 5% CO_2.
The data are obtained from Tables XI, XII, and XIII in Appendix.

Abbreviations	Products
	Organic acid fraction
G3P	Glyceraldehyde-3-phosphate
ISL	Isocitric lactone
MAL	Malic acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
GLY	Glyceraldehyde
POL	Polysaccharides
SUC	Sucrose
	Amino-acid fraction
ALA	Alanine
ASP	Aspartic acid
GLY	Glycine
SER	Serine

PRODUCTS OF 1 MIN FIXATION

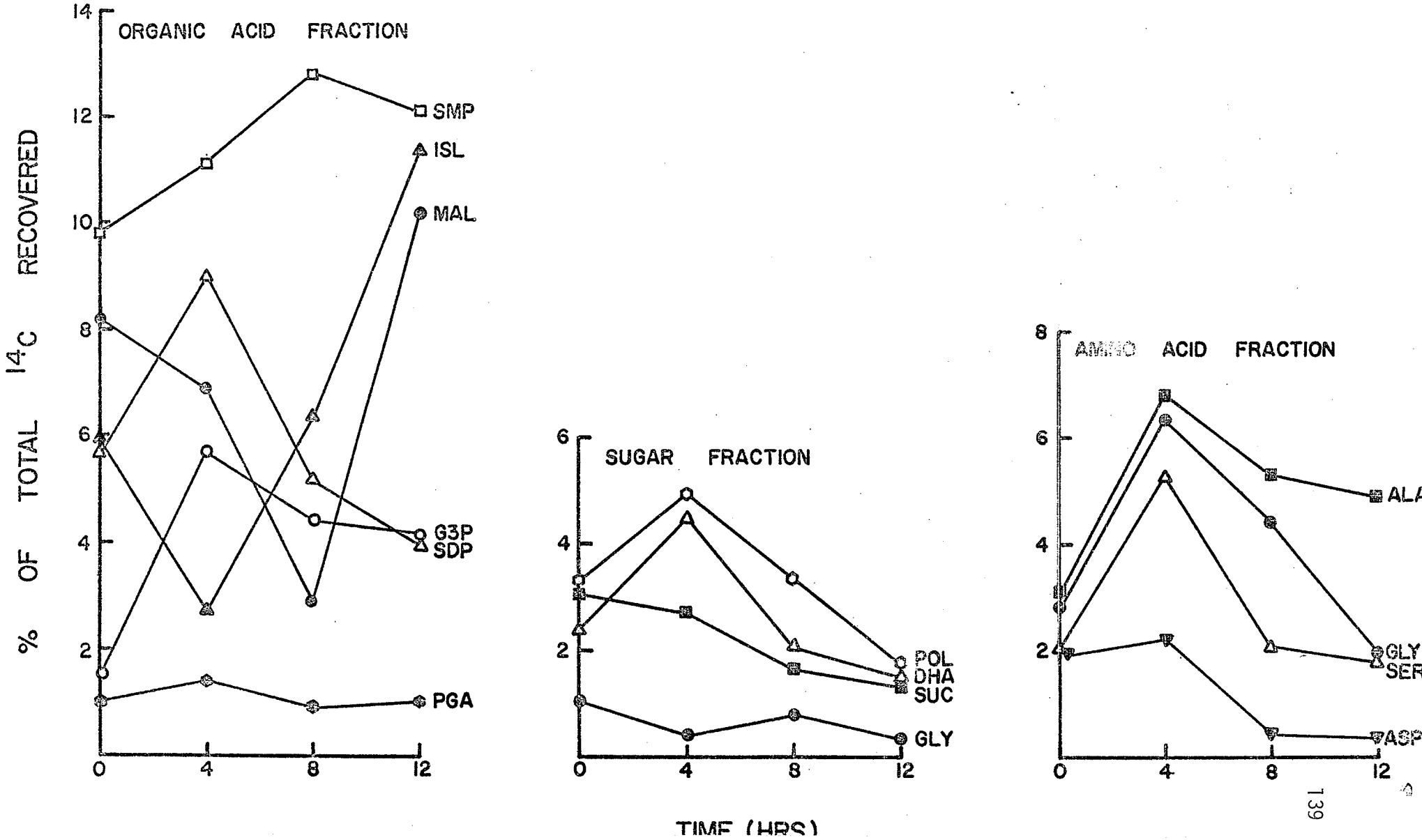
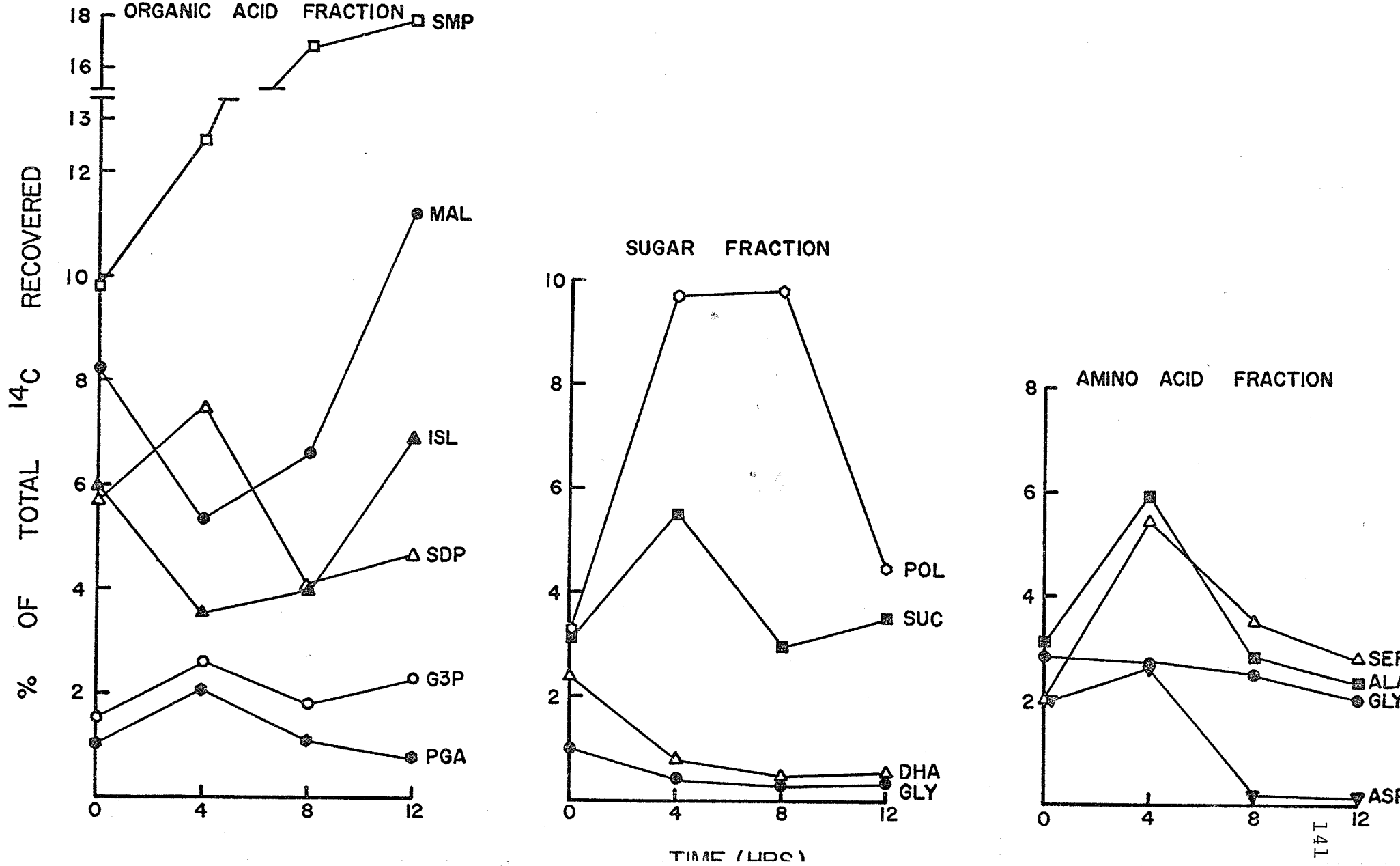


Figure 35. Photosynthetic products of one minute $^{14}CO_2$ fixation of zoospores produced in 5% CO_2 (Time:0) but grown in air during growth and S-phases.
The data are obtained from Tables XI, XII and XIII in Appendix.

Abbreviations	Products
	Organic acid fraction
G3P	Glyceraldehyde-3-phosphate
ISL	Isocitric lactone
MAL	Malic acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
GLY	Glyceraldehyde
POL	Polysaccharides
SUC	Sucrose
	Amino-acid fraction
ALA	Alanine
ASP	Aspartic acid
GLY	Glycine
SER	Serine


PRODUCTS OF 1 MIN FIXATION
ORGANIC ACID FRACTION
SMP
MAL
ISL
SDP
G3P
PGA
% OF TOTAL 14C RECOVERED
SUGAR FRACTION
POL
SUC
DHA
GLY
AMINO ACID FRACTION
SER
ALA
GLY
ASP
TIME (HRS)
0
4
8
12
2
4
6
8
10
12
13
16
18

shown in air grown cells as well as those previously observed in zoospores produced and grown in air (Figure 32). Regardless of the aeration with 5% CO_2 or air, the sugar monophosphates exhibited an increasing trend. The sugar diphosphates increased in both 5% CO_2 and air cells at the 4th hour and then declined. The amount present in 5% CO_2 grown cells was higher than that of air grown cells. In 5% CO_2 cultures, malate declined up to the 8th hour and then increased, whereas isocitrate lactone decreased at the 4th hour and then increased. The amount of malate was initially (0 - 4th hour) higher than isocitrate lactone after which this relation was reversed. Provision of air instead of 5% CO_2 did not alter the pattern of malate and isocitrate lactone with the exception that the decline at the 8th hour was not observed. Instead an increase was evident. Furthermore, the amount of malate at any one stage of the cell development was higher than isocitrate lactone. In 5% CO_2 grown cells, both glyceraldehyde-3-phosphate and 3-phosphoglycerate increased at the 4th hour and then declined, the amount present in the former being greater than the latter. In air, this pattern did not change. However, the amount of glyceraldehyde-3-phosphate was greatly reduced while only a slight increase in

3-phosphoglycerate was observed.

Polysaccharides, dihydroxyacetone and sucrose constituted the major sugars in 5% CO_2 grown cells and were present in decreasing amount in the order given. Both polysaccharides and dihydroxyacetone exhibit an increase at the 4th hour and then declined. Sucrose showed a continuous decline from the onset of illumination. Provision of air to zoospores previously produced in 5% CO_2 resulted in a vast increase in the amount of polysaccharides and sucrose, and a decrease in dihydroxyacetone and were present in decreasing amount in the order given. The pattern was also altered. Polysaccharides increased up to the 8th hour and then declined, whereas sucrose showed a decrease at the 8th hour. Dihydroxyacetone exhibited a continuous decrease during the 12 hour period.

Major incorporation of ^{14}C into the amino-acids occurred in alanine, glycine, serine and aspartate. They were present in decreasing amount in the order given in 5% CO_2 growing cells and all exhibited an increase at the 4th hour followed by a decline. Cells growing in air showed a slight increase in the amount of serine and a decrease in the amount of alanine and glycine. The amount of aspartate remained unchanged. The trend of alanine, serine

and aspartate remained unaltered as compared to 5% CO_2 cultures whereas glycine declined from the onset of growth. The amount present in air grown cells being alanine = serine > glycine > aspartate.

In all four types of cultures, glycerate and glycollate were detected in low amounts (Tables VIII and XI in Appendix). The amount of glycerate at any particular stage during growth and S-phase was higher than glycollate.

If the pathway of formation of products in either zoospores produced and grown in air (AA) or zoospores produced and grown in 5% CO_2 (CC) were basically the same, then the difference in the average percent of the products between them would be equal to the difference between (AA-AC) and (CC-CA) where AA = average percent of products in zoospores produced and grown in air, AC = average percent of products in zoospores produced in air but grown in 5% CO_2, CC = average percent of products in zoospores produced and grown in 5% CO_2, and CA = average percent of products in zoospores produced in 5% CO_2 but grown in air. Table VII showed that this was the case. It is obvious that malate plus isocitrate lactone in all 4 types of cultures were almost identical. This indicated that they were probably formed from the same oxaloacetate pool and the

Table VII. Difference between the average percent of various compounds formed either in air or 5% grown cells during the 12 hours of growth.

Compounds		(1) %		(2) %	(1 - 2) %
SMP + SDP	AA	23.52	AC	20.89	+2.63
	CC	17.40	CA	19.75	-2.35
	Difference	+6.12		+1.14	+4.98
Malate + Isocitrate-lactone	AA	13.53	AC	12.89	+0.64
	CC	13.68	CA	12.95	+0.73
	Difference	-0.15		-0.06	-0.09
Sucrose + Polysaccharides (POL)	AA	12.81	AC	9.30	+3.51
	CC	5.45	CA	10.57	-5.12
	Difference	+7.36		-1.27	+8.63
Amino-acids	AA	16.56	AC	23.08	-6.52
	CC	26.64	CA	21.47	+5.17
	Difference	-10.08		+1.61	-11.69

Data are obtained from Tables VIII to XIII in Appendix.

AA, air to air
CC, 5% CO_2 to 5% CO_2
AC, air to 5% CO_2
CA, 5% CO_2 to air

POL = polysaccharides

SMP, SDP = mainly sugar monophosphates and sugar diphosphates

difference between the amount of malate and isocitrate lactone in all 4 types of cultures were dependent upon whether malate or isocitrate lactone formation was favoured. In AA and CA cells, malate accumulation was favoured (and hence less protein), while isocitrate lactone formation was favoured in CC and AC cells (and hence probably greater protein synthesis).

V. KINETICS OF $^{14}CO_2$ INCORPORATION USING ZOOSPORES GROWN FOR 4 HOURS IN EITHER AIR OR 5% CO_2

The variations in the amounts of different products at different stages of cellular development suggest that different pathways are operative at different rates according to the stage of the cell cycle. Subsequently, the kinetics of ^{14}C incorporation into the various products were investigated using zoospores produced and grown for 4 hours in cultures bubbled with air or 5% CO_2. The results would indicate whether the path of carbon during the growth phase of *Chlamydomonas segnis* varied in response to changes in CO_2 concentration.

A. Distribution of ^{14}C in the Ethanol-water Soluble Fraction as a Function of Incubation Time

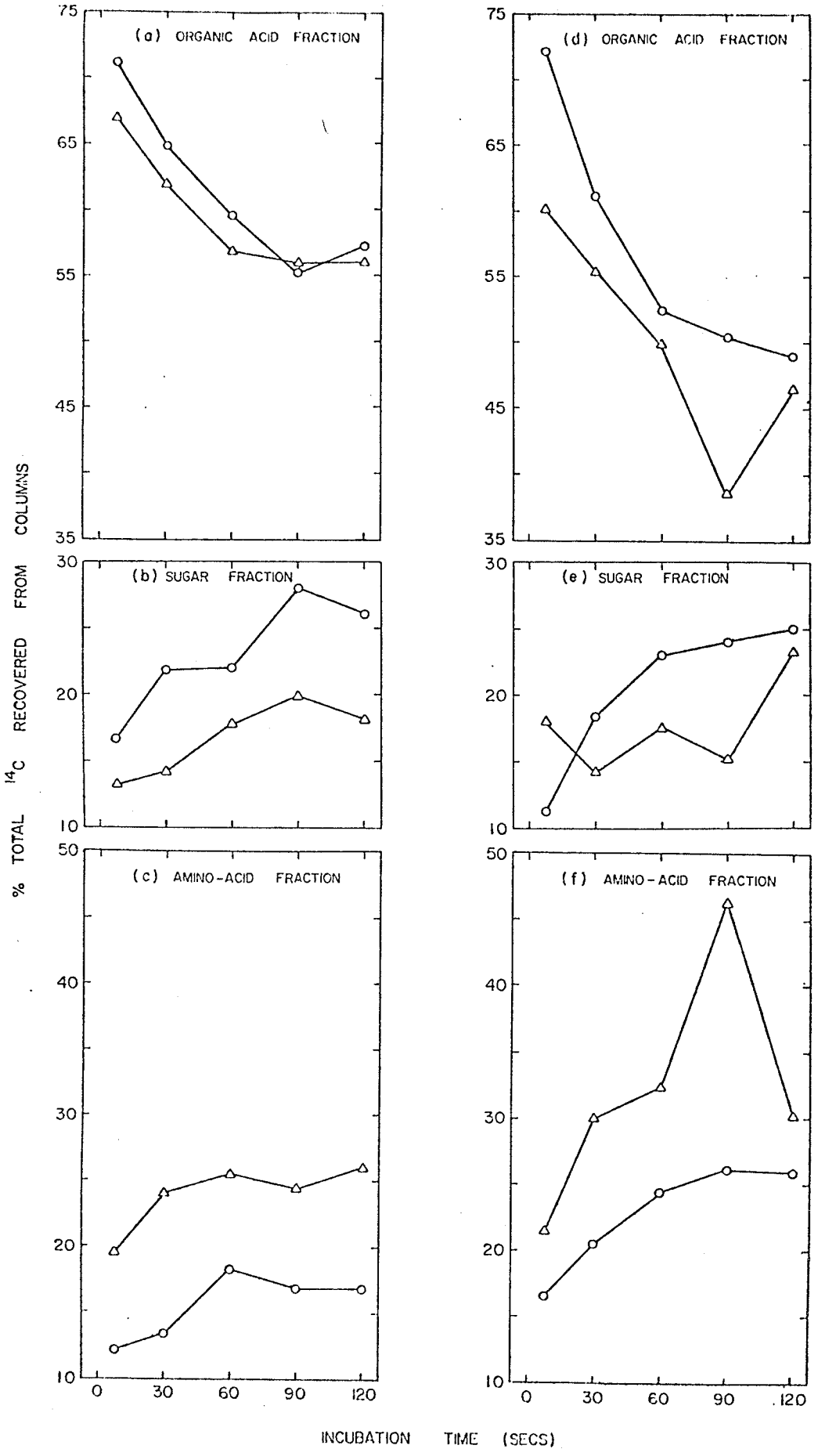
Figure 36a, b and c showed that in zoospores produced and grown in air the organic acid fraction decreased with incubation time. This was associated with simultaneous increases in sugars and amino-acids The former accumulated in relatively greater quantity than the latter. This pattern of ^{14}C incorporation did not change when the zoospores were provided with 5% CO_2 instead of air at the beginning of the growth phase. However, a significant increase in the level of amino-acids at the expense of sugars was evident.

On the other hand, in zoospores produced and grown in 5% CO_2 (Figure 36d, e and f) a greater increase in

Figure 36. Distribution of ^{14}C into the various fractions of the ethanol-water soluble fraction as a function of incubation time. The data presented are obtained from Table XIV in Appendix.

a, b and c: $^{14}CO_2$ fixation after 4 hours growth in air (o) or 5% CO_2 (Δ) of zoospores produced (Time:0) in air.

d, e and f: $^{14}CO_2$ fixation after 4 hours growth in air (o) or 5% CO_2 (Δ) of zoospores produced (Time:0) in 5% CO_2.


(a) ORGANIC ACID FRACTION
(b) SUGAR FRACTION
(c) AMINO-ACID FRACTION
(d) ORGANIC ACID FRACTION
(e) SUGAR FRACTION
(f) AMINO-ACID FRACTION
% TOTAL ^{14}C RECOVERED FROM COLUMNS
INCUBATION TIME (SECS)
75
65
55
45
35
30
20
10
50
40
0
30
60
90
120

amino-acids for 90 seconds was associated with a dramatic decrease in organic acid fraction. Thereafter, the drop in amino-acids accumulation was not accompanied by an equivalent increase in percentage of the organic acid fraction. Meantime, the sugars fluctuated and showed a much lower level as compared to amino-acids. Provision of air instead of 5% CO_2 during growth stimulated the flow of ^{14}C into sugars and amino-acids at the expense of the organic acid fraction. It was obvious that provision of air lessened the amino-acids formation and increased the synthesis of sugars.

In all cultures, the diversion of ^{14}C from the organic acid fraction to amino-acids was favoured in 5% CO_2 cultures and to sugars in air cultures.

B. Distribution of ^{14}C in the Products of the Organic Acid, Sugar and Amino-acid Fractions as a Function of Incubation Time

In order to determine the primary path of carbon, chromatographic and radioautographic identification of individual products from the various fractions were carried out. The results are given in Tables XV to XVIII (in Appendix) and are summarized in Figures 37 to 40.

1. Effect of 5% CO_2 Provided During Growth of Zoospores Previously Produced in Air

In zoospores produced and grown in air, the

incorporation of $^{14}CO_2$ occurred mainly via the Calvin cycle (Figure 37). This was shown by the fact that the major ^{14}C after 5 seconds was found in the sugar phosphates and 3-phosphoglycerate, both of which decreased with time. This was accompanied by an increase in malate up to 60 seconds after which the level remained unchanged. Meantime, isocitrate lactone showed a linear increase. The amount of malate was higher than isocitrate lactone which may suggest that the oxaloactetate formed by β-carboxylation was not actively used for amino-acids as shown by the very low level of aspartate (Table XV in Appendix). Of the amino-acids, high labelling of alanine was detected after 5 seconds which increased up to 30 seconds and then declined. This suggested that alanine was probably formed from the early products of photosynthesis via 3-phosphoglycerate. Serine appeared to occur via glycine which decreased after 30 seconds, while serine showed an increasing trend attaining a level higher than glycine only after 60 seconds photosynthesis.

Provision of 5% CO_2 during the growth of zoospores produced in air (Figure 38) did not alter the pathway of ^{14}C incorporation which was still predominantly the Calvin-type exemplified by the decrease in sugar monophosphates and 3-phosphoglycerate, and an

Figure 37. Distribution of ^{14}C in the photosynthetic products as a function of incubation time. Zoospores were produced (Time:0) and grown in air for 4 hours. Data are from Table XV in Appendix.

Abbreviations	Products
	Organic acid fraction
G3P	Glyceraldehyde-3-phosphate
ISL	Isocitric lactone
MAL	Malic acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
POL	Polysaccharides
SUC	Sucrose
	Amino-acid fraction
ALA	Alanine
GLY	Glycine
SER	Serine

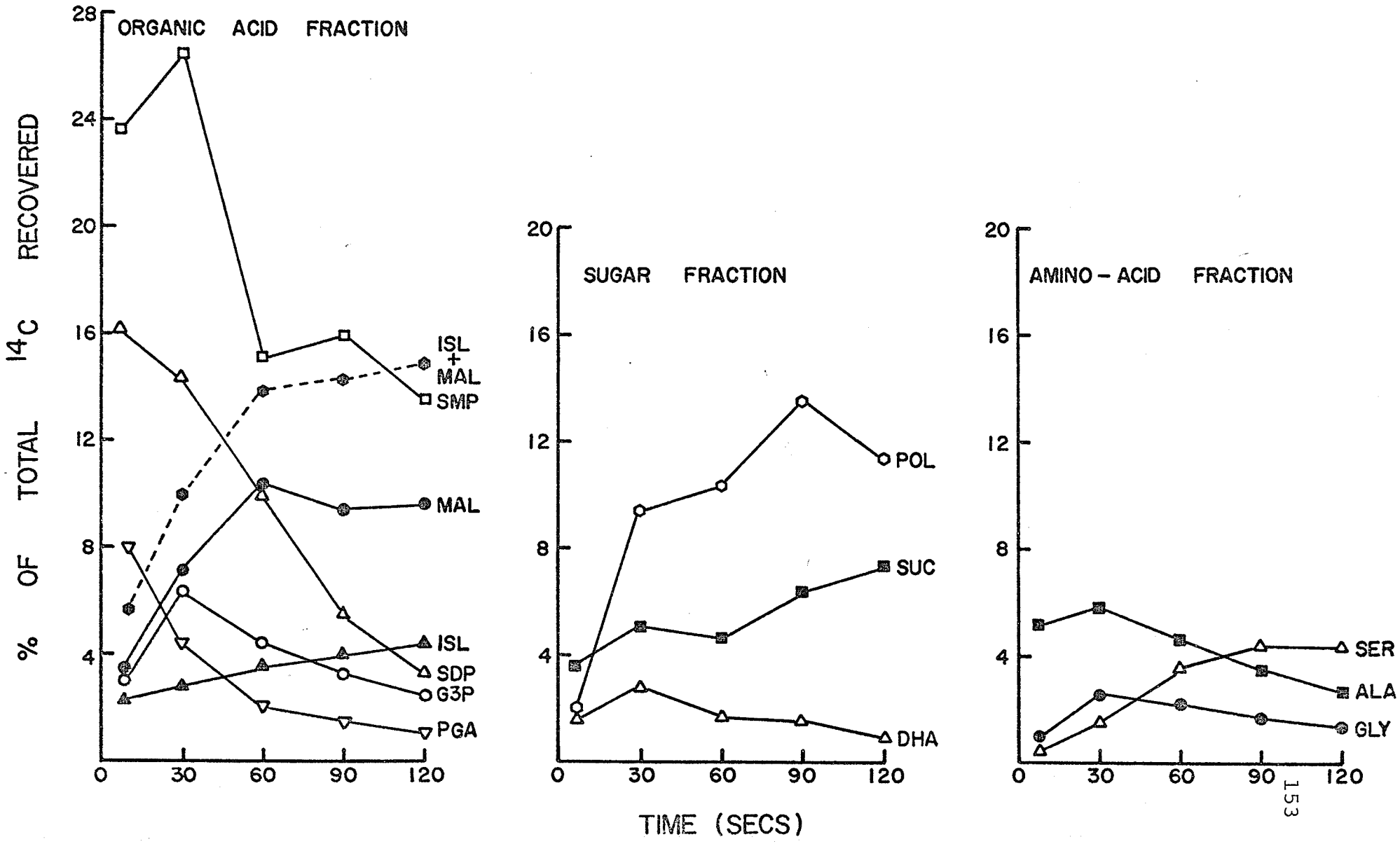
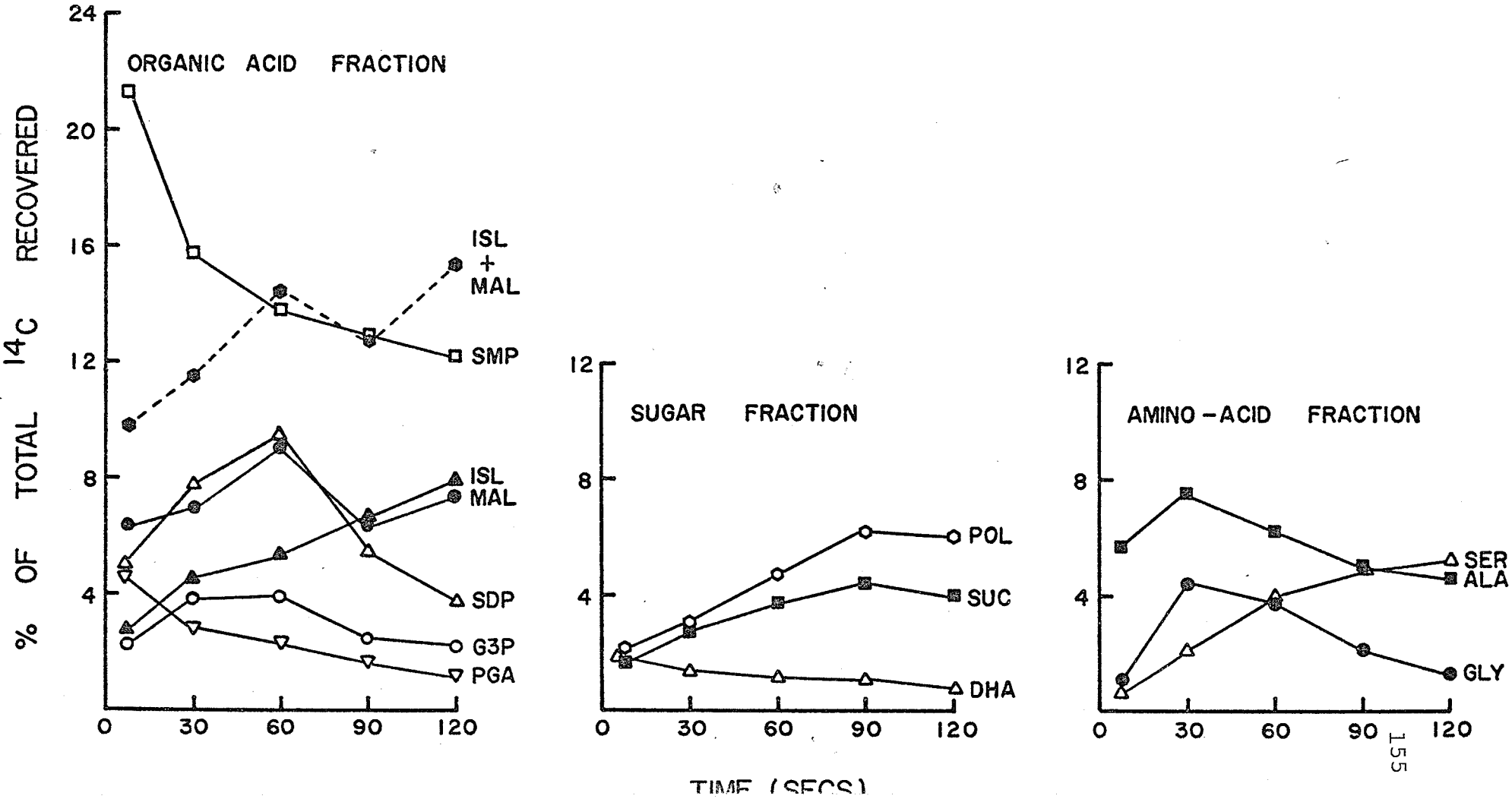

ORGANIC ACID FRACTION
SUGAR FRACTION
AMINO – ACID FRACTION
% OF TOTAL ^{14}C RECOVERED
TIME (SECS)
ISL + MAL
SMP
MAL
ISL
SDP
G3P
PGA
POL
SUC
DHA
SER
ALA
GLY
0
30
60
90
120
4
8
12
16
20
24
28

Figure 38. Distribution of ^{14}C in the photosynthetic products as a function of incubation time. Zoospores were produced (Time:0) in air but grown for 4 hours in 5% CO_2.
Data are from Table XVI in Appendix.

Abbreviations	Products
	Organic acid fraction
G3P	Glyceraldehyde-3-phosphate
ISL	Isocitric lactone
MAL	Malic acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
POL	Polysaccharides
SUC	Sucrose
	Amino-acid fraction
ALA	Alanine
GLY	Glycine
SER	Serine


ORGANIC ACID FRACTION
% OF TOTAL ^{14}C RECOVERED
24
20
16
12
8
4
ISL + MAL
SMP
ISL
MAL
SDP
G3P
PGA
0
30
60
90
120
SUGAR FRACTION
POL
SUC
DHA
AMINO-ACID FRACTION
SER
ALA
GLY
TIME (SECS)

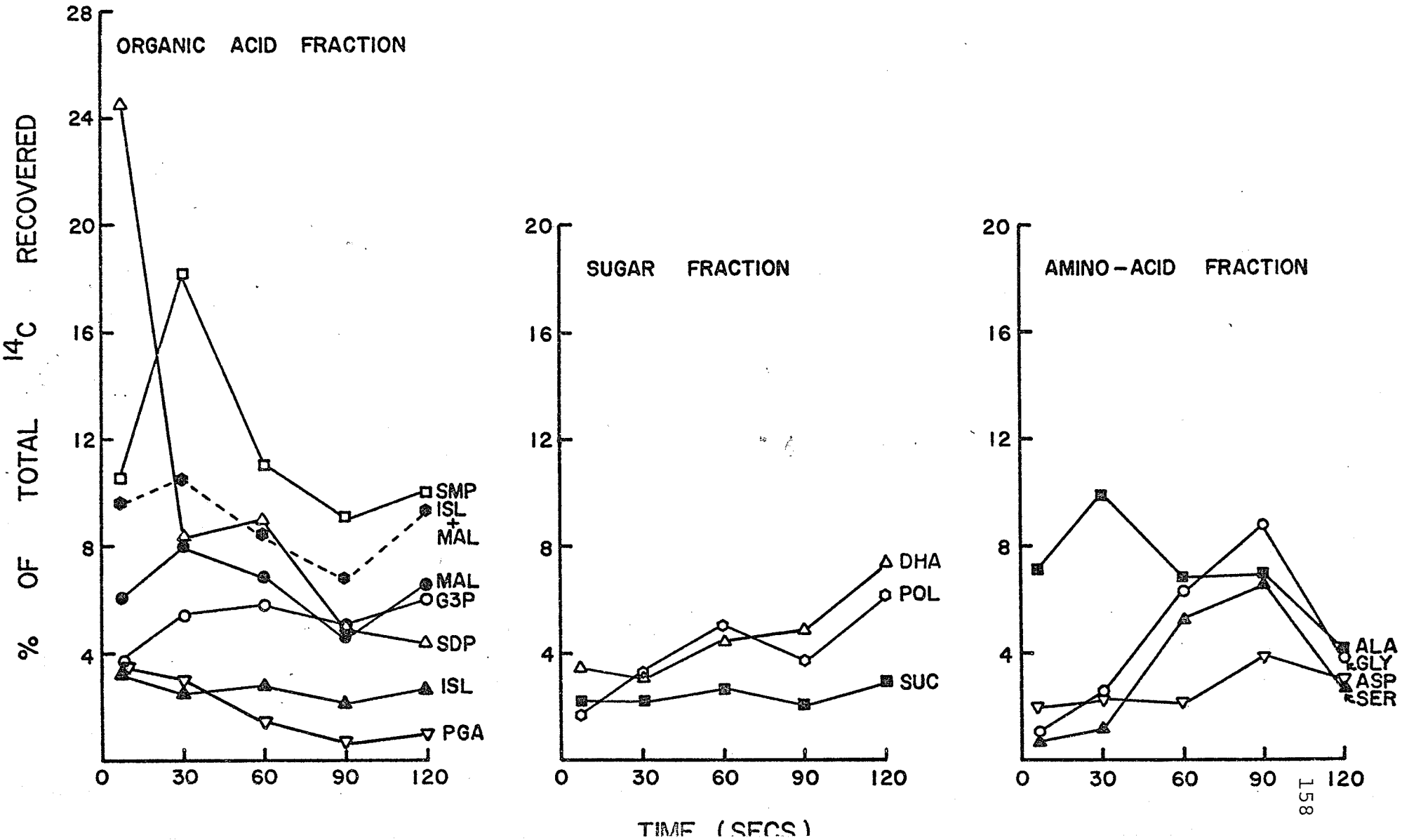
increase in malate and isocitrate lactone. The general pattern of ^{14}C incorporation into sugars was unaltered by provision of 5% CO_2 to zoospores produced in air. However, the effect of 5% CO_2 during growth was manifested by less ^{14}C incorporation into polysaccharides and sucrose, and an increased rate of incorporation into alanine, glycine and serine as compared to growth in air.

2. Effect of Air Provided During Growth of Zoospores Previously Produced in 5% CO_2

In zoospores produced and grown in 5% CO_2 (Figure 39), the major carboxylation pathway was again via the Calvin cycle as indicated by the high labelling in sugar phosphates plus 3-phosphoglycerate after 5 seconds photosynthesis and their continuous decline with incubation time. In addition ^{14}C incorporation via β-carboxylation was relatively stimulated as indicated by the appearance of aspartate associated with the decline in malate and isocitrate lactone. Furthermore, the decrease in malate and isocitrate lactone during 30 - 90 seconds period corresponded with increases in glycine and serine, both of which showed accumulation for 90 seconds. The pattern also indicated that glycine and serine formation occur via different pathways in 5% CO_2 grown cells. High and early

Figure 39. Distribution of ^{14}C in the photosynthetic products as a function of incubation time. Zoospores were produced (Time:0) and grown in 5% CO_2 for 4 hours. Data are from Table XVII in Appendix.

Abbreviations	Products
	Organic acid Fraction
G3P	Glyceraldehyde-3-phosphate
ISL	Isocitric lactone
MAL	Malic Acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
POL	Polysaccharides
SUC	Sucrose
	Amino-acid fraction
ALA	Alanine
ASP	Aspartate
GLY	Glycine
SER	Serine


ORGANIC ACID FRACTION
SUGAR FRACTION
AMINO-ACID FRACTION
% OF TOTAL ^{14}C RECOVERED
TIME (SECS)
SMP
ISL + MAL
MAL
G3P
SDP
ISL
PGA
DHA
POL
SUC
ALA
GLY
ASP
SER
0
30
60
90
120
4
8
12
16
20
24
28

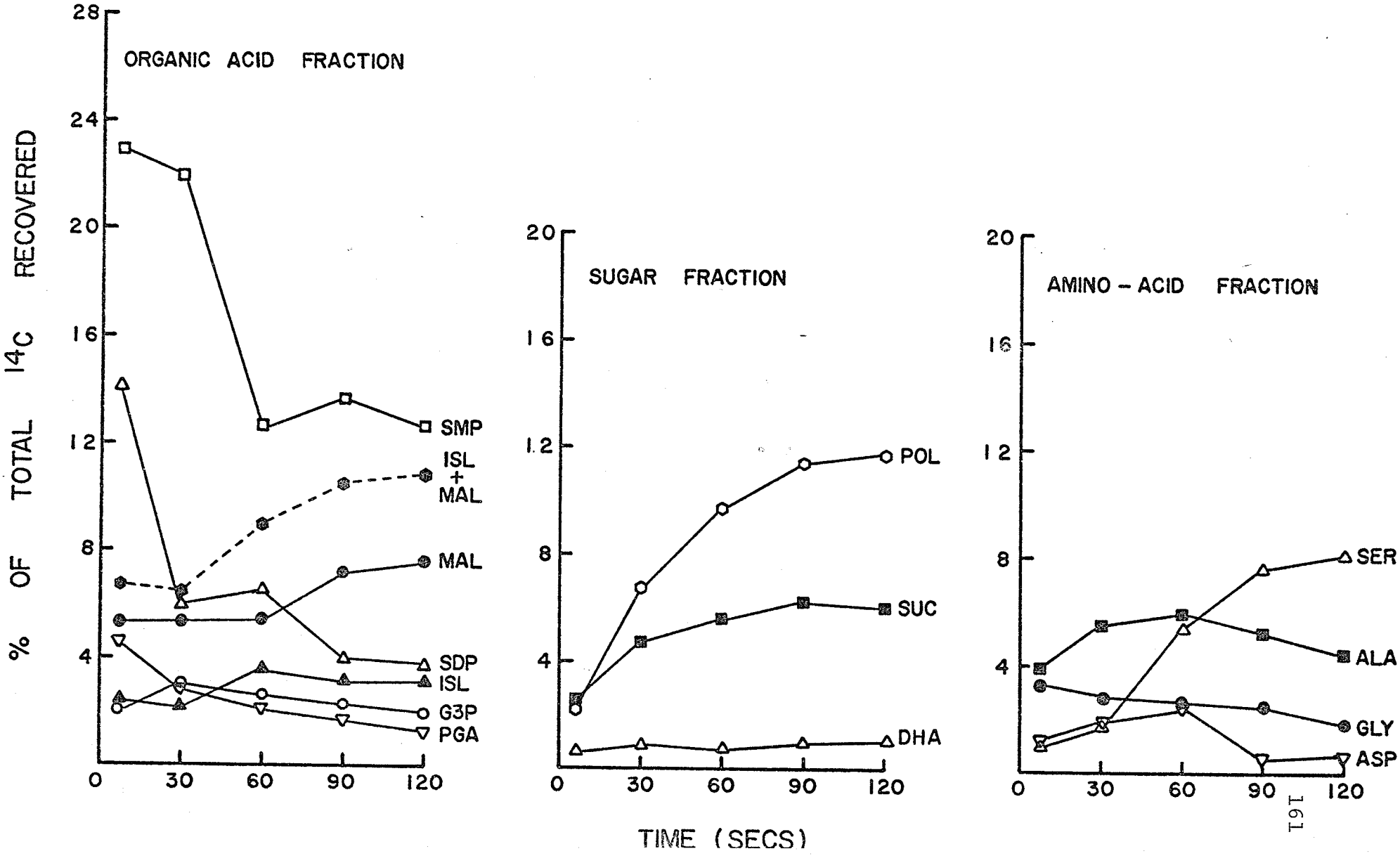
labelling of alanine which declined after 30 seconds indicated that it was formed from early products of photosynthesis and then directed to synthesis of cell proteins.

When the zoospores produced in 5% CO_2 were provided with air during growth (Figure 40), the path of carbon showed a preference towards the Calvin-type as indicated again by the decline in sugar phosphates and an increase in malate, sugars and amino-acids. The early high labelling in alanine was again detected. Serine exhibited a major increase after 30 seconds, while glycine exhibited a slight decrease with time. The decrease in glycine could not account for the increase in serine showing that serine was formed not only from glycine.

The results provided evidence that provision of the growing cells with 5% CO_2 stimulated the incorporation of $^{14}CO_2$ via β-carboxylation, presumably towards amino-acids formation and hence protein and sugars (Wang and Waygood, 1962), resulting in the increase in cell mass of zoospores produced in cultures bubbled with 5% CO_2.

Figure 40. Distribution of ^{14}C in the photosynthetic products as a function of incubation time. Zoospores were produced (Time:0) in 5% CO_2 but grown in air for 4 hours. Data are from Table XVIII in Appendix.

Abbreviations	Products
	Organic acid fraction
G3P	Glyceraldehyde-3-phosphate
ISL	Isocitric lactone
MAL	Malic acid
PGA	3-phosphoglyceric acid
SDP	Sugar diphosphates
SMP	Sugar monophosphates
	Sugar fraction
DHA	Dihydroxyacetone
POL	Polysaccharides
SUC	Sucrose
	Amino-acid fraction
ALA	Alanine
ASP	Aspartate
GLY	Glycine
SER	Serine


ORGANIC ACID FRACTION
SUGAR FRACTION
AMINO – ACID FRACTION
% OF TOTAL ^{14}C RECOVERED
TIME (SECS)
SMP
ISL + MAL
MAL
SDP
ISL
G3P
PGA
POL
SUC
DHA
SER
ALA
GLY
ASP
0
30
60
90
120
4
8
12
16
20
24
28

DISCUSSION

Although the effect of CO_2 concentration on the pH of nutrient medium was studied in detail by Galloway and Krauss (1961) and Sorokin (1971), no significant changes due to CO_2 concentrations used were observed in our case. The enriched phosphate nutrient medium maintained the pH almost around 6.7 throughout the cell cycle. In this respect Sorokin (1964) suggested that the delay in cell division might be due to the pH shifts caused by high CO_2 tension. This was discounted in our experiments because air grown cells when bubbled with 5% CO_2 during the division (i.e., at 12th hour light, Figure 7) did not delay the onset of zoospore release nor its completion. Soeder *et al.* (1966) also concluded that the delayed autospore release in *Chlorella* sp. was not due to pH differences. However, changes in the pH occurring within the cell and which can be induced by varying the CO_2 concentration of the medium (Neumann and Levine, 1971) cannot be ruled out. The possible role of carbonic anhydrase acting as a permease (Enns, 1967) and thereby regulating pH shifts in the cell (Atkins and Graham, 1971) is beyond the scope of this present work.

It is known that the assimilation of CO_2 or HCO_3^- during the photoautotrophic growth of unicellular green algae

is coupled with utilization of assimilatory power (NADPH and ATP) derived from light. On the other hand, dark respiration results in the production of carbon dioxide and ATP, and is shown to be inhibited by high CO_2 tension (Kidd, 1915; Soeder *et al.*, 1964). Therefore, if the profound inhibitory effect of aeration with 5% CO_2 in darkness on zoospore release (Figure 2) was attributable to a lack of energy then this energy could be provided by exposing the cultures to an interval of light (Figure 11 and Table I). The light effect in initiating the onset of zoospore release was substantially enhanced when CO_2 was removed by bubbling the zoospores (unreleased) with CO_2-free air (Table I). The latter effect indicated that CO_2 was probably competing for some of the energy required for zoospore release. This energy might be necessary to activate the kinetic apparatus for flagellum movement (Ettl, 1970) or to activate the "hatching enzyme" (Schlösser, 1966).

Although the onset and completion of zoospores release occurred in light (Figure 6) in 5% CO_2 cultures, the high CO_2 tension was still effective in delaying the onset of zoospores release from the tetrads for 4 hours as compared to 0.1% CO_2 cultures. This delay, however, could be overcome when the dividing cells were bubbled with air instead of 5% CO_2 (Figure 7). Considering the fact that the incipient cell division (first mitotic division) did occur irrespective of CO_2 concentration at the 12th hour of light,

then the observed delay in 5% CO_2 cultures maintained in continuous light ought to be due to an inhibitory effect on the second mitosis leading to the production of 4 daughter cells. Since the second mitotic division depends on the completion of the appropriate amount of DNA synthesized, the latter appeared to be delayed in 5% CO_2 than in 0.1% CO_2 or air culture. This was obvious when the length of the S-phase was calculated in 5% CO_2 cultures (Figure 10) which occupied a relative period of 0.62 of the cell cycle while that in air was 0.3. This delay could be explained by a competition that might have existed between CO_2 assimilation and DNA synthesis for the light energy. High CO_2 concentration might have imposed the continuation of carbon assimilation and related processes as indicated by the extended (beyond 8th hour) photosynthesis and a lower drop in photosystem II activity (Figures 13 and 15) as compared to the situation in air cultures. In the latter, photosynthesis appeared to be endogenously regulated when the low exogenous CO_2 concentration allowed the cells to do so.

The greater accumulation of macromolecules (Table II) i.e., RNA, protein and starch (starch granules observed in electron micrographs and not shown in this work) in zoospores produced in 5% CO_2 cultures as compared to air cultures seemed to be responsible for unequal cytoplasmic division in the former cultures resulting in the heterogeneity of the zoospores population. This was

indicated by the unequal response of the zoospore population to limited concentration of CO_2 (Figure 9). Therefore, aeration of cultures with relatively high CO_2 concentration may result in a more or less random culture rather than a synchronous one. Hence, "selection synchrony" (Mitchison, 1971; Molloy and Schmidt, 1970) using differential centrifugation followed by the provision of the appropriate CO_2 concentration in air to the culture would furnish the basis for a true synchronous culture for studies on the cell cycle.

Using the homogeneous zoospores with low initial mass formed in air cultures (Table II) to commence the cell cycle under low CO_2 available in air, the growth phase (G_1) could be sharply distinguished by the increased photosynthetic O_2-evolution, photosystem II and carbonic anhydrase activities (Figures 13, 15 and 17). These activities showed a sharp decline at the end of G_1 and thus its termination. If active photosynthetic O_2-evolution is a concomitant event of growth, which declines by the end of G_1 then 5% CO_2 must have forced the cells to maintain their photosynthetic capacity even during the S-phase (compare Figures 10 and 13). This appeared to be extra photosynthesis which was not required for the production of 4 zoospores because the latter could be produced in 0.1% CO_2 cultures (Figure 6). Therefore, the prolonged phase of O_2-evolution seemed to be imposed upon the cell by the unnatural high CO_2

concentration (5% CO_2) and not endogenously regulated. Under the appropriate or limited CO_2 concentration (air cultures), the cells managed to regulate photosynthesis by virtue of carbonic anhydrase which became repressed when the cells were provided with 5% CO_2 (Figure 23).

The pattern of photosynthetic O_2-evolution in air and 5% CO_2 cultures followed the general trend observed in other algae (Edmunds, 1965; Senger and Bishop, 1969; Bishop and Senger, 1971; Walther and Edmunds, 1973) and is believed to be an inherent characteristic of the cell cycle Thus, growth in different CO_2 concentration did not alter this inherent characteristic.

The observations concerning the decline in the photosynthetic capacity (as well as p-benzoquinone Hill reaction) during the mature and division phase of the cell cycle confirms the findings that this decline is due to the decrease in the amount of photosystem II activity (Senger and Bishop, 1967; Senger, 1970).

Examination of the trend of activities of RuDP- and PEP-carboxylases (Figures 18 and 19) showed no correlation between them and the observed pattern characteristic of the photosynthetic capacity. On the other hand, carbonic anhydrase, an enzyme implicated in photosynthesis (Graham *et al.*, 1971) showed similar pattern (compare Figures 13 and 17) as the photosynthetic O_2-evolution during the cell cycle indicating a possible regulatory relationship

between the latter and carbonic anhydrase. This may be supported by the observation that an alteration in the amount of enzyme synthesized (by transient changes of CO_2 concentrations) resulted in an alteration of the photosynthetic capacity (Figures 26 and 27).

Carbonic anhydrase may be responsible for provision of substrate to the carboxylating enzymes and therefore its high activity found in air grown cells may be responsible for the enhanced $^{14}CO_2$ fixation as also observed by other investigators (Graham and Whittingham, 1968; Graham *et al.*, 1971). However, in *Chlamydomonas segnis* the activity of carbonic anhydrase in air cultures was also associated with a higher specific activity of RuDP-carboxylase as compared to 5% CO_2 cultures. Therefore, it is not clear whether the enhanced $^{14}CO_2$ fixation would be primarily attributable to the high activity of carbonic anhydrase or rather to RuDP-carboxylase activity.

Besides its role as substrate for the carboxylating enzymes, HCO_3^- or CO_2 is required for the Hill reaction (Stemler and Govindjee, 1973; Abeles *et al.*, 1961; Stern and Vennesland, 1960). If carbonic anhydrase regulates the delivery of substrate (HCO_3^- or CO_2) to both the electron transport system (photosystem II) and the site of CO_2 fixation, then changes in carbonic anhydrase activity would result in the corresponding alteration of photosynthesis as demonstrated in Figures 26 and 27.

The data obtained from the analysis of $^{14}CO_2$ fixation products during the cell cycle showed that the incorporation of ^{14}C into various fractions of the cell varied according to the stage of the cell cycle (Smith *et al.*, 1961; Ahmed and Ries, 1969; Kanazawa *et al.*, 1970; Codd and Merrett, 1971) and reflected the periodic synthesis of cellular constituents (Ullrich, 1972; Hare and Schmidt, 1970; Tamiya, 1966). The nature and quantities of the products were also dependent upon the CO_2 concentration in which the zoospores were produced and/or grown. Zoospores produced and grown in air incorporated ^{14}C preferentially into sugars and sugar phosphates, whereas their counterparts in 5% CO_2 favoured the ^{14}C incorporation into amino-acids (Figure 31). This confirmed the observation that growth in high CO_2 concentration stimulated protein synthesis (Table II) in agreement with other investigators (Hiller, 1970; Graham and Whittingham, 1968). The increase in the incorporation of ^{14}C into sugars and sugar phosphates by air grown cells suggested a possible relationship between carbonic anhydrase and RuDP-carboxylase. Both enzymes indicated high activities in cells grown in air cultures which also indicated very low activity of PEP-carboxylase (Figures 17 and 18). Repression or inactivation of carbonic anhydrase by growth in 5% CO_2 coupled with higher activity of PEP-carboxylase seemed to be responsible for the enhanced amino-acids synthesis. The kinetics of $^{14}CO_2$ fixation at the 4th hour of the cell cycle (Figures 37 to 39)

revealed that the path of $^{14}CO_2$ incorporation in air grown cells was essentially via the Calvin cycle. Growth in 5% CO_2 stimulated β-carboxylation besides the existing carboxylation by RuDP-carboxylase via the reductive pentose phosphate cycle. This was also shown in *Chlorella pyrenoidosa* by Graham and Whittingham (1968). The operation of both pathways simultaneously in 5% CO_2 may be a compensatory mechanism of the cells in that repression or inactivation of carbonic anhydrase resulted in a lower incorporation via the Calvin cycle. This again reflected the close association of carbonic anhydrase and RuDP-carboxylase as well as the differential affinity of the enzymes RuDP-carboxylase and PEP-carboxylase to the substrate HCO_3^- or CO_2 (Cooper *et al.*, 1969; Waygood *et al.*, 1969; Cooper and Wood, 1971; Pocker and Ng, 1973).

Several investigators have shown that glycollate was a major product of $^{14}CO_2$ fixation (see Merrett and Lord, 1973). The ability to excrete glycollate in cells grown in high CO_2 but not in air grown cells has been attributed to lack of or low activity of glycollate dehydrogenase in cells grown in high carbon dioxide (Nelson and Tolbert, 1969; Merrett and Lord, 1973). The results presented in this investigation showed (Tables VIII and XI in Appendix) that glycollate was formed in low amounts in both air and 5% CO_2 grown cells. Coupled to this was the inability to demonstrate excretion of glycollate in the same species of alga (Badour

and Waygood, 1971a). In addition, the findings that glycine and serine (Figures 32 to 35) were two of the major amino-acids formed suggested that a rapid turnover of glycollate occurred in *Chlamydomonas segnis*. The slightly higher amount of glycollate in 5% CO_2 cells may reflect slight differences in the activity of glycollate dehydrogenase. Although this enzyme was not investigated in this present study the observations that glycollate was not excreted in the absence of inhibitors in *Chlorella fusca* (Goulding *et al.*, 1969; Merrett and Lord, 1973) may suggest that glycollate metabolism in *Chlamydomonas segnis* is similar to that of *Chlorella fusca*.

Of interest was the finding that isocitrate lactone, first identified by Chang and Tolbert (1970) in *Ankistrodesmus*, was one of the early products of $^{14}CO_2$ fixation in *Chlamydomonas segnis* whether grown in air or 5% CO_2 cultures. The average percent of malate plus isocitrate lactone (Table 7) were identical in both 5% CO_2 and air grown cells indicating that they were formed via a common pool, probably oxaloacetate. This observation may support the opinion that the low activity of PEP-carboxylase in air grown cells was the result of its inactivation in the cell free extract preparation. Undoubtedly, β-carboxylation must be operative in both air and 5% CO_2 grown cells to replenish the C_4-acids for the formation of glutamate. The greater accumulation of isocitrate lactone (dehydrated ring form of isocitrate) was associated in *Chlamydomonas segnis* with isocitrate lyase

activity (Badour and Waygood, 1971b; Foo *et al.*, 1971). This may suggest that isocitrate could be metabolized via the glyoxylate cycle and glycerate or glycine-serine pathways (Rabson *et al.*, 1962; Kornberg and Gotto, 1961; Reeves *et al.* 1967). In addition, the accumulation of isocitrate lactone (isocitrate) may protect the enzymes, isocitrate lyase and dehydrogenase, from being digested (Thurston *et al.*, 1973).

The high and early labelling of malate together with the findings that the activity of malate synthase was very low in *Chlamydomonas segnis* (Badour and Waygood, 1971b) may lend support to the view that malate was formed by malate dehydrogenase rather than via the glyoxylate cycle. The activity of NAD-linked malate dehydrogenase in this organism was found to be exceptionally high (about 7 μmoles/min/mg protein) in 5% CO_2 grown cells (Foo *et al.*, unpublished results from this laboratory).

In air grown cells, the higher amount of serine than glycine (Figure 32) indicated that serine was probably the end product of glycine as have been shown by many authors (Rabson *et al.*, 1962; Lord and Merrett, 1970b). This was confirmed by the observations that the loss in labelling of glycine resulted in an increase in the labelling of serine (Figure 37). However, in 5% CO_2 grown cells glycine formed was higher than serine (Figure 34) suggesting more than one pathway of serine formation, probably via 3-phosphoglycerate in addition to the glycollate pathway (Smith *et al.*, 1961;

Hess and Tolbert, 1967). This was confirmed by the kinetics experiments in which both glycine and serine showed increasing labelling with time (Figure 39).

Alanine appeared to be formed in cells grown in air and 5% CO_2 cultures from an early product of photosynthesis probably via 3-phosphoglycerate in accordance with the observations of many workers (Stange *et al.*, 1960; Hiller and Whittingham, 1964; Kanazawa *et al.*, 1970).

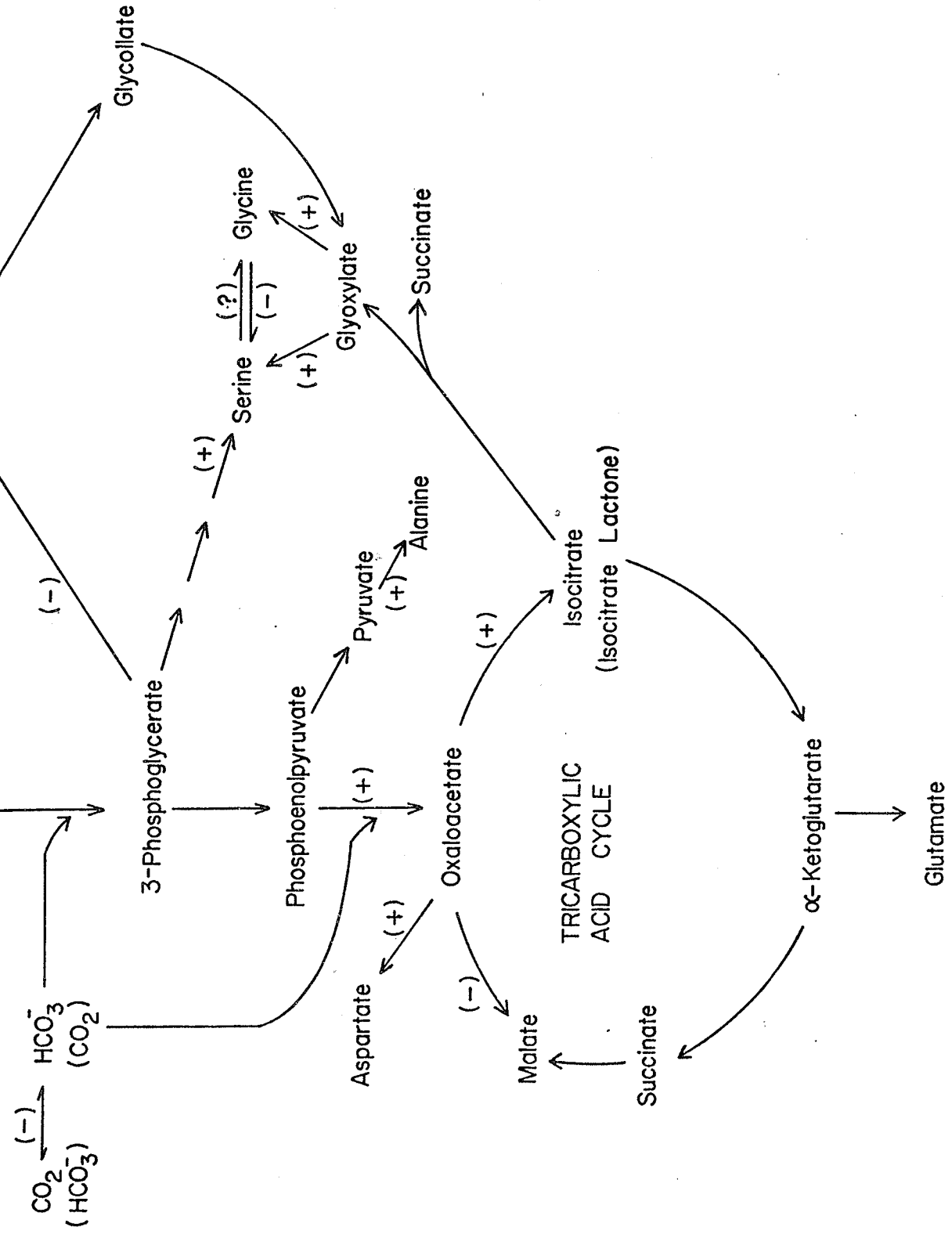
The metabolic pathways which were stimulated by growth in 5% CO_2 cultures as compared to growth in air cultures were summarized in Figure 41.

The present work provided evidence that the appropriate CO_2 concentration provided to the algal culture has to be considered as a major factor influencing the degree of synchrony of the culture and, hence, the characterization of the cell cycle. Mitchison (1971) pointed out that the early G_1 region is empty of information since most of the studies have been concentrated on the later parts of the cycle concerned with cell division. If the critical mass at which cells initiate DNA synthesis is determined by growth which is influenced by CO_2 concentration, then the latter should not be overlooked. Although Myers (1962) and Steemann-Nielsen (1955) concluded that 0.5% CO_2 or less in air is more than adequate to support growth and development of unicellular green algae of the *Chlorella* type, higher concentration have been used because of familiarity. Again

Figure 41. Schematic diagram of $^{14}CO_2$ fixation by air grown cells of *Chlamydomonas segnis*.

(+) Indicates activation of pathway by growth in 5% CO_2.

(-) Indicates inhibition of pathway by growth in 5% CO_2.


Glycollate
Sugars
(-)
Sugar Phosphates
Ribulose Diphosphate
(-)
3-Phosphoglycerate
(+)
Serine
(?)
(-)
Glycine
(+)
(+)
Glyoxylate
Succinate
Pyruvate
(+)
Alanine
Phosphoenolpyruvate
(+)
Isocitrate
(Isocitrate Lactone)
(+)
Oxaloacetate
TRICARBOXYLIC ACID CYCLE
α-Ketoglutarate
Glutamate
(+)
Aspartate
(-)
Malate
Succinate
(-)
CO_2 (HCO_3^-)
HCO_3^- (CO_2)

Mitchison (1971) pointed out that *Chlorella* and some other algae raise a problem about the relation of their cell cycles to those of most other cells. The 'normal' cell cycle has a two-fold increase followed by division into two daughters. However, in *Chlorella* an eight-fold increase followed by three rapid divisions into eight daughter cells is the conventional division number. There are two ways of regarding the *Chlorella* interphase. One of them is to look at it as equivalent to a single 'normal' cell cycle but with the difference that every synthetic event involves an eight-fold rather than a two-fold increase. Alternatively, it may be regarded as equivalent to three successive 'normal' cycles with their division dissociated and occurring at the end of the third cycle. This distinction is important for discontinuous events like synthesis of DNA and a step enzyme which may occur once in the normal cycle. Looking at the *Chlorella* cycle in the first way, it would be expected there to be a single period of synthesis but with more material being synthesized. Looking at it the second way, we would expect three separate periods of synthesis each with two-fold increase. Which of these ways is correct is at present unknown. However, using *Chlorella fusca* with four daughter cells produced simplified the situation and allowed more accurate studies of the cell cycle (John *et al.*, 1973). *Chlamydomonas segnis* grown and synchronized in air undergoes a normal cell cycle (as defined by Mitchison, 1971) and

formed only two daughter cells. Thus, it could prove to be a valuable tool in cell cycle studies rather than *Chlamydomonas reinhardti* which produces 16 zoospores (Lien and Knutsen, 1973). The latter authors found that in phosphorous deficiency, the cells produced 4 daughter cells after one cell cycle. Perhaps growth in air of this *Chlamydomonas* would result in the production of 2 zoospores and allow more refined studies to be carried out on the normal cell cycle.

The cell cycle of a homogeneous zoospore suspension of *Chlamydomonas segnis* bubbled with air could be characterized in the present work. The growth phase were marked by maximal carbonic anhydrase activity and photosynthetic capacity (Figures 15 and 17). Carbonic anhydrase was difficult to detect in 5% CO_2 cultures and, hence, appeared to be an essential enzyme in the normal cell cycle. The drop in photosystem II most likely terminated the growth phase. In conjunction with this was the observation that (Figure 32)sugar diphosphates and isocitrate lactone increased sharply from the 8th to 12th hour and, hence, provide another marker for the termination of G_1-phase. It may be speculated that the precursor of isocitrate lactone or its products (e.g., succinate) may replace H_2O as the electron donor and, hence, cause a drop in photosystem II (Healy, 1970; Stuart, 1971; Gaffron, 1972). During the G_1-period, a phase could be distinguished by the accumulation of RNA, amino-acids

and intermediates of the reductive pentose phosphate cycle (Figures 10, 11, 31 and 32). The step-wise increase in RuDP-carboxylase activity occurred after the termination of G_1 but prior to the onset of S-phase.

The use of a normal cell cycle in which growth is regulated towards the initiation of one mitotic division and production of two daughter cells may provide an invaluable tool for further studies on the sequence of events as regulated endogenously.

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APPENDIX

Figure 1. Sample radioautogram of the products of the organic acid fraction.

CIT	Citrate
FUM	Fumarate
G3P	Glyceraldehyde-3-phosphate
GLY	Glycerate
GCL	Glycollate
ISC	Isocitrate
ISL	Isocitrate lactone
MAL	Malate
PEP	Phosphoenolypyruvate
PGA	3-phosphoglycerate
SDP	Sugar diphosphates
SMP	Sugar monophosphates

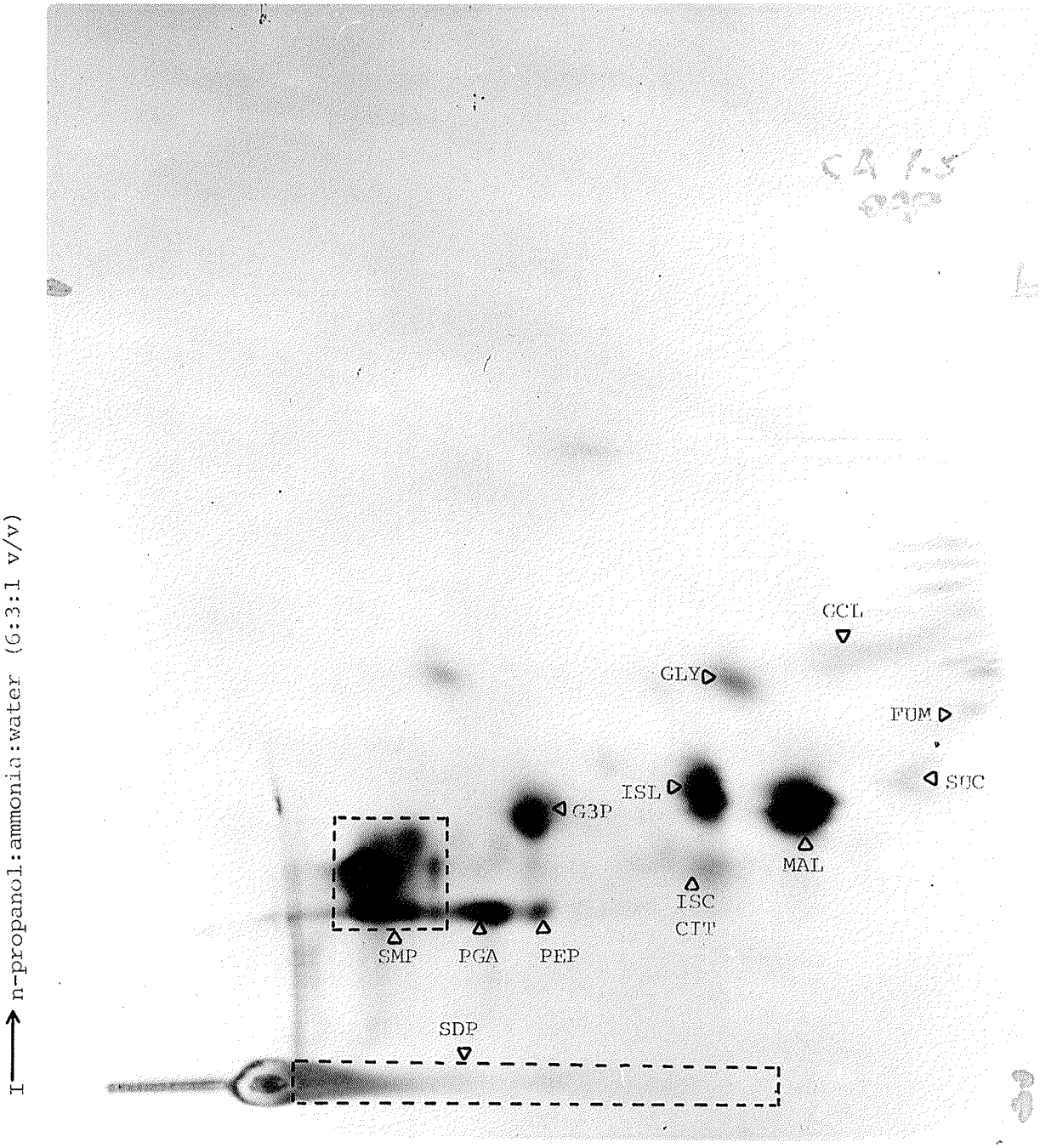
CCL
GLY
FUM
SUC
ISL
G3P
MAL
ISC
CIT
SMP
PGA
PEP
SDP
I ⟶ n-propanol:ammonia:water (6:3:1 v/v)
II ⟶ n-propyl acetate:formic acid:water (11:5:3 v/v)

Figure 2. Sample radioautogram of the products of the sugar fraction.

DHA	Dihydroxyacetone
FRU	Fructose
GLY	Glyceraldehyde
GLU	Glucose
MLT	Maltose
POL	Polysaccharides
RAF	Raffinose
RIB	Ribose
SUC	Sucrose
XYL	Xylose

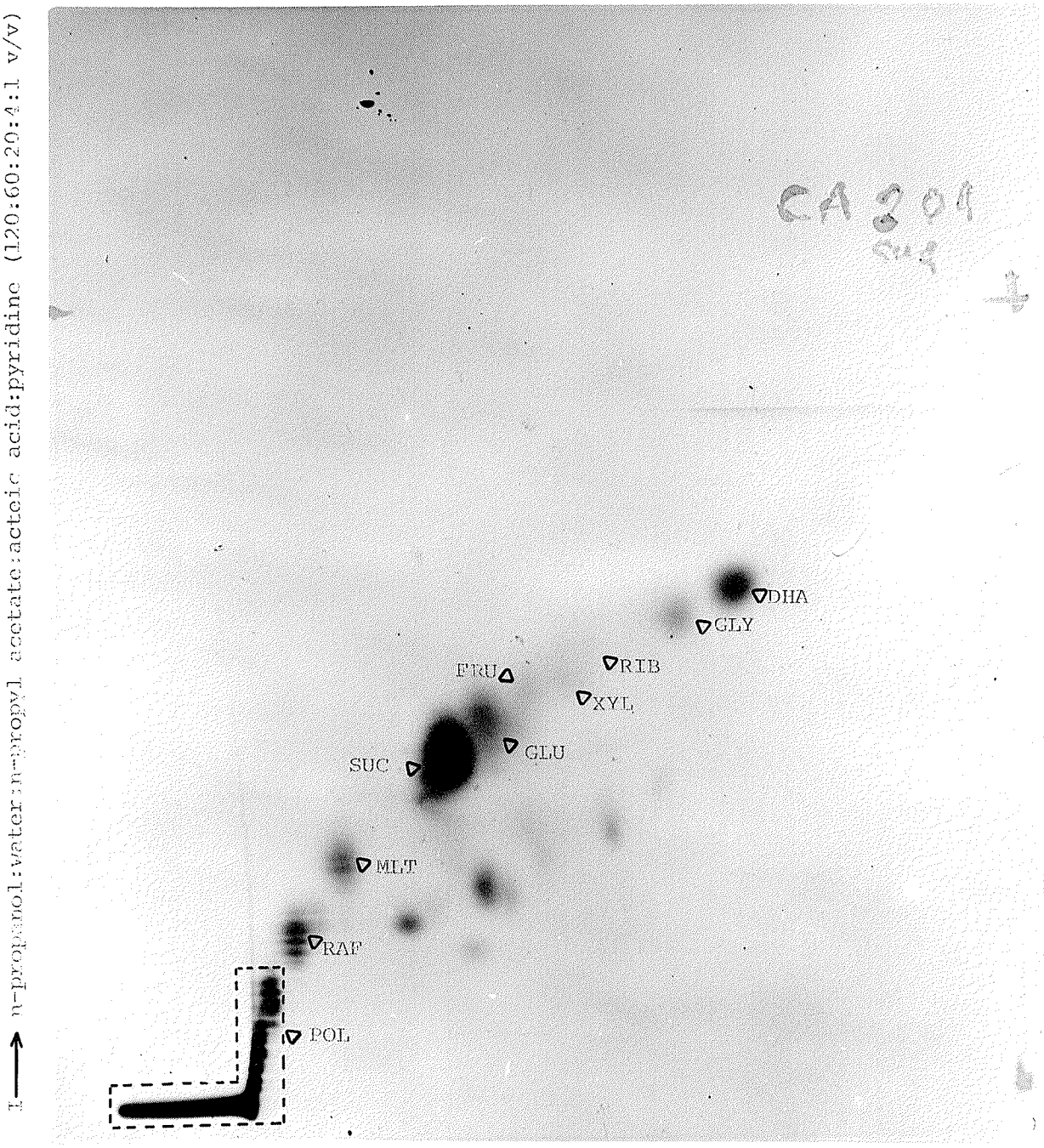
I ⟶ n-propanol:water:n-propyl acetate:acetic acid:pyridine (120:60:20:4:1 v/v)
II ⟶ n-butamol:acetic acid:water (12:5:3 v/v)
DHA
GLY
RIB
FRU
XYL
GLU
SUC
MLT
RAF
POL

Figure 3. Representative radioautogram of the amino-acid fraction.

ALA	Alanine
ASP	Aspartate
GLY	Glycine
GLT	Glutamine
GLU	Glutamate
SER	Serine
TYR	Tyrosine
UID	Unidentified

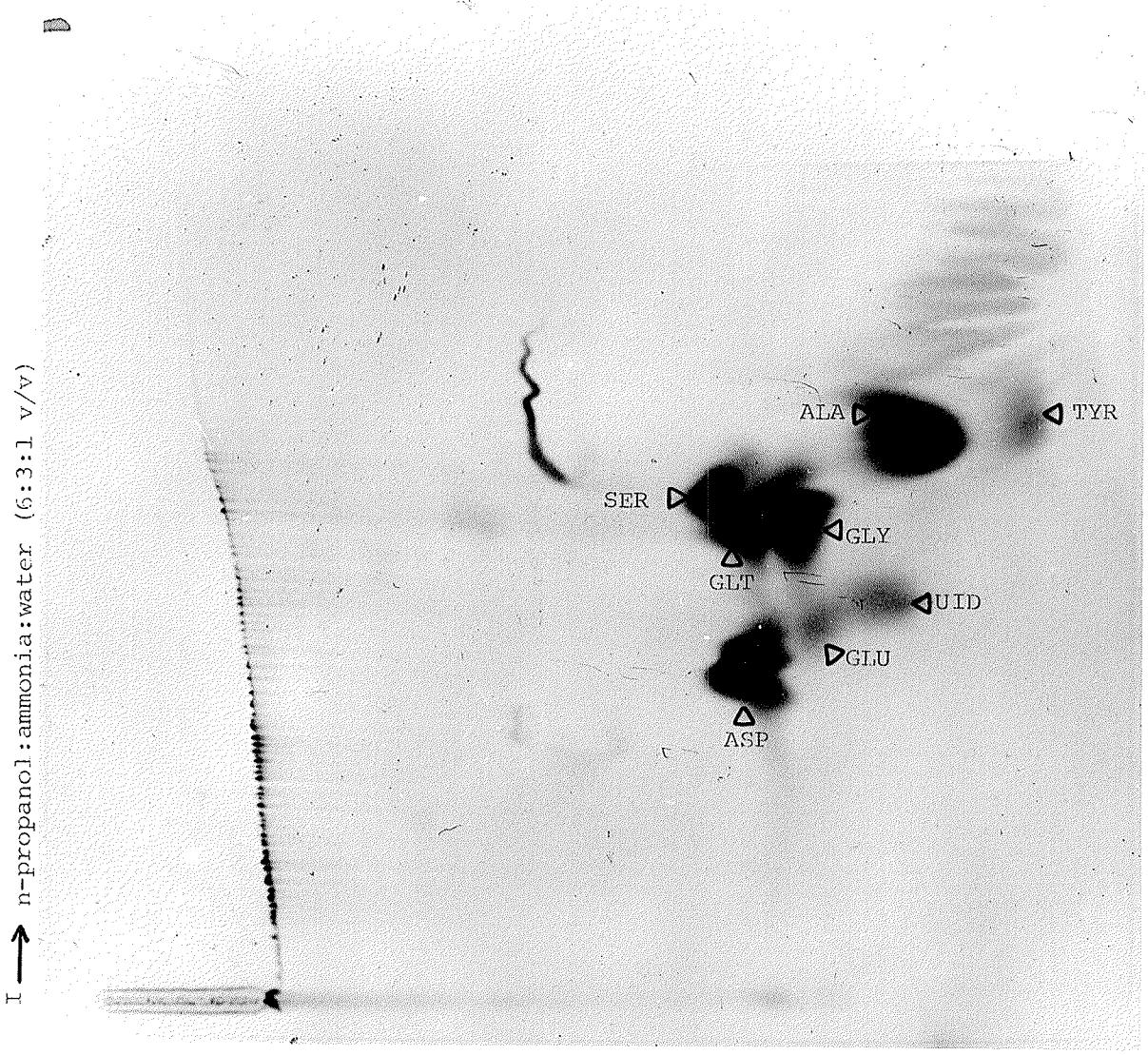
ALA
TYR
SER
GLY
GLT
UID
GLU
ASP
I ⟶ n-propanol:ammonia:water (6:3:1 v/v)
II ⟶ n-propyl acetate:formic acid:water (11:5:3 v/v)

Table I. Distribution of radioactivity in extracts from zoospores produced (Time:0) in cultures bubbled with air. 10 μCi $NaH^{14}CO_3$ (59 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$. The results are given as nmoles/20 mls suspension.

$^{14}CO_2$ Fixation (Time in seconds)	Fraction	nmoles/20 mls Suspension	Percent
5 - 10	Ethanol-water	0.492	93.5
	Residue + chloroform	0.034	6.5
	Total	0.526	100.0
30	Ethanol-water	1.658	85.5
	Residue + chloroform	0.280	14.5
	Total	1.938	100.0
60	Ethanol-water	3.400	75.9
	Residue + chloroform	1.080	24.1
	Total	4.480	100.0
90	Ethanol-water	4.100	63.1
	Residue + chloroform	2.400	36.9
	Total	6.500	100.0
120	Ethanol-water	5.300	57.0
	Residue + chloroform	4.000	43.0
	Total	9.300	100.0

Table II. Distribution of radioactivity in extracts from zoospores produced (Time:0) and grown in air for 8 hours. 10 μCi $NaH^{14}CO_3$ (59 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$. The results are given as nmoles/20 mls suspension.

$^{14}CO_2$ Fixation (Time in seconds)	Fraction	nmoles/20 mls Suspension	Percent
5 - 10	Ethanol-water	0.792	94.5
	Residue + chloroform	0.046	5.5
	Total	0.838	
30	Ethanol-water	2.670	86.8
	Residue + chloroform	0.412	13.4
	Total	3.082	
60	Ethanol-water	5.710	75.4
	Residue + chloroform	1.858	24.6
	Total	7.568	
90	Ethanol-water	6.800	65.9
	Residue + chloroform	3.520	34.1
	Total	10.32	
120	Ethanol-water	8.446	60.5
	Residue + chloroform	5.516	39.5
	Total	13.962	

Table III. Distribution of radioactivity in extracts from zoospores produced (Time:0) in air but grown in 5% CO_2 for 8 hours. 10 μCi $NaH^{14}CO_3$ (59 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_2$. The results are given as nmoles/20 mls suspension.

$^{14}CO_2$ Fixation (Time in seconds)	Fraction	nmoles/20 mls Suspension	Percent
5 - 10	Ethanol-water	0.286	96.0
	Residue + chloroform	0.012	4.0
	Total	0.298	
30	Ethanol-water	1.500	92.6
	Residue + chloroform	0.120	7.4
	Total	1.620	
60	Ethanol-water	3.140	88.2
	Residue + chloroform	0.420	11.8
	Total	3.560	
90	Ethanol-water	4.140	83.8
	Residue + chloroform	0.800	16.2
	Total	4.940	
120	Ethanol-water	5.780	79.0
	Residue + chloroform	1.540	21.0
	Total	7.320	

Table IV. Distribution of radioactivity in extracts from zoospores produced (Time:0) in 5% CO_2 but grown in air for 8 hours. 10 µCi $NaH^{14}CO_3$ (5 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$. The results are given as nmoles/20 mls suspension.

$^{14}CO_2$ Fixation (Time in seconds)	Fraction	nmoles/20 mls Suspension	Percent
5 - 10	Ethanol-water	0.673	90.1
	Residue + chloroform	0.074	9.9
	Total	0.747	
30	Ethanol-water	2.328	88.2
	Residue + chloroform	0.312	11.8
	Total	2.640	
60	Ethanol-water	4.157	77.6
	Residue + chloroform	1.200	22.4
	Total	5.357	
90	Ethanol-water	5.315	64.4
	Residue + chloroform	2.942	35.6
	Total	8.257	
120	Ethanol-water	6.564	57.3
	Residue + chloroform	4.883	42.7
	Total	11.447	

Table V. Distribution of radioactivity in extracts from zoospores produced (Time:0) in cultures bubbled with 5% CO_2. 10 µCi $NaH^{14}CO_3$ (59 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$. The results are given as nmoles/20 mls suspension.

$^{14}CO_2$ Fixation (Time in seconds)	Fraction	nmoles/20 mls Suspension	Percent
5 - 10	Ethanol-water	0.0230	93.9
	Residue + chloroform	0.0015	6.1
	Total	0.0245	
30	Ethanol-water	0.0760	92.0
	Residue + chloroform	0.0066	8.0
	Total	0.0826	
60	Ethanol-water	0.1600	85.9
	Residue + chloroform	0.0263	14.1
	Total	0.1863	
90	Ethanol-water	0.2360	83.2
	Residue + chloroform	0.0477	16.8
	Total	0.2837	
120	Ethanol-water	0.2950	75.6
	Residue + chloroform	0.0810	24.4
	Total	0.3760	

Table VI. Distribution of radioactivity in extracts of zoospores produced (Time:0) and grown in 5% CO_2 for 8 hours. 10 µCi $NaH^{14}CO_3$ (59 mCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$. The results are given as nmoles/20 mls suspension.

$^{14}CO_2$ Fixation (Time in seconds)	Fraction	nmoles/20 mls Suspension	Percent
5 - 10	Ethanol-water	0.1260	94.7
	Residue + chloroform	0.0070	5.3
	Total	0.1330	
30	Ethanol-water	0.4400	87.1
	Residue + chloroform	0.0650	12.9
	Total	0.5050	
60	Ethanol-water	0.9500	84.4
	Residue + chloroform	0.1756	15.6
	Total	1.1250	
90	Ethanol-water	1.4000	82.2
	Residue + chloroform	0.3030	17.8
	Total	1.7030	
120	Ethanol-water	1.7500	76.1
	Residue + chloroform	0.5495	23.9
	Total	2.2995	

Table VII. Distribution of ^{14}C in the various fractions of the ethanol-water soluble fraction as influenced by changes in CO_2 concentration during the phases of growth and DNA synthesis. 10 µCi $NaH^{14}CO_3$ (59 mCi/mM) were fed for one minute. The cells were killed, and radioactivity extracted and fractionated as described in Materials and Methods.

Time in Hours		0		4		8		12	
Changes in CO_2 Concentration	Products	DPM	%	DPM	%	DPM	%	DPM	%
Air (unchanged; control)	Amino-acid fraction	21448	14.90	37364	18.40	42552	19.20	10192	13.90
	Organic acid fraction	79148	54.80	121472	59.60	142632	64.50	51476	69.90
	Sugar fraction	43768	30.30	44828	22.00	36044	16.30	11920	16.20
	Total recovered	144364	100.00	203664	100.00	221228	100.00	73588	100.00
Air changed to 5% CO_2	Amino-acid fraction	-	-	38228	25.40	33948	26.50	26112	25.60
	Organic acid fraction	-	-	85548	56.80	76156	59.60	65468	64.10
	Sugar fraction	-	-	26808	17.80	17736	13.90	10544	10.30
	Total recovered			150584	100.00	127840	100.00	102124	100.00
5% CO_2 (unchanged; control)	Amino-acid fraction	2488	23.90	16126	32.40	15290	24.30	13172	22.10
	Organic acid fraction	6008	57.70	24957	50.00	31611	58.40	41404	69.40
	Sugar Fraction	1916	18.40	8792	17.60	7178	13.30	5048	8.50
	Total Recovered	10412	100.00	49875	100.00	54086	100.00	59624	100.00
5% CO_2 changed to air	Amino-acid fraction	-	-	72240	24.50	48368	21.20	32811	16.30
	Organic acid fraction	-	-	154817	52.50	131052	57.50	139027	69.10
	Sugar fraction	-	-	67908	23.00	48652	21.30	29336	14.60
	Total recovered			294965	100.00	338073	100.00	201174	100.00

Table VIII. Distribution of ^{14}C in the organic acid fraction during growth and DNA synthesis (0-12 hours). Data are also expressed as % of total ^{14}C recovered (Table VII in Appendix).

Time in Hours			0		4		8		12	
Changes in CO_2 Concentration of Cultures		Products	DPM	%	DPM	%	DPM	%	DPM	%
Air (unchanged, control)	1.	Glyceraldehyde-3-phosphate	3981	2.75	8989	4.41	7559	3.42	2677	3.63
	2.	Glycerate	1124	0.77	2126	1.06	2281	1.03	772	1.04
	3.	Glycollate	950	0.65	1458	0.71	1712	0.77	566	0.76
	4.	Isocitrate lactone	3737	2.58	7288	3.57	8415	3.80	4169	5.66
	5.	Malate	8186	5.63	21015	10.31	24105	10.90	8596	11.68
	6.	Phosphoenolpyruvate	633	0.43	2551	1.25	1284	0.58	437	0.59
	7.	3-phosphoglycerate	1100	0.76	4130	2.02	1712	0.77	669	0.90
	8.	Sugar diphosphates	9411	6.51	20528	10.07	11838	5.35	5302	7.20
	9.	Sugar monophosphates	24742	17.16	30854	15.14	36656	16.57	11839	16.08
Air changed to 5% CO_2	1.	Glyceraldehyde-3-phosphate	-	-	5903	3.91	2742	2.14	2500	2.44
	2.	Glycerate	-	-	1762	1.17	1599	1.25	1000	0.97
	3.	Glycollate	-	-	1120	0.74	1371	1.07	571	0.55
	4.	Isocitrate lactone	-	-	8127	5.39	3046	2.38	12142	11.88
	5.	Malate	-	-	13688	9.08	8149	6.37	8428	8.25
	6.	Phosphoenolpyruvate	-	-	1368	0.90	762	0.59	500	0.48
	7.	3-phosphoglycerate	-	-	3507	2.32	1218	0.95	1071	1.04
	8.	Sugar diphosphates	-	-	14201	9.43	8301	6.49	6143	6.01
	9.	Sugar monophosphates	-	-	20702	13.74	16754	13.10	11357	11.12

Table IX. Distribution of ^{14}C in the sugar fraction during growth and DNA synthesis (0-12 hours). Data are also expressed as percent of total ^{14}C recovered (Table VII in Appendix).

Time in Hours			0		4		8		12	
Changes in CO_2 Concentration of Cultures		Products	DPM	%	DPM	%	DPM	%	DPM	%
Air (unchanged, control)	1.	Dihydroxyacetone	1970	1.36	3451	1.69	1586	0.71	1442	1.95
	2.	Fructose + Glucose	1838	1.27	2286	1.12	2235	1.01	727	0.98
	3.	Glyceraldehyde	963	0.66	1703	0.83	1189	0.53	405	0.55
	4.	Maltose	1444	1.00	1031	0.50	937	0.42	238	0.32
	5.	Polysaccharides	20571	14.24	20845	10.23	12687	5.73	3023	4.10
	6.	Raffinose	2670	1.84	1389	0.68	1117	0.50	143	0.19
	7.	Sucrose	7134	4.94	9145	4.49	7317	3.30	3111	4.22
Air changed to 5% CO_2	1.	Dihydroxyacetone	-	-	1796	1.19	1117	0.87	1065	1.04
	2.	Fructose + Glucose	-	-	1501	0.99	763	0.59	475	0.46
	3.	Glyceraldehyde	-	-	1260	0.83	1135	0.88	633	0.61
	4.	Maltose	-	-	509	0.33	319	0.24	148	0.41
	5.	Polysaccharides	-	-	7130	4.73	4115	3.21	1645	1.61
	6.	Raffinose	-	-	670	0.44	337	0.26	137	0.13
	7.	Sucrose	-	-	5603	3.72	3370	2.63	2193	2.14

Table X. Distribution of ^{14}C in the amino-acid fraction during growth and DNA synthesis (0-12 hours). Data are also expressed as percent of total ^{14}C recovered (Table VII in Appendix).

Time in Hours			0		4		8		12	
Changes in CO_2 Concentration of Cultures		Products	DPM	%	DPM	%	DPM	%	DPM	%
Air (unchanged; control)	1.	Alanine	4268	2.96	9104	4.47	8000	3.62	1967	2.67
	2.	Aspartate	300	0.20	527	0.25	553	0.25	132	0.18
	3.	Glutamate	794	0.54	650	0.31	1064	0.48	224	0.30
	4.	Glycine	2108	1.46	4521	2.22	5617	2.54	1148	1.56
	5.	Serine	3263	2.26	7088	3.48	7234	3.27	1398	1.90
	6.	Tyrosine	987	0.68	1121	0.55	2212	1.00	265	0.36
	7.	Unidentified	866	0.60	1833	0.90	2434	1.10	294	0.40
Air changed to 5% CO_2	1.	Alanine	-	-	9328	6.20	9132	7.14	4935	4.83
	2.	Aspartate	-	-	428	0.28	509	0.40	653	0.64
	3.	Glutamate	-	-	497	0.33	563	0.44	715	0.70
	4.	Glycine	-	-	5581	3.70	1358	1.06	2507	2.45
	5.	Serine	-	-	5887	3.90	1188	0.93	1208	1.25
	6.	Tyrosine	-	-	1262	0.84	645	0.50	490	0.48
	7.	Unidentified	-	-	1280	0.85	1278	1.00	817	0.80

Table XI. Distribution of ^{14}C in the organic acid fraction during growth and DNA synthesis (0 - 12 hours). The data are also expressed as a percent of total ^{14}C recovered (Table VII in Appendix).

Time in Hours			0		4		8		12	
Changes in CO_2 Concentration of Cultures		Products	DPM	%	DPM	%	DPM	%	DPM	%
5% CO_2 (unchanged; control)	1.	Glyceraldehyde-3-phosphate	162	1.55	2845	5.70	2402	4.43	2484	4.16
	2.	Glycerate	162	1.55	1023	2.05	632	1.16	662	1.11
	3.	Glycollate	120	1.15	948	1.90	506	0.93	580	0.97
	4.	Isocitrate lactone	625	6.00	1372	2.75	3446	6.36	6790	11.38
	5.	Malate	859	8.25	3444	6.90	1581	2.91	6086	10.20
	6.	Phosphoenolpyruvate	102	0.98	424	0.85	474	0.87	331	0.55
	7.	3-phosphoglycerate	108	1.03	698	1.40	506	0.93	621	1.40
	8.	Sugar diphosphates	595	5.71	4495	9.01	2813	5.19	2360	3.95
	9.	Sugar monophosphates	1021	9.80	5540	11.10	6923	12.78	7204	12.08
5% CO_2 Changed to air	1.	Glyceraldehyde-3-phosphate	-	-	7740	2.62	4194	1.83	4588	2.28
	2.	Glycerate	-	-	5573	1.88	2621	1.41	2502	1.24
	3.	Glycollate	-	-	3560	1.20	3145	1.37	2642	1.31
	4.	Isocitrate lactone	-	-	10682	3.62	9043	3.96	13903	6.91
	5.	Malate	-	-	15791	5.35	15071	6.60	22383	11.12
	6.	Phosphoenolpyruvate	-	-	1857	0.62	1704	0.74	1529	0.76
	7.	3-phosphoglycerate	-	-	6347	2.15	2490	1.90	1668	0.82
	8.	Sugar diphosphates	-	-	22139	7.50	9536	4.13	9454	4.69
	9.	Sugar monophosphates	-	-	37156	12.59	38398	16.83	35730	17.76

Table XII. Distribution of ^{14}C in the sugar fraction during growth and DNA synthesis (0 - 12 hours). Data are also expressed as percent of total ^{14}C recovered (Table VII in Appendix).

Time in Hours			0		4		8		12	
Changes in CO_2 Concentration of Cultures		Products	DPM	%	DPM	%	DPM	%	DPM	%
5% CO_2 (unchanged; control)	1.	Dihydroxyactone	249	2.39	2250	4.51	1127	2.08	883	1.48
	2.	Fructose + Glucose	130	1.25	316	0.63	208	0.38	288	0.48
	3.	Glyceraldehyde	109	1.04	220	0.44	424	0.78	187	0.31
	4.	Maltose	100	0.95	132	0.26	136	0.25	101	0.16
	5.	Polysaccharides	341	3.27	2444	4.90	1780	3.28	888	1.49
	6.	Raffinose	114	1.09	202	0.40	215	0.39	101	0.16
	7.	Sucrose	318	3.05	1354	2.71	890	1.64	848	1.42
5% CO_2Changed to air	1.	Dihydroxyacetone	-	-	2377	0.80	1119	0.49	1144	0.56
	2.	Fructose + Glucose	-	-	2988	1.01	1752	0.76	1525	0.75
	3.	Glyceraldehyde	-	-	1358	0.46	730	0.31	704	0.34
	4.	Maltose	-	-	1562	0.52	1070	0.46	792	0.39
	5.	Polysaccharides	-	-	28725	9.73	22234	9.74	9153	4.54
	6.	Raffinose	-	-	2512	0.85	2491	1.09	1144	0.56
	7.	Sucrose	-	-	16298	5.52	6714	2.94	7070	3.50

Table XIII. Distribution of ^{14}C in the amino acid fraction during growth and DNA synthesis (0 - 12 hours). Data are also expressed as percent of total ^{14}C recovered (Table VII in Appendix).

Time in Hours			0		4		8		12	
Changes in CO_2 concentration of Cultures		Products	DPM	%	DPM	%	DPM	%	DPM	%
5% CO_2 (unchanged; control)	1.	Alanine	323	3.10	3403	6.82	2872	5.31	2924	4.90
	2.	Aspartate	212	2.03	1087	2.17	227	0.42	224	0.37
	3.	Glutamate	167	1.60	361	0.72	175	0.33	382	0.64
	4.	Glycine	289	2.77	3169	6.35	2385	4.41	1185	1.98
	5.	Serine	214	2.05	2628	5.27	1141	2.11	1159	1.85
	6.	Tyrosine	134	1.29	634	1.27	591	0.96	298	0.50
	7.	Unidentified	102	0.97	361	0.72	175	0.33	382	0.64
5% CO_2 Changed to air	1.	Alanine	-	-	17410	5.90	6481	2.84	4726	2.35
	2.	Aspartate	-	-	7513	2.54	353	0.15	0	0.00
	3.	Glutamate	-	-	1098	0.37	484	0.21	491	0.24
	4.	Glycine	-	-	8019	2.71	5702	2.50	4104	2.04
	5.	Serine	-	-	16037	5.43	7982	3.50	5596	2.78
	6.	Tyrosine	-	-	3684	1.24	2080	0.91	1588	0.79
	7.	Unidentified	-	-	7669	2.60	3239	1.42	1437	0.71

Table XIV. Distribution of ^{14}C in the various fractions of the ethanol-water soluble fraction. Kinetics of ^{14}C distribution.
Zoospores produced (Time:0) in either air or 5% CO_2 and then grown for 4 hours in both air and 5% CO_2.

Incubation Time (seconds)		5-10		30		60		90		120	
Changes in CO_2 Concentration of Cultures	Fractions	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%
Air (unchanged; control)	Organic acid	15832	71.1	61732	64.8	121472	59.6	196720	55.2	281948	57.2
	Amino-acid	2724	12.2	12776	13.4	37364	18.3	59944	16.8	83036	16.8
	Sugar	3700	16.6	20792	21.8	44828	22.0	100016	28.0	128264	26.0
	Total ^{14}C recovered	22256	99.9	95300	100.0	203664	99.9	356680	100.0	493248	100.0
Air changed to 5% CO_2	Organic acid	8796	67.0	33212	62.0	85548	56.8	128756	55.8	173996	56.1
	Amino acid	2560	19.5	12784	23.9	38228	25.4	56348	24.4	80496	25.9
	Sugar	1764	13.5	7588	14.2	26808	17.8	45732	19.8	55824	18.0
	Total ^{14}C recovered	13120	100.0	53584	100.1	150584	100.0	230836	100.0	310316	100.0
5% CO_2 (unchanged; control)	Organic acid	6696	60.3	11654	55.5	24957	50.0	41568	38.6	51790	46.5
	Amino acid	2391	21.5	6345	30.2	16126	32.3	49822	46.3	33603	30.2
	Sugar	2014	18.1	2983	14.2	8792	17.6	16195	15.1	25917	23.3
	Total ^{14}C recovered	11101	99.9	20982	99.9	49875	99.9	107585	100.0	111310	100.0
5% CO_2 changed to air	Organic acid	13730	72.2	70128	61.2	154817	52.2	238070	50.5	362348	49.1
	Amino acid	3149	16.6	23457	20.5	72240	24.5	121850	25.9	190427	25.8
	Sugar	2138	11.2	20997	18.3	67908	23.0	111449	23.6	185245	25.1
	Total ^{14}C recovered	19017	100.0	114582	100.0	249965	100.0	471369	100.0	738020	100.0

Table XV. Distribution of ^{14}C in the photosynthetic products as a function of time. Zoospores produced and grown in air for 4 hours.
Fixation, extraction, fractionation and chromatographic procedures are given in Materials and Methods. Data are expressed as percent of total ^{14}C recovered (Table XIV in Appendix).

Incubation Time (seconds)	5-10		30		60		90		120	
Products	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%
Organic Acid Fraction										
1. Glyceraldehyde-3 phosphate	649	2.91	6050	6.34	8989	4.41	11803	3.03	12406	2.51
2. Glycerate	326	1.46	1543	1.61	2162	1.06	4072	1.14	5921	1.20
3. Glycollate	352	1.57	1605	1.68	1458	0.71	2420	0.67	3665	0.74
4. Isocitrate lactone	507	2.27	2716	2.85	7288	3.57	17705	4.96	26503	5.37
5. Malate	776	3.48	6791	7.12	21025	10.31	33442	9.37	47367	9.60
6. Phosphoenolpyruvate	405	1.82	2037	2.13	2551	1.25	4328	1.21	3947	0.80
7. 3-Phosphoglycerate	1781	8.00	4136	4.33	4130	2.02	5508	1.54	4793	0.97
8. Sugar diphosphates	3593	16.14	13766	14.44	20528	10.07	19672	5.51	16635	3.37
9. Sugar monosphosphates	5272	23.68	25063	26.29	30854	15.14	56655	15.88	66258	13.43
Sugar Fraction										
1. Dihydroxyacetone	377	1.69	2682	2.81	3451	1.69	5701	1.59	4489	0.91
2. Fructose + Glucose	263	1.18	1520	1.59	2286	1.12	6501	1.82	8465	1.71
3. Glyceraldehyde	260	1.16	956	1.00	1703	0.83	3100	0.86	3335	0.67
4. Maltose	114	0.51	603	0.63	1031	0.50	2800	0.78	4233	0.85
5. Polysaccharides	496	2.22	8982	9.42	20845	10.23	48608	13.62	55795	11.31
6. Raffinose	140	0.62	935	0.98	1389	0.68	3100	0.86	6798	1.37
7. Sucrose	792	3.55	4886	5.12	9145	4.49	22803	6.39	35407	7.17
Amino-acid Fraction										
1. Alanine	1146	5.15	5394	5.66	9104	4.47	12307	3.45	13286	2.69
2. Aspartate	80	0.36	260	0.27	527	0.25	408	0.13	166	0.03
3. Glutamate	44	0.19	323	0.34	650	0.31	2098	0.58	3820	0.77
4. Glycine	227	1.02	2441	2.56	4521	2.22	5994	1.68	6726	1.36
5. Serine	100	0.45	1533	1.60	7088	3.48	15346	4.30	20925	4.24
6. Tyrosine	109	0.49	432	0.45	1121	0.55	1678	0.47	1661	0.33
7. Unidentified	245	1.10	1741	1.82	1850	0.90	1816	0.50	1412	0.28

Table XVI. Distribution of ^{14}C in the photosynthetic products as a function of time. Zoospores produced (Time:0) but grown for 4 hours in 5% CO_2.
Fixation, extraction, fractionation and chromatographic prodedures are given in Materials and Methods. Data are expressed as percent of total ^{14}C recovered (Table XIV in Appendix).

Incubation Time (seconds)	5-10		30		60		90		120	
Products	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%
Organic and Fraction										
1. Glyceraldehyde-3-phosphate	299	2.27	2059	3.84	5903	3.91	5794	2.51	6786	2.18
2. Glycerate	247	1.88	717	1.33	1762	1.17	2961	1.28	3688	1.18
3. Glycollate	274	2.09	797	1.48	1120	0.74	1674	0.72	2262	0.72
4. Isocitrate lactone	369	2.81	2457	4.58	8127	5.39	15193	6.58	22967	7.96
5. Malate	839	6.39	3720	6.94	13688	9.08	14549	6.30	22967	7.40
6. Phosphoenolpyruvate	223	1.70	597	1.11	1368	0.90	2961	1.28	2575	0.82
7. 3-phosphoglycerate	589	4.49	1395	2.60	3507	2.32	3863	1.67	3654	1.17
8. Sugar diphosphates	661	5.03	4185	7.80	14201	9.43	12746	5.52	11832	3.83
9. Sugar monophosphates	2808	21.40	8469	15.80	20702	13.74	29871	12.94	37931	12.22
Sugar Fraction										
1. Dihydroxyacetone	243	1.85	690	1.28	1796	1.19	2744	1.18	2456	0.79
2. Fructose + Glucose	134	1.02	432	0.80	1501	0.99	2927	1.26	3908	1.25
3. Glyceraldehyde	122	0.92	410	0.76	1260	0.83	2012	0.87	1507	0.48
4. Maltose	0	0.0	159	0.29	509	0.33	960	0.42	1284	0.41
5. Polysaccharides	268	2.04	1578	2.94	7130	4.73	14406	6.24	18589	5.99
6. Raffinose	0	0.0	243	0.45	670	0.44	1143	0.49	1563	0.50
7. Sucrose	229	1.74	1525	2.84	5603	3.72	10153	4.39	12225	3.93
Amino-acid Fraction										
1. Alanine	753	5.74	4001	7.46	9328	6.20	11664	5.01	14489	4.66
2. Aspartate	72	0.54	268	0.50	428	0.28	113	0.04	81	0.02
3. Glutamate	105	0.80	294	0.54	497	0.33	1690	0.73	2334	0.75
4. Glycine	129	0.98	2365	4.41	5581	3.70	5026	2.17	4073	1.31
5. Serine	95	0.72	1112	2.07	5887	3.90	11495	4.97	16180	5.21
6. Tyrosine	158	1.20	511	0.95	1262	0.84	1690	0.73	2334	0.75
7. Unidentified	266	2.02	614	1.14	1338	0.88	1747	0.75	885	0.28

Table XVII. Distribution of ^{14}C in the photosynthetic products as a function of time. Zoospores produced and grown in 5% CO_2 for 4 hours.
Fixation, extraction, fractionation and chromatographic procedures are given in Materials and Methods. Data expressed as percent of total ^{14}C recovered (Table XIV in Appendix).

Incubation Time (seconds)	5-10		30		60		90		120	
Products	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%
Organic Acid Fraction										
1. Glyceraldehyde-3-phosphate	408	3.67	1130	5.38	2845	5.70	5404	5.02	6681	6.00
2. Glycerate	234	2.11	315	1.49	1023	2.05	1288	1.19	1553	1.39
3. Glycollate	214	1.93	396	1.88	948	1.90	1870	1.73	2383	2.28
4. Isocitrate lactone	355	3.19	524	2.49	1372	2.75	2411	2.24	3159	2.83
5. Malate	683	6.15	1690	8.05	3444	6.90	5030	4.67	7302	6.56
6. Phosphoenolpyruvate	188	1.68	209	0.99	424	0.85	648	0.60	880	0.79
7. 3-phosphoglycerate	368	3.31	641	3.05	698	1.40	706	0.65	1087	0.97
8. Sugar diphosphates	2725	24.54	1748	8.33	4495	9.01	5071	4.71	4920	4.42
9. Sugar monophosphates	1171	10.54	3822	18.21	5540	11.01	9852	9.15	11187	10.04
Sugar Fraction										
1. Dihydroxyacetone	397	3.57	644	3.07	2250	4.51	5231	4.85	8501	7.63
2. Fructose + Glucose	87	0.78	101	0.48	316	0.63	615	0.57	803	0.72
3. Glyceraldehyde	143	1.28	149	0.71	220	0.44	340	0.31	363	0.32
4. Maltose	0	0	101	0.48	132	0.26	356	0.33	414	0.37
5. Polysaccharides	207	1.86	662	3.15	2444	4.90	4146	3.85	6920	6.22
6. Raffinose	0	0.00	143	0.68	202	0.40	567	0.52	492	0.44
7. Sucrose	244	2.19	465	2.21	1354	2.71	2316	2.15	3292	2.95
Amino-acid Fraction										
1. Alanine	795	7.16	1859	8.85	3403	6.82	7424	6.90	4637	4.16
2. Aspartate	218	1.96	482	2.29	1087	2.17	4085	3.79	3226	2.89
3. Glutamate	72	0.64	165	0.78	361	0.72	797	0.74	706	0.63
4. Glycine	112	1.01	533	2.54	3169	6.35	9466	8.79	4234	3.80
5. Serine	74	0.66	244	1.16	2628	5.27	7124	6162	2957	2.65
6. Tyrosine	237	2.00	747	3.55	634	1.27	1495	1.38	1008	0.90
7. Unidentified	335	3.01	406	1.93	774	1.55	996	0.92	470	0.42

Table XVIII. Distribution of ^{14}C in the photosynthetic products as a function of time. Zoospores produced (Time:0) 5% CO_2 but grown in air for 4 hours.
Fixation, extraction, fractionation and chromatographic procedures are given in Materials and Methods. Data are expressed as percent of total ^{14}C recovered (Table XIV in Appendix).

Incubation Time (Seconds)	5-10		30		60		90		120	
Products	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%
Organic Acid Fraction										
1. Glyceraldehyde-3-phosphate	398	2.09	3436	2.99	7740	2.62	10951	2.32	14494	1.96
2. Glycerate	480	2.52	1753	1.53	5573	1.88	8808	1.86	13769	1.86
3. Glycollate	288	1.50	1683	1.46	3560	1.20	5951	1.26	8696	1.17
4. Isocitrate lactone	467	2.45	2525	2.20	10682	3.62	15236	3.23	23915	3.24
5. Malate	1002	5.27	6101	5.32	15791	5.35	34044	7.22	55801	7.56
6. Phosphoenolpyruvate	370	1.94	1472	1.28	1857	0.62	2380	0.50	4348	0.58
7. 3-phosphoglycerate	865	4.54	3225	2.81	6347	2.15	7618	1.61	8696	1.17
8. Sugar diphosphates	2663	14.00	6732	5.87	22139	7.50	18331	3.88	28263	3.82
9. Sugar monophosphates	4352	22.88	25106	21.91	37156	12.59	64517	13.68	93848	12.71
Sugar Fraction										
1. Dihydroxyacetone	125	0.65	945	0.82	2377	0.80	4567	0.96	7965	1.07
2. Fructose + Glucose	112	0.59	903	0.78	2988	1.01	6798	1.44	9632	1.30
3. Glyceraldehyde	75	0.39	672	0.58	1358	0.46	2563	0.54	3705	0.50
4. Maltose	32	0.17	546	0.47	1562	0.52	3120	0.66	4631	0.62
5. Polysaccharides	410	2.15	7727	6.74	28725	9.73	53941	11.44	86694	11.74
6. Raffinose	45	0.23	924	0.80	2512	0.85	4458	0.94	6669	0.90
7. Sucrose	485	2.54	5459	4.76	16298	5.52	29199	6.19	44644	6.04
Amino-acid Fraction										
1. Alanine	734	3.86	6263	5.46	17410	5.90	24614	5.22	32563	4.41
2. Aspartate	230	1.20	2111	1.85	7513	2.54	2072	0.43	2285	0.30
3. Glutamate	72	0.38	563	0.49	1098	0.37	3290	0.69	6475	0.87
4. Glycine	624	3.28	3286	2.87	8019	2.71	12550	2.66	13208	1.78
5. Serine	202	1.05	1900	1.66	16037	5.43	35946	7.46	59604	8.08
6. Tyrosine	246	1.29	2158	1.88	3684	1.24	5179	1.09	7617	1.03
7. Unidentified	189	0.99	1126	0.99	1878	0.63	2559	0.54	3828	0.52