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TITLE ....HETEROTROPHIC UTILIZATION OF D-GLUCOSE BY THE GREEN ALGA,

ANKISTRODESMUS BRAUNII (NAEG.)

THESIS ....Ph.D., 1984

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HETEROTROPHIC UTILIZATION OF D-GLUCOSE BY THE GREEN ALGA, *ANKISTRODESMUS BRAUNII* (Naeg.)

A Thesis

Submitted to

The Faculty of Graduate Studies

University of Manitoba

In Partial Fulfillment

of the Requirements of the Degree

Doctor of Philosophy

by

Roderick C. Bollman

August 1983

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a Ph.D. thesis entitled: Heterotrophic Utilization of D-Glucose by the Green Alga, Ankistrodesmus Braunii (Naeg.)

submitted by Roderick C. Bollman

in partial fulfilment of the requirements for the Ph.D. degree.

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Date of oral examination: November 23rd, 1983

The student has satisfactorily completed and passed the Ph.D. oral examination.

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Chairman of Ph.D. Oral*

(*The signature of the Chairman does not necessarily signify that the Chairman has read the complete thesis.)

HETEROTROPHIC UTILIZATION OF D-GLUCOSE BY THE GREEN ALGA, ANKISTRODESMUS BRAUNII (NAEG.)

by

Roderick C. Bollman

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

To

Jill and Philip

- some sacrifice can never be measured -

- some gratitude never expressed -

ABSTRACT

The green alga, Ankistrodesmus braunii (Naeg.) was found to be a facultative heterotroph. The alga possesses an active transport system for d-glucose, which is not saturated at high glucose concentrations. The system appears to be biphasic and follows the model of two independent, permease proteins. The high affinity component had a V_{max} of 1.2×10^{-7} pmol glucose.cell^{-1}.min^{-1} at a K_t of 7.7 nM. This transport rate is similar or faster than transport rates of glucose attributed to bacteria. The low affinity component of the system had a V_{max} of 10.78×10^{-4} pmol glucose.cell^{-1}.min^{-1} at a K_t of 16.39 μM which is comparable to transport kinetics found in other algae or attributed to natural algal populations.

The total glucose transport resulted in a 50.9% conversion to assimilated algal carbon and resulted in a doubling time of 31.4 h in 0.1 mM glucose, as compared with 15.7 h for photoautotrophic growth in continuous light of 5000 lux. In the light, transport of glucose was inactivated or repressed and photosynthesis was almost the entire source of energy and biosynthetic carbon, as the addition of glucose was neither additive or synergistic during light growth.

A reduction of CO_2 to a level which could not sustain photosynthesis reduced the inhibiting effects of light on glucose transport, permitting light, heterotrophic growth, with a doubling time of 99.6 h at 0.1 mM and 36.1 h at 1.0 mM glucose. External glucose was not required to induce or activate the transport system.

The transport system was directly dependent on temperature with a $Q_{10(10-20°C)}$ of 1.9-2.4 but was not strongly influenced by pH functioning at approximately the same rate through a pH range of 5-9. While the system is capable of using ATP from any source of phosphorylation, the glucose does not appear to be phosphorylated prior to transport. The ionophores monensin, valinomycin, oubain and gramcidin only minimally inhibited transport, but inhibitors of substrate, oxidative and photophorylation exhibited marked inhibition.

Electron micrographs revealed that, in the latter stages of the cell cycle, the photosynthetic capacity would be reduced due to a reduction in the chloroplast lamella and the stored starch was depleted from the cells. This information coincides with the reduction of photosynthetic rates of over 70% which were observed during these stages of the cell cycle. Glucose transport did not decrease by this amount, even in the light, suggesting an active heterotrophic role during the division stages of the cell cycle.

Heterotrophy is therefore capable of making a significant, competitive contribution to the overall metabolism of the green alga Ankistrodesmus braunii (Naeg.).

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INTRODUCTION

It has been known for several decades that some algae are capable of dark, heterotrophic growth (Bristol-Roach 1928; Algéus 1948, 1949; Griffiths et al. 1961; Parker et al. 1961; Danforth 1962). Other algae appear able to utilize dissolved organic material to enhance growth but only in the light (Pringsheim and Wiessner 1960). Droop and McGill (1966), working on 39 strains of algae suggested, in fact, that all algae could be divided into two major groups, facultative heterotrophs and obligate autotrophs. The heterotrophs could be further subdivided into photoorganotrophs and chemoorganotrophs. The results of this early work then created the idea that the algae, in aquatic ecosystems, were utilizing the dissolved organic materials to sustain or enhance their photoautotrophic growth.

Unfortunately, these growth experiments, conducted to determined the heterotrophic potential of algae did not reflect natural conditions. The cultures were usually axenic, thus eliminating any of the natural competition that would occur between algae and bacteria for the organic substrates. Also, the concentrations of organic substrates used to generate the heterotrophic growth (Algéus 1948; Griffiths et al. 1961; Theriault 1965; Droop and McGill 1966) were far higher than are normally found in natural waters (Vallentyne 1957; Duursma 1966; Hobbie 1966; Sloan and Strickland 1966; Allen 1976; Hama and Handa 1980; Lambou and Hern 1983).

In an attempt to reconcile these differences between laboratory experiments and natural situations and to better predict the actual rate

of assimilation of organic substrates, a series of papers (Hobbie and Wright 1965a; 1965b, Wright and Hobbie 1965, 1966) were published. Using modifications of radiotracer techniques described by Steeman-Neilson (1952) and Parsons and Strickland (1962), they were able to determine the rate of transport of selected organic compounds by natural populations. The technique also provided a means of estimating the natural concentration of these compounds, the most effective natural concentration at which the transport occurred and the turnover time of the compounds. It was further suggested that a distinction could be made between algal and bacterial utilization of the organic compounds (Hobbie and Wright 1965b; Allen 1969). This use of radioisotopes to determine uptake rates and thus predict heterotrophic potential has since become the most widely used method of determining unialgal and natural mixed population heterotrophy (Droop 1974; Neilson and Lewin 1974; Sepers 1977; Raven 1980). This technique has since been employed to investigate the mechanisms of transport (e.g. Komor et al. 1977; Hellebust 1978; Raven 1980). It has also been used to study the impact of various environmental parameters on the transport capabilities of algae (e.g. Karlander and Krauss 1966; Cooksey 1972; Berman et al. 1977; Vincent 1980; Shah and Syrett 1982).

The results of the radiotracer studies involving transport kinetics of organic substrates, supplied at realistic concentrations, suggest that the algae would be unable to compete with bacteria for available organic compounds in natural waters. Either the transport velocities are too slow (e.g. Wright and Hobbie 1966; Allen 1969; Sepers 1977; Berman and Gerber 1980; Ellis and Stanford (1982) or the rapid transport systems are

efficient at such high substrate concentrations that they would not likely be present in natural waters (Komor and Tanner 1974; Hellebust 1978; Lewin and Hellebust 1978). Only a few reports (e.g. Mayfield and Inniss 1978; Saks and Kahn 1979; Vincent and Goldman 1980) suggest that, at least some algae, are competitive with bacteria in transporting natural organic nutrients. The weight of the evidence then suggests that very little, if any, natural algal heterotrophy occurs. However, evidence is available, for the existence of active transport systems for organic substrates in algae (Raven 1980). It is hardly conceivable that an alga would evolve a complex system to transport organic compounds and not be able to utilize that system in natural environments. This is especially true when it is realized that such transport systems utilize energy and organic material in their biosynthesis and action.

The general discrepancy in the two major directions of heterotrophy research stimulated this investigation. The first problem was to determine whether algae could not compete with bacteria for natural substrates or whether the discrepancy was simply due to variations or errors in methodology. A second question involved the relationship between the heterotrophic capabilities of the alga, the overall metabolism and the environment. There have been few attempts to determine the actual significance of heterotrophy to the growth and metabolism of a particular alga (e.g. Lewin and Hellebust 1975; Lewin and Hellebust 1976; Hellebust and Lewin 1977) and these were reported primarily using marine diatoms. Also, the vast majority of studies involving unialgal cultures were concerned with one or two environmental parameters so that, at best, only an isolated inference could be drawn on the potential heterotrophic

capabilities in varying environmental conditions. The major parameter tested as to its effect on transport was light intensity (e.g. Karlander and Krauss 1966; vanBaalen et al. 1971; Cooksey 1972; Berman et al. 1977; Darley et al. 1981; Richardson and Fogg 1982). A few researchers have examined such parameters as salinity (Hellebust 1978; McLean et al. 1981), CO_2 and O_2 levels (e.g. Nilsen and Johnson 1982), phosphate levels (Berman et al. 1977; Mayfield and Inniss 1978), nitrogen availability (e.g. North and Stephans 1971; Peak et al. 1980; Shah and Syrett 1982) and pH (Komor and Tanner 1974). The results of these isolated studies tends to indicate that there are many environmental factors which can influence or control algal heterotrophy.

A third aspect of algal heterotrophy involves study of the actual transport mechanism. This includes the type of transport system, the source of energy to power the transport and the metabolic control of the transport system. There have been several models proposed for the actual type of transport system of organic substrates by algae. Wright and Hobbie (1966) and Allen (1969) suggested that any meaningful transport is by diffusion and only at high substrate concentrations. Most recent work suggests the transport of organic substrates is active. The systems described appear to be constitutive, substrate induced or alternate (light, CO_2) substrate repressed (Raven 1980). The biochemical energy source for transport appears to be ATP but the involvement of ATP may be direct or indirect. Komor and Tanner (1973) proposed a complex model of H^+-sugar cotransport for the green alga Chlorella vulgaris. Hellebust (1978) involved Na^+ as the cotransport ion in Cyclotella cryptica. The ATP involvement would then be to maintain a H^+ or Na^+ gradient across

the cell membrane.

The direction of this research was to extensively examine the heterotrophic capabilities of one alga using only one organic substrate. It was reasoned that, if sufficient aspects of heterotrophy were examined a comprehensive model of the potential mechanisms and probability of meaningful competitive utilization of the substrate could be estimated.

The organism chosen for this study was the Chlorophycophyta alga, Ankistrodesus braunii (Naeg.). This organism was chosen for several reasons. A. braunii is known to be a photoautotrophic alga with an extremely wide distribution. The genus has been found in such habitats as small pools, damp soils and sewage treatment lagoons where it may reach bloom proportions (Prescott 1961; Bold and Wynne 1978). It has also been identified in samples from oligotrophic, eutrophic, acidic and alkaline bodies of water but it is more prevalent in eutrophic waters (Prescott 1961) where the potential for organic compounds at a concentration suitable for heterotrophic growth would occur. This ubiquitous and obviously successful distribution suggests that the alga is well adapted to a range of environments and its ability to reach bloom proportions indicates a very competitive metabolism. Since areas where organic substrates are abundant, seem to be the habitats of preference for A. braunii, it would be beneficial if the alga could utilize these organic substrates to augment its growth. This would be a beneficial supplement to photoautotrophic growth or a method of continued growth when photosynthesis is absent.

A second rationale for the choice of A. braunii is the fact that it is neither a flagellate alga or a blue green alga, both groups, which at

least through evolutionary considerations, are suspected of being more closely related to recognized heterotrophs. Such an organism is desirable if extrapolations were to be made to heterotrophy by algae. A third consideration was the evidence that A. braunii had at least some heterotrophic capabilities as a facultative heterotroph (e.g. Bollman & Robinson 1977, Tanner 1969; Mayfield & Inniss 1978). Other, more artificial reasons for selecting this alga are: ease of procurement and maintenance in axenic culture; ability to grow on a completely defined, inorganic medium so that any organic substrate may be added without interference by some unknown extraneous compounds.

The organic substrate used throughout the entire investigation was d-glucose. The reasons for the selection of this substrate were as follows: its occurrence at low levels in natural aquatic ecosystems; the fact that it is a metabolite of algae; its availability as high specific activity U-C^{14} glucose; and its common usage in other heterotrophic experiments by both phycologists and bacteriologists.

Four major areas concerning the heterotrophic potential and transport mechanisms were investigated. The first study involved the determination of the growth potential and transport kinetics of d-glucose by A. braunii. The second study was an attempt to compare the relative efficiencies of photoautrophic and heterotrophic growth, to determine the factors controlling the transport system and environmental conditions most probably to promote heterotrophy. The third investigation was an attempt to characterize the transport system and a final series of experiments were designed to demonstrate the contribution heterotrophy could make to the cell metabolism at different stages during the cell cycle.

Chapter One

Heterotrophic Growth Potential of

Ankistrodesmus braunii (Naeg.)

INTRODUCTION

The heterotrophic potential of algae has been the topic of considerable research for several decades (e.g., Droop 1974; Neilson and Lewin 1974; Raven 1980). Original emphasis of this research was placed on straight forward growth experiments. Organic substrates were added to an inorganic medium, inoculated with algae and incubated under various conditions to ascertain the effects of the organic substrates on the growth of the algae (Bristol-Roach 1928; Finkle et al. 1950; Killam and Myers 1956; Griffiths 1963, 1965). The results of these experiments suggested that many species of algae are capable of growing and sustaining populations on a variety of organic substrates. Perhaps the greatest criticism of this work is the fact that experiments were conducted using extremely high substrate concentrations. This has created the question of whether these algae could exist on environmentally meaningful levels of organic compounds.

During the 1960's a technique was developed which could possibly answer this question. Hobbie and Wright (1965a) expanding on the radioisotope methods of Steeman-Nielson (1952) and Parsons and Strickland (1962) developed a technique of determining the rate of movement of labelled organic compounds into cells. By considering a carbon replacement time, an estimate of the heterotrophic potential of an organism could be calculated. Since this time, research has proliferated in this area. Studies have been done on both natural populations (Wright and Hobbie 1965; Hamilton and Austin 1967; Vaccaro 1967; Saks and Kahn 1979;

Vincent and Goldman 1980), and on unialgal cultures which is probably best exemplified by the work of Hellebust and Lewin (1971, 1977), and Lewin and Hellebust (1976, 1978) on three species of diatoms, and Komor, Tanner and their coworkers in a series of papers (1968 to 1978) on heterotrophy in Chlorella vulgaris.

The results of this radioisotope work have led to three widely accepted conclusions. Firstly, many algae have active transport systems capable of removing organic substances from water, even if the compounds are in low concentrations. Secondly, that these systems are not capable of competing with bacterial uptake systems for naturally occurring substrates. Finally, that the velocity of uptake by these systems could not support substantial algal growth. Only recently have a few researchers showed any evidence to the contrary. Lewin and Hellebust (1978) demonstrated that Nitzschia laevis had an uptake system for glutamate and glucose capable of accounting for the observed heterotrophic growth rates on these substrates. Saks and Kahn (1979) showed that Cylindrotheca closterium could compete successfully with a bacterium for low molecular weight organic compounds in a natural environment.

The purpose of this investigation was to answer three questions. Could the green alga Ankistrodesmus braunii grow heterotrophically on the organic compound d-glucose? Secondly, did it possess an active uptake system for glucose capable of accounting for this growth? Finally, was this uptake system efficient enough to compete with bacteria at natural substrate levels. To answer these questions, a growth analysis was done on A. braunii cells which were grown in the presence of glucose. Using U-C^{14} glucose, uptake kinetics were established and the results compared with literature results for bacterial uptake.

MATERIALS AND METHODS

1. Source and Maintenance of Algal Cultures:

The green alga Ankistrodesmus braunii (Naeg.) Bruunthaler 1915 was obtained from the Algal Culture Collection at the University of Texas in Austin as Ankistrodesmus braunii (Naeg.) no. 245. Axenic cultures of the alga were maintained on slants containing the following major mineral salts ($g.L^{-1}$: $Ca(NO_3)_2$ (0.4); K_2HPO_4 (0.1); $MgSO_4.7H_2O$ (0.25); Na_2CO_3 (0.2); Na_2SiO_3 (0.586); and $FeCl_3$ - EDTA (0.00016). Trace elements were added in the following concentrations ($mg.L^{-1}$): H_3BO_4(0.093); $ZnSO_4.2H_2O$ (0.12); $MnSO_4.4H_2O$ (0.02); $(NH_4)_6\ Mo_7O_{24}$ (0.006); $CuSO_4$ (0.005); and $CoCl_2.6H_2O$ (0.008). The medium also contained d-glucose (1.0mM) and was prepared with either 1.2% nutrient agar or 1.2% proteose peptone agar (Difco). The latter was used to monitor cultures for the presence of bacteria. The final pH was adjusted to 7.0 by the addition of 0.1N HCl.

All liquid cultures used in the entire thesis were grown on a medium containing the same major and trace mineral salt concentrations. They were also at a pH of 7.0. These cultures were illuminated under cool white and Sylvania Gro-Lux at an intensity of 4500 to 5000 lux. Cultures were grown in controlled environment chambers at a constant temperature of 22°C. All cultures were aerated with air which was passed through a sterile glass wool filter, a saturated $CuSO_4$ solution and one sterile distilled deionized water flask. The entire apparatus was autoclaved for 20 minutes at 115°C and 15 psi. before each new culture was begun. The rate of air flow was kept sufficient to cause enough agitation to keep

the algae from settling. Cells used from these cultures were harvested between 3 and 5 days from the time of inoculation. This time was previously determined to be within the exponential growth phase.

2. Growth Experiments:

To test the ability of A. braunii to grow on glucose, three test conditions were established. Since the organic substrate could either support growth in the dark through heterotrophism or enhance growth in the light through photoassimilation or both, the following conditions were employed. Cultures were grown under 5000 lux continuous light, a regime of 12 h light and 12 h dark or in constant darkness. Aeration, temperature, pH and mineral salt concentrations were the same as above.

Cells from an exponentially growing culture which contained no glucose were added to 250 mL erlenmeyer flasks containing 150 mL of sterile medium. The initial cell concentrations were 4.3×10^3 cells.mL^{-1} (12-12 light regime), 2.5×10^4 cells.mL^{-1} (dark regime) and 1.2×10^4 cells.mL^{-1} (continuous light regime). To test the ability to grow in the dark, 2 flasks had no glucose, 3 flasks had 0.1 mM glucose and 3 flasks had 1.0 mM glucose. To investigate the possibility of organic enhancement of light growth, 4 flasks had no glucose and 4 flasks had 1.0 mM glucose in each light regime. At the same time each day, 5 mL of culture were removed aseptically from each flask. The cell number and cell volumes were determined using a Model B Coulter Counter. Maximum obtainable yields, growth constants and doubling times were calculated for each condition using the method outlined by Fogg (1975).

3. Uptake Kinetics of Glucose:

All cells used in these experiments were grown under the conditions described above, except that they were grown under continuous light for 4 days then placed in the dark for 24 h prior to the experiments. The medium also contained 2.0 mM glucose. For each experiment the cells were grown in axenic culture. Cells were harvested, washed and centrifuged 4 times in sterile, glucose free medium to remove any residual or adsorbed glucose. They were then added to fresh sterile medium at a final concentration of 1×10^8 cells.L^{-1}. Cell counts were again determined on a Model B Counter Counter. The new culture was then dispensed as 15 mL samples into 25 mL culture tubes. These tubes were then placed in the dark until the experiment began. Equivalent, heat killed, dead cell controls were also established.

The actual uptake velocities were determined by adding labelled glucose to the cultures, incubating for one minute, removing the cells by filtration and counting the amount of radioactive glucose in the cells by liquid scintillation. The final concentrations in each experiment ranged, in 17 steps, from 1.2 nM to 12.2 μM. d-[C^{14}(U)]glucose. All radioactive glucose was purchased from New England Nuclear. For 1.2 nM to 122.2 nM glucose concentrations, the specific activity was 291.6 mCi/mM. For concentrations ranging from 159.0 nM to 12.2 μM glucose, the specific activity was 4.5 mCi/mM. The filters were 0.45 um GA (Gelman) cellulose acetate membrane. Filters were prewashed with filter-sterilized (0.22 μm filters) medium containing 2.0 μM glucose. Immediately following the filtration, using approximately 75mm Hg vacuum pressure,

the filters were washed with 30 mL of medium and each placed into 10 mL of scintillation cocktail (Bray 1960)). Counts were made on a Picker Liquimat 200 liquid scintillation counter using the channels ratio method (Wang and Willis 1965). The counter was set for 10 minute counts with a preset statistic standard deviation of 1.5 as an acceptable limit. The calculations of the kinetic parameters were based on four separate experiments, each having four replicate samples at each of the 17 glucose concentrations. All values obtained for the uptake velocities at each substrate concentration were subjected to a correction by subtracting an apparent uptake found in dead cell controls.

4. Calculation of the Kinetic Parameters:

The atypical uptake velocity versus substrate concentration curve obtained (Fig. 1-5) was assumed to have resulted from the interaction of two kinetically distinguishable uptake systems. Therefore the observed velocities (v) at each substrate concentration (S) would actually result from a combination of these two components and could be described by the following equation.

$$v = \frac{V_1 S}{K_1 + S} + \frac{V_2 S}{K_2 + S}$$

where v is the velocity of uptake at any substrate concentration S; V_1 and V_2 are the maximum velocities of uptake for the two systems; and K_1 and K_2 are the substrate concentrations at which half of these maximum velocities are reached. The values for V_1, V_2, K_1 and K_2 were calculated using methods outlined by Wright and Hobbie (1965) combined with a modification of the successive approximation method

described by Akedo and Christensen (1962) and Reid et al. (1970). See Appendix I.

5. Determination of Algal Organic Carbon:

The organic carbon content of cells was determined from the wet-oxidation (Strickland and Parsons 1972) of known numbers of cells.

RESULTS

1. Dark Growth on Glucose

Ankistrodesmus braunii demonstrated the ability to grow heterotrophically on glucose in the absence of light (Fig. 1-1a and 1-1b). Cells grown in the absence of light and glucose failed to double twice in the entire 10 day period. In the dark, in the presence of 0.1 mM glucose, cell numbers and total cell volume increased exponentially from day 1 to day 4. In the presence of 1.0 mM glucose, this occurred from day 1 to day 6. If cell number measurements were used, there was a lag period of one day at both glucose concentrations but, if cell volumes were used, this lag period did not exist. The maximum yields were significantly different ($P<0.01$) in each of the three treatments (Table 1-1). Doubling times based upon cell numbers were 31.4 h on 0.1 mM glucose and 22.11 h on 1.0 mM glucose. Those based on cell volumes (Table 1-2) were 42.45 h and 25.8 h on 0.1 and 1.0 mM glucose respectively.

2. Glucose Enhancement of Photo-autotrophic Growth:

There did not appear to be any enhancement of growth in the light when 1.0 mM glucose was added to cultures (Figs. 1-2a and b, 1-3a and b). Tables 1-1 and 1-2 illustrate the differences between the three experimental light conditions. The doubling times in the presence of light with or without glucose are shorter than those in dark grown cells in 1.0 mM glucose, being only 14.0 h and 14.6 h respectively. Also, the major difference appears to be in the cell volumes (Fig. 1-4). Cells grown in

Figure 1-1a: The effect of glucose on the growth of A. braunii in the dark. The growth curves were determined by plotting the log of cell no. against time. Lines represent different amounts of glucose added at time zero.

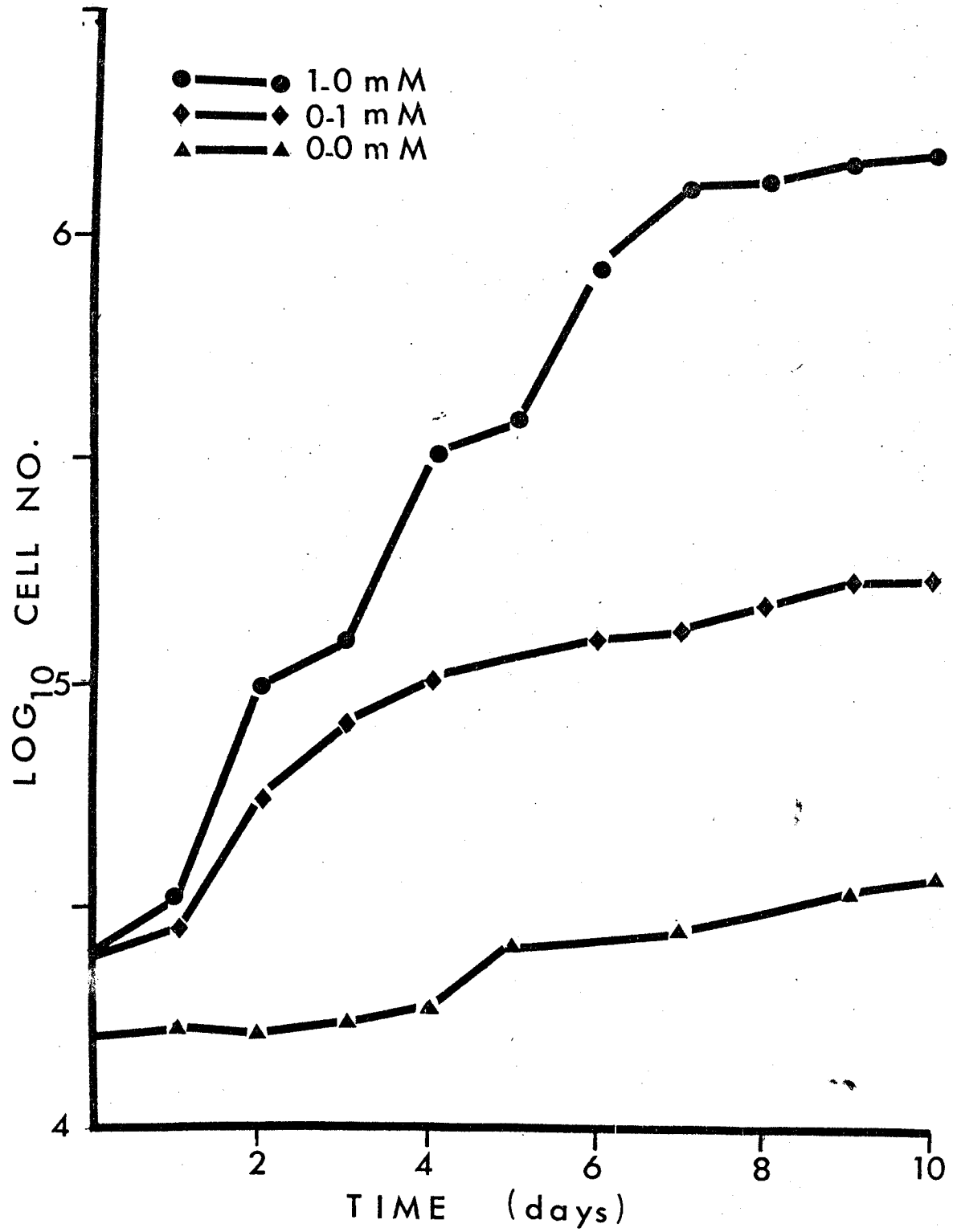

1.0 m M
0.1 m M
0.0 m M
LOG10 CELL NO.
6
5
4
2
4
6
8
10
TIME (days)

Figure 1-1b: The effect of glucose on the growth of A. braunii in the dark. The growth curves were determined by plotting the log of cell volumes (μm^3) against time. Lines represent different amounts of glucose added at time zero.

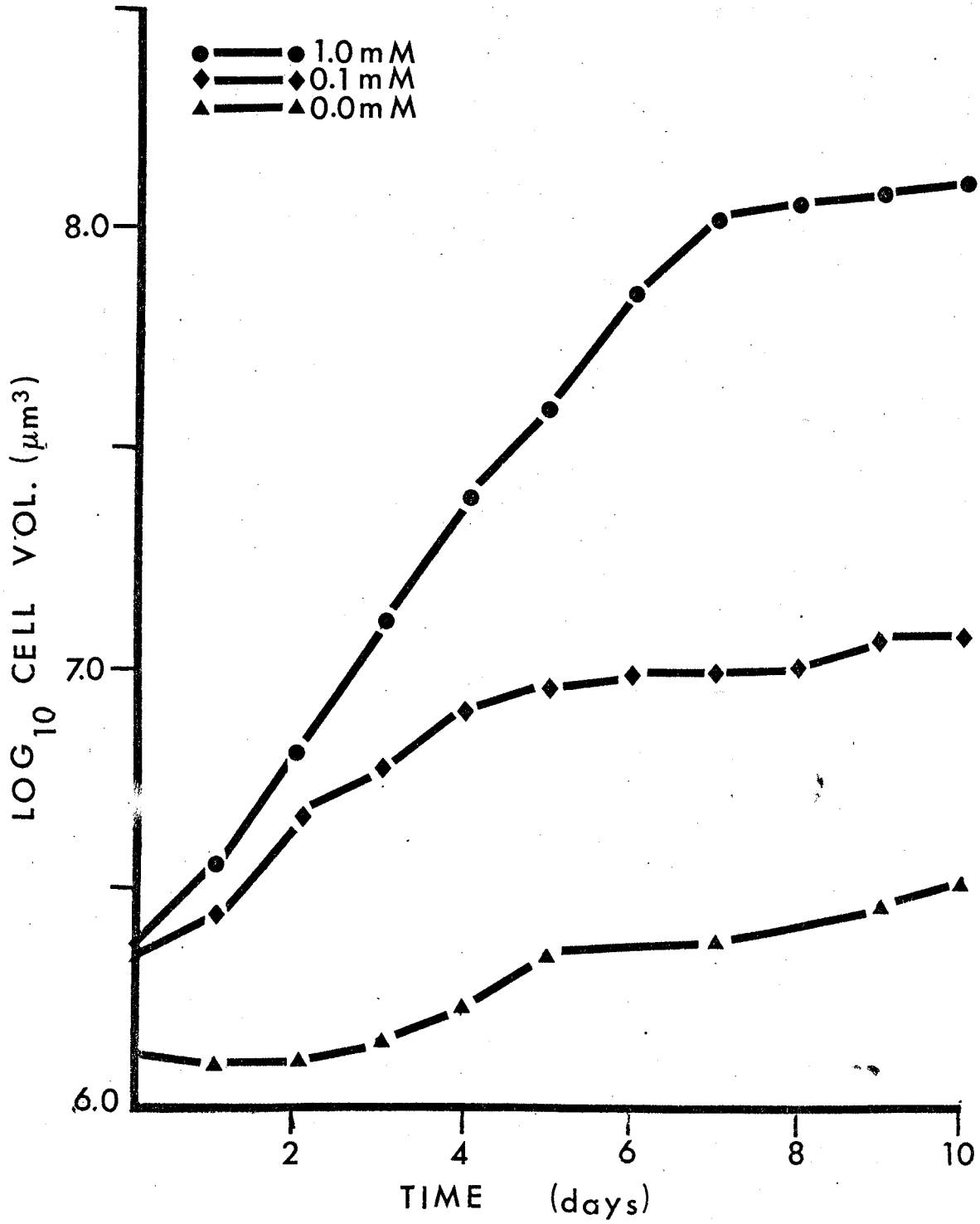

1.0 mM
0.1 mM
0.0 mM
8.0
7.0
6.0
LOG10 CELL VOL. (μm3)
2
4
6
8
10
TIME (days)

TABLE 1-1

Growth potential of Ankistrodesmus braunii grown on d-glucose. All results are based on calculations using cell numbers.

Light Treatment	Glucose Concentration	k'[1]	Doubling Time (h)	Maximum[2] Yield	Days to[3] Max. Yield
continuous	0.0	0.495	14.59	3.16×10^6	8
	1.0 mM	0.515	14.03	2.34×10^6	8
12 light/12 dark	0.0	0.625	11.56	4.27×10^6	11
	1.0 mM	0.565	12.79	4.57×10^6	11
dark	0.0	0.034	190.10	3.89×10^4	9
	0.1 mM	0.230	31.41	1.31×10^5	9
	1.0 mM	0.327	22.11	1.46×10^6	10

1. k' is the specific growth constant
2. The highest number of cells reached in each culture (cells per mL).
3. The days required to reach the maximum yield.

TABLE 1-2

Growth potential of Ankistrodesmus braunii grown on d-glucose. All results are based on calculations using cell volumes.

Light Treatment	Glucose Concentration	k'[1]	Doubling Time (h)	Maximum[2] Yield	Mean Cell[3] Volume
continuous	0.0	0.460	15.70	3.81×10^8	117.1 ± 0.7%
	1.0 mM	0.530	13.63	3.24×10^8	140.9 ± 0.6%
12 light/12 dark	0.0	0.595	12.14	5.02×10^8	96.2 ± 4.5%
	1.0 mM	0.565	12.79	5.62×10^8	111.9 + 1.8%
dark	0.0	0.052	138.90	3.47×10^6	85.1 ± 1.9%
	0.1 mM	0.170	42.49	1.23×10^7	79.1 ± 2.4%
	1.0 mM	0.280	25.80	1.32×10^8	89.5 ± 1.4%

1. k' is the specific growth constant
2. The highest volume of cells reached in each culture (μm^3 of cells per mL).
3. The mean cell colume (μm^3).

FIGURE 1-2a: The effect of glucose on the growth of A. braunii in continuous light based on the log of cell no. against time. Lines represent different amounts of glucose added at time zero.

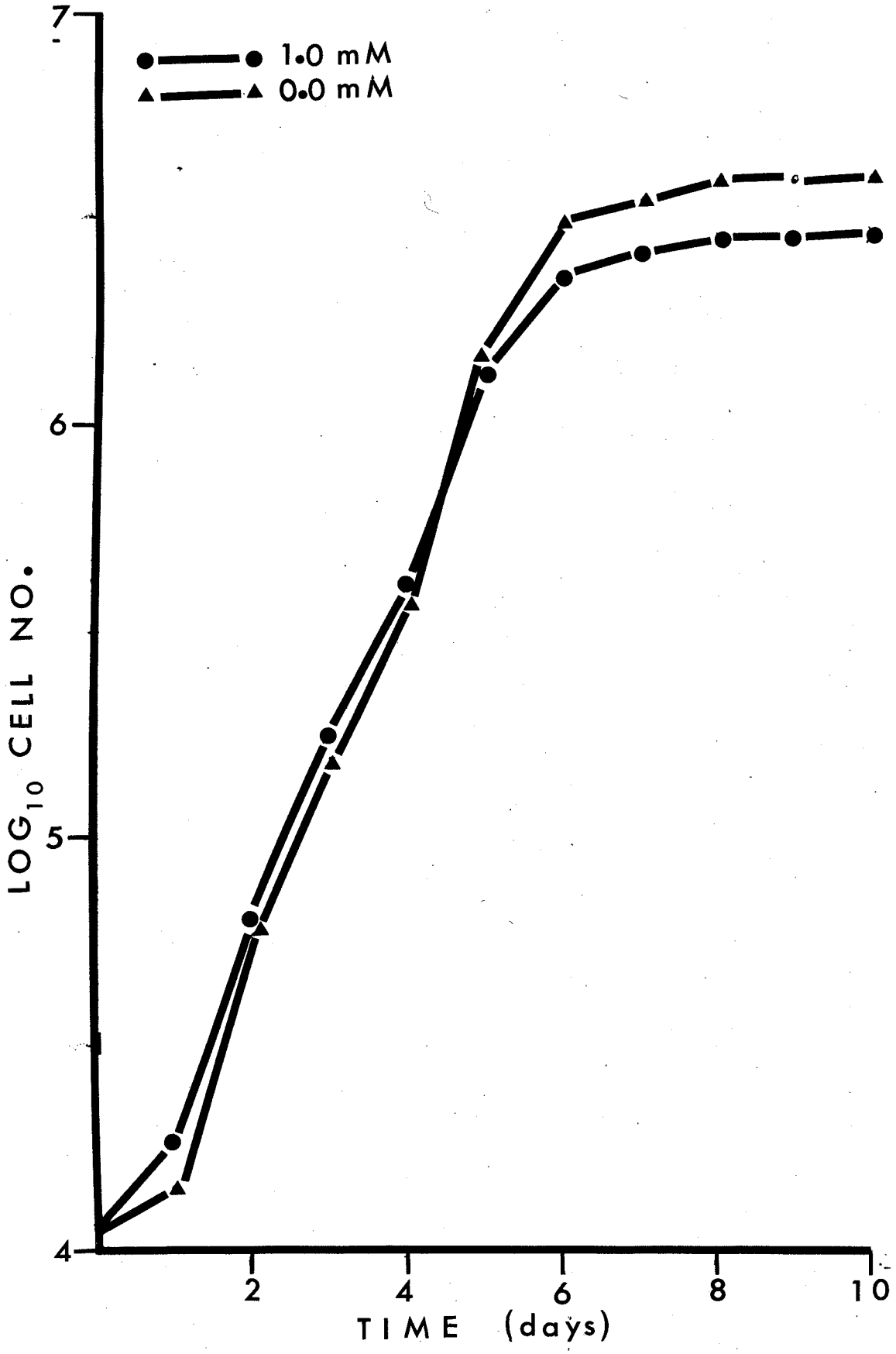

1.0 mM
0.0 mM
7
6
5
4
LOG10 CELL NO.
2
4
6
8
10
TIME (days)

FIGURE 1-2b: The effect of glucose on the growth of A. braunii in continuous light (5000 lux) based on the log of cell volume (μm^3) against time. Lines represent different amounts of glucose added at time zero.

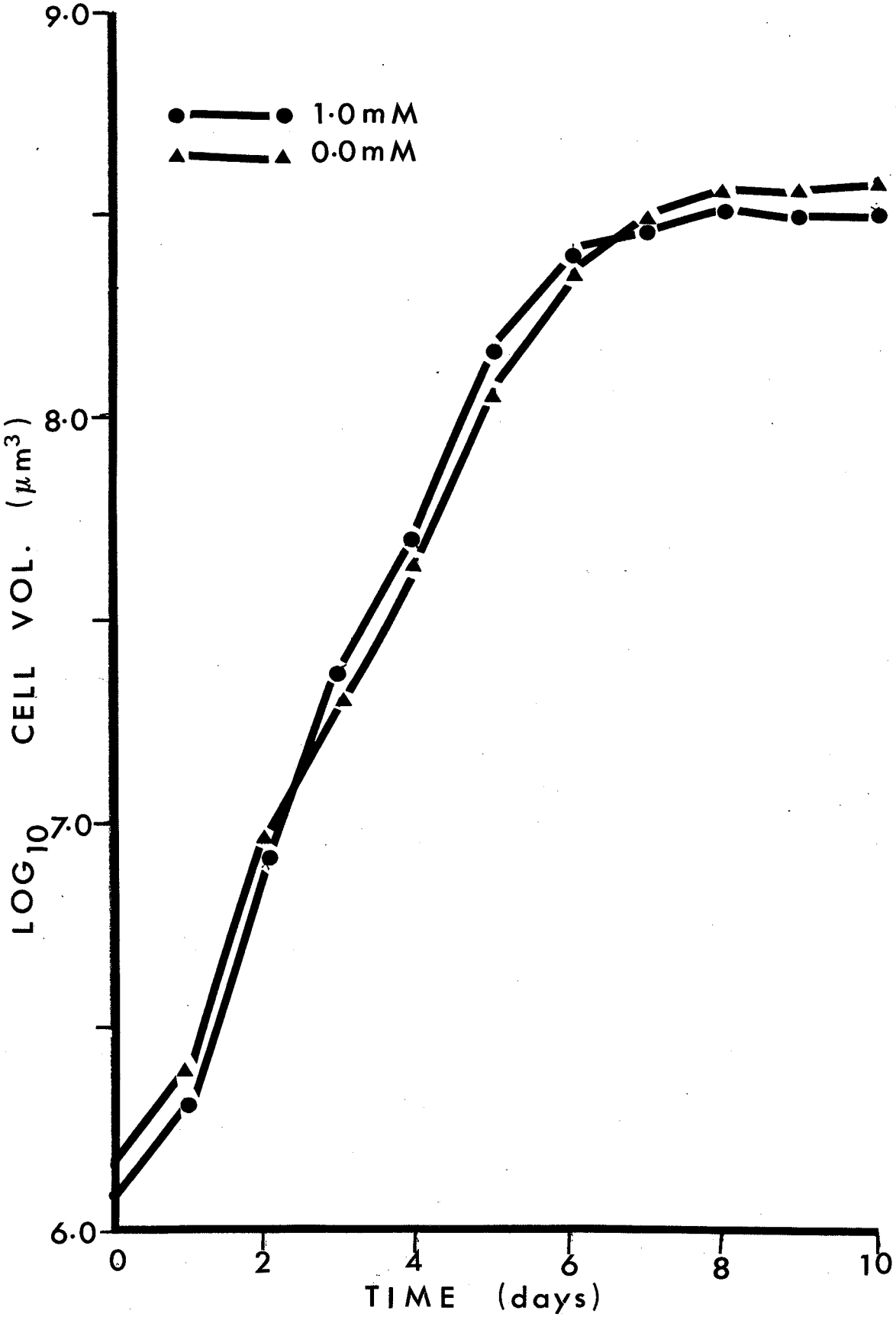

9·0
8·0
7·0
6·0
1·0 mM
0·0 mM
LOG10 CELL VOL. (μm3)
0
2
4
6
8
10
TIME (days)

FIGURE 1-3a: The effect of glucose on the growth of A. braunii in a 12h light/12h dark regime based on the log of cell no. against time. Lines represent different amounts of glucose added at time zero.

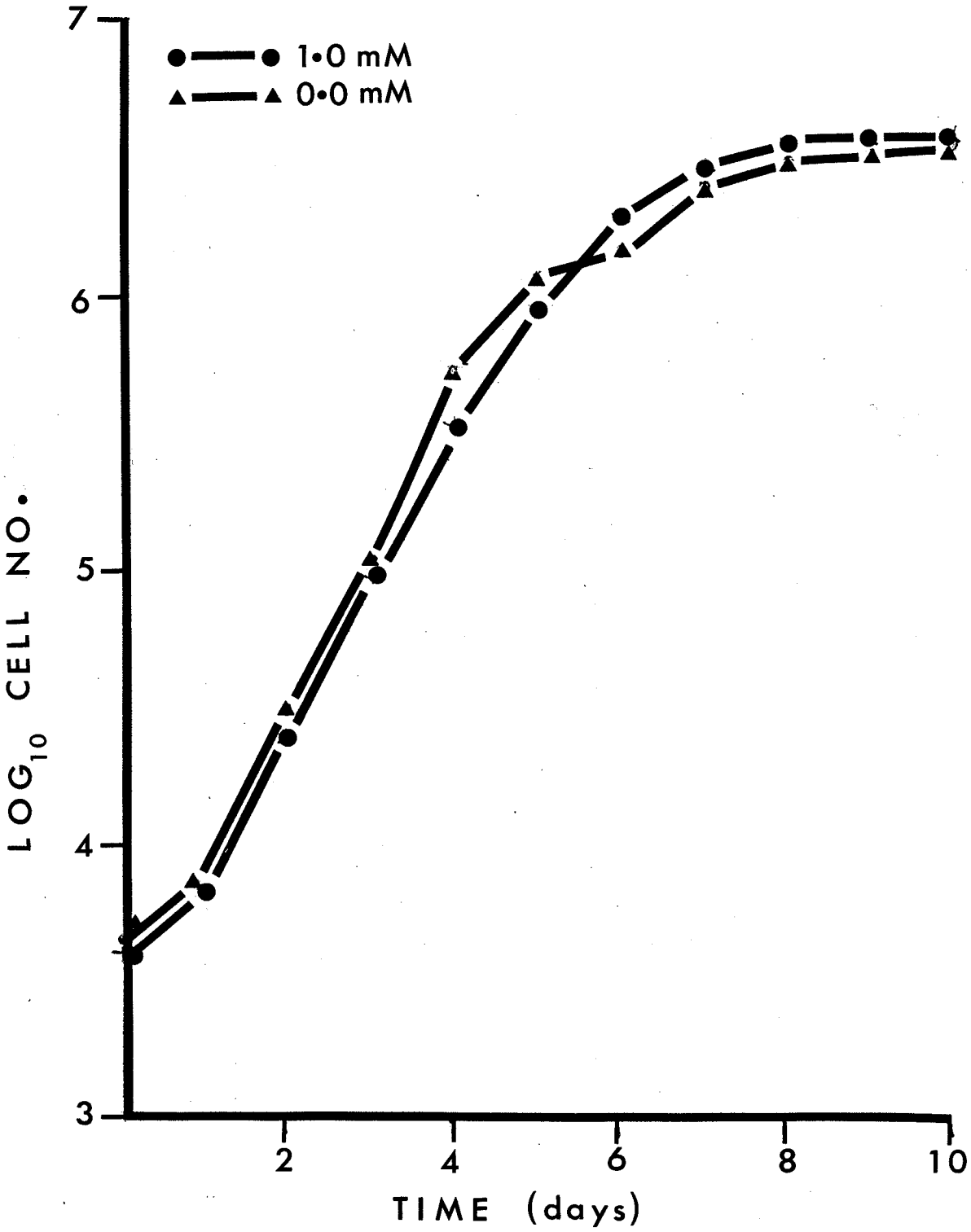

1·0 mM
0·0 mM
7
6
5
4
3
LOG10 CELL NO.
2
4
6
8
10
TIME (days)

FIGURE 1-3b: The effect of glucose on the growth of A. braunii in a 12 h light/12 h dark regime based on the log of cell volume (μm^3) against time. Lines represent different amounts of glucose added at time zero.

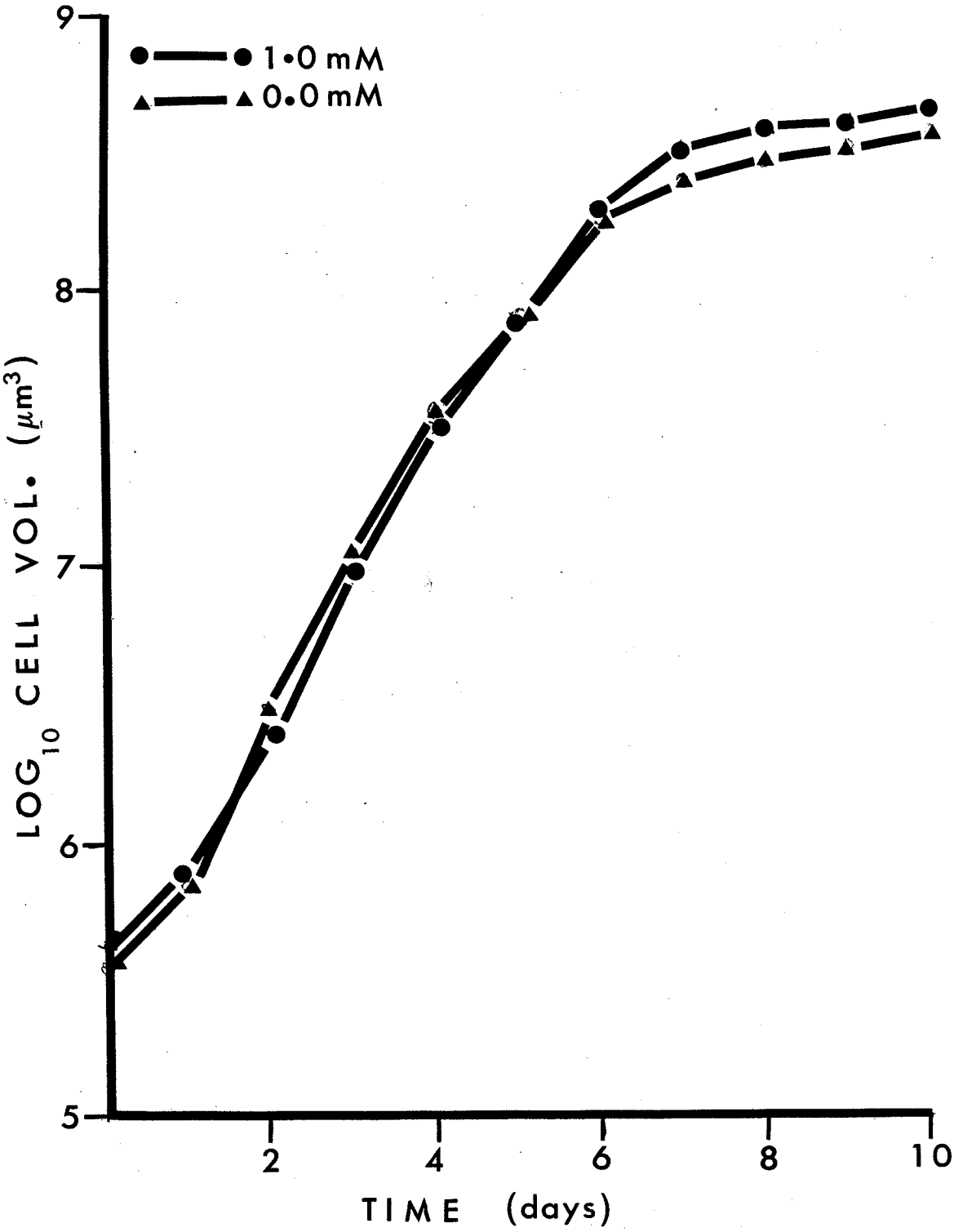
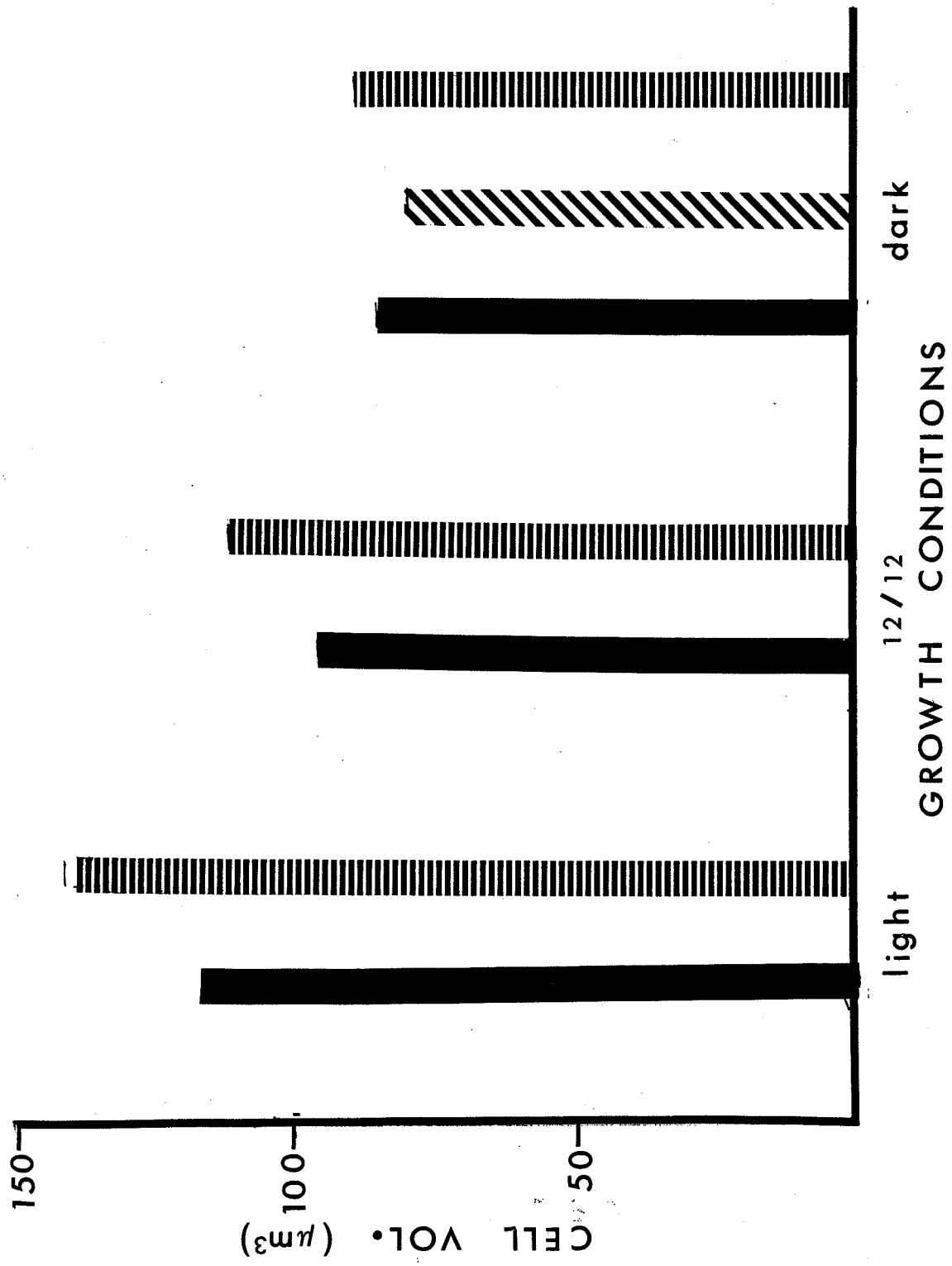

1•0 mM
0•0 mM
9
8
7
6
5
LOG10 CELL VOL• (μm3)
2
4
6
8
10
TIME (days)

FIGURE 1-4: The effect of glucose on the mean cell volume of A. braunii when grown under various light conditions.

- no glucose added 
- 0.1mM glucose added 
- 1.0mM glucose added 


CELL VOL. (μm³)
150
100
50
light
dark
12/12
GROWTH CONDITIONS

continuous light and the 12/12 light/dark regime had a 14% and 17% greater mean volume respectively in the presence of 1.0 mM glucose. Cells grown in the 12/12 regime with glucose had a 20% greater volume than those grown in the dark on the same glucose concentration, while those grown in continuous light had a 36.5% greater volume.

3. Uptake Kinetics:

The uptake of glucose over the entire range of concentrations showed an typical curve when the s/v values were plotted against (s) and when 1/v values were plotted against 1/s (See Appendix I, Figs. 5-1, 5-2). The curve instead showed the typical shape of one described for a two component uptake system (Reid et al. 1970). Figure 1-5 illustrates this response. When a line was calculated using successive approximations of two least squares equations (Appendix I, Table 5-3), the calculated values all fell within 10% of the observed velocity of uptake values. The uptake kinetics calculated from these equations for the two components revealed two markedly different systems. A high affinity system, which functions most significantly at the low substrate concentrations, had a K_t of 7.7 nM glucose and a V_{max} of 1.2 x 10^{-10} nmol glucose.cell^{-1}.min^{-1}. A low affinity system, which is more important at higher substrate concentrations, had a K_t of 16.39 μM and a V_{max} of 10.78 x 10^{-10} μmol.cell^{-1}min^{-1}

4. Efficiency of Conversion of Glucose to Cell Carbon:

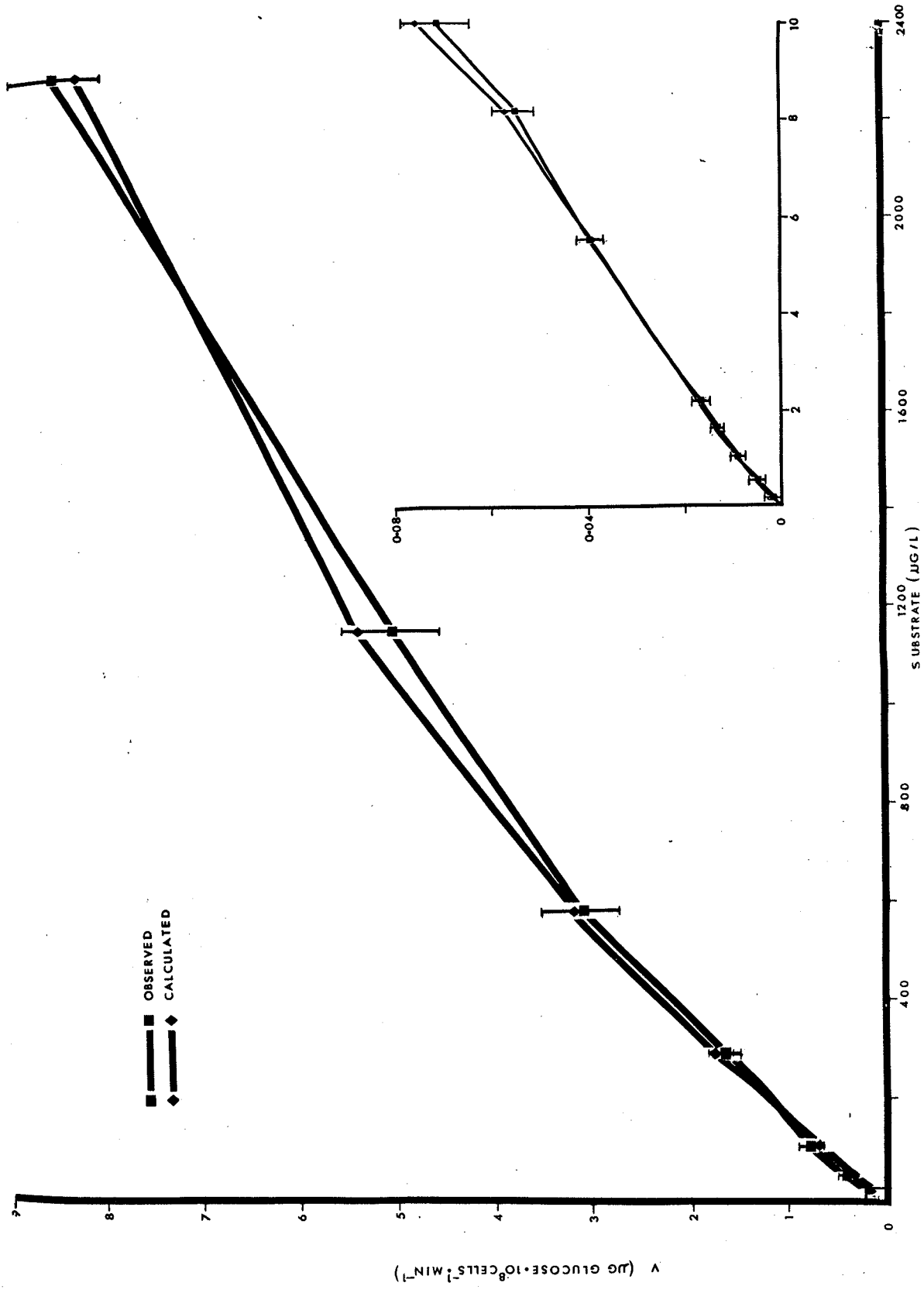
The maximum cell production in the dark growth experiments was 1.31 x 10^5 cells.mL^{-1} with 0.1 mM glucose and 1.46 x 10^6 cells.mL^{-1}

with 1.0 mM glucose. Assuming glucose was the limiting factor of growth in each case, then the amount of carbon used to produce these cells was 7.2 $\mu g.mL^{-1}$ and 72.0 $\mu g.mL^{-1}$ at 0.1 mM and 1.0 mM respectively. Therefore, the amount of carbon required to produce one cell in a culture containing 0.1 mM glucose is 54.94 pg. The amount required to produce one cell in a culture containing 1.0 mM glucose is 49.32 pg. The carbon content of one cell, as determined by the wet acid oxidation method was 28.0 pg. The efficiency of conversion of glucose in the 0.1 mM glucose culture was then calculated to be 50.91%, while the efficiency of conversion in the 1.0 mM solution was 56.77%.

5. Uptake Rates Required to Produce the Observed Doubling Times:

It is possible to calculate the theoretical rates of glucose uptake required to produce the observed doubling times found in the dark cultures grown with 0.1 mM and 1.0 mM glucose. To double the number of cells at 0.1 mM glucose would require 54.94 $pg.cell^{-1}$ carbon. To reach this value in 31.41 hours, the cells would have to take up carbon at a rate of 0.02916 $pg.cell^{-1}.min^{-1}$ or, $4.05 \times 10^{-10} \mu mol.cell^{-1}.min^{-1}$. To double the number of cells at 1.0 mM glucose would require 49.32 pg carbon $cell^{-1}$. To reach this value in 22.11 hours, the cells would be required to take up glucose carbon at a rate of 0.03718 $pg.cell^{-1}.min^{-1}$ or $5.16 \times 10^{-10} \mu mol.cell^{-1}.min^{-1}$. Table 1-3 summarizes these results, and compares them with the uptake rates determined through the uptake kinetics experiments. From this table, it is evident that the uptake kinetics could account for the required rate

FIGURE 1-5: The observed (with error bars) and calculated uptake velocities at increasing substrate levels. The lines were calculated using the method outlined in Appendix I. Insert represents uptake at glucose concentrations from 0.25-10.0 $\mu g.L^{-1}$.



velocity-
calcu-
Insert
25-10.0

TABLE 1-3

Comparison of the results of the growth experiments and the uptake kinetic experiments. All results are based on calculations using cell numbers. The results only include the dark growth experiments.

Glucose concentration	Doubling Times (h)	Carbon[1,2] Required/Cell	Percent[3] Efficiency	Uptake Rate[4] Reqd. cell/min	Calc (v)[5] From Kinetics
0.1 mM	31.41	54.94 pg	50.9	4.05×10^{-10} µmol	4.28×10^{-10} µmol
1.0 mM	22.11	49.32 pg	56.8	5.16×10^{-10} µmol	4.48×10^{-10} µmol

1. Determined by dividing total carbon utilized by total number of cells produced.
2. Assumes glucose is the limiting factor in all the cultures.
3. Determined by dividing the amount of carbon/cell by the previous column.
4. Determined by dividing doubling times (min.) into required carbon/cell.
5. Determined by uptake kinetics experiments. Values based on cell/min.

of uptake at 0.1 mM glucose but they are slightly too slow to account for the uptake required to produce the observed growth rate at 1.0 mM glucose.

DISCUSSION

Three questions concerning the heterotrophic capabilities of Ankistrodesmus braunii were asked. The first question concerned the ability of A. braunii to exist under a completely heterotrophic mode of nutrition. The results of the dark growth experiments clearly indicate that A. braunii can in fact exist as a facultative heterotroph. The doubling capacity of 22.11 hours in 1.0 mM glucose and 31.41 hours in 0.1 mM glucose are rapid enough to offer the alga ability to sustain itself in the absence of light sufficient for photosynthesis. The fact that the maximum yields increased by an order of magnitude with an order of magnitude increase in the glucose levels indicate that the growth curves are a reflection of a response to the glucose concentration. The assumption that glucose was the limiting factor is probably correct. The doubling times themselves are close to those reported for Nitzschia laevis by Lewin and Hellebust (1978). They found the N. laevis doubled every 24 hours on 1.2 mM glucose and 36 h on any glucose concentration between 0.0375 mM and 0.6 mM.

The utilization of glucose to enhance light growth appeared to be minimal (Tables 1-1, 1-2). Doubling times were not decreased nor maximum yields increased. The only effect glucose appeared to have on light grown cultures was to increase the volume of the cells. If cells excrete up to 70% of their fixed carbon (Fogg et al. 1965; Nalewajko and Schindler 1976), the uptake of glucose may replace some of this and allow a greater increase of algal biomass between divisions. The reduction in cell volume between light and dark growth (Table 1-2) suggests

that photosynthesis is not only sufficient for maximum cell division rates but also provides the alga with an increased biomass beyond its requirements for survival. This excess of organic material is not available during dark growth. The presence of a lag period in cell division of one day suggests the need to induce the transport system. The lack of this lag period when examining the cell volumes dispells this notion.

The efficiency of conversion of glucose to cell carbon of 50% at 0.1 mM and 56.8% at 1.0 mM glucose closely corresponds to the value of 48% found in N. laevis grown on 1.2 mM glucose (Lewin and Hellebust 1978). The lack of a 100% efficiency could be due to an energy expenditure needed to drive the uptake system and other metabolic processes such as biosynthesis and cell division. It may also result from subsequent excretion of glucose which was absorbed (Komor et al. 1973).

The question as to whether A. braunii possesses an active uptake system capable of accounting for the observed growth is answered affirmatively. Table 1-3 indicates that the uptake rates calculated through kinetic studies could definitely account for the growth at 0.1mM glucose. At the 1.0 mM glucose concentration, the uptake rate calculated from the kinetics appears slightly low. In fact, both values of estimated glucose carbon required per cell may be too high. The cell carbon values were determined using light grown cells. As seen in Table 1-2, these cells have anywhere from 20-36% greater volume than dark grown cells. If there is a positive correlation between cell volume and cell weight, the amount of carbon estimated to be required per cell will be too high. The value of 28.0 pg glucose carbon per cell calculated by wet-acid oxidation may also be too high. Mayfield and Inniss (1978) determined the dry weight

of another clone of A. braunii to be 12.3 pg in cells with a mean volume of 64 μM^3.

The presence of a biphasic uptake system for organic molecules has been speculated for some time. Akedo and Christensen (1962) postulated this mechanism for the transfer of amino acids across the intestinal membrane of rats. Reid et al. (1970), Hoban and Lyric (1976) and Hoban (1977) found biphasic systems for transporting amino acids, and glucose across bacterial cell membranes. Liu and Hellebust (1974) reported the existence of two carrier systems for proline uptake in the diatom Cyclotella cryptica. Komor and Tanner (1975) found high and low affinity uptake systems for hexose sugars in the green alga Chlorella vulgaris. The relative contribution of the system to the overall uptake capacities is dependent on the substrate concentrations available (Reid et al. 1970). At extremely low substrate levels the high affinity system would dominate, while at higher substrate levels the low affinity system would dominate. The kinetic parameters of the low affinity system found in A. braunii of 16.39 μM (K_t) and 10.78 x 10^{-10} $\mu mol.cell^{-1}min.^{-1}$ (V_{max}) correspond well with the literature values for algal uptake systems (Hellebust and Lewin 1977, Sepers 1977). The high affinity system kinetic values correspond more closely to those values reported to be for bacteria in natural populations (Sepers 1977). The high affinity system, which is determined by the velocities at the low substrate concentrations is subject to the greatest experimental error and is therefore perhaps an under estimate of the actual V_{max} attained by this system in A. braunii. More work could be done at these concentrations to ensure that the results are accurate.

The question as to the competitiveness of the uptake system in A.

braunii is far more speculative. Comparisons must be made with reported literature results of uptake by natural populations. These results seldom lead to direct comparisons because: the bacterial biomass was either not determined; or was reported in such a form as to exclude comparisons; or the methodology was very different.

The majority of the researchers have concluded that algae cannot compete successfully with bacteria for organic substrates in the natural environment (Hobbie and Wright 1965a, 1965b; Wright and Hobbie 1966; Munroe and Brock 1968; Allen 1969). In a review of some of the reported literature values for bacterial and algal uptake, Sepers (1977) concludes that bacteria are far more efficient. Only a few workers have suggested that algae can compete successfully with bacteria (Saks and Kahn 1979; Kuzmenko 1980; Vincent and Goldman 1980). Mayfield and Inniss (1978) found A. braunii could compete successfully with Flavobacterium but not Pseudomonas.

For A. braunii to compete successfully with natural populations of bacteria, it must have an uptake system as efficient as that of the bacteria at glucose levels of the same order of magnitude as are found in natural bodies of water. The levels of glucose in natural waters, both marine and limnetic have been determined at between 4.4 nM and 1.1 uM (Hobbie and Wright 1965a; Hobbie 1967; Vaccaro 1967; Vaccaro et al. 1968; Allen 1969; Cavari and Hadas 1979; and Seki et al. 1980). They are certain to be even higher in polluted or effluent rich waters. As demonstrated above, A. braunii is capable of transporting glucose through this entire range with the high affinity system effective at the low concentrations and the low affinity system effective at higher glucose levels.

To make a meaningful comparison between A. braunii transport and that of isolated bacteria or natural bacterial populations, the kinetics of the transport systems must be compared. Both the specific transport constant (K_t) and the velocity of transport (V_{max}) must be examined. A. braunii must not only be capable of transporting glucose at a rate more rapid than bacteria, due to its larger volume, but it must also be able to conduct this transport at similar glucose concentrations to the bacteria. The V_{max} value allows a comparison of the former while the K_t value provides an indication of the latter.

While the K_t value for the low affinity system is too high to suggest an efficient uptake at the more prevalent low natural substrate concentration, the K_t value of the high affinity system (7.7 nM) is well within the range of the more efficient natural populations (Table 1-4). Any increase in natural glucose concentrations would be transported by the low affinity system thus increasing the overall transport velocity.

It is more difficult to compare the V_{max} values. The V_{max} values reported in Table 1-4 are for all the organisms in a liter of water. The number of bacteria in natural waters in highly variable. Collins (1960) found bacterial concentrations of 2.4×10^6 cells. L^{-1} in Lake Windermere and Esthwaite Water. Koning and Dorgelo (1982) found seasonal ranges from 0.3 to 43.7 $\times 10^8$ cells.L^{-1} in euphotic waters of Lake Maarsseveen I. Spring populations in Lake Mossø were from 11.0 to 18.0 $\times 10^9$ cells.L^{-1} (Riemann et al. 1982). Ellis and Stanford (1982) reported bacterial concentrations of 1.3×10^8 cells. L^{-1} in the photic zone of the oligotrophic Flathead Lake and 9.5 x

10^{-8}cells.L^{-1} in the photic zone of the eutrophic Lake Texomo.

If it is assumed that all bacteria were counted, which is unlikely because of the errors of the various counting methods, then a conservative estimate of a lake population would be 10^8 cells.L^{-1}. From Table 1-4 the V_{max} values ranged from 0.00035 - 2.23 nmol.L^{-1}.min^{-1}. Using the estimate of 10^8 bacterial cells per liter, the V_{max} values would then range from 4 x 10^{-9} pmol.$cell^{-1}$.min^{-1} to 2.2 x 10^{-5} pmol.$cell^{-1}$.min^{-1}. The V_{max} for the high affinity system of A. braunii is 1.2 x 10^{-7} pmol. $cell^{-1}$.min^{-1} and is in the center of the bacterial range, but the K_t is at the low end of the range of values reported in Table 1-4. These direct comparisons are difficult, since the temperature glucose concentrations and other environmental conditions are quite variable. For example, the high value of 2.2 x 10^{-5} pmol.$cell^{-1}$.min^{-1} was determined using high glucose concentrations (K_t = 278.0 nM) where the low affinity system of A. braunii would be operative and adding to the V_{max} value

Two studies, where more direct comparisons can be made, were done by Dietz et al. (1976) and Seki et al. (1980). Dietz et al. (1976), using a glucose concentration range of 1.0 to 100 nM found, in a freshwater pool at 19.5°C, a V_{max} of 3.0 x 10^{-9} pmol.$cell^{-1}$.min^{-1} in a natural bacterial population. The K_t of this transport was 77.0 nM. Seki et al. (1980) using a glucose concentration range of 0-555.6 nmol, reported a V_{max} of 1.0 to 3.0 X 10^{-9} pmol.$cell^{-1}$.min^{-1} in the 12°C waters of Patricia Bay. The K_t of this population was 127 nM. A comparison with these results suggests that A. braunii is a more efficient heterotroph

TABLE 1-4

Uptake of glucose by natural populations. K_t values are in nM, V_{max} values are in nmol.liter^{-1} min^{-1}.

Location	K_t	V_{max}	Reference
River in Fraser Valley	1.1 - 94.4	0.00035 - 0.0648	Albright and * Wentworth
Estuary	11.8 - 278.0	0.0138 - 2.23	Crawford et al.*
Pacific Ocean	122 - 466	0.00036 - 0.0022	Seki et al.*
Phillipine Sea	350	0.001	Seki et al.*
Tokyo Bay	122 - 372	0.2 - 1.665	Seki et al.*
Booth Bay Maine	72	0.041	Wright & Shaw*
Kiel Fjord	9.2 - 34.2	0.053 - 0.1966	Gocke*
German Streams	376.6 (K_t + Sn)	0.0037 - 1.481	Marxsen (1980)
Pacific Ocean	13.8 - 105.5 (K_t + Sn)	0.00239 - 0.0037	Dietz et al. (1976)
Simon Fraser Pool	41.5 - 77.7 (K_t + Sn)	0.0015 - 0.0063	Dietz et al. (1976)
Lake 227	6.66	1.34	Nalewajko and Schindler (1976)

* Values reported by Sepers (1977)

than either of these natural populations.

Moaledj and Overbeck (1982) isolated four strains of bacteria from the PluBsee. The heterotrophic potential of two pseudomonads and 2 Actinomycetes was examined at 15°C and a glucose concentration range of 333.3 nM to 2666.6 nM. The K_t values for the bacteria ranged from 227.0 to 1211.0 nM and the V_{max} values ranged from 2.6×10^{-8} to 2.04×10^{-7} pmol.cell^{-1}L^{-1} A. braunii could apparently compete successfully with these organisms for natural glucose.

There are several other points that should be considered when speculating on the heterotrophic potential of A. braunii. This report has attempted to illustrate the heterotrohpic capabilities of the alga based on glucose levels alone. The uptake systems for glucose may not be specific and in fact may transport any hexose sugar or other organic substrates. The alga may also have other uptake systems. Cohen and Monod (1957) suggest evidence for 30 to 50 different permeases in Escherichia coli. Algae have also been shown to be able to live on a variety of different organic substrates (Droop and McGill 1966; Sloan and Strickland 1966; and Hellebust 1970,1971). If other uptake systems are present, and since total disolved organic compounds can range from 12.9 to 15.5 mgC.L^{-1} (Storch and Saunders 1978), then the likelihood of A. braunii being able to survive heterotrophically seems quite high.

SUMMARY

1. Ankistrodesmus braunii is capable of surviving heterotrophically in the dark.

2. The doubling times are 1.4 and 2 times slower than phototoautotrophic growth in continuous light in the presence of 1.0 mM and 0.1 mM glucose respectively.

3. The organic substrate d-glucose is actively transported by a biphasic, low and high affinity transport system.

4. The high affinity system had a K_t of 7.7 nM and a V_{max} of 1.2 $x10^{-7}$ pmol.cell^{-1}.min^{-1}. The low affinity system had a K_t of 16.39 μM and a V_{max} of 10.78 x 10^{-4}pmol.cell^{-1}.min^{-1}.

5. The uptake rates would more than account for the observed heterotrophic growth of A. braunii.

6. A. braunii could apparently compete with bacteria for naturally occurring glucose.

CHAPTER TWO

POSSIBLE SIGNIFICANCE OF HETEROTROPHY IN THE METABOLISM OF *Ankistrodesmus braunii* (Naeg.)

INTRODUCTION

The green alga Ankistrodesmus braunii (Naeg.) is capable of surviving in the dark with the organic substrate d-glucose as a sole carbon and energy source. From the results presented in Chapter One, Fig.1-1 and Table 1-1,it is evident that this heterotrophic growth, while not as efficient as photoautotrophic, can still provide a reasonable alternative for the survival of the organism. Although this has been clearly illustrated and is known in other algae (Lewin 1974, Lewin and Hellebust 1978), the exact role of heterotrophy in the natural existence of the alga is still not evident.

From Fig. 1-2 and Table 1-1 it is apparent that heterotrophy does not act in an additive or synergistic fashion with photosynthesis in continuous light. What is not evident is whether glucose transport occurs at all while the cells are photosynthesizing. If uptake can occur during photosynthetic periods, there is a question as to whether it contributes significantly to total growth or cell production at this time. While growth rate is only minimally enhanced by glucose in continuous light, it does lead to a greater total yield of cells, both in number and cell volume (Table 1-1).

The fate of the assimilated glucose in the light or dark has not yet been explained. It was shown in Chapter One that the rate of heterotrophic growth could only account for the utilization of approximately 50% of the glucose transported. The other 50% must either be respired or excreted. Both the respiration of assimilated organic compounds (Hobbie and Crawford 1969) and the excretion of these compounds or their metabolic

products (Komor et al. 1973; Nalewajko and Schindler 1976) have been demonstrated in other algae.

Heterotrophy may be reduced or absent in the light, since transport, if active, would by definition, require expenditure of cell energy. If photosynthesis was adequate to meet the specific growth constant under the prevailing environmental conditions, it would be wasteful to expend this energy when the organic substrates were not required. Instead, it would be more efficient if the cell had some mechanism for insuring maximum transport at times when it was required, and reducing or stopping transport when it was not necessary. The uptake capacity may, on the other hand, be coupled with the energy availability of the cell. Cells with extremely low energy reserves may reduce transport and direct their energy expenditures to other activities such as biosynthesis or cell division.

There are several conditions where heterotrophy would be an asset to the alga. The most obvious is under conditions where light is insufficient to sustain photosynthesis. These conditions would exist at night, under winter ice with a heavy snow cover, in extreme Northern latitudes in the winter, under prolonged heavy cloud cover, deep in the photic zone or aphotic zone and in extremely turbid waters. In situations where CO_2 is limiting, photosynthesis may be reduced, even in adequate light (Wright 1964; Schindler and Fee 1973; Cohen et al. 1981). When cells are dividing and reconstituting their photosynthetic apparatus, heterotrophy could supply additional energy and organic carbon to supplement the photosynthetic process when energy demands are high.

A series of experiments was conducted to investigate the involvement

of heterotrophy in the complete metabolism of A. braunii. The first experiments involved the determination of photosynthetic rates and subsequent excretion of fixed carbon. This was done to determine how closely the photosynthetic capacity correlated with the known growth capacity. The second experiments involved determination of glucose uptake rate and respiration over a three hour period in an attempt to account for at least a portion of the 50% loss of transported glucose (Chapter One). The third group of experiments involved determination of transport rates under combinations of four environmental conditions. These conditions included light availability during preconditioning of the cells, glucose availability during preconditioning of the cells, CO_2 availability and light and dark uptake. These experiments were conducted to determine what environmental conditions promote uptake. The final series of experiments involved the use of metabolic inhibitors with the hopes of determining the energetics of the transport system. By combining the results of these experiments, an attempt was made to describe at least a portion of the significance that heterotrophy had on the survival of A. braunii.

MATERIALS AND METHODS

1. Source and Maintenance of Algal Cultures:

Ankistrodesmus braunii (Naeg.) cells were obtained and maintained in the same manner as outlined in Chapter One. The only variations from this method occurred when experiments were conducted requiring a change in the prehistory of the cells. All cells were harvested in the exponential growth phase, washed four times in sterile, organic-free medium and resuspended in fresh sterile medium at a final concentration of 2×10^7 cells/L or 1×10^8 cells/L. All results not expressed on a per cell basis are reported on a 1×10^8 cells/L basis. The results of experiments conducted at 2×10^7 cells/L were extrapolated 1×10^8 cells/L. From the results of an experiment on the effects of cell numbers on glucose transport (Appendix V) it was believed that this extrapolation would not influence the results.

2. Determination of Uptake Rates:

All uptake experiments, unless otherwise stated, were conducted in the following manner. For each different treatment, 15 mL samples of algal suspension were dispensed into culture tubes. Transport was allowed for one minute. One of the tubes had cells that were previously heat killed for 5 minutes at 80°C (Hellebust and Lin 1978). After one minute, the cell suspension was filtered onto 0.45 µm cellulose acetate membrane filters and washed with 30 mL of prefiltered medium containing 2.0 µM unlabelled glucose. The filters were placed into Bray's scintillation cocktail (Bray 1960) and counted after 24 hours in the dark to eliminate

any errors due to fluoresence caused by exposure of the fluor to light. All uptake rates were calculated by the same method as outlined in Chapter One. Uptake was conducted at 22°C and 500 lux light intensity unless otherwise stated. The heat killed cells were used as controls and the values obtained from these cells substracted from the average results of the other three tubes.

3. Determination of Photosynthetic Rates:

All cells used in these experiments were taken from cultures during the exponential growth phase (grown under continous light at 5000 lux intensity). All experiments were also conducted in a mixture of cool white and Sylvania Gro-Lux bulbs at an intensity of 5000 lux. The temperature was 22°C. For each experiment, 15 culture tubes were supplied with 15 mL of algal suspension. Labelled carbon (0.2μCi/mL) was added in the form of $NaHC^{14}O_3$ (New England Nuclear - specific activity of a 1 μCi/μg). At time intervals of 1, 15, 30, 60 and 180 minutes, three of the cultures were filtered onto 0.45 um membrane filters. The filters were washed with 20 mL of sterile medium, acidified over concentrated HC1 and added to Bray's scintillation fluor. The amount of inorganic carbon available for photosynthesis was determined by the method of Strickland and Parsons (1968). The amount of fixed carbon was determined using the method outlined in APHA Standard Methods (1965). All assimilation values were expressed as pg carbon.cell^{-1}. min^{-1}. Doubling times were calculated using a value of 28 pg carbon/cell.

4. Excretion of Fixed Carbon:

The experiments to determine the amount of organic carbon excreted following photosynthesis were done concurrently with the assimilation experiments. The filtrates were acidified to pH of 2-3 using 0.1 N HCl. Each was then bubbled with air for 20 minutes to drive off any labelled inorganic carbon. Three, 0.5 mL subsamples were removed from each filtrate and added to vials of fluor. The samples were counted and the values converted to excreted carbon .cell^{-1}.min^{-1}.

5. Respiration of Transported U-C^{14} glucose:

These experiments were conducted concurrently with the photo- synthesis and excretion experiments. The cells used for these experiments were taken from the same cultures as the above experiments. Uptake of U-C^{14} glucose and respiration of the assimilated glucose were also measured at 1, 15, 30, 60 and 180 minutes. The experiments were conducted under bench lighting of 500 lux or in the dark. Forty-five, 10 mL samples of cell suspension were dispensed into vials fitted with serum caps. A syringe needle had been inserted through each cap from below. U-C^{14} glucose (specific activity 291.6 μCi/μM) was added at a final concentration of 1 μg glucose/L to 15 vials and 10 μg glucose/L to another 15 vials. U-C^{14} glucose (specific activity 4.5 μCi/μM) was added to the remaining 15 vials at a final concentration of 100 μg glucose/L. After the appropriate time interval(1, 15, 30, 60 or 180 minutes), sufficient 0.1 N HCl was injected through the syringe into the suspension to reduce the pH to 1-3. This was done to stop transport (Chapter Three) and to drive off any inorganic $C^{14}O_2$ that would

have been produced from respiration. The syringe projecting into the cell suspension was hooked up to air line and air was gently bubbled through the suspension to displace any labelled CO_2. The inverted syringe needle was connected to two successive vials of 5 mL of phenethylamine. It was assumed that any $C^{14}O_2$ displaced by acidifying and bubbling would be trapped in the phenethylamine. The second vial was to catch any $C^{14}O_2$ the first trap missed. After bubbling for 15 minutes two, 0.5 mL subsamples of each of the phenethylamine traps were removed and added to Bray's fluor and counted using the liquid scintillation counter. All counts were corrected for colour quenching of phenethylamine. The counts were converted to μg carbon.10^8 cells^{-1}. min^{-1}. The contents of the acidified suspension were filtered as in the previous uptake experiments, and counted to determine the amount of U-C^{14} glucose assimilated by the cells. These results were also expressed as μg carbon.10^8 cells^{-1} . min^{-1}.

6. Effect of Removal of CO_2 on Cell Growth and Glucose Uptake

a) Growth of Ankistrodesmus braunii

Growth experiments were conducted using the same methods as in Chapter One. The light intensity was 5000 lux and temperature was 22°C. Two major modifications were applied to remove CO_2 from the growth medium of the algae. The aeration system was modified by placing three chromatography columns, packed with NaOH pellets, in series between the $CuSO_4$ flask and the second distilled water flask. This was done to remove any CO_2 from the air entering the flasks. The second modification was the omission of $NaHCO_3$ from the culture medium. A third modification was the addition of Tris phosphate buffer, pH 7, to the medium. The pH of the culture was determined before and after the growth experiments and no flask exhibited a pH change of over 0.5. Six

replicate experiments were done for each of two external glucose concentrations (0.1 mM and 1.0 mM glucose).

b)Transport of U-C^{14} glucose:

The effects of removal of CO_2 on glucose transport were measured using three different light conditions. These included a light intensity of 5000 lux, 500 lux and no light. The experimental cultures were placed in these light conditions and a CO_2-free aeration system 5 hours prior to the time of experimentation. The medium was buffered with Tris phosphate buffer to pH 7.0, contained no added $NaHCO_3$ and had an algal concentration of 2 x 10^7 cells/L. At the time of experimentation, sufficient U-C^{14} glucose (specific activity 4.5 μCi/μM) was added to the cultures to bring the final concentration to 10 or 100 μg glucose/L. After a 1 minute incubation period, 15 mL samples were removed, filtered and counted as in the previous uptake experiments.

7. Effect of Different Preconditioning Conditions on Uptake:

A series of experiments was conducted to determine the effect of light and glucose preconditioning on transport of external U-C^{14} glucose. Light preconditioned cells were grown in continuous light (5000 lux) until the time of experimentation. Dark preconditioned cells were subjected to a 24 h dark period immediately prior to experimentation. In both light and in dark preconditioning, the cells were either provided with no glucose or 0.01 mM glucose. Uptake was measured in both the light and in dark for all the above combinations. The following list represents the series of test conditions:

a. light plus glucose preconditioning - light uptake
b. light plus glucose preconditioning - dark uptake
c. light minus glucose preconditioning - light uptake
d. light minus glucose preconditioning - dark uptake

e. dark plus glucose preconditioning - light uptake
f. dark plus glucose preconditioning- dark uptake
g. dark minus glucose preconditioning - light uptake
h. dark minus glucose preconditioning - dark uptake

Concentrations of U-C^{14} glucose were 10 µg glucose/L (specific activity 291.6 µCi/µM) and 100 µg glucose/L (specific activity 4.5 µCi/µM. The transport rates were determined as outlined above. The results are discussed as they pertain to the total cell energetics. Some of the results are again discussed in Chapter Three, as they pertain to the mechanism of uptake.

8. Effect of Metabolic Inhibitors on Transport:

Cells used in these experiments were subjected to the same range of preconditioning as the cells in the previous section. All inhibitors were applied 15 minutes prior to the actual uptake experiments. Those inhibitors that were only slightly soluble or insoluble in water were first dissolved in ethanol. An experiment was conducted to determine the effect of applied ethanol concentrations on the glucose transport (Appendix IV). The results showed that ethanol, at the levels used, had no effect on transport.

The inhibitors used have all been demonstrated to affect some area of cell energy metabolism. Appendix III lists all the inhibitors used throughout the entire thesis, as well as giving their abbreviations, effective concentrations, and sites of inhibition. The specific inhibitors employed in this chapter were Amytal (10^{-3} and 10^{-4}M); CCCP (10^{-5} and 10^{-6}M); DCMU (10^{-5} and 10^{-6}M); DNP (10^{-4} and 10^{-5}M); Imidazole (10^{-3} and 10^{-4}M); KCN (10^{-4} and 10^{-5}M); Quercitin

(10^{-4} and 10^{-5} M); and Salicylaldoxime (10^{-2} and 10^{-3} M). All concentrations applied were of the order of magnitude reported in the literature required to cause inhibition (Appendix IV). As well, a second application, one order of magnitude more concentrated was used. All uptake experiments followed the method and conditions outlined above. The concentrations and specific activities of the U-C^{14} glucose were the same as in the previous section. The results were discussed as they pertain to the energy involvement associated with glucose transport. Some of the results are discussed again in Chapter Three, as they pertain to the specific mechanism of glucose uptake.

RESULTS

1. Photosynthesis and Excretion:

The photosynthetic accumulation of carbon and the subsequent excretion of fixed carbon is illustrated in Figures 2-1 and 2-2 and in Table 2-1. These results suggest that photosynthetic rates are at first quite rapid, but by one hour have slowed to a constant rate of approximately 0.1 pg carbon.cell^{-1}.min^{-1}. This stable rate appears to be about 50% of the initial rate. If these values are converted to rates of carbon assimilation and a value of 28 pg carbon/cell is the average cell weight (Chapter One), then the theoretical doubling time of the cells at the stable photosynthetic rate would be about 4.75 hours. The initial doubling time would be 2.23 hours.

The rate of excretion of fixed carbon appears to follow the same pattern as the photosynthetic rates. There appears to be a greater proportion of the fixed carbon excreted during the first 15 min. than at 30, 60 and 180 min. Where photosynthetic rates are stabilized after 60 min., the excretion rates are stabilized after 30 min. The stable rate of excretion is approximately 3.5% of the total fixed carbon.

2. Respiration of Transported U-C^{14} Glucose:

The results of these experiments are illustrated in Figures 2-3, 2-4, and 2-5 and Table 2-2. The rate of glucose uptake at all external glucose concentration decreased sharply with time during the first 30 min., but stabilized at approximately 25% (Table 2-2) of the rate of uptake at 1 min. Respiration of the assimilated glucose followed a different pattern. At 1 min. there was no measurable respiration of

FIGURE 2-1: Photosynthetic rates of A. braunii during a 3 hour experiment. Experimental and preconditioning light intensity was 5000 lux.

FIGURE 2-2: Photosynthetic rates corresponding to Fig. 2-1 and converted to potential cell doubling times during a 3 hour experiment.

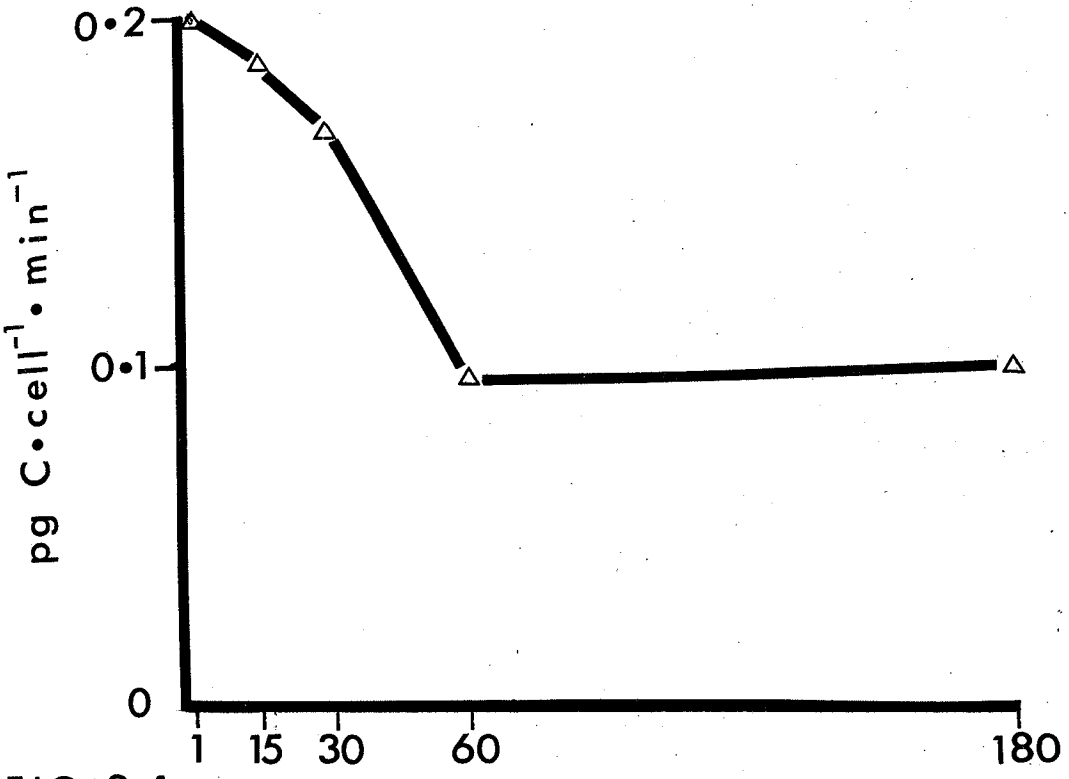

0•2
0•1
0
pg C•cell^{-1}• min^{-1}
1
15
30
60
180

FIG:2-1

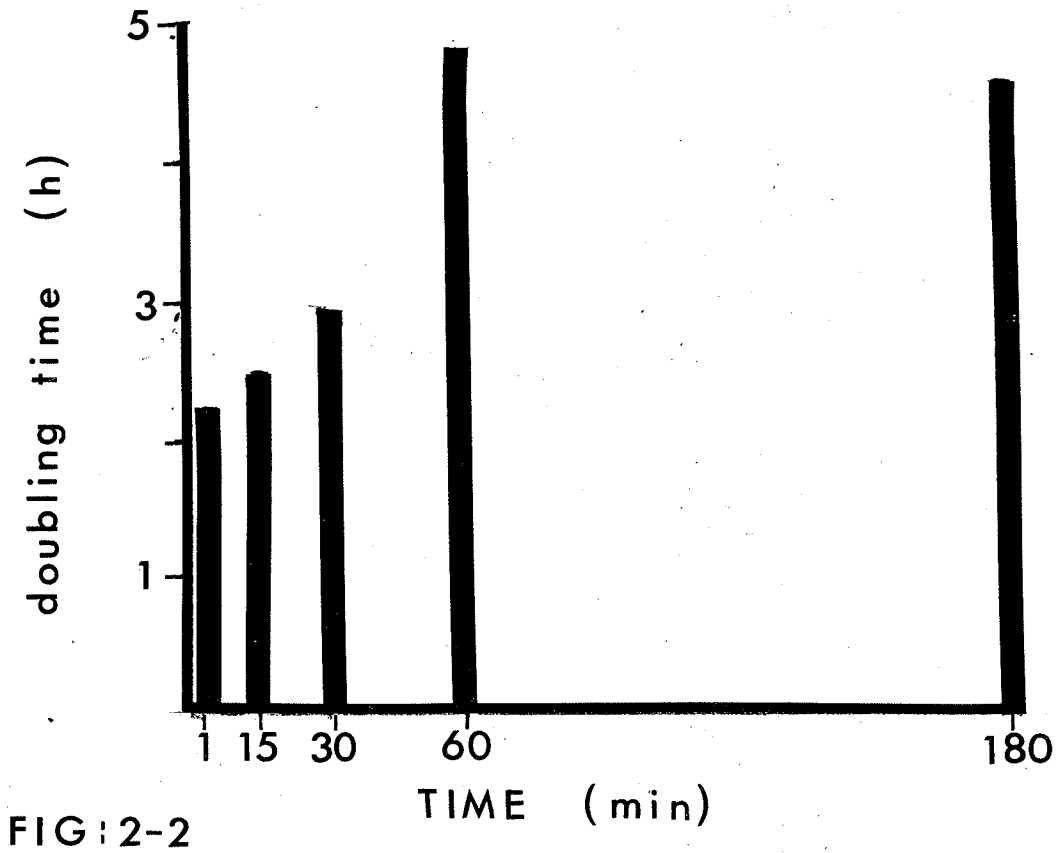

5
3
1
doubling time (h)
1
15
30
60
180
TIME (min)

FIG:2-2

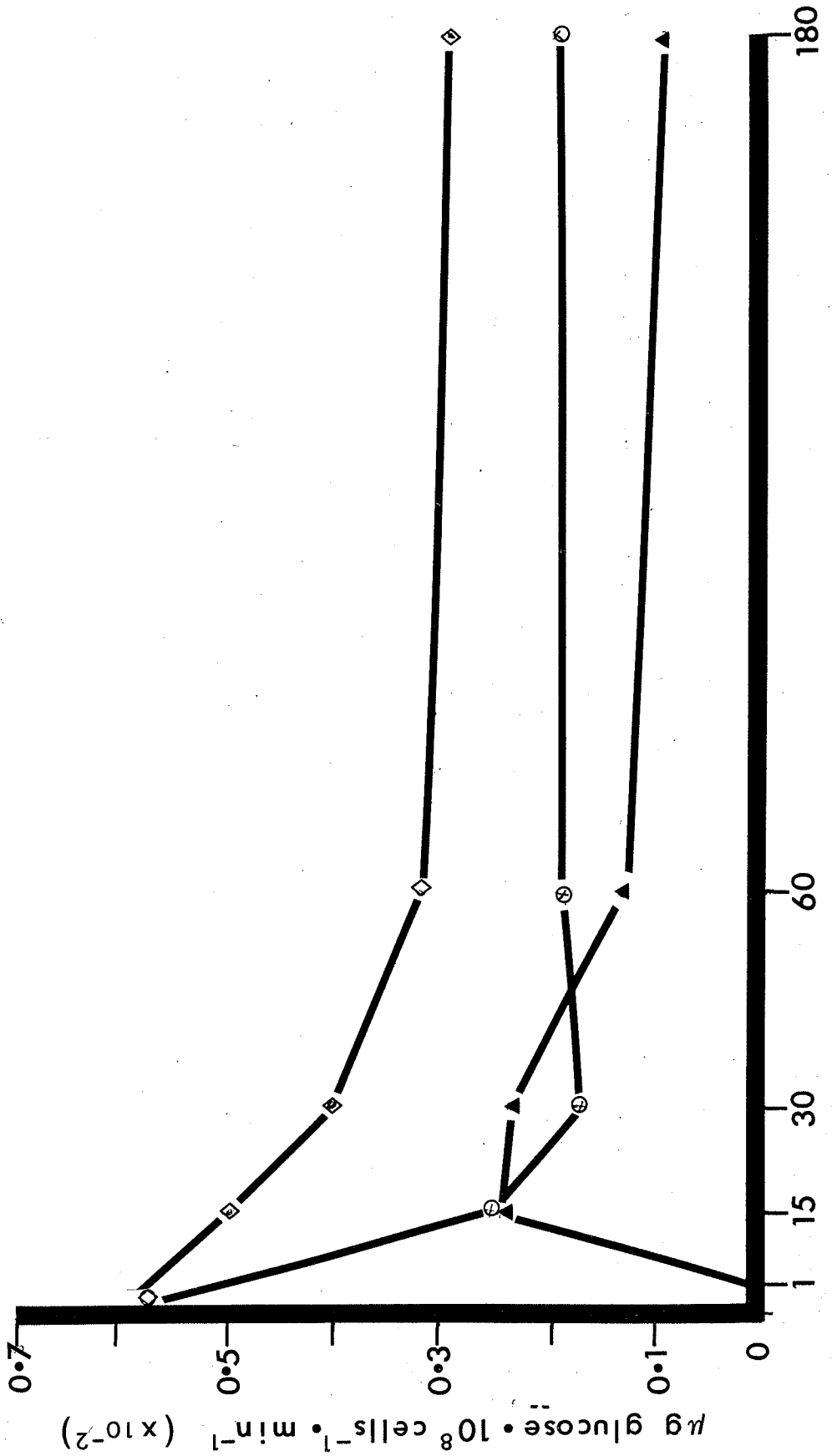
TABLE 2-1

Photosynthetic rates, excretion rates and doubling times of A. braunii. Results are expressed in pg carbon.cell^{-1} min^{-1}.

Time	Photosynthetic Rate	Excretion Rate	Total Fixed Carbon	Percent Excretion	Doubling Times (hr)
1 min.	0.21 ± 11.9%	0.047	0.257	18.42	2.23
15 min.	0.19 ± 13.9%	0.027	0.216	12.67	2.48
30 min.	0.17 ± 6.7%	0.006	0.175	3.69	2.77
60 min.	0.10 ± 4.3%	0.004	0.101	3.84	4.83
180 min.	0.10 ± 4.4%	0.004	0.107	3.43	4.53

FIGURE 2-3: Uptake and respiration of U-C^{14} Glucose during a three hour experiment. Total uptake line is a combination of net uptake and respiration rates. External glucose concentration applied time 0 was 1.0 µg U-C^{14} glucose/L.

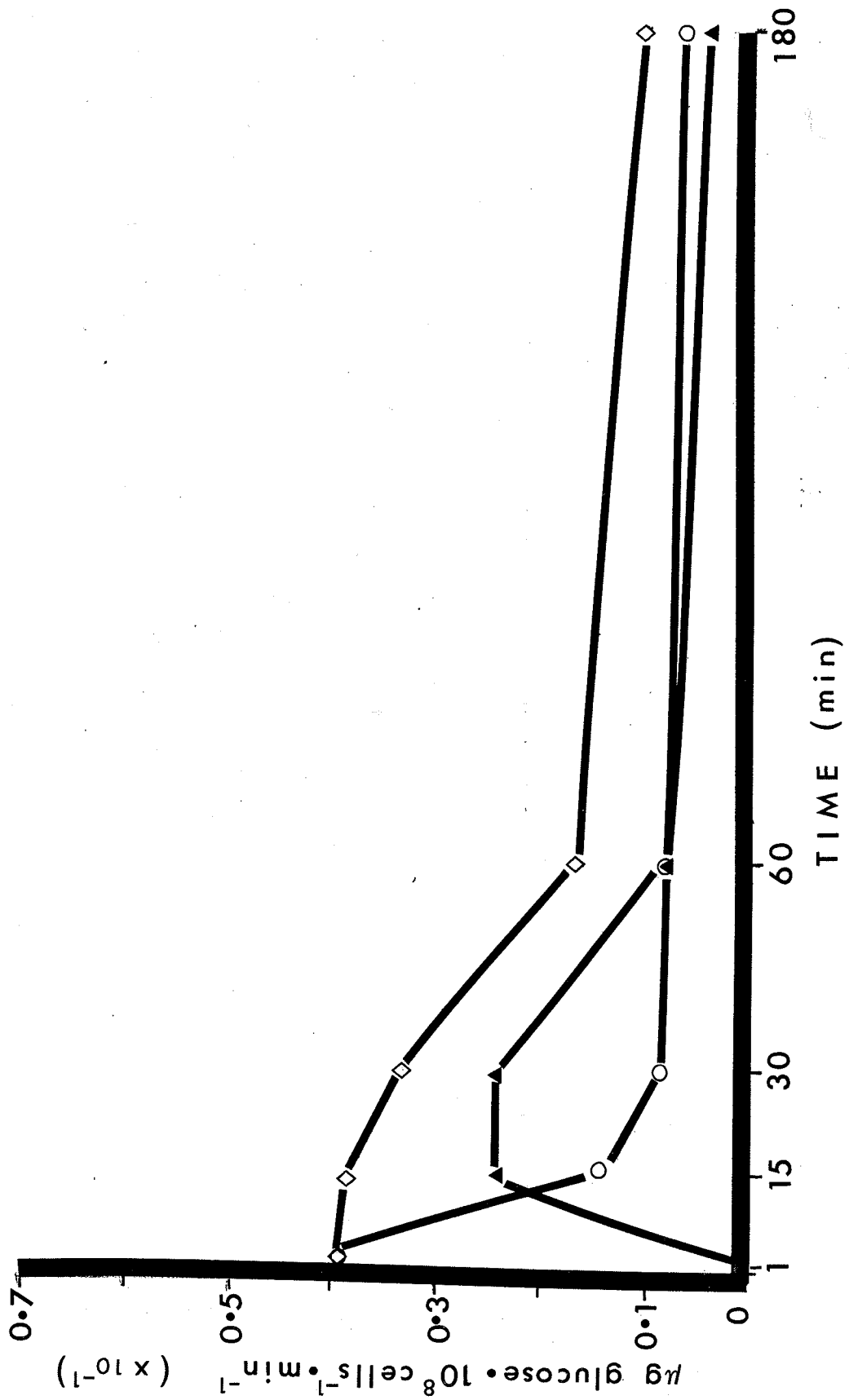
- assimilation and respiration ◇—◇
- assimilation ○—○
- respiration ▲—▲



three
of net
entra-

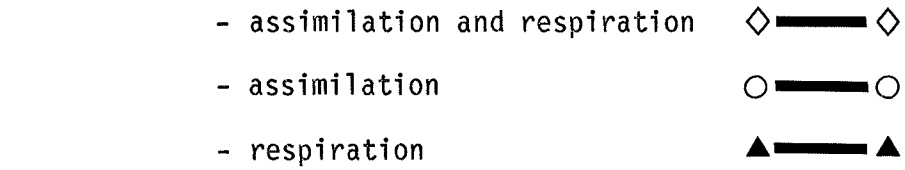
FIGURE 2-4: Uptake and respiration of glucose. External glucose concentration applied at time 0 was 10.0 µg U-C^{14} glucose.L^{-1}.

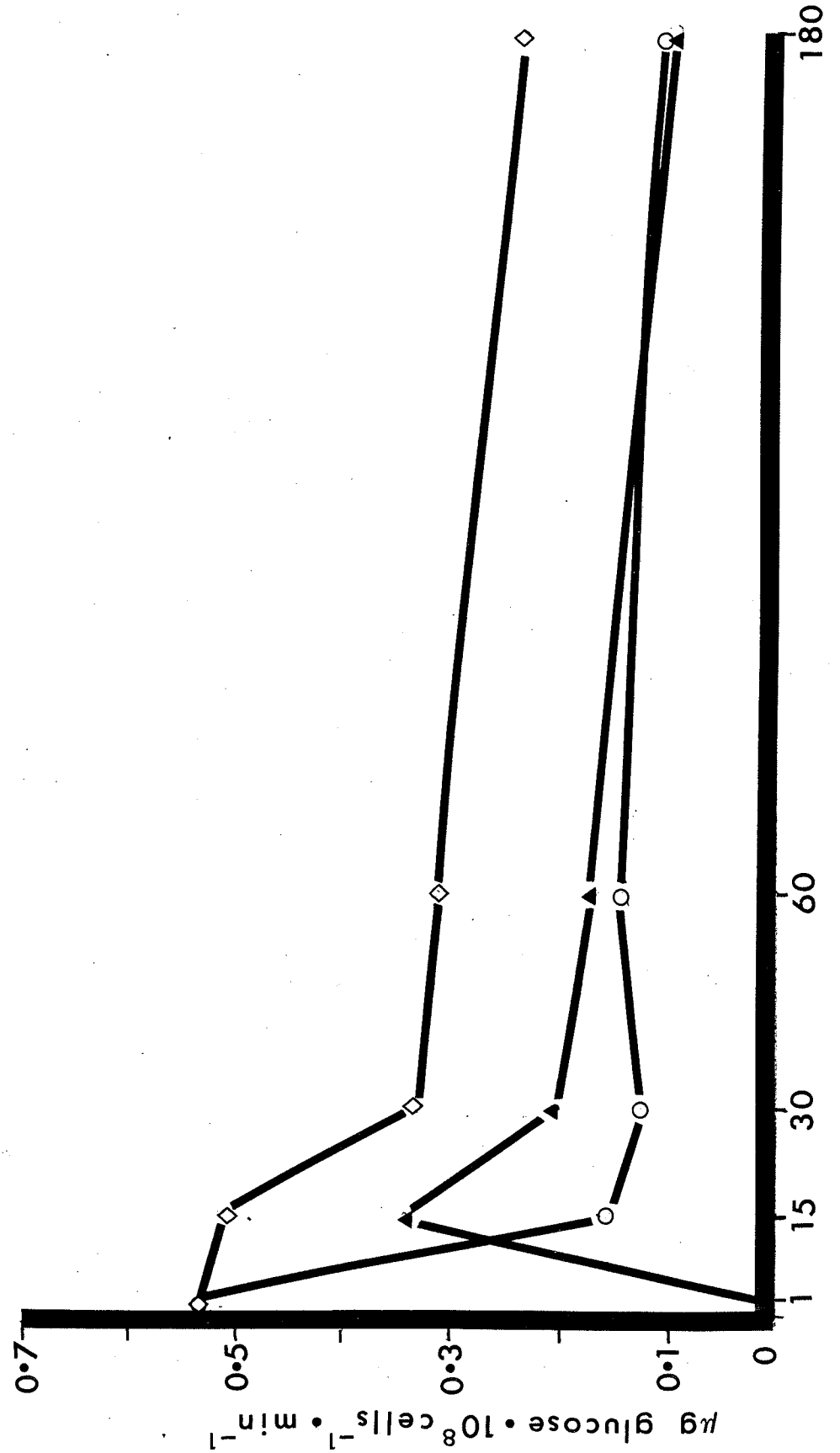
- assimilation and respiration ◇——◇
- assimilation ○——○
- respiration ▲——▲


μg glucose • 10⁸ cells⁻¹ • min⁻¹ (x 10⁻¹)
0
0·1
0·3
0·5
0·7
1
15
30
60
180
TIME (min)

lucose
U-C14

FIGURE 2-5: Uptake and respiration of glucose. External glucose concentration applied at time 0 was 100.0 µg U-C^{14} glucose.L^{-1}.





concen-
U-C14

TABLE 2-2

Uptake and respiration of U-C^{14} glucose. All cells used in these experiments were preconditioned for 4 hr in the dark in the presence of 0.01 mM glucose. Experiments were conducted at a light intensity of 500 lux. All results are expressed in μg glucose.10^8cells^{-1}.min^{-1}.

Time(min)	External Glucose ugL^{-1}	Uptake	Uptake % of 1 min.	Respiration	Total Uptake	Percent Resp.	Total Percent
1	1.0	0.01 ± 2.1%	100.0	0	0.006	0	100.0
	10.0	0.04 ± 6.5%	100.0	0	0.041	0	100.0
	100.0	0.54 ± 10.1%	100.0	0	0.535	0	100.0
15	1.0	0.003 ± 20.4%	41.9	0.002 ± 31.2%	0.005	49.3	82.8
	10.0	0.01 ± 1.2%	35.8	0.024 ± 26.6%	0.039	62.5	95.5
	100.0	0.16 ± 16.1%	29.7	0.349 ± 28.4%	0.509	68.7	94.4
30	1.0	0.002 ± 1.2%	27.9	0.002 ± 22.5%	0.004	58.3	66.9
	10.0	0.009 ± 22.1%	21.8	0.025 ± 18.8%	0.034	73.7	82.8
	100.0	0.12 ± 18.7%	22.7	0.208 ± 19.3%	0.330	63.2	61.7
60	1.0	0.002 ± 2.6%	31.4	0.001 ± 14.5%	0.003	40.7	52.9
	10.0	0.008 ± 2.3%	20.0	0.008 ± 21.3%	0.016	49.9	39.9
	100.0	0.15 ± 28.2%	27.0	0.170 ± 20.5%	0.316	53.8	59.1
180	1.0	0.002 ± 2.1%	32.8	0.001 ± 28.1%	0.003	31.6	47.9
	10.0	0.006 ± 3.8%	15.8	0.004 ± 11.6%	0.011	40.5	26.6
	100.0	0.12 ± 1.0%	19.8	0.099 ± 8.4%	0.205	48.3	38.3

* Total uptake as a percent of 1 min. total uptake.

assimilated glucose, but by 15 min the highest respiration rate was attained. At external glucose concentrations of 1.0 and 10.0 $\mu g.L^{-1}$ this rate of respiration continued for 30 min and then dropped to a reasonable stable rate. At an external glucose concentration of 100.0 $\mu g.L^{-1}$, the high respiration rate dropped immediately (Figures 2-3, 2-4, and 2-5). When net uptake rates and respiration rates were combined, the total uptake as a percent of the 1 min rates, dropped approximately 60% by 180 minutes.

Only one experiment was conducted with the uptake and respiration occuring in the dark. The values were similar to the above and well within the majority of the standard error limits of the values obtained for the experiments done under 500 lux light.

3. Effect of CO^2 Depletion:

a) Heterotrophic Growth:

The results of the growth experiments on 0.1 mM and 1.0 mM glucose are illustrated in Figures 2-6 and 2-7. These figures indicate that 0.1 mM had only minimal stimulation of light growth in the absence of CO^2 . Glucose concentrations of 1.0 mM promoted growth in the light in the absence of CO^2. When these values were converted to cell doubling times (Table 2-3), and compared with the doubling times reported in Chapter One, it became apparent that heterotrophic growth was limited in the light even though photoautotrophic growth was inhibited by a lack of CO^2.

b) Transport of U-C^{14} Glucose:

Under all light condition, the absence of CO^2 appears to inhibit the uptake of glucose (Figure 2-8 and Table 2-4).

4. Effect of Light and Glucose Preconditioning on Transport:

The results of these experiments are illustrated in Figures 2-9, 2-10 and 2-11. They are also listed in Appendix VI. The results are somewhat inconclusive but some trends appear evident. Firstly, regardless of any other condition tested, cells grown in continuous light of 5000 lux had a lower uptake rate than those grown in dark for 24 h prior to experimentation. The reduction in transport rates was from 4.9% to 65.5%. The presence of light at the time of experimentation slightly enhanced uptake under all preconditioning treatments. Cells preconditioned in the dark showed a much greater enhancement by light uptake (average 26.21%) than those preconditioned in continous light (average

FIGURE 2-6: Heterotrophic growth in the absence of CO_2. Light was 5000 lux and temp. 22°C. The results are based on changes in cell number. Lines represent different amounts of glucose added at time zero.

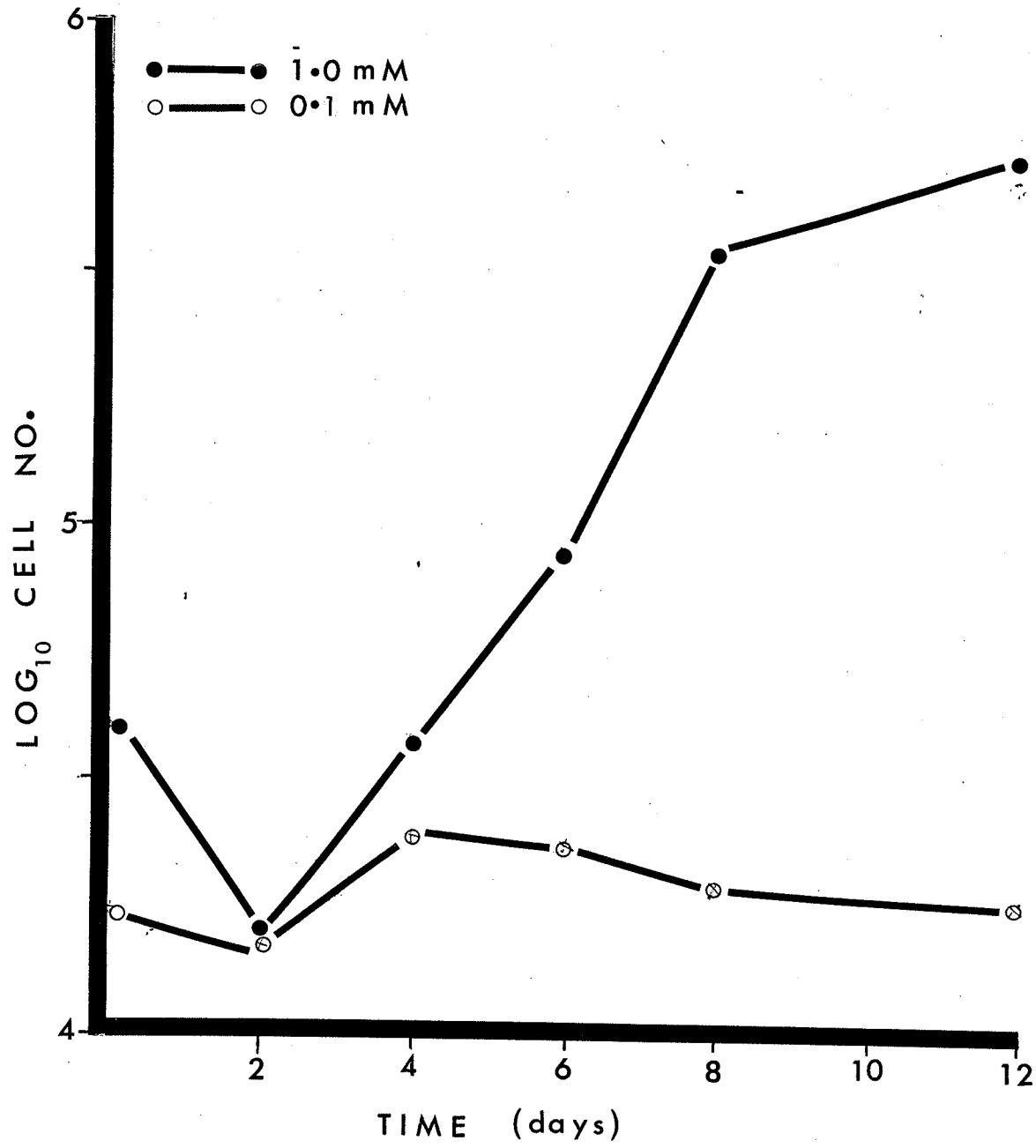

1·0 mM
0·1 mM
6
5
4
LOG10 CELL NO.
2
4
6
8
10
12
TIME (days)

FIGURE 2-7: Heterotrophic growth in the absence of CO_2. Results expressed by changes in cell volume. Lines represent different amounts of glucose added at time zero.

Results
present

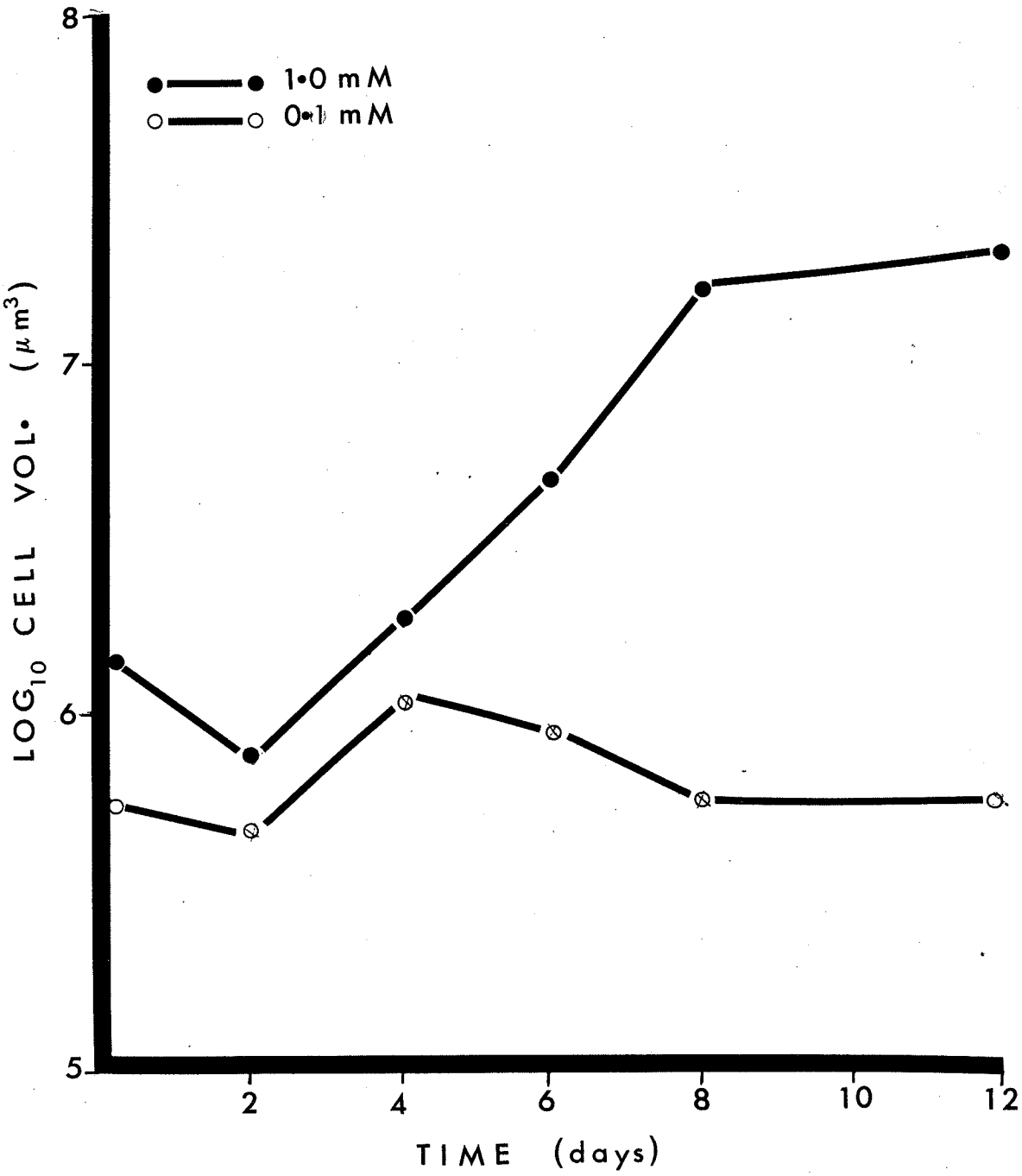


TABLE 2-3

Effect of removal of CO^2 on the heterotrophic growth of A. braunii as estimated by doubling times.

Glucose Concentration	Continuous Light (0.0 CO^2)	Continuous * Light (+CO^2)	Dark * (+CO^2)
0.0 mM	-	15.70 hr.	138.90 hr.
0.1 mM	99.64 hr.	-	42.49 hr.
1.0 mM	36.12 hr	13.63 hr.	25.80 hr.

* Results from experiments in Chapter One.

TABLE 2-4

Effect of removal of CO^2 on the transport of glucose. All values expressed as µg glucose.10^8 cells^{-1}min^{-1}.

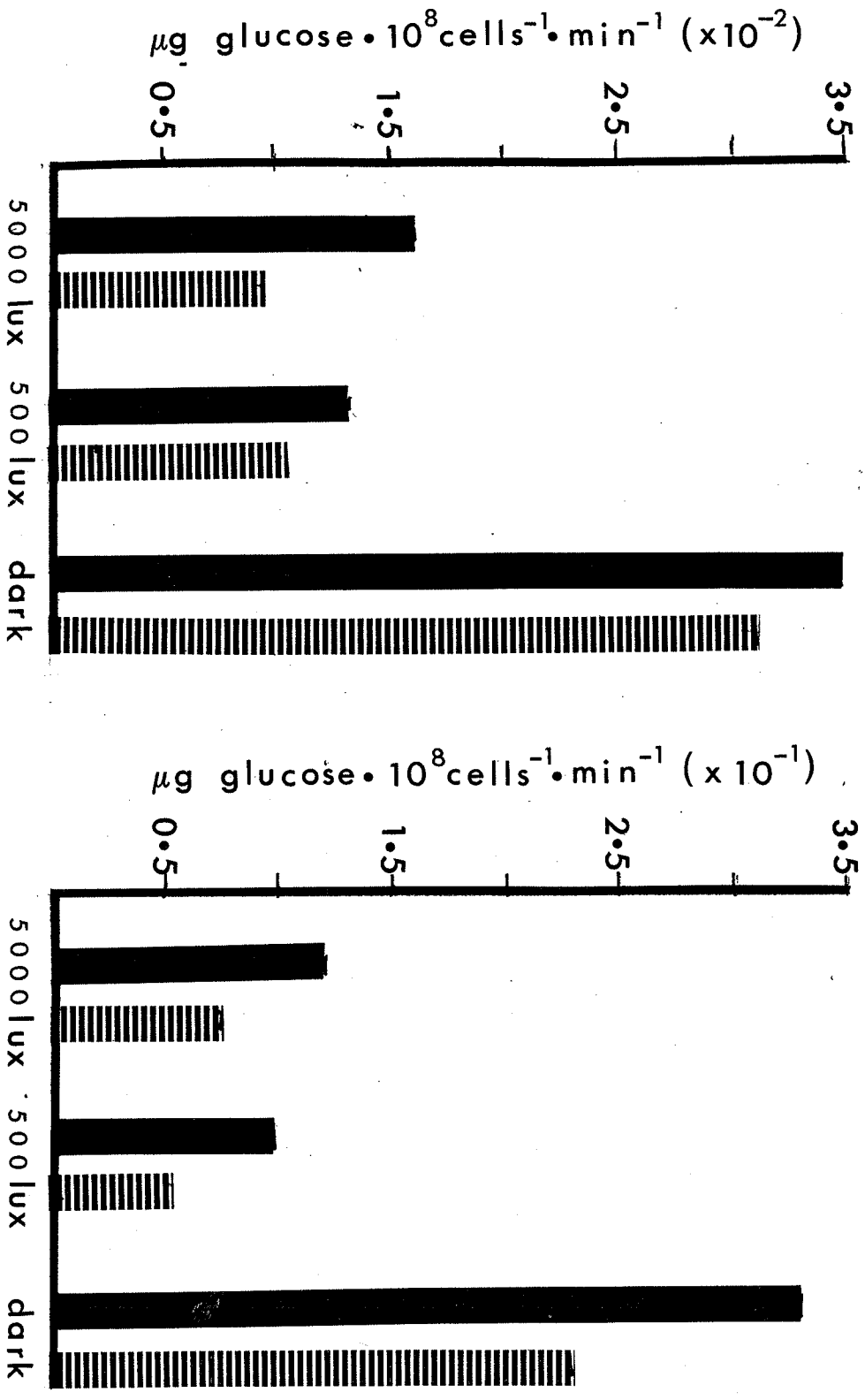
Experimental* Light & CO^2 Conditions	External Glucose 10 µg.L^{-1}	Percent Inhibition	External Glucose 100 µg.L^{-1}	Percent Inhibition
5000 lux + CO^2	0.016		0.122	
5000 lux - CO^2	0.009	41.2	0.075	38.7
500 lux + CO^2	0.013		0.097	
500 lux - CO^2	0.010	24.5	0.051	47.8
dark + CO^2	0.035		0.330	
dark - CO^2	0.031	11.1	0.227	31.0

* These conditions prevailed during the uptake experiments.

FIGURE 2-8: Effect of CO_2 on the uptake of glucose. Measurements were made using three different light conditions and two different glucose concentrations. Histogram A was at an external glucose concentration of 10 $\mu g.L^{-1}$. Histogram B was at an external concentration of 100 $\mu g.L^{-1}$.

- plus CO_2

- minus CO_2



LIGHT PRECONDITIONS

10.33%). The presence of glucose during preconditioning demonstrated a mixed response. Cells preconditioned in continuous light and glucose always had a lower rate of transport than those without glucose. Cells preconditioned in the dark with glucose showed an enchancement of uptake rates in all but one instance.

5. Effects of Metabolic Inhibitors on Glucose Transport:

The results of the inhibitor experiments are illustrated in Figures 2-12, 2-13, 2-14, and 2-15. They are also listed as percent inhibition in Tables 2-5, 2-6, 2-7 and 2-8. The percent inhibition is expressed relative to the results in the previous experiments. Any reduction in transport caused by the preconditioning or light conditions at the time of uptake, would then be taken into account. In describing the inhibition in this section and through the discussion portion of this chapter, four categories are used. Transport that was inhibited by 75-100% is described as being strongly inhibited. A 50-74% inhibition is described as being partially inhibited, while 25-49% inhibition is termed mildly inhibited. If the inhibition was less than 25%, then it was considered not to have been inhibited. Although it is realized that the separation into these categories is somewhat arbitrary and may be misleading, the volume of data dictated that some condensation be made for ease of discussion. The results are presented according to the principal site or most frequently cited area of inhibition. Other sites of inhibition for each of the inhibitors, will be considered in the discussion. The inhibitor of PS II, DCMU, partially or mildly inhibited glucose transport in the light, except when the cells were preconditioned in the light without glucose. There was no inhibition of dark uptake at 10 $\mu g.L^{-1}$ but at

FIGURE 2-9: Effect of light preconditioning on the transport of glucose. Histogram A is at an external glucose concentration of 10 $\mu g.L^{-1}$ and histogram B is at an external glucose concentration of 1000 $\mu g.L^{-1}$ Letters directly below the bars represent the pretreatment. The combination of letters below this represents the glucose pretreatment and light (500 lux) conditions during the uptake. (eg. D+G is dark uptake and glucose pretreatment).

- continuous light preconditioning

- 24h dark preconditioning

glucose.
n of 10
concen-
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letters
d light
is dark

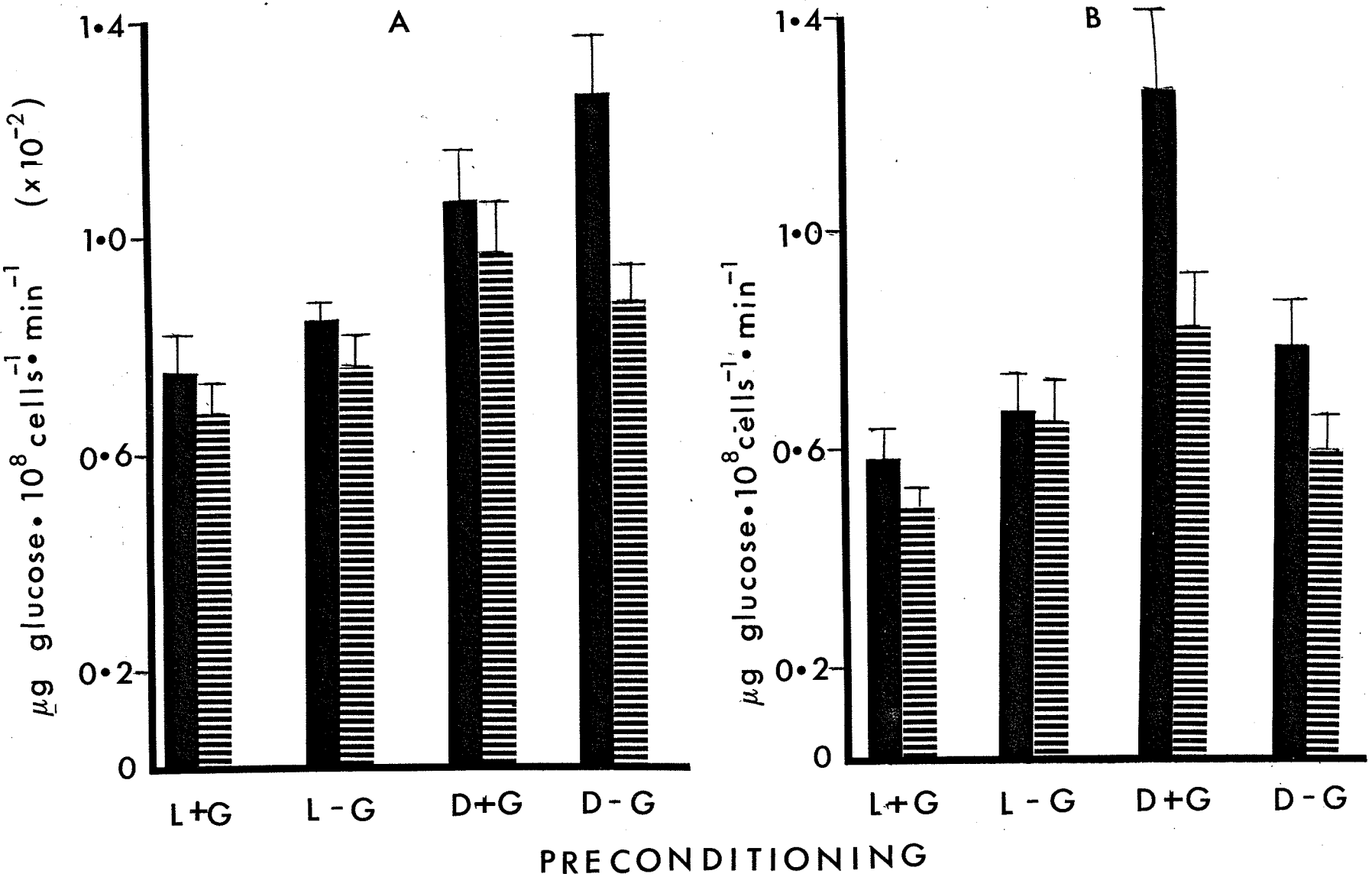
A
B
1•4
1•0
0•6
0•2
0
μg glucose • 10^8 cells^-1 • min^-1 (x10^-2)
L+G
L-G
D+G
D-G
PRECONDITIONING

FIGURE 2-10: Effect of light at the time of uptake, Histogram A is at an external glucose concentration of 10 $\mu g.L^{-1}$ and histogram B is at an external glucose concentration of 1000 $\mu g.L^{-1}$. Letters directly below the bars represent the experimental conditions (D=dark) while the combination of letters represents the pretreatment. (eg. D+G is dark and glucose pretreatment)

- uptake in the light 

- uptake in the dark 

FIGURE 2-11: Effect of glucose pretreatment on the transport of glucose. Histogram A is at an external glucose concentration of 10 $\mu g.L^{-1}$. Symbols directly below the bars represent the type of glucose pretreatment. (eg. + = 0.01 mM glucose) The combination of letters represents the pretreatment and experimental light conditions. (eg. D+D is dark pretreatment and dark uptake) Abbreviations:

- plus glucose preconditioning

- minus glucose preconditioning

lucose.
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ose)
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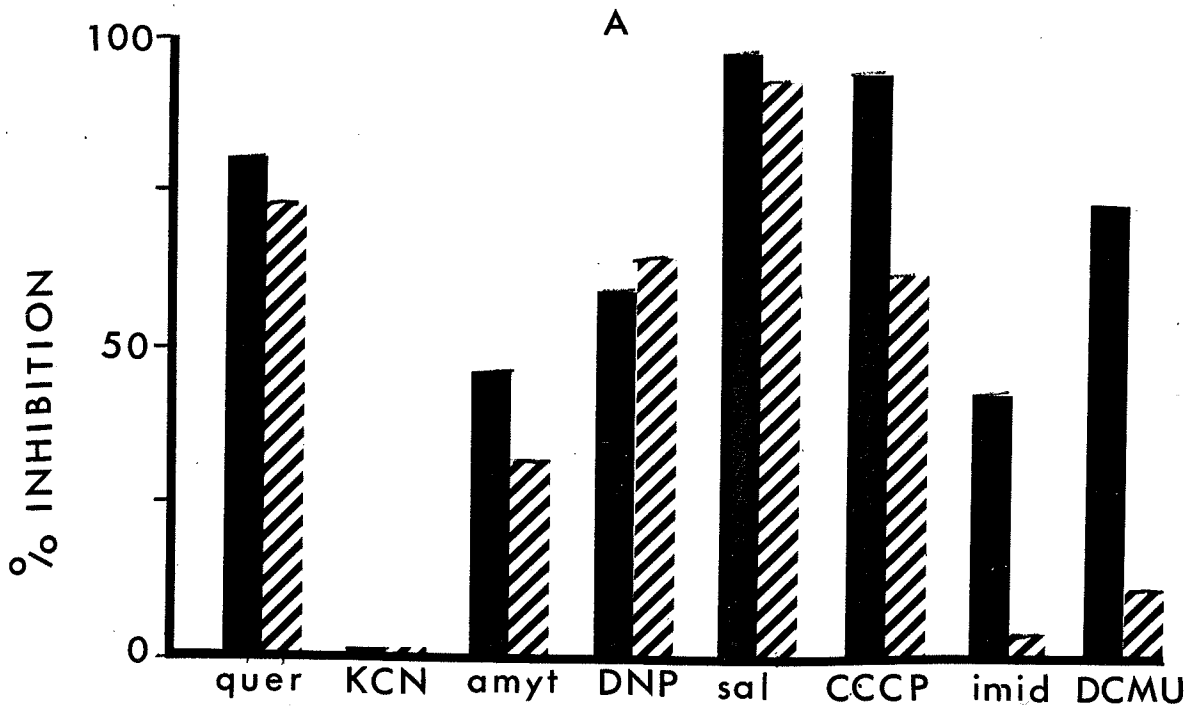
FIGURE 2-12: Effect of inhibitors on cells preconditioned in the dark with no glucose. Histogram A represents uptake at a glucose concentration of 10 $\mu g.L^{-1}$. Histogram B is at a glucose concentration of 1000 $\mu g.L^{-1}$.

quer. = Quercitin
KCN = potassium cyanide
amyt = Amytal
DNP = 2'4' dinitrophenol
Sal = salicylaldoxime
CCCP = carbonyl cyanide-m-chlorophenyl hydrazone
Imid = imidazole
DCMU = dichloromethyl urea

- light uptake

- dark uptake


A
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

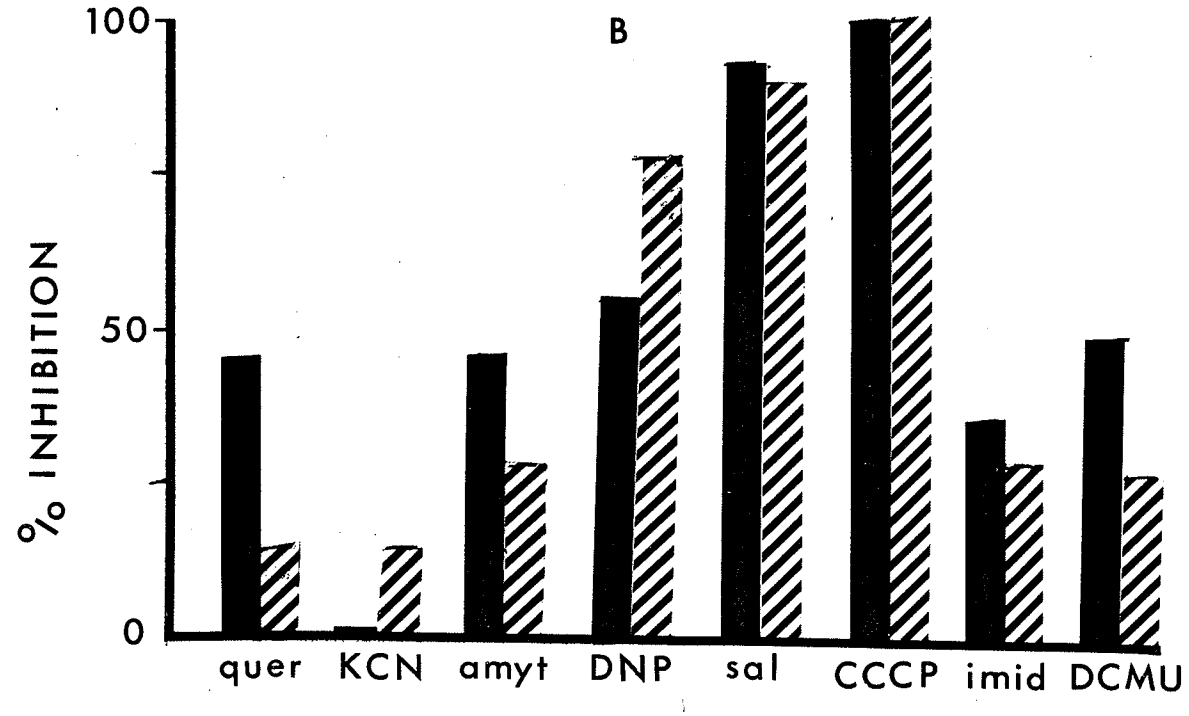
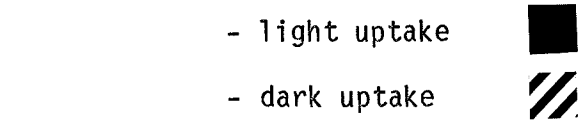
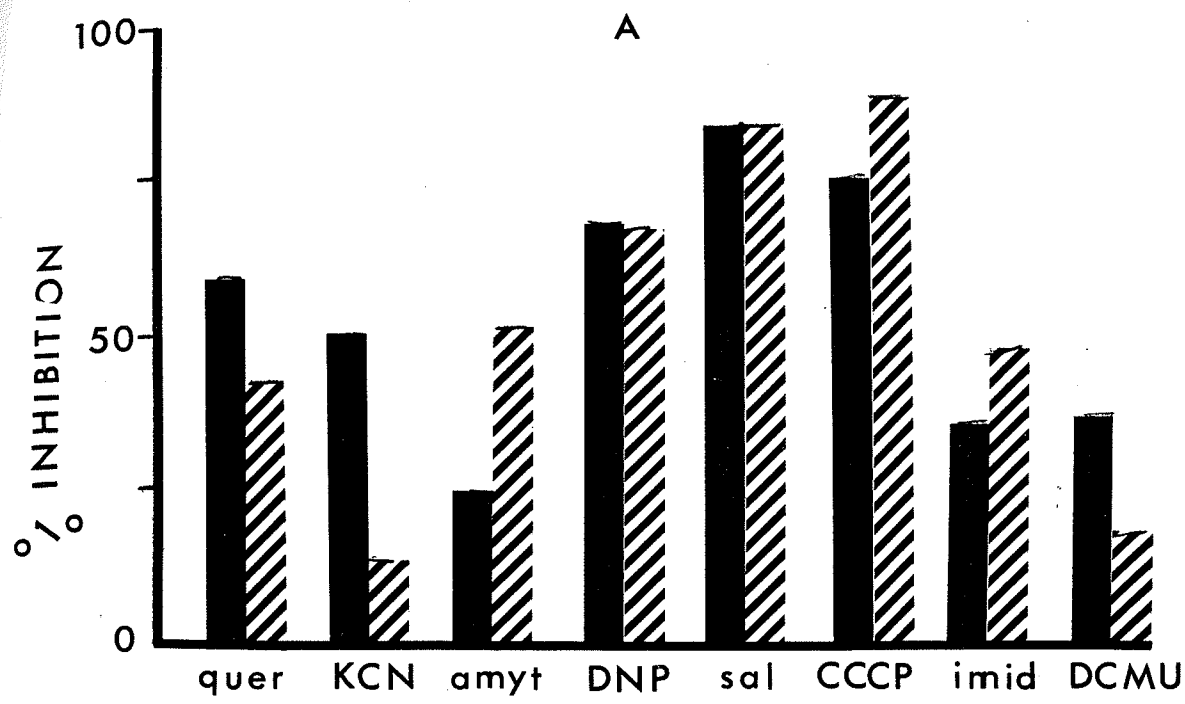

B
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

FIGURE 2-13: Effect of inhibitors on transport by cells preconditioned in the dark with glucose. Symbols and histograms are the same in Fig. 2-12.




A
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

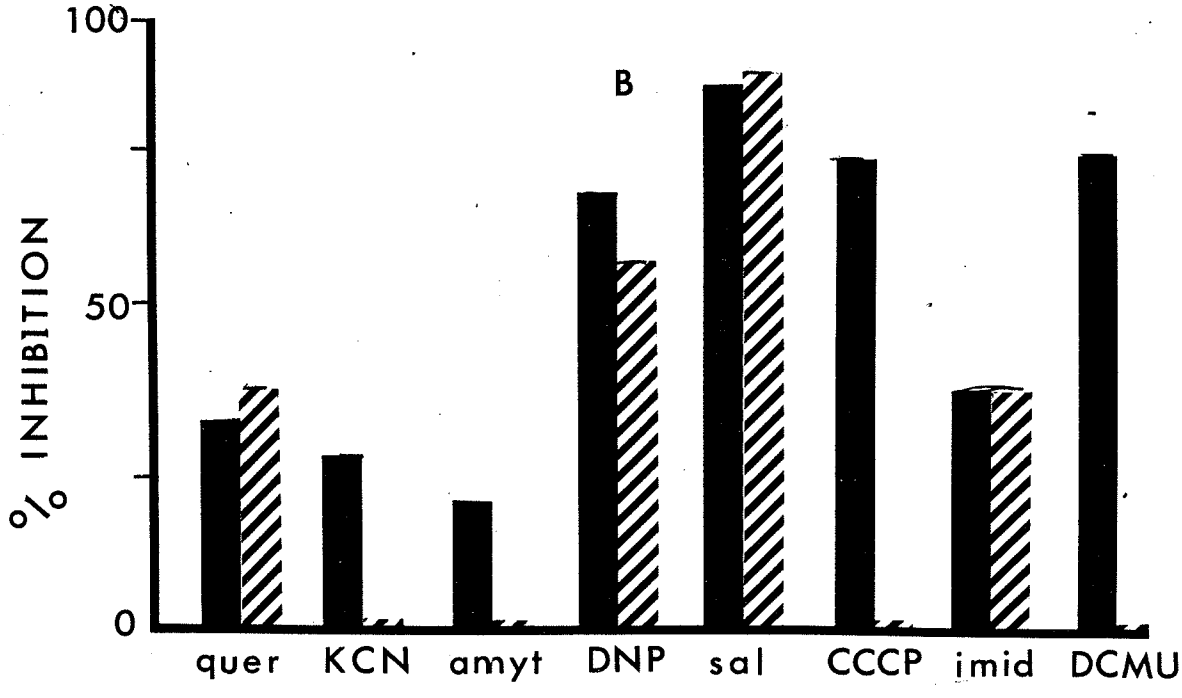
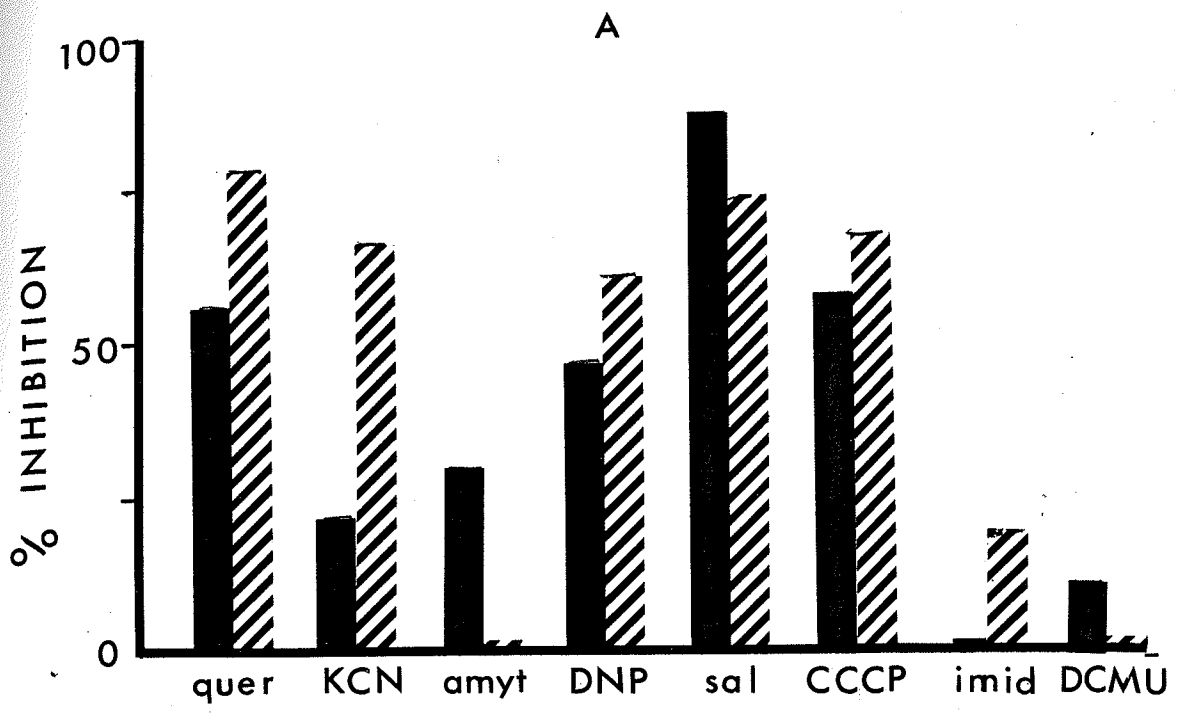

B
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

FIGURE 2-14: Effect of inhibitors on the transport by cells preconditioned in the light without glucose. Symbols and histograms are the same as in Fig. 2-12

- light uptake 

- dark uptake 


A
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

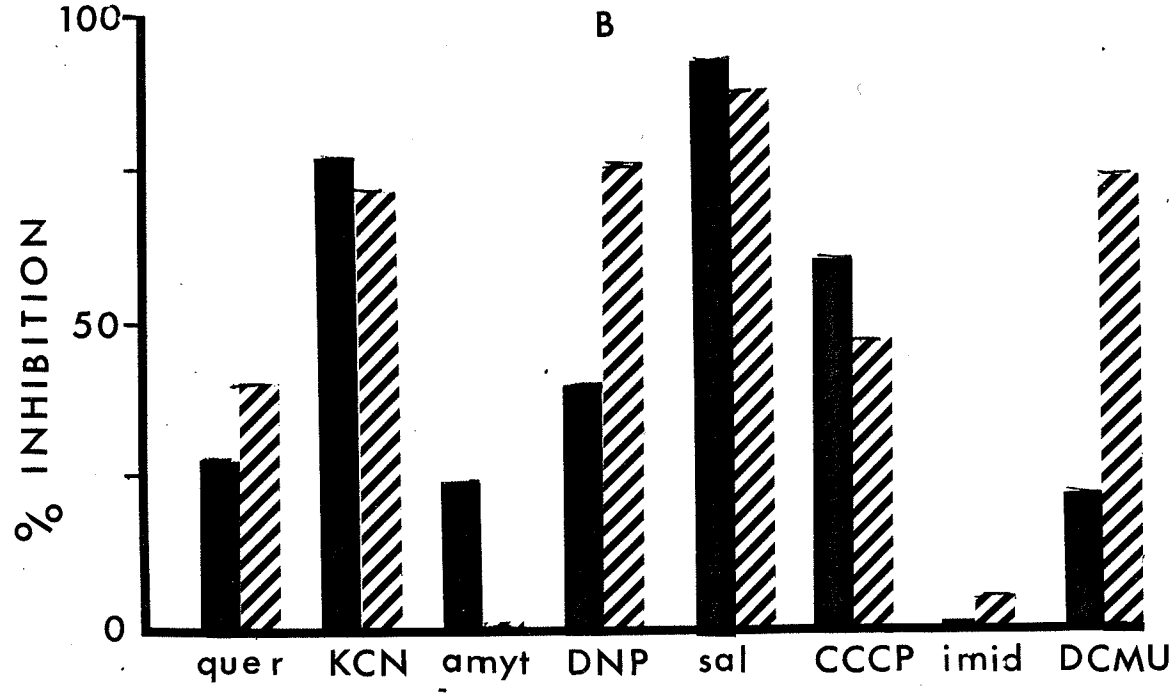
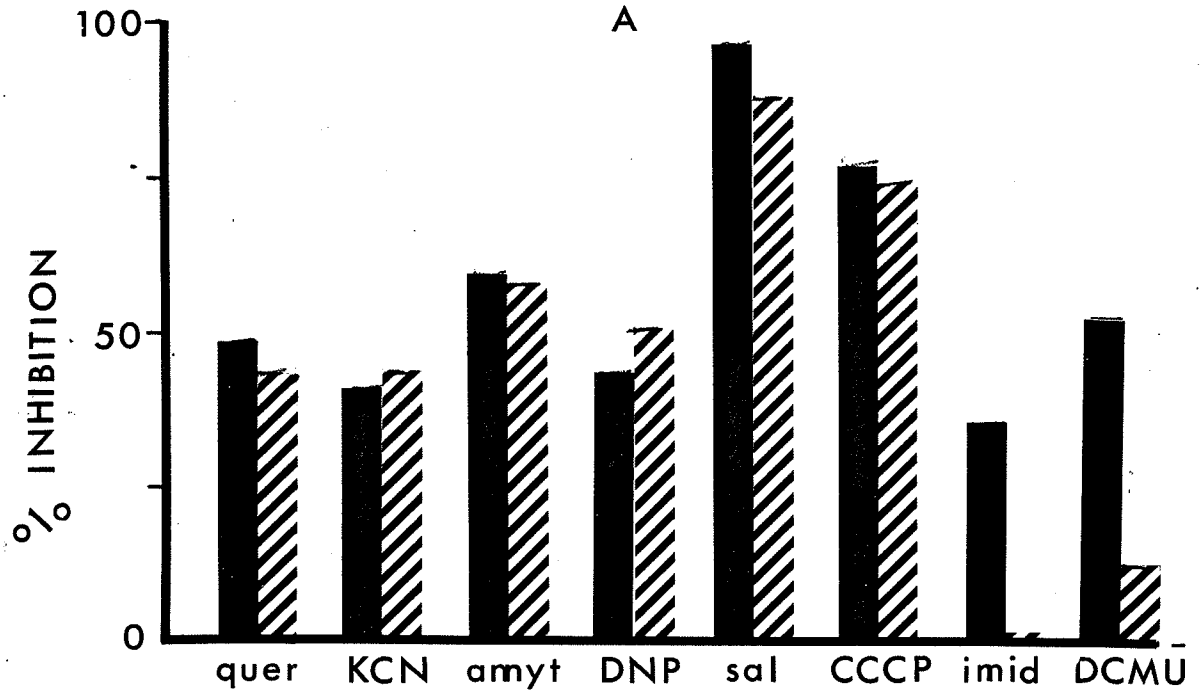

B
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

FIGURE 2-15: Effect of inhibitors on transport by cells preconditioned in the light with glucose. Symbols and histograms are the same as in Fig. 2-12.

- light uptake 

- dark uptake 


A
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

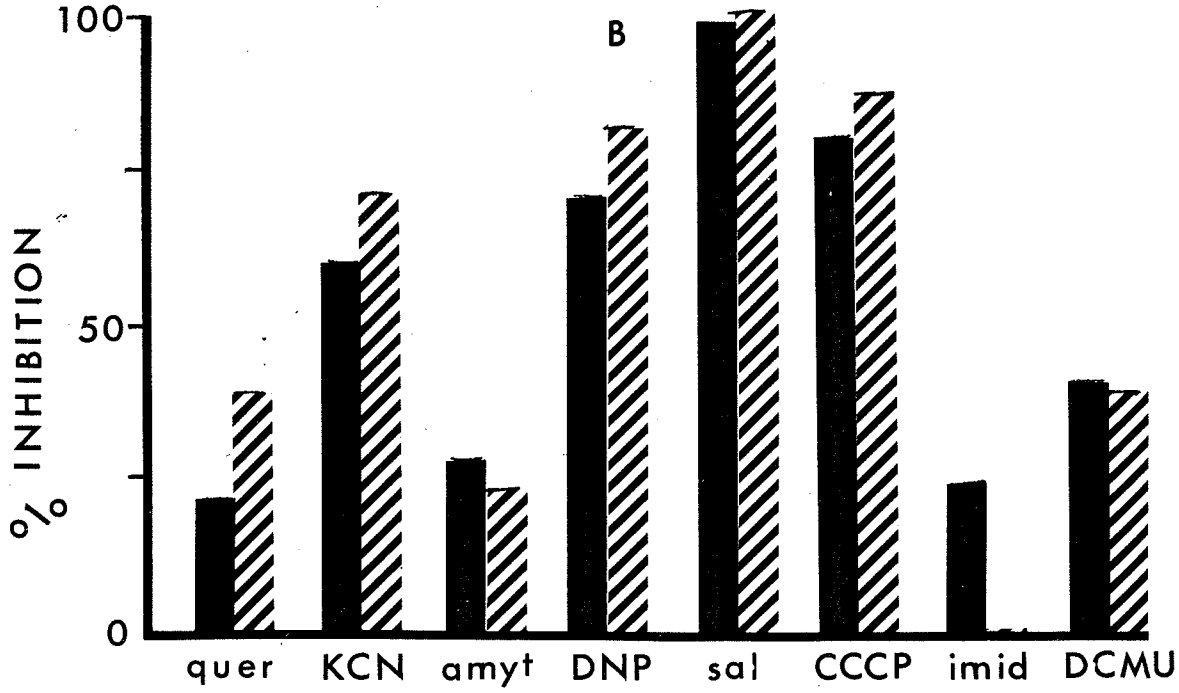

B
100
50
0
% INHIBITION
quer
KCN
amyt
DNP
sal
CCCP
imid
DCMU

TABLE 2-5

Effect of inhibitors on transport by cells preconditioned in the dark with no glucose. The top number in each pair represents dark uptake, while the bottom no. represents light uptake. Values are of percent inhibition.

Inhibit. Conc.	Amyt.	CCCP	DCMU	DNP	Imid.	KCN	Quer.	Sali.
10 µg glucose.L^{-1}								
10^{-6}M	-	46.3D 48.0L	24.2D 64.9L	-	-	-	-	-
10^{-5}M	-	61.6D 94.1L	11.6D 73.1L	30.1D 33.9L	-	28.6D 0.0L	38.2D 42.8L	-
10^{-4}M	32.3D 31.4L	- -	- -	64.4D 58.9L	0.0D 40.3L	0.0D 0.0L	72.9D 80.4L	-
10^{-3}M	7.1D 46.7L	-	-	-	3.9D 43.3L	-	-	71.1D 84.1L
10^{-2}M	-	-	-	-	-	-	-	92.4D 97.8L
1000 µg glucose.L^{-1}								
10^{-6}M	-	48.8D 30.2L	0.0D 17.3L	-	-	-	-	-
10^{-5}M	-	100.0D 100.0L	26.3D 49.3L	17.5D 7.4L	-	0.0D 0.0L	0.0D 4.5L	-
10^{-4}M	12.5D 23.4L	-	-	77.8D 55.5L	0.0D 27.6L	15.3D 0.0L	14.4D 45.8L	-
10^{-3}M	27.4D 46.8L	-	-	-	28.6D 36.2L	-	-	82.4D 74.2L
10^{-2}M	-	-	-	-	-	-	-	90.3D 93.7L

TABLE 2-6

Effect of inhibitors on transport by cells preconditioned in the dark with glucose. The letter D represents dark uptake, while the letter L represents light uptake. Values are of percent inhibition.

Inhibit. Conc.	Amyt.	CCCP	DCMU	DNP	Imid.	KCN	Quer.	Sali.
				10 μg glucose.L^{-1}				
10^{-6}M	-	61.5D 50.0L	32.3D 46.1L	-	-	-	-	-
10^{-5}M	-	88.9D 75.3L	18.3D 37.6L	39.1D 39.2L	-	0.0D 28.9L	25.8D 31.9L	-
10^{-4}M	51.6D 27.9L	- -	- -	67.1D 68.1L	58.0D 41.2L	14.1D 50.1L	42.6D 59.1L	-
10^{-3}M	51.6D 24.4L	-	-	-	47.9D 36.9L	-	-	61.3D 62.6L
10^{-2}M	-	-	-	-	-	-	-	82.7D 82.7L
				1000 μg glucose.L^{-1}				
10^{-6}M	-	59.3D 40.3L	0.0D 79.4L	-	-	-	-	-
10^{-5}M	-	100.0D 100.0L	0.0D 76.7L	0.0D 23.6L	-	0.0D 0.0L	13.1D 9.0L	-
10^{-4}M	0.0D 2.3L	-	-	59.1D 70.1L	33.9D 17.0L	0.0D 28.9L	39.8D 33.9L	-
10^{-3}M	0.0D 20.1L	-	-	-	39.8D 39.5L	-	-	44.1D 33.5L
10^{-2}M	-	-	-	-	-	-	-	89.9D 86.8L

TABLE 2-7

Effect of inhibitors on transport by cells preconditioned in the light with no glucose. The letter D represents dark uptake, while the letter L represents light uptake. Values are of percent inhibition.

Inhibit. Conc.	Amyt.	CCCP	DCMU	DNP	Imid.	KCN	Quer.	Sali.
			10 μg glucose.L^{-1}					
10^{-6}M	-	5.4D 42.6L	48.8D 28.2L	-	-	-	-	-
10^{-5}M	-	68.8D 58.8L	1.2D 11.2L	39.6D 47.7L	-	57.3D 45.2L	27.3D 35.5L	-
10^{-4}M	52.3D 21.3L	- -	- -	61.3D 9.6L	40.0D 16.4L	66.4D 22.7L	78.4D 56.5L	-
10^{-3}M	0.0D 30.9L	-	-	-	19.1D 0.0L	-	-	46.7D 44.5L
10^{-2}M	-	-	-	-	-	-	-	73.3D 86.9L
			1000 μg glucose.L^{-1}					
10^{-6}M	-	8.9D 23.3L	36.7D 0.0L	-	-	-	-	-
10^{-5}M	-	47.1D 60.0L	74.7D 23.0L	16.7D 11.7L	-	27.6D 24.4L	0.0D 0.0L	-
10^{-4}M	0.0D 15.9L	-	-	75.7D 40.5L	0.0D 0.0L	71.5D 76.5L	39.9D 28.9L	-
10^{-3}M	0.0D 24.2L	-	-	-	6.1D 0.0L	-	-	54.8D 39.4L
10^{-2}M	-	-	-	-	-	-	-	91.8D 86.7L

TABLE 2-8

Effect of inhibitors on transport by cells preconditioned in the light with glucose. The letter D represents dark uptake, while the letter L represents light uptake. Values are of percent inhibition.

Inhibit. Conc.	Amyt.	CCCP	DCMU	DNP	Imid.	KCN	Quer.	Sali.
				10 µg glucose.L^{-1}				
10^{-6}M	-	48.2D 36.5L	48.0D 68.8L	-	-	-	-	-
10^{-5}M	-	73.6D 77.3L	12.2D 52.1L	32.9D 37.1L	-	21.7D 40.2L	25.8D 37.1L	-
10^{-4}M	57.3D 59.1L	- -	- -	50.2D 42.8L	0.0D 35.1L	43.5D 31.7L	42.6D 47.8L	-
10^{-3}M	41.4D 7.2L	-	-	-	0.0D 14.8L	-	-	38.4D 64.7L
10^{-2}M	-	-	-	-	-	-	-	87.2D 96.3L
				1000 µg glucose.L^{-1}				
10^{-6}M	-	17.7D 12.2L	35.5D 0.0L	-	-	-	-	-
10^{-5}M	-	86.9D 80.2L	39.2D 41.7L	17.7D 14.9L	-	0.0D 7.5L	13.1D 0.0L	-
10^{-4}M	0.4D 13.5L	-	-	81.9D 70.9L	0.0D 5.8L	71.5D 60.2L	39.8D 22.1L	-
10^{-3}M	23.3D 28.0L	-	-	-	0.0D 24.4L	-	-	7.3D 57.2L
10^{-2}M	-	-	-	-	-	-	-	100.0D 97.8L

1000 $\mu g.L^{-1}$ UDCM partially inhibited uptake by cells preconditioned with light but not glucose. DCMU also mildy inhibited transport by cells preconditioned in the light with glucose and the dark without glucose. The uncoupler of cyclic photophosphorylation, CCCP, strongly or partially inhibited uptake under all conditions. Salicylaldoxime, an inhibitor that complexes with heavy metals in either photosynthetic or respiratory electron transport chains, strongly inhibited all transport. Imidazole, an uncoupler of electron transport chains, demonstrated either no inhibition or only mild inhibition. Another uncoupler, DNP, showed a partial inhibition at all uptake conditions except were the cells were preconditioned in the light with or without glucose and subjected to light uptake at $10\mu g.L^{-1}$ and cells preconditioned in the light without glucose and subjected to light uptake at 1000 $\mu g.L^{-1}$.

The inhibitor of NADH oxidase, amytal, demonstrated either mild inhibition or none at all in all conditions except where cells were preconditioned in the dark and subjected to dark uptake. Quercitin, an inhibiter of mitochondrial ATPase, showed strong or partial inhibition under most conditions at 10 $\mu g.L^{-1}$, but only mild or no inhibition at 1000 $\mu g.L^{-1}$. KCN, an inhibitor of cytochrome oxidase, also demonstrated a mixed effect on glucose transport. At external concentrations of $10\mu g.L^{-1}$, it strongly or partially inhibited transport by cells that were preconditioned in the light, but showed only mild, or no inhibition of cells preconditioned in the dark.

DISCUSSION

When examining the contribution of heterotrophy to the total metabolism of an alga, two overriding questions must be considered. Firstly, under what environmental and physiological conditions will heterotrophy make a significant contribution to the survival of the cell? Secondly, how significant is the heterotrophy relative to the photoautotrophic metabolism of the alga and how efficient is the conversion of transported organic substrate to algal biomass.

From Chapter One, it is evident that Ankistrodesmus braunii can exist as a photoautotrophic organism. Growth rate or doubling time in continuous light or in 12 h light/12 h dark regime is the same in the presence or absence of glucose. Only cell volume increases in the presence of glucose in the above light conditions. Because of the apparent advantages of photosynthesis such as the lack of a need to expend energy to transport a metabolizable substrate, the heterotrophic capabilities would be significant if photosynthesis only marginally met the cell demands for organic carbon and energy. In this instance, if an inhibition of photosynthesis occurred, a facultative heterotroph would have a selective advantage over obligate photoautotrophs. The fact that cell volume increases in the presence of glucose in the light provides indirect support for this justification of a transport system.

The results of the photosynthesis experiments do not indicate a marginally adequate system. In fact, the initial rates of net assimilation of fixed carbon would result in a potential doubling time of 2.2 h (Table 2-1). Even after an apparent stablization period of 1 to 3 hours, the projected doubling times were only 4.5 to 4.8 h. It is expected that

after this period of time, excretion and respiration would have reached a constant level (Nalewajko and Schindler 1976). Comparing these potential doubling times to the actual doubling time, in continuous light, of 15.7 h (Chapter One), the alga would be able to greatly exceed this observed growth rate by photosynthesis alone.

To determine whether respiratory and excretory losses were, in fact, stabilized in one to three hours, excretion and respiration were measured for this time interval. Fogg (1966), suggested that excretion of fixed carbon may reach as high as 50% or greater. Others, such as Nalewajko and Lean (1972), Nalewajko et al.(1976) and Berman and Gerber (1980) suggest excretion rates of closer to 10% of the total fixed carbon are more realistic. Mague et al.(1980) question the high excretion rates and suggest that much of the apparent excretion is due to artifacts of the methodology. Excretion of fixed carbon by A. braunii (Table 2-1) followed a pattern reported by Nalewajko and Schindler (1976). The excretion rate was high for the first 15 min. (18.4% and 12.7% of total fixed carbon for the first 1 min. and 15 min. samples respectively) and then stabilized between 30 min. and 180 min. at approximately 3.5%. A reason for the high initial excretion rate may be that A. braunii required the establishment of an equilibrium between inside and outside the the cell with respect to some organic metabolite such as glycollate (Fogg 1975). Until such equilibrium is established, the cells will more rapidly excrete the metabolite. If this was the case for A. braunii, the quasi-equilibrium would apparently be reached at about 3.5% or 0.0036 pg carbon.cell^{-1}min^{-1}. Excretion of fixed carbon, therefore, would not likely increase to a level where net photosynthetically fixed

carbon would be only enough to account for the observed doubling times (Chapter One) of photoautorophically grown A. braunii. It would not even account for the apparent decline in net photosynthesis between 1 min. and 180 min. (Table 2-1). Mague et al. (1980) found much higher rates of excretion (30% of fixed carbon) under high light intensities. Watanabe (1980) found excretion rates of 54% with high light. Since the experiments were conducted under only moderate light intensities (5000 lux) perhaps excretion could play a more significant role in reducing the rate of net photosynthetic production in these circumstances.

Experiments to determine the rate of assimilated organic carbon transported and subsequently respired suggest a dark respiration rate of 46.0% in the dark and 48.0% in low light (500 lux). This similarity between dark and low light respiration consistant with the results reported by Graham (1980). Even if the respiration rates, including photorespiration and dark respiration combined, were as high as those respiration rates in the dark, the combined fixed carbon loss of 46% for respiration and 3-4% for excretion would only account for approximately 50% of the total fixed organic carbon. These results explain the increase in projected doubling time from 2.2 to 4.5 h after 30 min. experiments, since net photosynthesis is reduced by 50%, but they do not indicate a marginal or limiting net asssimilation. It is realized that converting net assimilation rates to doubling times is erroneous, even in continuous light, since photosynthesis will not occur throughout the cell cycle (Tamiya 1966). Also, high light intensities may decease net accumulation of fixed organic carbon in a natural diurnal cycle, since dark respiration rates may increase following periods of prolonged or high

intensity light (Stone and Ganf 1980).

The photosynthesis, excretion and respiration rates indicate that A. braunii does not require heterotrophic metabolism to sustain growth under normal environmental conditions. The observed facultative heterotrophic capabilities of the alga must therefore only be required during periods when photosynthesis is limiting or absent. The most obvious environmental conditions which would neccessitate heterotrophy would be during periods where insufficient light is availiable for photosynthesis. This could occur: at night; in turbid waters; during extensive cloudy periods; or when the alga sinks below the photic zone. Other, less frequently occurring times would be during periods of CO^2 limitation or periods in the cell cycle when photosynthesis is reduced. As heterotrophy is an energy expending process, it would also be beneficial to the cell, if transport was reduced or absent during periods of active photosynthesis.

It has been demonstrated above, that photosynthesis could more than account for light growth. It is still not apparent as to whether glucose transport occurs in the light. From the results of the tests of the effects of preconditioning on transport, it was demonstrated that transport occurred in both the light and the dark, but is slower in light preconditioned cells (Fig 2-9 and 2-10).

Three variables were examined when testing the effects of light on glucose transport. These included light preconditioning, uptake in the light and glucose preconditioning. It was assumed that the uptake system was either constitutive, inducible or some combination of both. If it is constitutive, uptake should occur at the same rate no matter what the preconditioning, providing the cell has sufficient energy and the system

is not metabolically inactivated. If it is one of the other alternatives, uptake will only proceed if the system is induced and will wholly or partially cease when induction is stopped or repression occurs. As stated above, it would be inefficient if the system was completely constituitive. If the system was at least partially inducible, then it is logical to assume the induction method is at least indirectly involved with the energy or organic carbon demands of the cells. If it is constituitive, then transport will only be altered if the energy availability to drive the transport system is altered.

To test these hypotheses, it was assumed that if energy demand was the sole inducing force, perhaps through the reduction of negative-feedback repression, then cells grown in the dark, with no glucose would have the highest rate of uptake. Cells grown in the dark with glucose would be next, while cells grown in the light with glucose would have the lowest uptake rate. This would only apply if glucose was not required for induction or lifting repression and photophosphorylation ATP would not significantly alter the energy availability to drive the transport system. The first of these conditions was shown to be correct in Chapter Three. If energy availability was the determining factor in the uptake rates, then the reverse of the above situation would apply. That is, cells preconditioned in the light with glucose should have the highest amount of available energy and therefore the most rapid uptake rate. Cells grown in the dark without glucose would have the lowest amount of available energy and therefore the slowest uptake rate. Again, if photophosphorylation could enhance the transport, the model would have to be altered. Finally, two other possibilities could occur, where a combination of energy and organic carbon demand and available energy determined the uptake rate, but they alternate in their relative importance.

Appendix VI describes the models tested in detail. From the results reported in the Appendix, it appears as if the uptake system is partially inducible. This is also supported by the results of Chapter Three. It also appears as if light represses the transport during preconditioning, but photophosphorylation can provide energy for the transport. Glucose preconditioning provided mixed results. It apparently enhanced uptake under some conditions of preconditioning but at other times had no affect. Perhaps its only role is to provide energy for transport. The uptake of glucose from these experiments appears to be controlled by a mixture of energy demands and available energy with the energy demand dominating the control and light as a high energy source repressing transport. This argument is further substantiated by the results of experiments using cycloheximide (Chapter Three).

The results of the experiments reducing the availability of CO_2 for photosynthesis also support these findings. When cells were grown in continuous light (5000 lux), in the absence of CO_2 heterotrophic growth differed from dark heterotrophy. The results in Table 2-3 indicate that the doubling time was increased by more than 100% at an external glucose concentration of 0.1 mM and approximately 50% at an external glucose concentration of 1.0 mM. These results indicate that firstly, even though the CO_2 may not have been completely eliminated, it was certainly reduced to a level that could not support photoautotropic growth. Secondly, the amount of O_2 available for oxidative phosphorylation or light available for photophosphorylation would be sufficient to provide the energy for glucose transport at a rate comparable

to dark transport. Because this rate was not attained, it is suggested that light is probably inhibiting glucose transport or repressing the formation of a transport protein. Since the pH was monitored at the end of the experiment and found to have been within one unit of the starting pH (7.2), and also since pH over a wide range (5-9) did not alter uptake rates (Chapter Three), then this could not have been responsible for the reduced uptake in the absence of CO_2.

Tolbert (1980) suggested that photorespiration increases with decreasing CO_2 availability and increasing O_2 availability, both conditions which should have been present in these experiments. If this is the case in A. braunii, and photorespiration results in a lower availability of ATP than dark respiration due to a less efficient use of the transported glucose, then this would account for the reduction in growth in the presence of glucose but absence of CO_2. The drop in uptake rate in the dark is less than in the light when CO_2 is eliminated. This reduction, due to CO_2 removal, was 11.1% in the dark and 41.2% in the light at 10 $\mu g.L^{-1}$ external glucose and 31.0% in the dark and 38.7% in the light at 100 $\mu g.L^{-1}$. This would support the theory of reduced efficiency of photorespiration. It could also support the contention that photophosphorylation, particularly cyclic photophosphorylation, (since non-cyclic photophosphorylation would be inhibited with the blocking the Calvin cycle) can be used to drive the transport system but light itself tends to inhibit or repress the transport system.

To further determine the influence heterotrophy can exert on the survival of A. braunii, a series of inhibitors known to interfere with

energy metabolism, were applied prior to uptake. The major processes through which algal cells obtain their high energy ATP are: non-cyclic and cyclic photophosphorylation; oxidative phosphorylation; and to a lesser extent substrate phosphorylation. At least one of these processes must directly or indirectly provide the energy for transport. Specific inhibitors were applied to block each of these processes individually and under different preconditioning and uptake conditions.

It was hoped that the results of these experiments would at least partially answer several questions. Firstly, can all sources of ATP be used to supply the energy for transport? Secondly, can the prehistory of the cell alter the rate of glucose transport? Finally, is there a difference between the two systems responsible for the transport (Chapter One) in terms of their energy requirements or their ability to utilize different energy sources?

Moreland (1980), separates photochemical and mitochondrial inhibitors into five categories. These categories are: i. electron transport inhibitors; ii. uncouplers; iii. energy transfer inhibitors; iv. inhibitory uncouplers; and v. electron acceptors. The fourth category, the inhibitory uncouplers are compounds that at one condition or concentration, uncouple the phosphorylation process from the electron transport chain and at other conditions or concentrations, block the chain. Moreland (1980) defines the electron transport inhibitors as substances which inactivate one or more intermediate electron transport carriers. These carriers are found in both photochemical and mitochondrial systems. Inhibitors of this type used in the study were DCMU, salicylaldoxime, amytal, quercitin and KCN.

Uncouplers dissociate electron transport from ATP generation by preventing the membrane from reaching an energized state. One uncoupler, CCCP, was used. Energy transfer inhibitors act directly on phosphorylation in coupled systems. Their site of action is removed from that of uncouplers but they still prevent the formation of ATP. While no inhibitor of this type was used in this section, DCCD was used in another study (Chapter Three). Inhibitory uncouplers interfere with reactions affected by both electron transport inhibitors and uncouplers. They may inhibit cyclic and non-cyclic photophosphorylation and oxidative phosphorylation. They usually act as uncouplers at lower molar concentrations. This study involved the use of two such inhibitors, imidazole and DNP.

Electron transport, electron transfer inhibitors and uncouplers were once thought to be specific in their sites of action. It has more recently been suggested that all categories utilized can function at more than one site (Raven 1976). Their specificity is restricted to the type of reactions from the electron transport chain in both the chloroplasts and the mitochondria. Because of this lack of specificity, the results lead to very broad conclusions without giving answers to specific contributions of the various phosphorylating reactions.

It has been noted that light preconditioning tended to reduce transport but light during 1 min. uptake enhanced transport. This would suggest that light itself somehow inhibits or represses the transport system, but that photophosphorylation can provide at least some of the energy for transport. To test this hypothesis, the inhibitors DCMU and CCCP were applied. If these inhibitors are specific, they should

inhibit light but not dark uptake. DCMU inhibited light uptake more than dark uptake in all preconditions except where the cells were preconditioned in the light without glucose and supplied with 1000 ug.L^{-1} experimental glucose. In general the inhibition of DCMU was greater in cells preconditioned in the dark than in the light. This may be due to a higher available energy level in the cells grown in the light so that alternate sources of ATP would be available. CCCP showed strong to partial inhibition under all light conditions. There was very little distinction between light and dark uptake although where difference did occur, it tended to be greater in light transport. Since CCCP is an uncoupler, it is likely that its inhibition will be acting on a number of sites of ATP formation. This would mean that, not only would it act on photo-and oxidative phosphorylation, but it would produce a combined inhibition that was greater than that of DCMU under all circumstances. This was found to be true when comparing the results of the two inhibitors.

Other inhibitors reported to inhibit photophosporylation, salicylaldoxime and DNP, also inhibited light transport, but their influence is also exerted on oxidative phosphorylation so the results are somewhat inconclusive.

To determine whether oxidative phosphorylation and substrate phosphorylation could energize transport, dark uptake in the presence of quercitin, KCN and amytal were compared with dark uptake without inhibitors. Quercitin, an inhibitor of mitochondrial ATPase and pyruvate kinase in animal tissue, was added to determine the influence of subtrate phosphorylation. When preconditioning was in the dark, inhibition was greater in light uptake, but when cells were preconditioned in the

light, inhibition was greater during dark uptake. This would suggest that respiration can drive uptake, at least immediately after moving from dark to light conditions. Also, cells grown in the light, and then placed in the dark can respire quickly after being transferred. The inhibition during light uptake was less than that of the inhibitors that effect photophosphorylation alone or a combination of oxidative and photophosphorylation. This would be anticipated if ATP generated from photosynthesis was more important than that from respiration to cells that are low in energy (dark grown cells). As shown from efficiency calculations in the next section, cells grown heterotrophically, in the dark, are almost 100% efficient therefore they would have very few of their resources tied up as starch. Photophosphorylation would then become more important as an energy source and inhibition by a substance which acts on respiration would have a lesser effect.

KCN and amytal demonstrated only mild inhibition under most of the pretreatments. Another explanation for the low inhibition of these compounds is that they are all known inhibitors of animal respiration but they may not work as efficienctly on algae. Combining the results it is apparent that any source of ATP can be used to drive the transport system.

The results of the inhibitor study also seem to indicate that preconditioning can alter the uptake rate. The addition of glucose in the preconditioning could have two effects on the uptake rate. Firstly, it could induce the synthesis of the transport protein and secondly, it could act as a substrate for oxidative phosphorylation. The first consideration is dealt with in Chapter Three. It is apparent from this

section and Chapter Three that glucose is not required to induce the system. If glucose was used as an energy source, then cells grown in the dark without glucose should have very little organic carbon reserves which could be oxidized to produce ATP. In this instance, light uptake should be strongly inhibited. CCCP and DCMU both illustrated a strong inhibition of glucose transport with light.

The two parts of the transport system did not demonstrate a different response to the inhibitors. The few times there appeared to be any difference may have been due to some experimental error.

From the results of the above experiments, a model can be suggested for the role of heterotrophy in the metabolism of A. braunii. When light and CO_2 are sufficient for photoautotrophic growth, the total accumulation of organic compounds will be derived from both photosynthesis and organic transport. The portion of the assimilated carbon from the external environment will be quite low. The gross rate of organic carbon accumulation (GCA) could be described by equation 1.

$$GCA = PCA + TCA \quad (1)$$

Where PCA is the rate of photosynthetic carbon assimilated and TCA the rate of transported carbon accumulation. When the temperature is 22°C, light intensity 5000 lux, pH 7.2, inorganic medium not limiting and the preconditioning of the cells in a 24 hour dark period prior to transport, the GCA for A. braunii would be:

$$GCA = 0.10298 \text{ pg carbon.cell}^{-1}\text{min.}^{-1} + 0.00122 \text{ pg carbon.cell}^{-1}\text{min}^{-1}$$

$$GCA = 0.10420 \text{ pg carbon.cell}^{-1}\text{min}^{-1}$$

When the external glucose concentration was 100 ug.L^{-1} heterotrophy accounted for less than 2% of the GCA.

Since there were losses due to excretion and respiration these values must be incorporated into equation 1. The result is equation 2 which determines the net carbon accumulation (NCA) rate.

$$NCA = GCA - Ex. - Rs. \quad (2)$$

From the above experiments, excretion rates were determined as 3.5% of GCA or 0.00724 pg carbon.cell^{-1}.min^{-1}. The respiration rates can only be extrapolated from the respiration rates determined in low light. This was approximately 50% of the GCA or 0.05210 pg carbon.cell^{-1}.min^{-1}. It is likely that this results in an overestimation of the respiration rate (Graham 1980). The NCA is then calculated to be 0.04486 pg carbon.cell^{-1}.min^{-1}. If this value is used to determine the potential doubling time of A. braunii under these conditions, the doubling time would be 10.4 hour. The actual doubling time was 15.7 hours. The efficiency of growth under these conditions would then be 66.3%. If the organic carbon content of the medium increased or the light intensity or CO_2 level decreased, the NCA would decrease but the relative contribution of the heterotrophic carbon would increase.

In the dark, the equation would change to:

$$NCA = TCA - Ex. - Rs \quad (3)$$

At an external glucose concentration of 1.0 mM, the TCA is 0.077033 pg carbon.cell^{-1}.min^{-1}. Assuming an excretion rate of 3.5% and a respiration rate of 48%, the NCA would be 0.03736 pg carbon.cell^{-1}.min^{-1}. Also assuming it takes 28.0 pg carbon to

produce one cell, then the doubling time should be 12.5 hours. The comparison with the actual doubling time indicates an efficiency of 56.5% during heterotrophic growth. It appears that heterotrophy has a negligible role in light metabolism but it is an important and efficient energy and biosynthetic carbon source in the dark.

CHAPTER THREE

MECHANISM OF ACTION OF AN ACTIVE TRANSPORT SYSTEM FOR GLUCOSE IN *Ankistrodesmus braunii* (Naeg.)

INTRODUCTION

Knowledge of the actual mechanism of organic substrate transport by cell is essential. Such knowledge may lead to the understanding of the importance of this transport to the cell. It may also permit prediction of environmental influences on uptake rates. Such knowledge may further permit an accounting for variations among transport experiments conducted using different organisms and different substrates but perhaps the single most important use of such information is that it may elucidate the actual potential of a transport system to meet the requirements of the cell for the substrate transported.

Several different mechanisms have been described to account for the movement of organic molecules, across cell membranes. Hamilton (1975), Simoni and Postma (1975) and Dills et al. (1980) reviewed the mechanisms of organic solute transport across bacterial membranes. Nisson (1974) described transport in plant cells in general, and Raven (1976, 1980) reviewed algal transport specifically. It is evident that transport systems can initially be separated into two fundamentally different types. The first type requires no cellular expenditure of energy and is driven by the physical laws pertaining to diffusion. This transport is passive and includes simple diffusion and facilitated diffusion. The second type involves the coupling of expenditure of cellular energy with transport and is active. Simple diffusion of organic solutes across cell membranes does not result in any net concentration difference of solute across the membrane. In extremely low external solute concentrations, such as would be found in natural environments, this method of uptake would be extremely inefficient and could not likely meet cellular

requirements for any high demand substrate. Facilitated diffusion involves the use of a transport system to permit the rapid establishment of a solute equilibrium across the cell membrane, but it does not permit transport against a concentration gradient. Dills et al. (1980) suggest that glycerol is the only organic substrate known to be transported by this process by microorganisms although red blood cells, transport glucose across the cell membrane by this process.

There are several models of active transport. The most prominent of these are a redox model, a permease model, a group translocation and transport model and a chemiosmotic model (Hamilton 1975; Dills et al. 1980). Hamilton (1975) describes the redox model as one in which the carrier responsible for substrate translocation can also function as a redox or electron carrier. The carrier exists in either a reduced or oxidized state and varies in its affinity for the substrate and its orientation within the cell membrane. The oxidized form, with an S-S disulfide bond has a high affinity for the substrate at the outer membrane. On reduction, reorientation of the carrier and a decrease in affinity results in the release of the substrate at the inner surface of the membrane. The model contends that the redox energy supplied to the carrier is derived from coupling with the redox energy of the electron transport chain. To be applicable to eukaryotic cells, this coupling must be indirect and involve the linking of reduced NAD from glycolysis to some redox system in the cell membrane (Hamilton 1975).

Group translocation and transport models presume that the transport of a substrate is coupled with an exergonic metabolic reaction (Hamilton 1975). Membrane bound enzymes would catalyze the chemical modification

of the substrate at the surface of the cell. This modified molecule would then diffuse across the membrane with a concentration gradient and immediately be converted back to the original form or utilized directly in metabolism. An extension of the model might include a chemical reaction at the inside surface of the cell membrane, which would modify the substrate, thus preventing it from reducing the concentration gradient of the original extracellular substrate.

The permease model assumes that the substrate binds with a carrier protein (permease) on the outside of the membrane. The permease-substrate complex then either diffuses across the membrane or undergoes a physical reorientation so that the substrate is directed towards the inside of the cell. The substrate is then split off the permease and released to the inside of the cell. The involvement of cellular energy could be either to enhance the association or dissociation of the substrate with the permease or to enhance the rate of translocation of the permease-substrate complex or free permease (Hamilton 1975).

The chemiosmotic model of transport couples the primary transport of protons or some cation such as Na^+ or K^+ with the secondary transport of the substrate. The energy involvement in this process centres around the maintenance of a proton or cation gradient across the membrane, such that the outside of the membrane has a positive charge and an excess of these ions relative to the inside. This electrochemical gradient then exerts a force (proton motive force, PMF) or energy supply across the membrane which can be used to drive the ions and substrate into the cell (Hamilton 1975). This coupling of proton or cation transport with substrate transport has been referred to as a proton or cation symport

(Hamilton 1975; Komor and Tanner 1973; Eddy 1982). Otto et al. (1980) describes the symport as a bifunctional carrier which translocates both anions and neutral substances and one or more protons or cations. The electrochemical potential of the H^+ or cation gradient across the membrane is made to do the osmotic work of accumulating uncharged molecules.

There have been several different mechanisms of substrate transport described for algae (Wiessner 1970; Komor and Tanner 1974; Pope 1974; and Hellebust and Lewin 1977). When narrowing the literature to transport of smaller organic molecules, there are still several mechanisms suggested. Because of the slow uptake velocities observed and the direct correlation between uptake velocity and substrate concentration, Allen (1969) and Pope (1974) suggest that organic substrate transport may occur by simple diffusion. Given the slowness of diffusion and the low levels of substrate of natural systems, it is unlikely that algae could rely extensively on diffusion of organic substrates for their metabolism.

There are however, descriptions of a number of different active or energy utilizing mechanisms to account for more rapid transport of small organic molecules. North and Stephens (1972) and Lylis and Trainor (1973) have simply stated that certain algae take up organic substrates actively, while making no attempt to describe the type of transport system involved. Komor and Tanner (1974) describe an active transport system involving a carrier-mediated proton symport in the green alga Chlorella vulgaris, and Hellebust (1971) suggests the possibility of some pre or post-phosphorylating mechanism necessary for the transport of glucose by Cyclotella cryptica. Other mechanisms such as a simple

carrier-mediated transport system or some type of facilitated diffusion, have also been suggested (Raven 1980).

This chapter describes experiments which were conducted to determine the actual mechanism of the active uptake of glucose by Ankistrodesmus braunii. The possibility of simple diffusion or facilitated diffusion playing a major role in glucose transport has already been negated (Chapter One) and the temperature dependence of uptake (Appendix II) supports the contention of active uptake. The study was, however, complicated by the biphasic nature of the uptake system (Chapter One). The apparent presence of high and low affinity component of the system may be interpreted in one of four ways (Hoban and Lyric 1977). The system could have two distinct transport carriers, one operating primarily at low glucose concentrations and the other at high; the system is possibly mediated by an allosteric enzyme which loses sensitivity at high substrate levels and thus never becomes saturated; transport may be mediated by an enzyme at low glucose concentration and by diffusion at higher levels; or finally a portion of the transported substrate may subsequently be excreted, either by the same carrier or by diffusion. Each of these possibilities would result in different interpretations of the mechanism of uptake and must be considered.

MATERIALS AND METHODS

1. Source and Maintenance of Algal Cultures:

Ankistrodesmus braunii (Naeg.) cells were obtained and maintained as outlined in Chapter One. The only variation from this method occurred when experiments were conducted on effects of the prehistory of cells on the capacity of that transport system. All experimental cells were harvested in the exponential growth phase, washed four times in sterile, organic - free medium and resuspended in fresh sterile medium at a final concentration of 1×10^8 cells/L. With all inhibitor studies except cycloheximide, cells were exposed to inhibitor for 20 min. prior to the actual experiment. Since several of the inhibitors were weakly soluble or insoluble in water they had first to be dissolved in ethanol, consequently, the influence of applied ethanol concentrations on the glucose transport was also assessed (Appendix IV) and it was shown that ethanol levels used had no influence on transport.

2. Determination of Uptake Rates:

All uptake experiments, unless otherwise stated, were conducted in the following manner. For each treatment, 15 mL of algal suspension, prepared as above, was dispensed into each of 3 culture tubes. Another 15 mL of heat killed (5 min. at 80°C) cells was dispensed into a fourth tube. Following one minute in the presence of substrate the cell suspensions were filtered onto 0.45 μm cellulose acetate membrane filters and washed with 30 mL of prefiltered medium containing 2.0 μm unlabelled glucose. The filters were then placed into scintillation cocktail (Bray 1960) and counted after 24 hours in the dark to avoid chemiluminescence

error. Radio-activity of cells was determined as described in Chapter One. All uptake rates were calculated as outlined in Chapter One. Experiments were conducted at 22°C and 500 lux. Heat killed cells were used as controls and the values obtained from these cells subtracted from the mean of the other three tubes.

3. Requirement for Cellular Energy for Glucose Transport:

Several inhibitors, which are known to disrupt photosynthesis, respiration or both, were added to the algal suspension prior to the uptake experiments. Methods and results of such experiments are outlined in Chapter Two. Results are discussed again in this chapter only as they support the evidence for or against specific transport mechanisms. All inhibitors used throughout the entire study, their abbreviations, effective concentrations and sites of inhibition are listed in Appendix III.

4. Effect of p-Chloro mercuribenzene Sulfonate on Glucose Transport:

Cells were preconditioned by placing them in the dark in the presence of 0.01 mM glucose for 24 hours prior to experimentation. The inhibitor PCMBS was added at final concentrations of 10^{-5}M and 10^{-4}M. The transport of glucose was measured under two external concentrations of U-C^{14} glucose. The low concentration was 10 µg/L (55.6 nM) (specific activity = 310.3 mCi/mmol) and the high concentration was 1000 µg/L (5.6 µM) (specific activity 3.8 mCi/mmol).

5. Effect of Phloretin and Phlorizin on Glucose Transport:

Cells were preconditioned as above. Both inhibitors were added at

a final concentration of 10^{-5}M and 10^{-4}M. Glucose concentrations and specific activities were as above.

6. Effect of Specific Ionophores on Glucose Transport:

Cells were preconditioned as above. Four separate ionophores were utilized. These were gramcidin, monensin, ouabain and valinomycin. Each ionophore was chosen for its ability to block the establishment of a cation or proton gradient across cell membranes (Appendix III). Gramcidin was added at final concentrations of 10^{-7}M and 10^{-6}M. Monensin was added at final concentrations of 10^{-6}M and 10^{-5}M. Ouabain was added at final concentrations of 10^{-6}M and 10^{-5}M and valinomycin at final concentrations of 10^{-7}M and 10^{-6}M. Glucose concentrations were as above.

7. Effect of pH on the Transport of Glucose:

Cells were preconditioned as above. Prior to experimentation the cells were further acclimatized by placing aliquots in each of eight different pH solutions for 15 minutes. Solutions of pH 3-5 were buffered with 0.025M Na-citrate. Solutions of pH 6-8 were buffered with 0.025M Na-phosphate and solutions of pH 9 and 10 were buffered with 0.025M Tris buffer (Komor and Tanner, 1974). The pH of suspensions was tested immediately prior to and following the experiments. Uptake was measured at external glucose concentrations of 1 µg/L, 10 µg/l, 100 µg/L and 1000 µg/L. For experiments using 1 and 10 µg/L U-C^{14} glucose, the specific activity was 291.6 mCi/mmol and for 100 and 1000 µg/L the specific activity was 4.5 mCi/mmol.

8. Effect of Preconditioning on Glucose Transport:

Cells were subjected to four different combinations of environmental conditions for 24 hours prior to the uptake experiments. These included, dark with 0.01 mM glucose; dark with no glucose; 5000 lux light with 0.01 mM glucose; and 5000 lux light with no glucose. Glucose concentrations tested were as in the previous section. Experiments were conducted as above.

9. Effect of Cycloheximide on Glucose Transport:

The protein synthesis inhibitor cycloheximide was added at a final concentration of 10^{-5}M three hours prior to experimentation. Two different prehistories were tested. One culture was placed in the dark with 0.1 mM glucose for 21 hours then the cycloheximide was added and they were replaced in the dark for 3 hours. The second culture was kept in 5000 lux light with 0.01 mM glucose for 21 hours, the cycloheximide added and this followed by replacement in the dark for three hours. Experiments were then conducted using the same glucose concentration as in the previous experiments on preconditioning.

10. Effect of Time on the Uptake of Glucose by Cells with Different Prehistories:

Cells were preconditioned using the same four different environmental conditions as in part 9. Uptake was then measured at 1, 5, 10, 15, 30, 60 and 120 minute intervals. During this time period, the cells were exposed to 10 µg/L U-C^{14} glucose (specific activity of 291.6 mCi/m mol) and kept in the dark.

RESULTS

1. Requirement for Cellular Energy:

The results of the inhibitor studies are outlined in Tables 3-1a and 3-1b. These results represent a portion of the metabolic inhibitors tested in Chapter Two. All of these inhibitors affect the ability of the cells to produce energy in the form of ATP. The uncouplers CCCP and DNP reduced uptake by up to 100% and 70.1% respectively. The electron transport chain inhibitors KCN and salcylaldoxime inhibited transport by up to 67.3% and 86.8% respectively. The ATPase inhibitor DCCD reduced uptake by 90.6%. The inhibitor of photosystem II, DCMU reduced transport in the light by up to 76.7%.

2. Effect of PCMBS on Glucose Transport.

The inhibitor PCMBS acts on membrane proteins by altering the -SH bonds in the proteins. Application of this inhibitors resulted in a decrease in glucose transport of 85.2% at an external glucose concentration of 10 µg/L and 70.6% at a glucose concentration of 1000 ug/L.

3. Effect of Phloretin and Phlorizin on Glucose Transport:

The addition of these two inhibitors, which are known to block the action of phosphorylase enzymes, had very little effect on the transport rates of glucose. At a glucose concentration 10 µg/L, phloretin reduced uptake by 35.2%, while phlorizin inhibited uptake by only 32.0%. At a glucose concentration of 1000 ug/L, phloretin inhibited uptake by 73.2%, while phlorizin, the stronger of the two inhibitors, reduced uptake by only 19.1%.

TABLE 3-1a: Percentage inhibition of d-glucose[1] assimilation by A. braunii[3] caused by metabolic inhibitors

Inhib. Conc.(M)	CCCP	DCCD	DCMU	DNP	KCN	Salcylaldoxime
10^{-6}	50.0	-	46.1	-	-	-
10^{-5}	75.3	-	37.6	39.2	28.9	-
10^{-4}	-	45.2	-	68.1	50.1	-
10^{-3}	-	90.6	-	-	66.4	62.6
10^{-2}	-	-	-	-	-	82.7

TABLE 3-1b: Percentage inhibition of d-glucose[2] assimilitation by A. braunii[3] caused by metabolic inhibition

Inhib. Conc.(M)	CCCP	DCCD	DCMU	DNP	KCN	Salcylaldoxime
10^{-6}	40.0	-	79.4	-	-	-
10^{-5}	100.0	-	76.7	23.6	0.0	-
10^{-4}	-	38.1	-	70.1	28.6	-
10^{-3}	-	90.2	-	-	67.3	33.5
10^{-2}	-	-	-	-	-	86.8

1. Final concentration of glucose 10 µg/L (55.6 nM).
2. Final concentration of glucose 1000 µg/L (5.6 µM).
3. Cells preconditioned with 24 hr. dark and 0.02 mM glucose.

4. Effects of Specific Ionophores on Glucose Transport:

The four ionophores added to the cultures prior to the uptake experiments, demonstrated a minimal inhibition of glucose uptake (Tables 3-2a and 3-2b). Only the ionphore monensin, which disrupts Na^+ / H^+ exchange across membranes, reduced uptake by more than 50% (57.8%), at an external glucose concentration of 1000 µg/L. Even this inhibitor only reduced transport by 32.6% at an external glucose concentration of 10 µg/L.

5. Effect of pH on the Transport of Glucose:

The results of the effects of altering the external pH on the rate of transport of glucose are illustrated in Figures 3-1a and 3-1b. These suggest that there is very little difference in uptake rates of glucose within a pH range of 5 to 9 at 1 µg/L and 10 µg/L external glucose. There is also little difference within a pH range of 5 to 8 at a glucose concentration of 100 µg/L and 1000 µg/L. Beyond these ranges of pH, glucose transport was severely curtailed.

6. Effect of Preconditioning on Glucose Transport:

The results of these experiments are listed in Table 3-3 and illustrated in Figures 3-2a and 3-2b. From these results, and others reported in Chapter Two, it is evident that the prehistory of the cells can play an important role in determining the transport capacity of the cells. The results indicate that the most important aspect of preconditioning is the absence of light. Cells preconditioned in the dark had from 2.2 to 5.3 times greater uptake velocities than those which were

TABLE 3-2a: Percentage inhibition of d-glucose[1] assimilation by A. braunii[3] caused by membrane ionophores

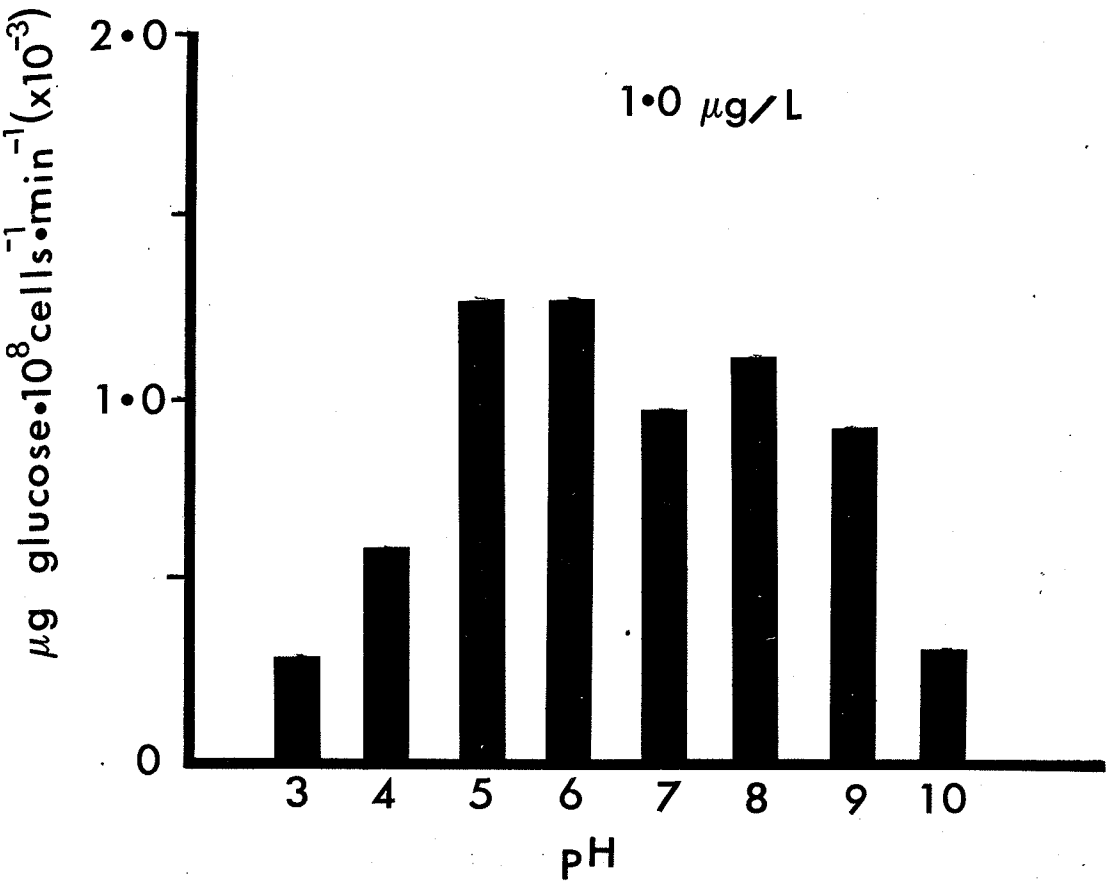
Ion. Conc.(M)	Gramcidin	Monensin	Ouabain	Valinomycin
10^{-7}	29.9	-	-	8.2
10^{-6}	14.9	26.4	4.2	39.8
10^{-5}	-	32.6	31.9	-
10^{-4}	-	-	-	-

TABLE 3-2b: Percentage inhibition of d-glucose[2] assimilation by A. braunii caused by membrane ionophores

Ion. Conc.(M)	Gramcidin	Monensin	Ouabain	Valinomycin
10^{-7}	0.0	-	-	22.9
10^{-6}	41.4	5.1	11.0	23.3
10^{-5}	-	57.8	22.0	-
10^{-4}	-	-	-	-

1. Final concentration of glucose 10 µg/L (55.6nM).
2. Final concentration of glucose 1000 µg/L (5.6 µM).
3. Cells preconditioned with 24 hr. dark and 0.02 mM glucose.

FIGURE 3-1a: The effects of pH on the transport rates of glucose at external glucose concentrations of 1 µg/L (5.6 nM) and 10 µg/L (55.6 nM).


1•0 μg/L
μg glucose•10^8cells^-1•min^-1(x10^-3)
2•0
1•0
0
3 4 5 6 7 8 9 10
pH

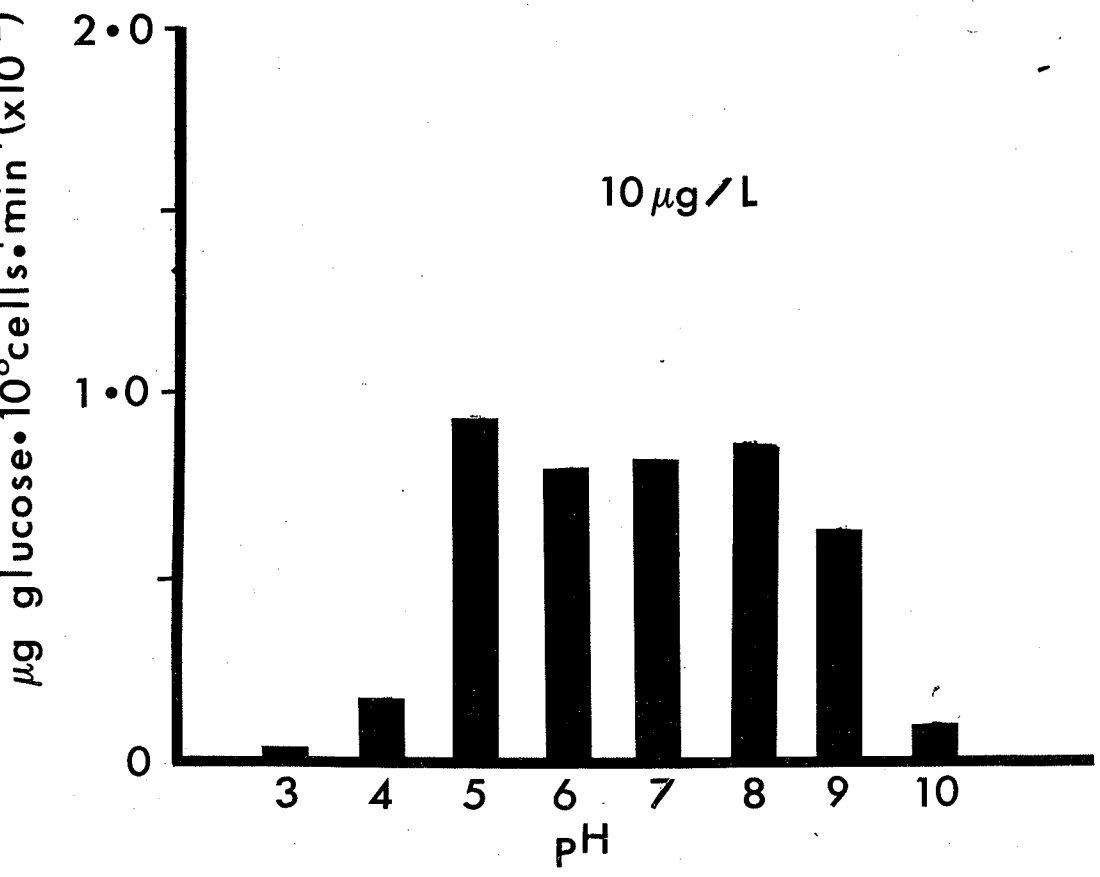
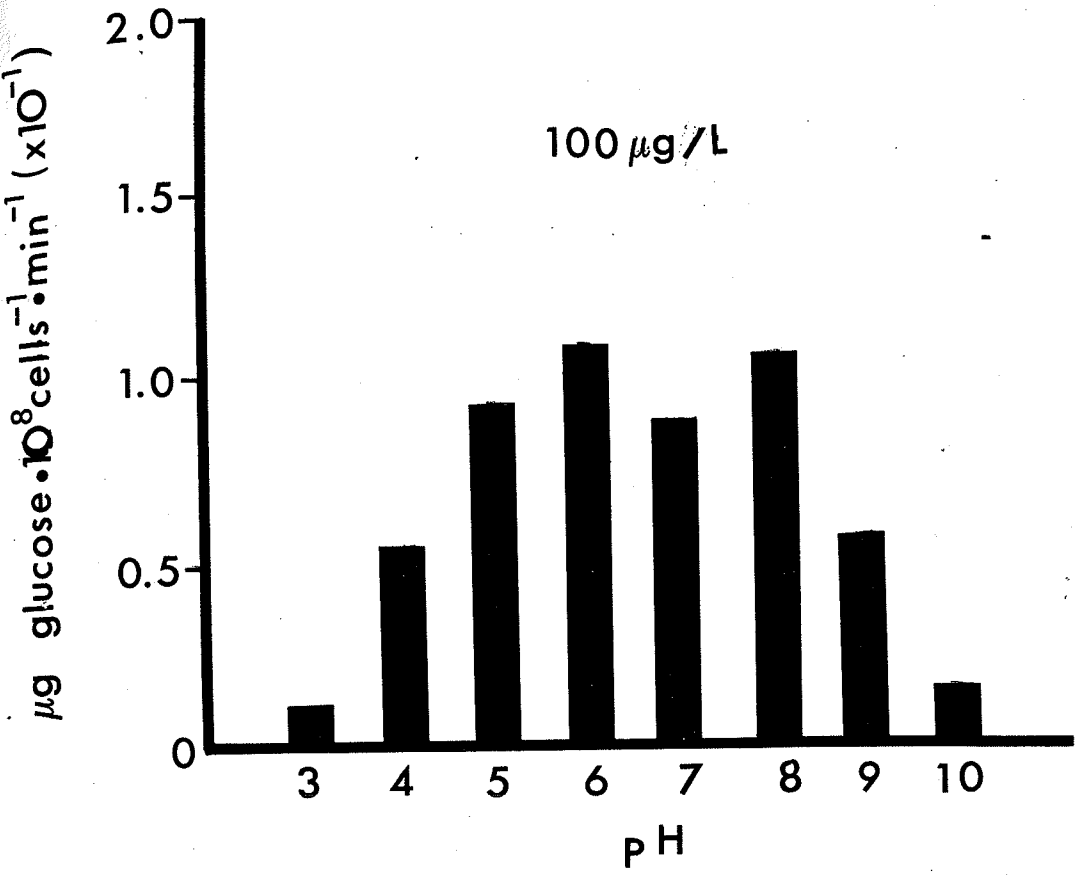
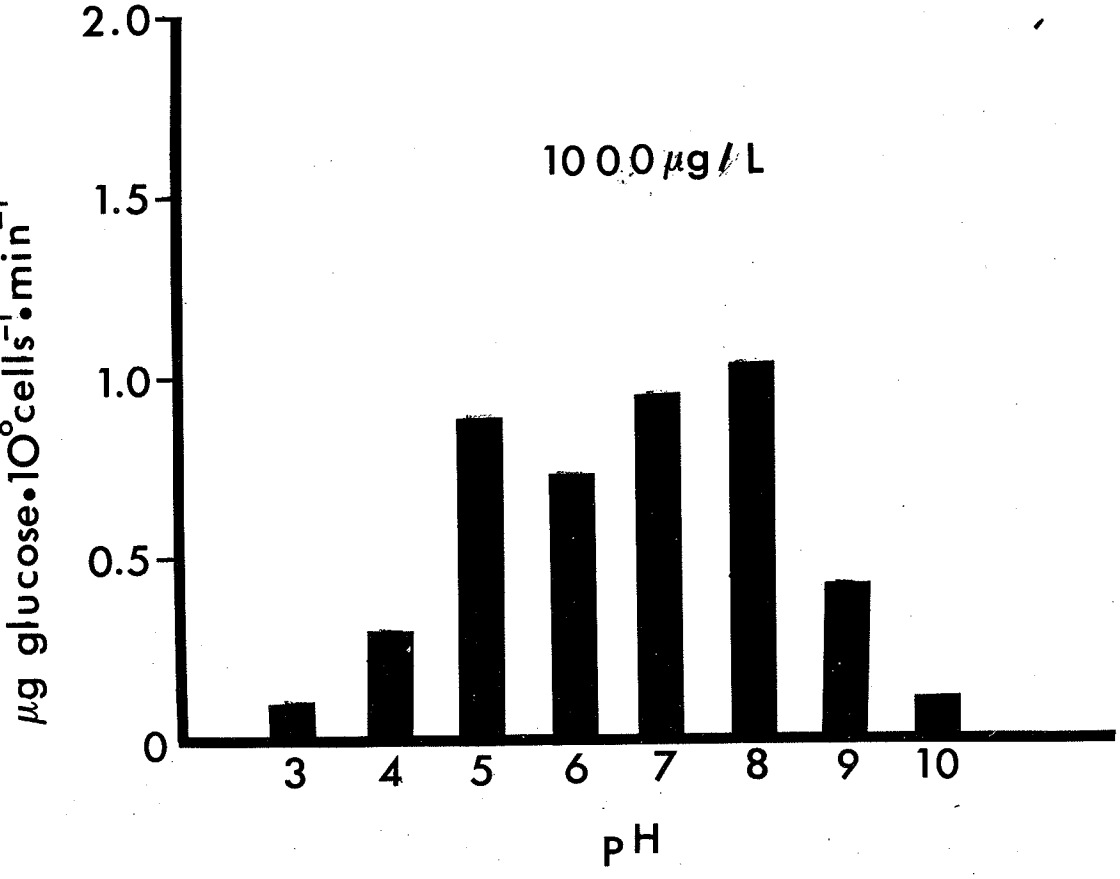

10 μg/L
μg glucose•10^8cells^-1•min^-1(x10^-2)
2•0
1•0
0
3 4 5 6 7 8 9 10
pH

FIGURE 3-1b: The effects of pH on the transport rates of glucose at external glucose concentrations of 100 μg/L (0.56 μM) and 1000 μg/L (5.56 μM).


100 μg/L
μg glucose•10^8cells^-1•min^-1 (x10^-1)
2.0
1.5
1.0
0.5
0
3 4 5 6 7 8 9 10
pH


1000 μg/L
μg glucose•10^8cells^-1•min^-1
2.0
1.5
1.0
0.5
0
3 4 5 6 7 8 9 10
pH

preconditioned in the light. Preconditioning with glucose in the light or dark had little effect on the uptake rates of the cells, and in fact those cells that were preconditioned with glucose had a slightly lower uptake rate than those without glucose.

7. Effect of Cycloheximide on Glucose Transport:

The effect of adding cycloheximide, a known inhibitor of protein synthesis, was to reduce the transport rate of dark grown cells by 74.5% to 82.4% (Figures 3-2a and 3-2b). There was no effect on the transport of glucose by light grown cells. The reduction of transport in dark preconditioned cells brought down their transport rates to those of light grown cells.

8. The Effect of Time on the Uptake of Glucose:

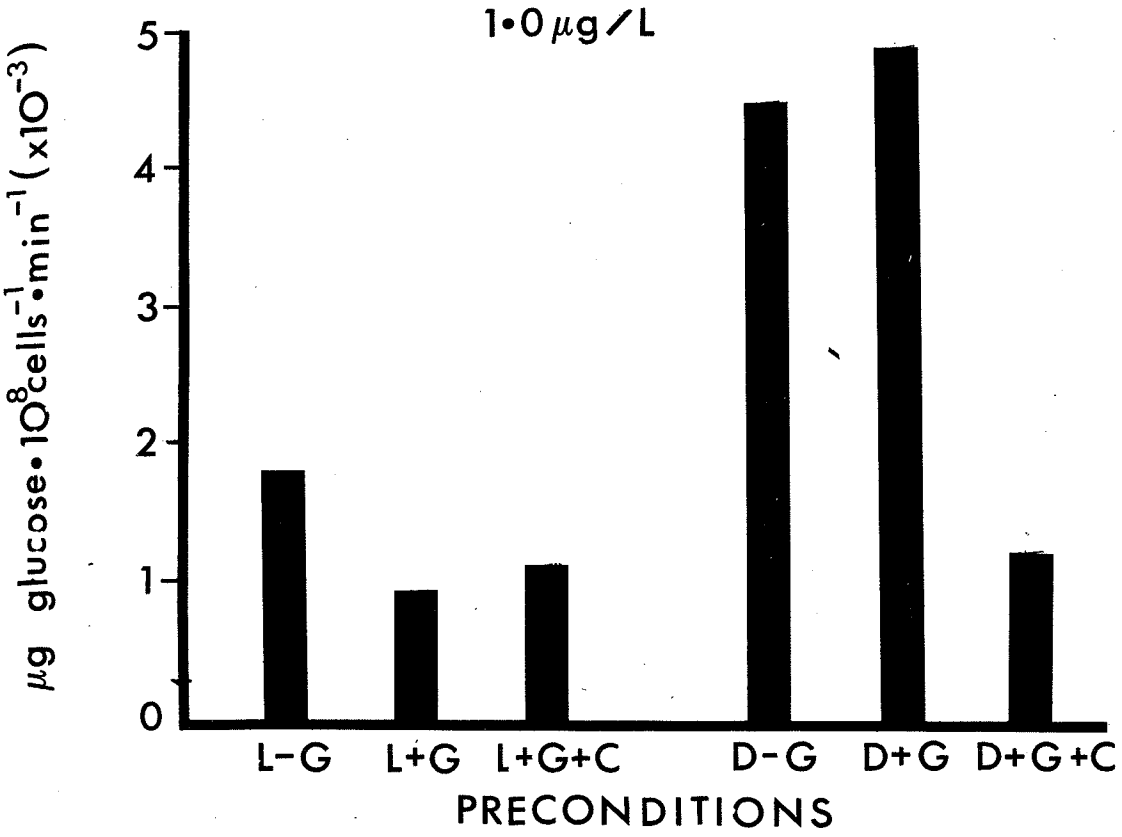
The results of these experiments are illustrated in Figure 3-3. Cells preconditioned in the dark,with or without glucose, began to transport immediately, although initially, (1-2 min.) cells preconditioned with glucose had a slightly higher uptake rate. There was very little difference in uptake rates over time in any cells preconditioned in the dark. Cells grown in the light and 0.01 mM glucose had a slow initial transport rate but this increased considerably after 10-15 min. Cells grown in the light, with no glucose, did not begin rapid glucose uptake until after 30-60 min.

TABLE 3-3: The effect of different preconditioning treatments on the uptake of d-glucose by A. braunii. All results are expressed as μg glucose.10^8 cells^{-1}.min^{-1}. Bracketed numbers are percent standard errors.

Substrate level provided μg/L	Light	Light + glucose[1]	Dark	Dark + glucose[1]
1.0	0.002 (±6.7)	0.001 (±8.2)	0.004 (±6.1)	0.005 (±13.4)
10.0	0.010 (±4.9)	0.006 (±18.2)	0.226 (±3.0)	0.019 (± 5.5)
100.0	0.158 (±4.7)	0.090 (±8.4)	0.353 (±5.7)	0.406 (± 5.7)
1000.0	0.949 (±5.7)	0.483 (±6.5)	2.238 (±3.3)	2.221 (±13.7)

1. Glucose used in preconditioning was at a concentration of 0.01 mM.

FIGURE 3-2a: Effects of preconditioning and the inhibitor cycloheximide on the uptake of glucose at a final concentration of 1 μg/L and 10 μg/L. Preconditions were: light minus glucose (L-G); light plus glucose (L+G); light plus glucose and cycloheximide (L+G+C); dark minus glucose (D-G); dark plus glucose (D+G); dark plus glucose and cycloheximide (L+G+C).


1•0 μg/L
μg glucose • 10^8 cells^-1 • min^-1 (x10^-3)
5
4
3
2
1
0
L-G
L+G
L+G+C
D-G
D+G
D+G+C
PRECONDITIONS

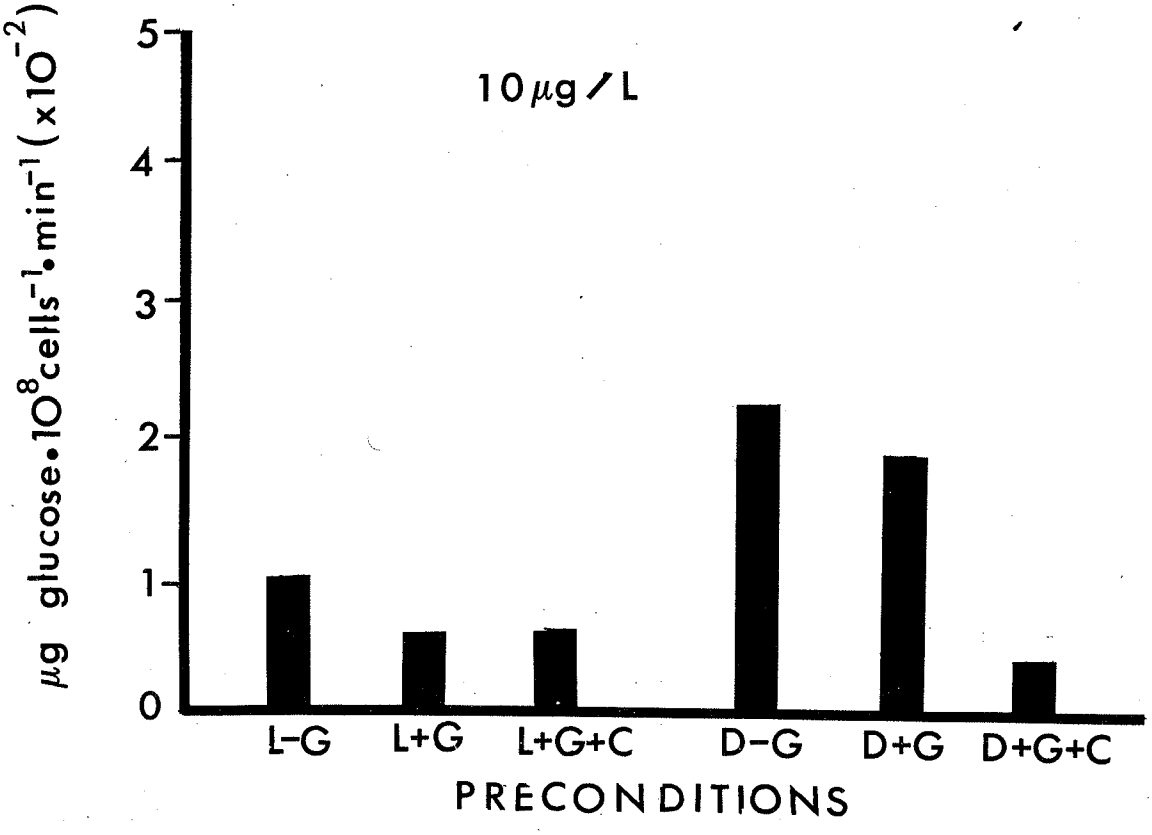
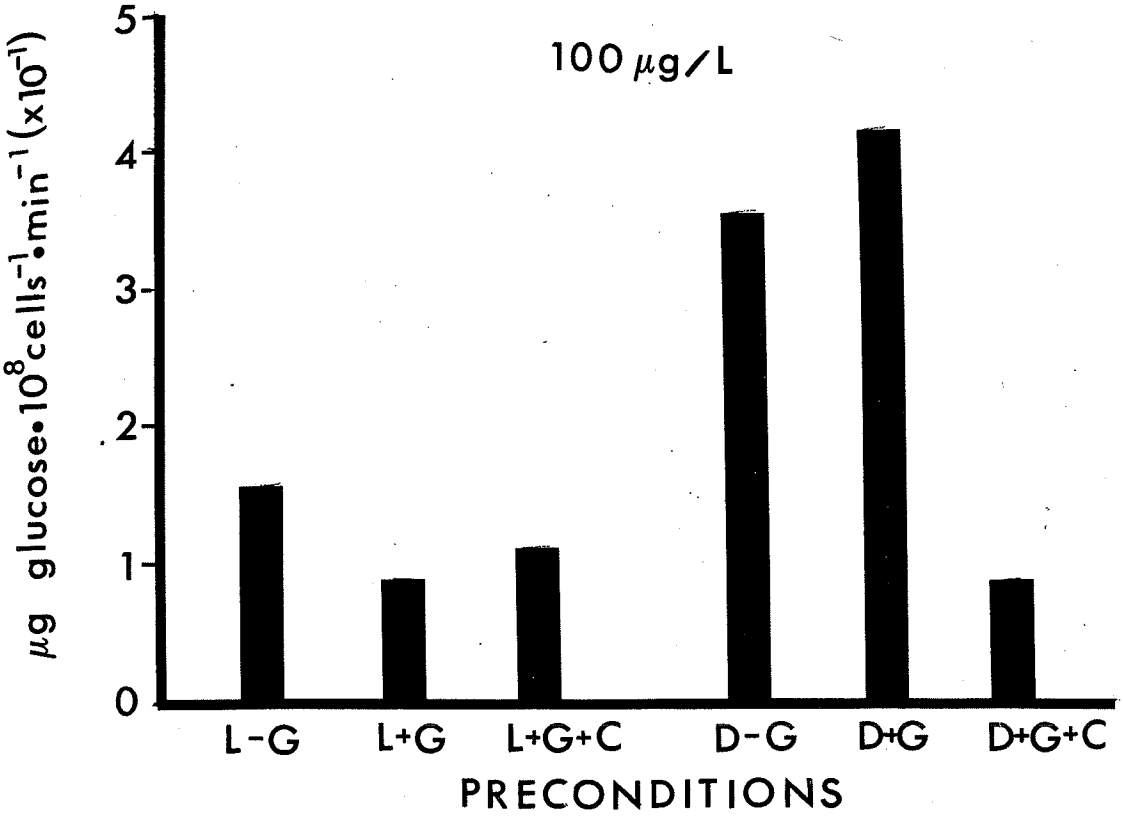

10 μg/L
μg glucose • 10^8 cells^-1 • min^-1 (x10^-2)
5
4
3
2
1
0
L-G
L+G
L+G+C
D-G
D+G
D+G+C
PRECONDITIONS

FIGURE 3-2b: Effects of preconditioning and the inhibitor cycloheximide on the uptake of glucose at a final concentration of 100 µg/L and 1000 µg/L. Preconditions were as in Fig. 3-2a.


100 μg/L
μg glucose•10^8cells^-1•min^-1(x10^-1)
5
4
3
2
1
0
L-G
L+G
L+G+C
D-G
D+G
D+G+C
PRECONDITIONS

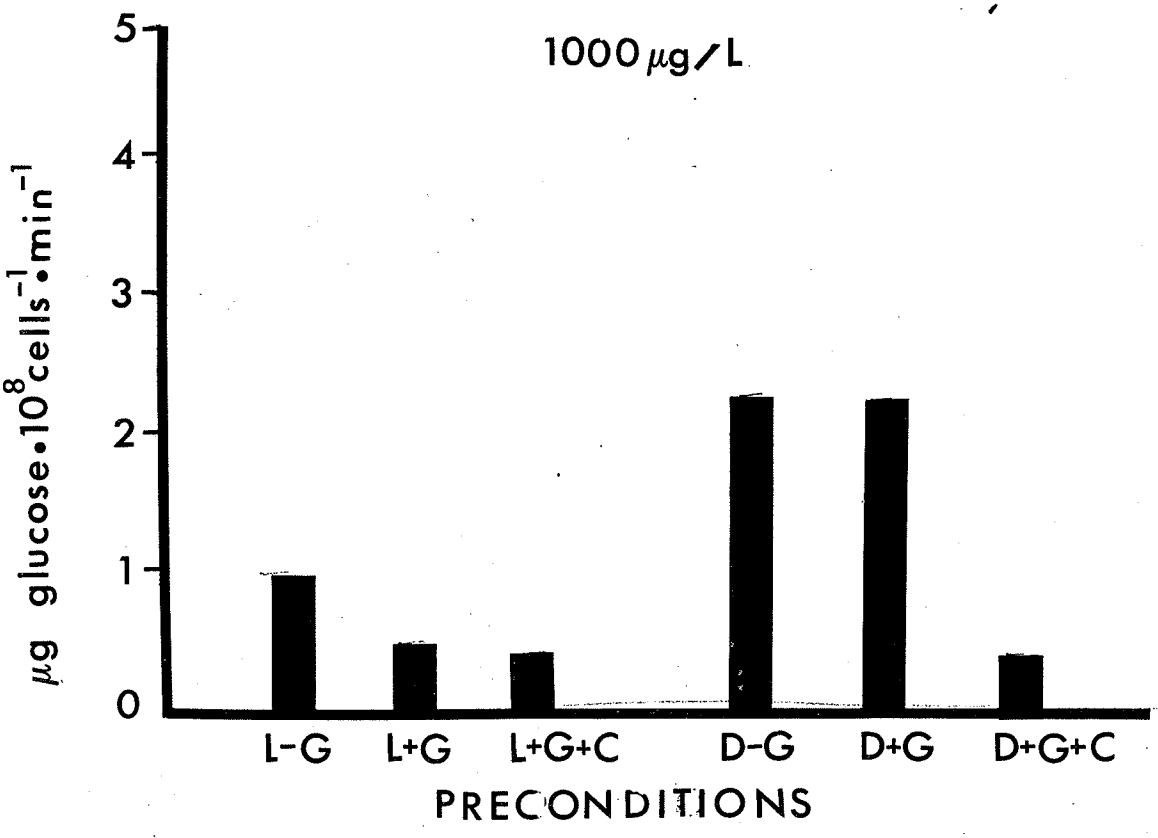
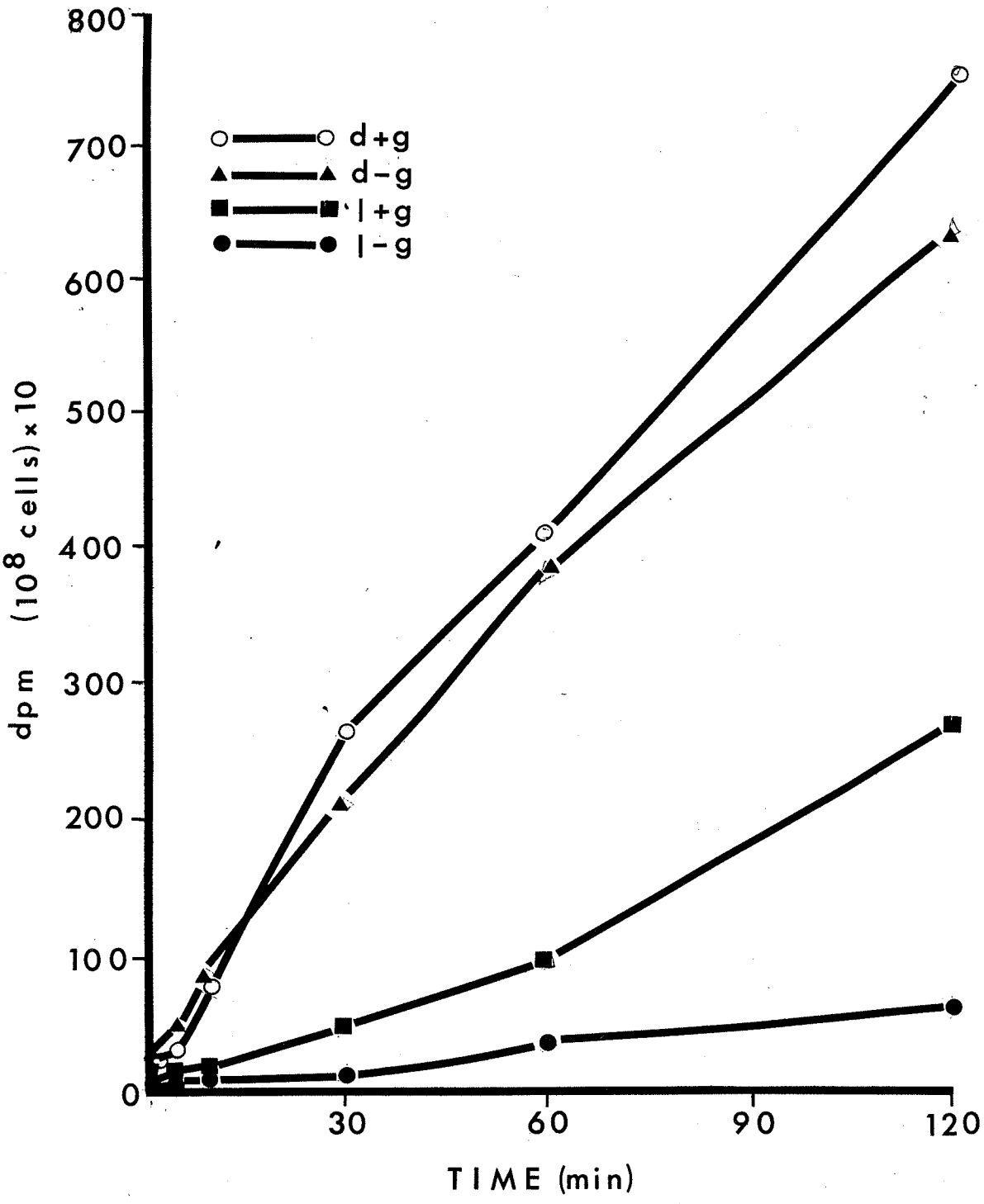

1000 μg/L
μg glucose•10^8cells^-1•min^-1
5
4
3
2
1
0
L-G
L+G
L+G+C
D-G
D+G
D+G+C
PRECONDITIONS

FIGURE 3-3: Effects of time on the uptake of glucose (10 μg/L) by cells that have undergone different preconditioning treatments. Preconditions were 0.01 mM glucose plus dark (d+g), 0.01 mM glucose plus light (l+g), no glucose plus dark (d-g) and no glucose plus light (l-g).


800
700
600
500
400
300
200
100
0
d+g
d−g
l+g
l−g
dpm (10⁸ cells) × 10
30
60
90
120
TIME (min)

DISCUSSION

The first possible mechanism for glucose transport by A. braunii tested, was passive uptake by simple diffusion and/or facilitated diffusion. Five points of evidence suggest that neither of these mechanisms could account for the uptake of glucose by Ankistrodesmus braunii. Firstly, the rates of transport (Chapter One) were too rapid to be accounted for by simple diffusion. They were far more rapid than those attributed to algal diffusion of glucose and acetate (Wright and Hobbie, 1965b and Allen, 1969). Secondly, the ability to concentrate glucose at levels greater than extracellular glucose is contrary to the potentials of either diffusion or facilitated diffusion. A. braunii cells were found to contain labelled glucose at approximately 50 times external levels after 2h. Thirdly, if active transport was eliminated in the heat-killed dead cell controls then any label found in these cells must be due to a combination of accumulation in the cell wall or unstirred water layer (Winne 1973; Podesta 1977) and diffusion. It was found that the amount of glucose in these cells ranged from 0.1 to 0.001 that in the intact cells. A fourth consideration is the effect of temperature change on glucose transport (Appendix II). The rate of uptake of glucose at 30 µg/L and 825 µg/L external glucose concentration showed a considerable decline between 20 and 0°C. The $Q_{10(10-20)}$ and $Q_{10(0-10)}$ values were 1.9 and 2.4 respectively. These values correspond favourably with Q_{10} values reported for active glucose uptake (Hellebust 1971 Komor et al. 1971). Finally, the dependence of transport on metabolic energy (Chapter Two) provides conclusive evidence in support of an active

transport mechanism. Based then upon an assumption of active transport, the results of the remaining experiments were examined with the intent of determining the actual mechanism involved.

The first potential mechanism examined followed the redox model. The possibility that this mechanism is responsible for glucose uptake by A. braunii is remote. It relies on the coupling of an electron doner with S-S disulfide bonds and reducing these bonds to SH-SH bonds (Dills et al. 1980). There are several points of evidence to suggest that this mechanism is not operative in A. braunii. Firstly, the only source of this reducing power available to surface membrane proteins would be from glycolysis. If this limited source of energy was sufficient to account for the uptake of glucose, then inhibitors of photosynthesis should have had no effect on uptake. DCMU, an inhibitor of photosystem II reduced uptake by up to 79.4% (Table 3-1b). Uncouplers of photophosphorylation and oxidative phosphorylation should also have had no effects, but the uncouplers CCCP and DNP reduced transport by 100% and 70% respectively (Table 3-1b). ATPase inhibitors also should have a negligible effect on glucose transport, but DCCD an inhibitor of ATPase activity, reduced uptake by 90.6%.

The only evidence to support this model as the mechanism for glucose transport was the inhibition by PCMBS. This inhibitor, a sulfhdryl reagent, which acts on membrane protein -SH bonds reduced uptake by 85.2% and 70.6% at glucose concentrations of 10 and 1000 µg/L respectively. Since these bonds are essential to the model (Hamilton 1975), then this inhibition could be interpreted as preventing the reduced form of the carrier from being produced and thus preventing uptake (Lepley and

Mukkada 1983). This inhibition could also be explained if any membrane protein involved in any type of transport, had -SH bonds. Since these bonds are responsible for the tertiary structure of proteins, then their disruption would result in configurational changes in the proteins which would probably render them inactive. From this evidence, it seems highly unlikely that a redox carrier model can provide an appropriate explanation for glucose transport by A. braunii.

The group translocation model also seemed to have limited, if any, application to glucose transport by A. braunii. The key difference between this mechanism and other active systems is that the substrate itself becomes modified and is transported into the cell in the modified state. The enzyme or enzymes responsible for the modification of the substrate would have to be located at the outer surface of the membrane (Dills et al. 1980). This mechanism has largely been described for bacteria (Dills et al. 1980) and mammalian intestinal cells (Hamilton 1975). Hellebust (1971) and Nisson (1974) reported examples of plant translocation in which the sugar translocated had been rapidly phosphorylated (99.4% glucose transported in 10 min. Hellebust 1971). A suggestion was made that this phosphorylation occurred prior to uptake but no direct evidence was cited. The most popular type of group translocation involves the membrane phosphorylation of the substrate. The source of the phosphate and energy for this reaction is phosphoenolpyruvate (PEP) from glycolysis. This PEP phosphotransferase system (PTS) has been reported to be involved in sugar transport in obligate and facultative anaerobic bacteria (Dills et al. 1980). The PEP dependent transferase systems are insenitive to cyande and unaffected by uncouplers of oxidative

phosphorylation (Wood and Kelly 1982). To test for this mechanism of glucose uptake, the diphenolic glucoside inhibitor phlorizin and its aglucone phloretin were added to the cells. At its highest concentration, phlorizin showed 32% and 19% inhibition of uptake at glucose levels of 10 and 1000 µg/L respectively. Phloretin reduced transport by 35.2% and 73.2% at these glucose levels. Since these inhibitors act on phosphorylase enzymes, and since except in one instance, they demonstrated limited inhibition of uptake, it appears unlikely that this mechanism is responsible for glucose transport by A. braunii. Another point of evidence against this mechanism lies in the source of energy. Since two glucose molecules could be transported for every glucose molecule converted to PEP via the Embden-Meyerhof pathway, then this uptake should be self-regenerating. Inhibitors such as DCMU and KCN should not effect the transport and yet they were shown to reduce uptake by 76.7% and 67.3% respectively (Table 3-1b). Also, quercitin, an inhibitor of pyruvate kinase, inhibited glucose uptake by 85% (Chapter Two). This should not have effected the availability of PEP. It is more likely to have inhibited transport by preventing oxidative phosphorylation. Thus it appears highly improbable that group translocation is the mechanism responsible for glucose uptake by A. braunii.

The two most probable mechanisms for glucose uptake are the chemiosmotic or permease models. Both of these models have been suggested (if not in name) as the mechanism for glucose uptake in algae. Tanner and his coworkers in a series of papers (Tanner 1969; Komor and Tanner 1971; Komor and Tanner 1973a; Komor and Tanner 1973b; Haass and Tanner 1974; Komor and Tanner 1974; Komor and Tanner 1975; Grunebeck and

Komor 1976; Komor and Tanner 1976; Fenzl et al. 1977; and Komor et al. 1978) demonstrated that glucose uptake by the freshwater alga Chlorella vulgaris is mediated through a proton symport mechanism. These workers thoroughly outlined a biphasic, pH dependent transport system. Hellebust and Lewin (1977) and Hellebust (1978) described a somewhat different system of uptake for the marine diatom Cyclotella cryptica, Although they did not actually postulate the exact mechanism of uptake, these authors implied the presence of a carrier mediated system or systems similar to the permease model of Hamilton (1975). Hellebust (1978) did demonstrate a strong Na+ dependency for glucose transport. He attributed this dependency to either a Na+ requirement for effective binding of the organic solute to the carrier as in a permease model or the need for a Na+ gradient which would establish a type of chemiosmotic force as in the symport model.

Both these works and others (Nisson 1974) suggest glucose is transported by a biphasic or multiphasic system operating with different kinetics at different substrate concentrations. This could be the result of two or more separate transport proteins, one allosteric transport protein or one transport protein and simple diffusion at higher substrate concentrations (Hoban and Lyric 1977).

To test the applicability of the chemiosmotic model to glucose transport by A. braunii, the uptake was measured at different pH from 3-10. The results (Figure 3-1a and 3-1b) indicated that uptake was not dependent on pH any more than any enzyme reaction would be. Since external proton concentration did not effect the rate of uptake, it seems unlikely that a proton motive force is responsible for transport. The

application of monensin, an ionophore specific for Na+/H+ exchange across membranes, had only a minimal effect on uptake (Table 3-2a and 3-2b). This again supports the argument that a chemiosmotic model is not appropriate for glucose transport by A. braunii. The minimal inhibition by the ionophores gramcidin, ouabain and valinomysin (Table 3-2a and 3-2b) also suggest that glucose transport is not coupled with transport of another cation. While there is some inhibition by all these ionophores, it is more likely that they are acting at the chloroplast or mitochondrial membrane level inhibiting ATP formation. This system then differs from that reported in Chlorella vulgaris by not depending on a proton gradient. It also differs from that reported in Cyclotella cryptica by not showing a strong Na+ dependency. This would be ecologically sound, since C. cryptica is a marine alga, living in an environment of high Na+ levels and high pH. A. braunii is an ubiquitous freshwater alga existing in both alkaline and acidic waters.

The strong inhibition by uncouplers of oxidative and photophosphorylation (Table 3-1a and 3-1b) and ATPase enzymes suggest that glucose uptake is dependent on metabolic energy in the form of ATP derived from photosynthesis and/or respiration. Quercitin, which also inhibits transport ATPases reduced glucose uptake by up to 85% (Chapter Two). All of the evidence appears, by the process of elimination, to support the permease model as the most likely mechanism for glucose transport by A. braunii. Since the uptake is affected in the same fashion and usually to the same degree at both high and low substrate levels, it is unlikely that the biphasic nature of the system is due to the presence of two enzymes. The rapid uptake and inhibition at high external glucose levels

tends to negate the possibility of facilitated diffusion operating at this level. While the allosteric permease systems appear to be a viable alternative, the transport kinetics do not follow the pattern of described allosteric reactions. The fourth alternative is that a portion of transported substrate is subsequently excreted. This excretion could be through diffusion, or carrier-mediated. The carrier could be independent of the uptake system or the same carrier that initially transported the glucose into the cell. Since the system does not appear to be saturated, then diffusion appears to be more probable.

The form of the carrier system also differs somewhat from that of Chlorella vulgaris (Komor and Tanner 1973a) and Cyclotella cryptica (Hellebust 1978) with respect to its formation. Both of these algae appear to have inducible transport systems, although the stimulus for induction is different. This implies that their transport proteins are relatively short lived and they depend on particular environmental conditions to keep them continually supplied. Chlorella vulgaris requires external glucose and a 1h dark period for induction of the system (Komor and Tanner 1971). Cyclotella cryptica requires only an extended dark period with no external glucose (Hellebust 1971). A. braunii appears to have a certain amount of constitutive carrier protein that is always presents, even in light of 5000 lux and no external glucose (Figures 3-2a and 3-2b). The cells can be induced to produce additional protein in the dark (Fig. 3-2a and 3-2b). This is further substantiated by the effects of cycloheximide an inhibitor of protein synthesis. The addition of this inhibitor had little effect on the uptake of light grown cells, in the presence of glucose, but it reduced the uptake of dark grown cells to the

level found in the light grown cells (Fig. 3-2a and 3-2b). Time-course studies (Fig. 3-3) support this argument for dark grown cells. Cells preconditioned with a 24h dark period with or without glucose began rapid uptake of glucose immediately and proceeded at almost the same rate for 2h. Transport by light preconditioned cells over a 2h time period was somewhat inconsistant with the other results concerning induction. Those grown in the light with glucose had slow initial transport rates, but increased rapidly after 10-15 min. in the dark. This would suggest a light inactivation of the carrier. Cells preconditioned with light and no glucose, did not begin rapid uptake until 30-60 min. after being placed in the dark. This time is consistent with the time required for induction of a carrier system. The only explanation for the discrepancy in these cells is either experimental error, or a greater proportion of the cells were in a portion of their life cycle which was not conducive to rapid uptake (Chapter Four).

SUMMARY

1. Ankistrodesmus braunii has an active transport mechanism for the uptake of glucose.

2. The transport mechanism appears to be a biphasic permease carrier.

3. A. braunii can utilize oxidative and/or photophosphorylation to generate the ATP necessary to drive the transport system against a concentration gradient.

4. The permease carrier is maintained at a certain low active threshold during periods conducive to photosynthesis but this threshold can be greatly increased either through activation or induction of synthesis during periods of minimal photosynthesis.

CHAPTER FOUR

CONTRIBUTIONS OF HETEROTROPHIC GROWTH DURING THE CELL CYCLE OF *Ankistrodesmus braunii* (Naeg.)

INTRODUCTION

As a cell develops through its life cycle, various anatomical and physiological changes take place which reduce the effectiveness of the photosynthetic apparatus (Tamiya 1964; Pickett-Heaps 1975). Consequently, the role of heterotrophy during the stages of the cell cycle of Ankistrodesmus braunii is worthy of investigation. It has been noted, that as a cell moves from an active growth phase to a division phase, there is a severe curtailment of its rate of photosynthesis (Tamiya, 1964; Takeda and Hirokawa 1982). Young cells, newly produced from mother cells, are most active photosynthetically. As cells reach their first and second mitotic division states and early cytokinesis, photosynthesis is lowest and endogenous respiration is high (Tamiya 1964). This would suggest that the cells would have a high energy demand while photophosphorylation energy and carbon fixation are at their lowest level. Starch reserves or some low molecular weight organic carbon pool may satisfy this demand, but there is a possibility that heterotrophy could be significant at this time in the cell cycle. Consequently, transport of organic materials could increase at this time in the cycle either through the removal of the light repression or inactivation of the transport system, or stimulation of synthesis of new transport proteins. Since the nucleus is involved in mitosis at this time, the latter is less likely. Different transport rates for substances such as uracil occur during the cell cycle (Knutsen 1972) but since this compound is directly involved in nucleic acid synthesis, this would be expected.

Komor et al. (1973), reported a variability of almost a factor of

two in the uptake kinetics of various batch cultures of Chlorella vulgaris, but fully reproducible results between samples of the same batch culture. This phenomenon was also noted in this study. If there is a variation in uptake rates at different stages in the cell cycle, and different batch cultures had different proportions of cells at different phases of growth, the variability might be explained.

In an attempt to further increase the understanding of the potential importance of heterotrophy, this study examined the cells at different stages of the cell cycle. Cultures were synchronized, then examined throughout a complete cycle. The cycle itself is described, transport and photosynthetic measurements were conducted at different stages and an electron microscopy study was done. The changes in ultrastructure were then related to the uptake and photosynthesis results.

MATERIALS AND METHODS

1. Source and Maintenance of Algal Cultures:

Ankistrodesmus braunii (Naeg.) cells that were used in the initial inoculum for all the experiments in the Chapter were obtained and maintained in the same manner as outlined in Chapter One. The cultures were always used in their exponential growth phase. Periodic examinations of the purity of the cells were conducted according to the methods in Chapter One.

2. Establishment of Synchronous Culture:

The cells in which synchrony was attempted were grown in the medium described in Chapter One. The cultures were started with an initial inoculum of 5×10^4 cells.mL^{-1} in a total volume of 1 liter. The cells were grown in 2.5 L flat bottom culture flasks, in continuous light at an intensity of 5000 lux. When the culture density reached approximately 5×10^6 cells.mL^{-1} the cells were subjected to a 14 h light 10h dark regime. After one day, 750 mL of culture was discarded and 750 mL of fresh sterile medium added to each culture. This was repeated for 4 - 8 days or until a 24 h yield was four times the cell count at the beginning of the 24 h period. Cells were routinely examined during this time to determine the relative proportion of dividing to non-dividing cells. At this time it was ascertained that the cultures were synchronized and assumed the cycle would free-run for at least 24 h.

3. Description of the Cell Cycle:

Cells were examined at all stages of the cell cycle and photographed at a X1000 magnification using a Leitz microscope. The different phases of the cell cycle appeared to match the description of the cell cycle of Chlorella ellipsoidea (Tamiya 1964) and the same terms were used to describe the different phases of the cell cycle of A. braunii.

4. Photosynthetic Rates Through the Cell Cycle:

Photosynthetic rates were determined using the same methods as described in Chapter One. All rates were determined through 15 min. runs. Cultures were programmed so that the end of the 10h dark period occurred at 12:00 noon. At this time the cultures were placed in 24h continuous light. At 1:00 PM the first photosynthesis experiments were conducted. Three replicates were done at 4h time intervals ending at 1:00 PM the following day. Cultures were examined prior to each successive experiment and the number of cells dividing (L_1 - L_4 stage) were compared with the number of cells not apparently dividing (D_n - D~L stage). The percentage of cells in the D_n - D~L stages was determined and cultures were grouped into 4 categories (0-25%, 26-50%, 51-75% and 76-100%). Final photosynthetic rates were expressed according to these categories.

5. Transport Rates Throughout the Cell Cycle:

Cells used in these estimations were from the same cultures and harvested at the same time as for photosynthesis measurements. The culture medium contained no glucose during the growth of the cells.

Transport rates were determined using the same methods as described in Chapter One. External U-C^{14} glucose concentrations were 1 $\mu g.L^{-1}$, 10 $\mu g.L^{-1}$, 100 $\mu g.L^{-1}$ and 1000 $\mu g.L^{-1}$. Specific activities were the same as in Chapter One. All incubation times were one minute.

6. Ultrastructure of Ankistrodesmus braunii:

Cells were harvested in the D_a, L3 and L4 stages of the cell cycle. The source of cells was the same as for photosynthesis and transport studies.

a) Fixation of Cells:

Thirty mL samples of the algal cultures were centrifuged and the supernatant discarded. Five mL of 4% gluteraldehyde in 0.1M Na-cacodylate buffer (pH 7.4) and a drop of Photo-flo/H_2O (50:50) was added to the algal pellet. The cells were fixed for 1.5 h. at room temperature. The fixative was then removed using 4 successive centrifugation washes with 10 mL of 0.1M Na-cacodylate buffer (pH 7.4). After the final centrifugation, the supernatant was discarded and 5 mL of 2.5% buffered Noble Agar (Difco) added to the pellet in the centrifuge tube. The tube was placed at a slant and when the agar solidified the embedded algae were cut into 1 mm^3 cubes and placed into small sample vials (Gillespie, personal communication).

b) Post Fixation and Staining:

Two mL of 1% O_sO_4 in 0.1M Na-cacodylate buffer (pH 7.4) was added to the agar cubes. Cells were post-fixed for 2h at 4°C. The solution was removed and the cubes washed 4 times (15 min./wash) in 5 mL of double distilled deionized water. After the last wash the cubes were transferred to a stain solution containing 0.5% aqueous uranyl acetate

for 16 h at room temperature. The uranyl acetate was removed by pipette and the cells placed in 5 mL double distilled, deionized water for 10 min. Cells were dehydrated by immersion for 10 min. in each of a graded ethanol series consisting of 30%, 50%, 70% and 90% ethanol. Two changes of absolute ethanol (30 min. each) preceded embedding.

c) Embedding:

The agar blocks containing the cells were immersed in a 1:1 mixture of Spurr's resin and absolute ethanol for 1 hour, followed by a 3:1 mixture for another hour. Cubes were then immersed in fresh, undiluted Spurr's resin for 16 h and finally in fresh resin for a further 8 h. Cubes were then embedded in plastic (Beem type) capsules using fresh Spurr's resin. The capsules were placed in an oven at 60°C for 16 h, stored for 24 h., then sectioned.

d) Sectioning and Microscopy:

Embedded cells were sectioned on a Sorvall Porter-Blum MT-1 ultramicrotome using a diamond knife. Sections that appeared silver to silver-gold colour were floated out on distilled water and flattened with xylene fumes. Sections were picked up on TAAB HT 300 copper grids and restained for 3 min with Reynold's lead citrate (Reynolds 1963). Sections were rinsed by rapid repetitive dipping in distilled water that had been boiled and quickly cooled to room temperature. The sections were then blotted dry on clean filter paper and viewed in a Philips EM 200 electron microscope at an accelerating voltage of 60 kv.

RESULTS

1. Description of the Cell Cycle:

Cells were examined using a phase contrast, oil emersion lens at a final magnification of 1000x. The cell cycle appears to follow the same sequence as that described for Chlorella ellipsoidea (Tamiya 1964). The life cycle is illustrated in Plate 4-1. Because of the apparent similarity between Ankistrodesmus braunii and Chlorella ellipsodea, the same terms were used to describe its life cycle (Note legend with Plate 4-1). Cell volumes, determined by microscopic examination of D_n cells, were compared with volumes of L_1, L_2 and L_3 cells. There was a 3.97x increase in the latter cells. The L_1 cells were 3.74x larger than the D_n cells. This would suggest that most of the structural biosynthesis is almost complete at this stage of the life cycle. After two successive nuclear divisions, the cells immediately began cytokinesis producing three diagonal cross walls which result in four autospores (L_3 stage). This is consistant with the observations of Pickett-Heaps, (1975) for Ankistrodesmus falcatus. At this point, the autospores are short stubby cells with pointed ends. After this period (L_3) the short cells elongate into the typical needle-shaped autospores released just prior to the D_n stage. This morphogenesis is inconsistant with A. falcatus (Pickett-Heaps 1975). The cytokinesis of A. falcatus results in the needle shaped cells with no need for a period of morphogenesis. This could be explained if one accepts that Ankistrodesmus braunii is actually Monoraphidium braunii (Na.Kutz) (Kom-Leg.), (Komârkovâ-Legnerovâ 1969; Kessler 1980).

PLATE 4-1: Life Cycle of Ankistrodesmus braunii (Naeg.)*

Dn cells - (nascent D cells), young cells newly produced (in the dark) from mother L cells.

Da cells - (active D cells), photosynthetically active cells produced from Dn cells when illuminated.

D~L cell - cell of transient stage between D and L.

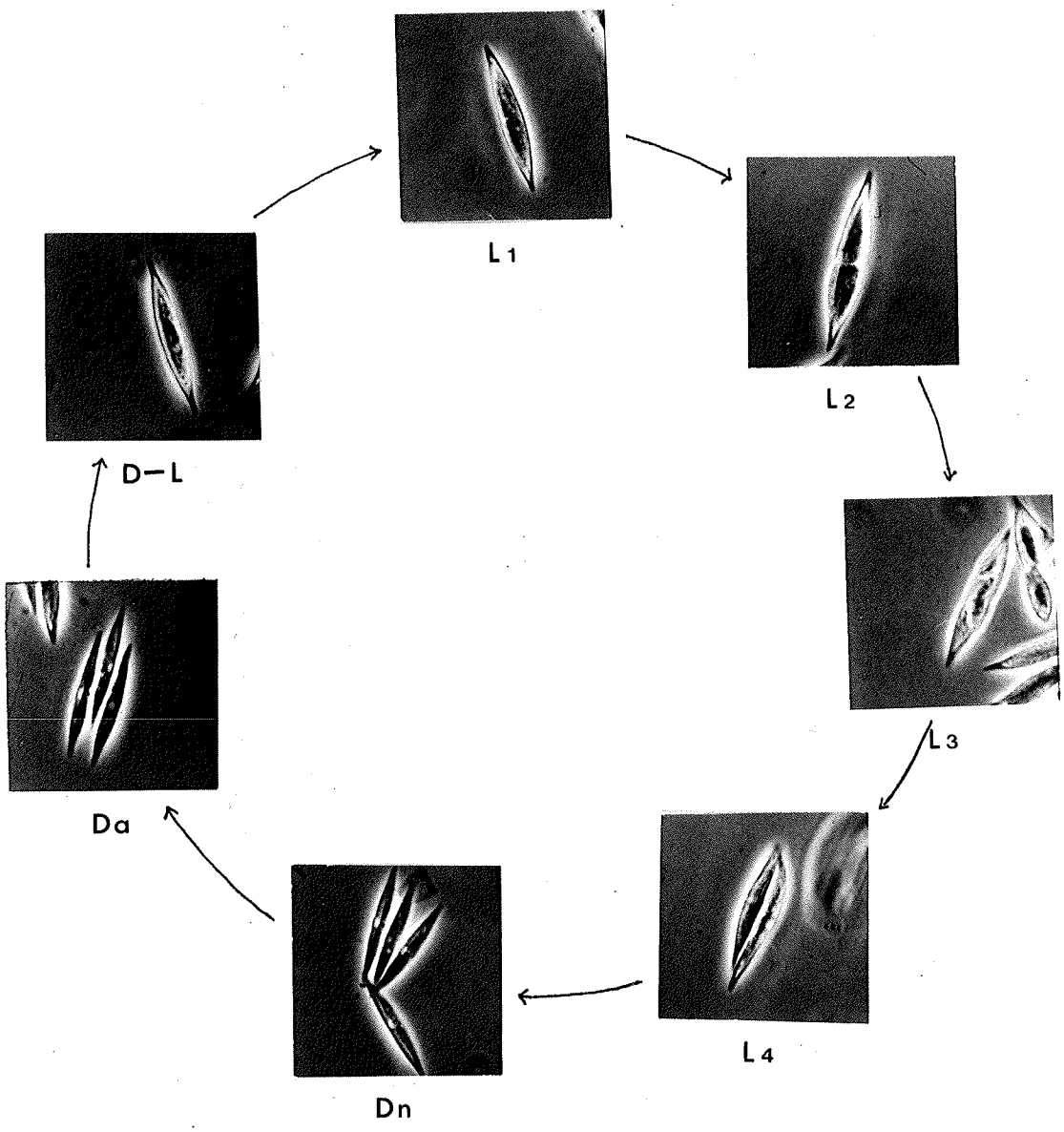
L1 cell - (unripened L cell) large size and undergoing the first mitotic division.

L2 cell - (half ripened L cell), cell undergoing the second mitotic division.

L3 cell - (ripened L cell) cell which are undergoing cytokinesis.

L4 cell - (fully matured L cell) cell completing the morphological changes to the characteristic autospores.

* Description primarily from Tamiya (1964).



Life Cycle of Ankistrodesmus braunii

2. Synchronization of Cultures:

After several attempts, absolute synchrony was not attained. At the end of every four hour period, the cells were counted and examined to obtain the relative proportion of cells at the different stages of the cycle. The cultures that gave the outward appearance of being the most synchronized had approximately 90% of the cells divide within a 4 hour time period. Consequently, all the results in this Chapter are expressed in terms of the percent cells in the Dn to D∼L stage.

3. Photosynthetic Rates During the Cell Cycle:

The results of the photosynthetic rates experiments are listed in Table 4-1 and illustrated in Figure 4-1a and b. Since there was a change in the cell volume as a greater proportion of the cells reached the L1 stage, the results were expressed as functions of both cell number and cell volume. From these results, it is apparent that the photosynthetic rate declines as a greater proportion of the cells reach the division phase of the cell cycle. When the results are expressed as cell number, the photosynthetic rate of cultures which have 75% or more cells dividing is approximately 35% of cultures which have less than 25% of the cells dividing. Based on cell volume, this value drops to approximately 28% of the rate where 25% or fewer cells are dividing.

4. Transport Rates During the Cell Cycle:

Rates of glucose transport are reported on a per cell basis in Table 4-2 and Fig. 4-2 and on a unit of cell volume basis in Table 4-3 and

FIGURE 4-1a: Change in photosynthetic rates with an increasing proportion of the culture dividing. Results expressed on a per cell basis.

1 = Fewer than 25% dividing cells

2 = 26-50% dividing cells

3 = 51-75% dividing cells

4 = more than 75% dividing cells

FIGURE 4-1b: Change in photosynthetic rates with an increasing proportion of the cells in the culture dividing. Results expressed on a per unit volume basis. Bars are the same as Fig. 4-1a.

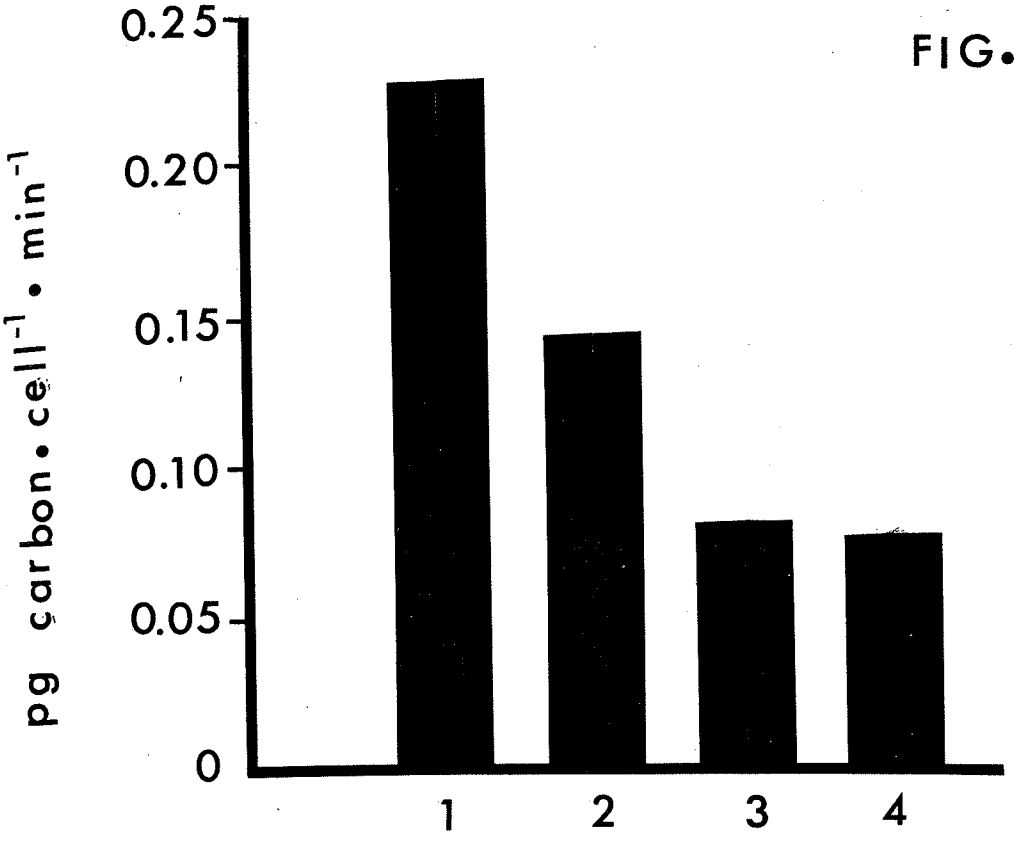


FIG. 4-1a

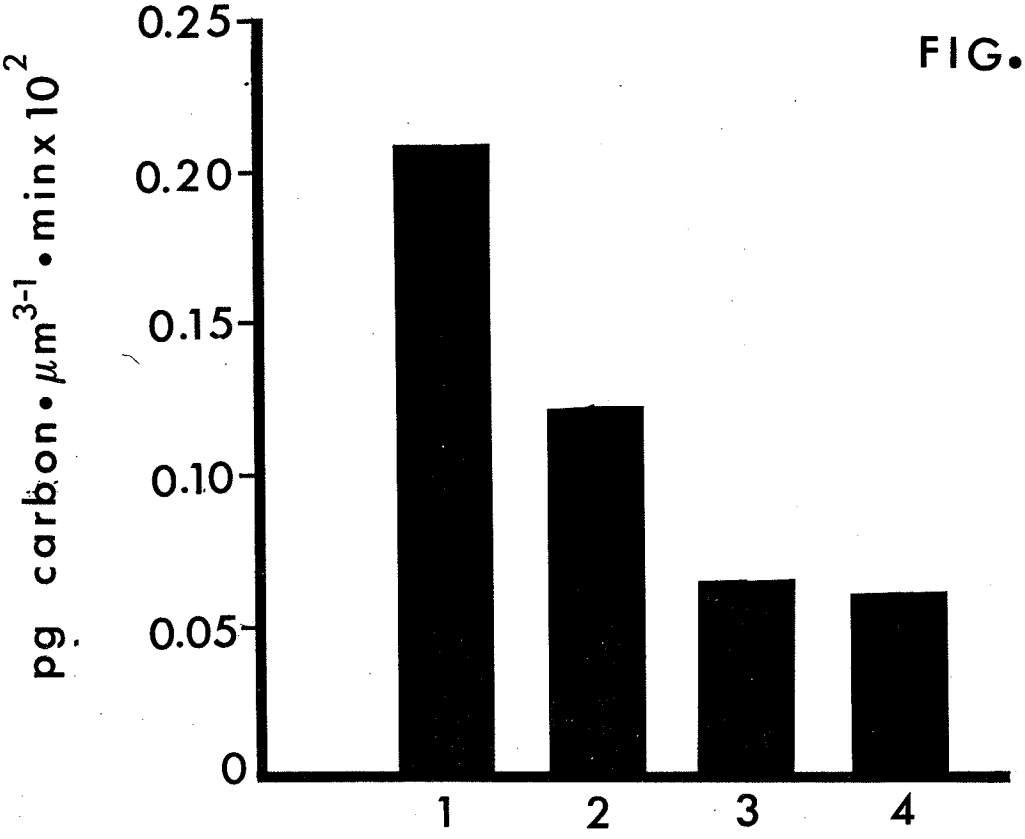


FIG. 4-1b

TABLE 4-1: Effect of cell division on the photosynthetic rate of *Ankistrodesmus braunii*. All results expressed as pg carbon.cell^{-1}.min^{-1} or pg carbon.$\mu m^3{}^{-1}$.min^{-1}.

Percent Cells in Dn to L1 Stage	Photosynthetic Rate per Cell	Percent of 76-100% Rate	Photosynthetic Rate per μm^3	Percent[1] Photosynthesis
76 to 100%	0.227	100.0	0.0021	100.0
51 to 75%	0.144	63.1	0.0012	57.7
26 to 50%	0.083	36.3	0.0006	30.8
0 to 25%	0.080	35.1	0.0006	27.9

1. Percentage values are expressed in comparison with the photosynthetic rates when 76 to 100% of the cells are in the Dn to L1 stage of the cell cycle.

FIGURE 4-2: Variations in transport rates when different proportions of the cells in the culture are dividing. External glucose concentrations are 1 $\mu g.L^{-1}$ (A), 10 $\mu g.L^{-1}$ (B), 100 $\mu g.L^{-1}$ (C) and 1000 $\mu g.L^{-1}$ (D). Results are expressed as μg glucose.10^8 cells.$^{-1}$min.$^{-1}$.

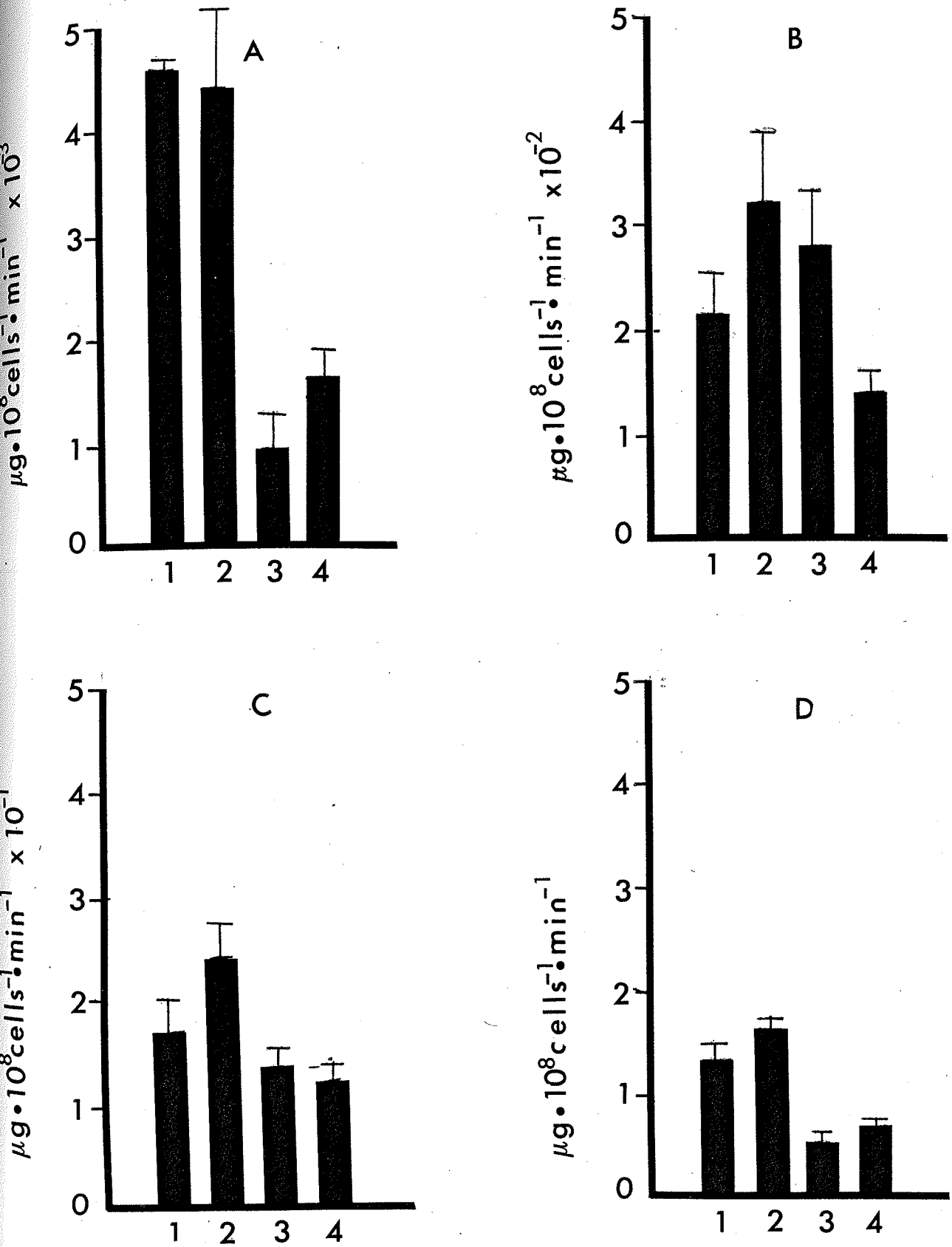

A
B
C
D
$\mu g \cdot 10^8 cells^{-1} \cdot min^{-1} \times 10^{-3}$
$\mu g \cdot 10^8 cells^{-1} \cdot min^{-1} \times 10^{-2}$
$\mu g \cdot 10^8 cells^{-1} \cdot min^{-1} \times 10^{-1}$
$\mu g \cdot 10^8 cells^{-1} \cdot min^{-1}$
0
1
2
3
4
5

FIGURE 4-3: Variations in transport rates when different proportions of the cells in the culture are dividing. External glucose concentrations are 1 $\mu g.L^{-1}$ (A), 10 $\mu g.L^{-1}$ (B), 100 $\mu g.L^{-1}$ (C) and 1000 $\mu g.L^{-1}$ (D). Results are expressed as μg glucose.10^{8}cell vol.$^{-1}$min^{-1} x 10^{-4}. Numbers of bars are the same as in Fig. 4-2.

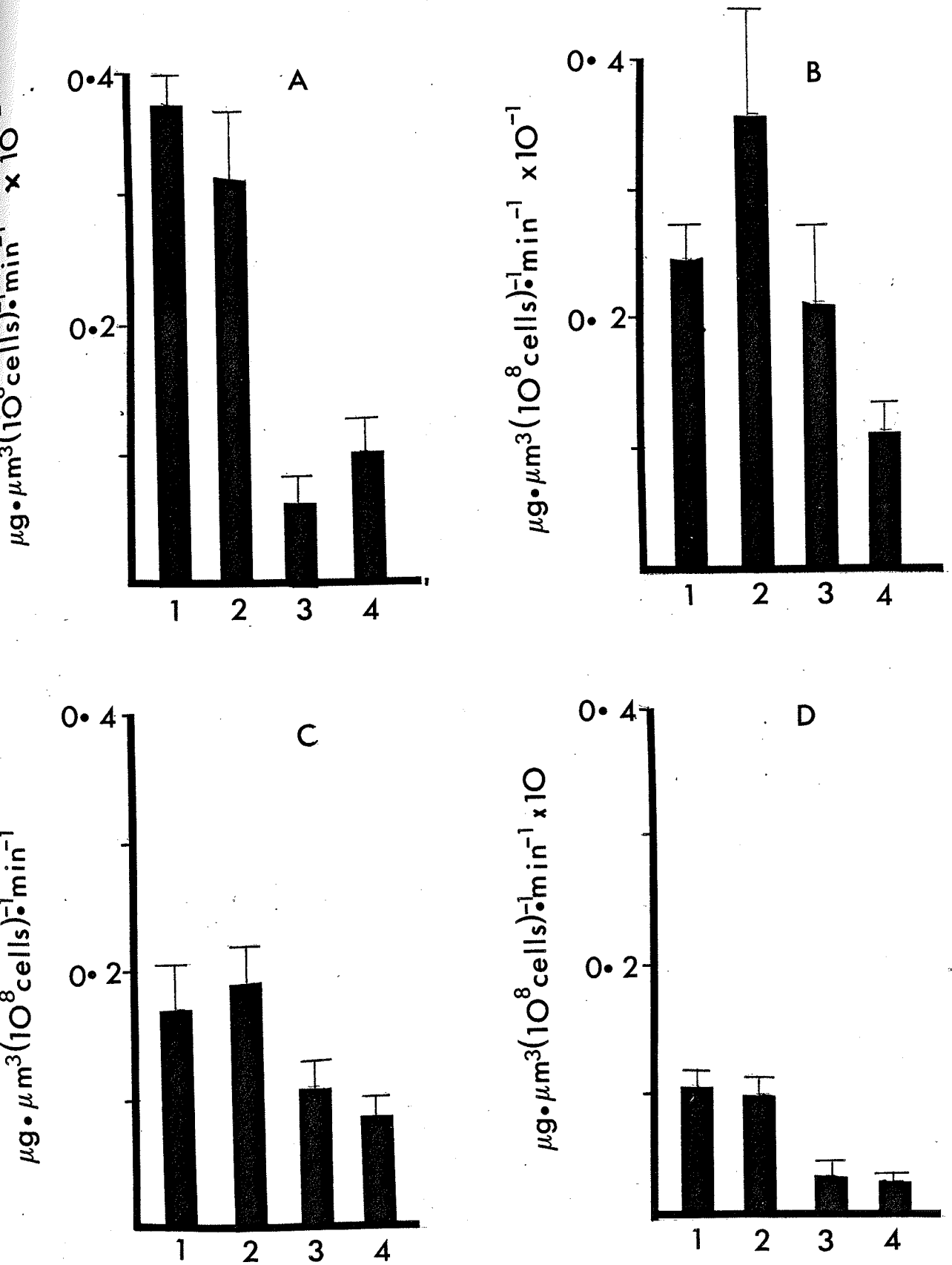

A
B
C
D
0•4
0•2
1
2
3
4
$\mu g \cdot \mu m^3 (10^8 cells)^{-1} \cdot min^{-1} \times 10^{-2}$
$\mu g \cdot \mu m^3 (10^8 cells)^{-1} \cdot min^{-1} \times 10^{-1}$
$\mu g \cdot \mu m^3 (10^8 cells)^{-1} \cdot min^{-1}$
$\mu g \cdot \mu m^3 (10^8 cells)^{-1} \cdot min^{-1} \times 10$

Fig. 4-3. The uptake rates generally declined as a greater number of the cells in the culture were dividing. As the number of cells dividing increased from less than 25% to 26 to 50%, the uptake rate decreased slightly (4.0%) at an external glucose concentration of 1.0 $\mu g.L^{-1}$ but increased at higher glucose concentrations. As the number of dividing cells increased to greater than 75%, the transport rates declined but not as rapidly as the photosynthetic rates.

5. Ultrastructure of Cells During the Cell Cycle:

a) Cells at the Da Phase:

Cells in the Da phase (Plate 4-2) show a single, well developed chloroplast. The chloroplast has extensive photosynthetic lamellae and a large accumulation of starch. The cytoplasm has a dense concentration of ribosomes but they do not appear to be associated with endoplasmic reticulum. There were no golgi bodies seen in any section of this phase.

b) Cells at the L3 Phase:

The micrograph of a cell at L3 (Plate 4-3) illustrate that cytokinesis is nearly complete. Morphogenesis has not yet begun. The chloroplasts are still well developed, with extensive lamellae and large starch deposits. The dense cytoplasmic ribosomes now appear to be associated with endoplasmic reticlum.

c) Cells at the L4 Phase:

At the L4 (Plate 4-4) the chloroplasts are distinctly different, devoid of starch and with a reduction of lamellae. Golgi complexes are now evident and cytoplasmic ribosomes appear to be fewer and associated with endoplasmic reticulum.

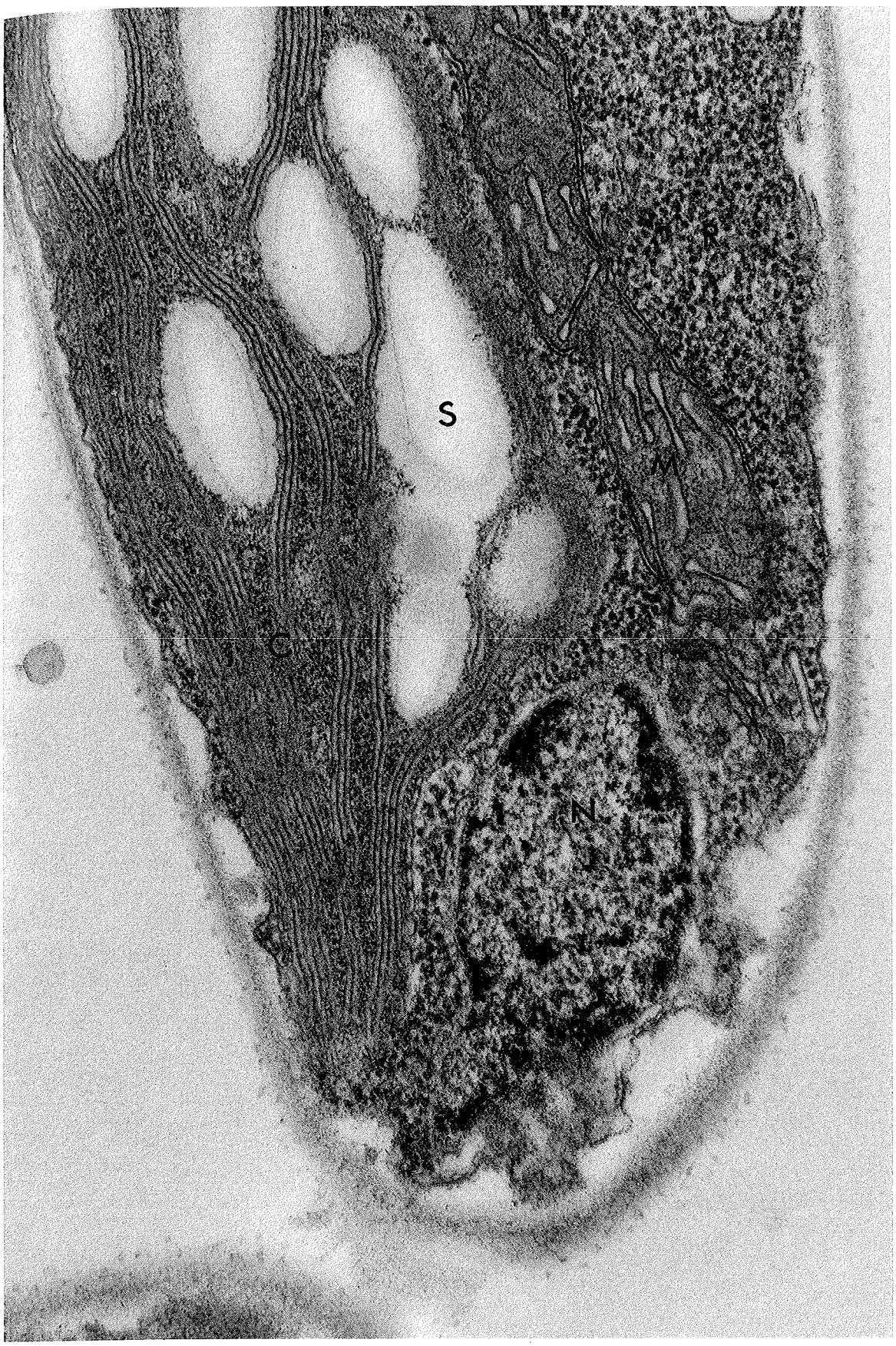
TABLE 4-2: Effect of cell division on uptake rate of U-C^{14} glucose. Results expressed as µg glucose.10^8 cells^{-1}min^{-1}.

Percent Cells in Dn to L1 stages.	External Glucose Concentration 1 µg.L^{-1}	+ %SE.	10 µg.L^{-1}	+ %SE.	100 µg.L^{-1}	+ %SE.	1000 µg.L^{-1}	+ %SE.
76 to 100%	0.005	2.4	0.022	17.2	0.17	17.7	1.35	10.0
51 to 75%	0.004	19.1	0.032	23.8	0.24	14.4	1.61	9.0
26 to 50%	0.001	33.9	0.028	22.2	0.14	13.4	0.52	10.1
0 to 25%	0.002	20.9	0.014	18.2	0.12	14.5	0.69	8.0

TABLE 4-3: Effect of cell division on uptake rate of U-C^{14} glucose. Results expressed as µg glucose.vol. of 10^8 cells^{-1}min^{-1} x 10^{-4}.

Percent Cells in Dn to L1 stages.	External Glucose Concentration 1 µg.L^{-1}	+ %SE.	10 µg.L^{-1}	+ %SE.	100 µg.L^{-1}	+ %SE.	1000 µg.L^{-1}	+ %SE.
76 to 100%	0.37	6.8	2.47	9.4	17.02	19.8	106.90	9.0
51 to 75%	0.31	16.3	3.57	24.0	18.90	15.5	100.89	10.0
26 to 50%	0.06	36.0	2.21	24.2	11.06	17.1	34.90	8.1
0 to 25%	0.10	24.8	1.13	21.8	8.73	16.5	29.50	10.8

PLATE 4-2: Electron micrograph of Da cell l.s showing chloroplast (C), mitochondriun (M), nucleus (N), ribosomes (R), and starch deposits (S). (x 49,100)



(C),
starch

PLATE 4-3: Electron micrograph of L3 cell l.s. showing chloroplast (C) mitochondriun (M), nuclei (N), endoplasmic reticulum (E) and starch deposits (S). (x 58,000)

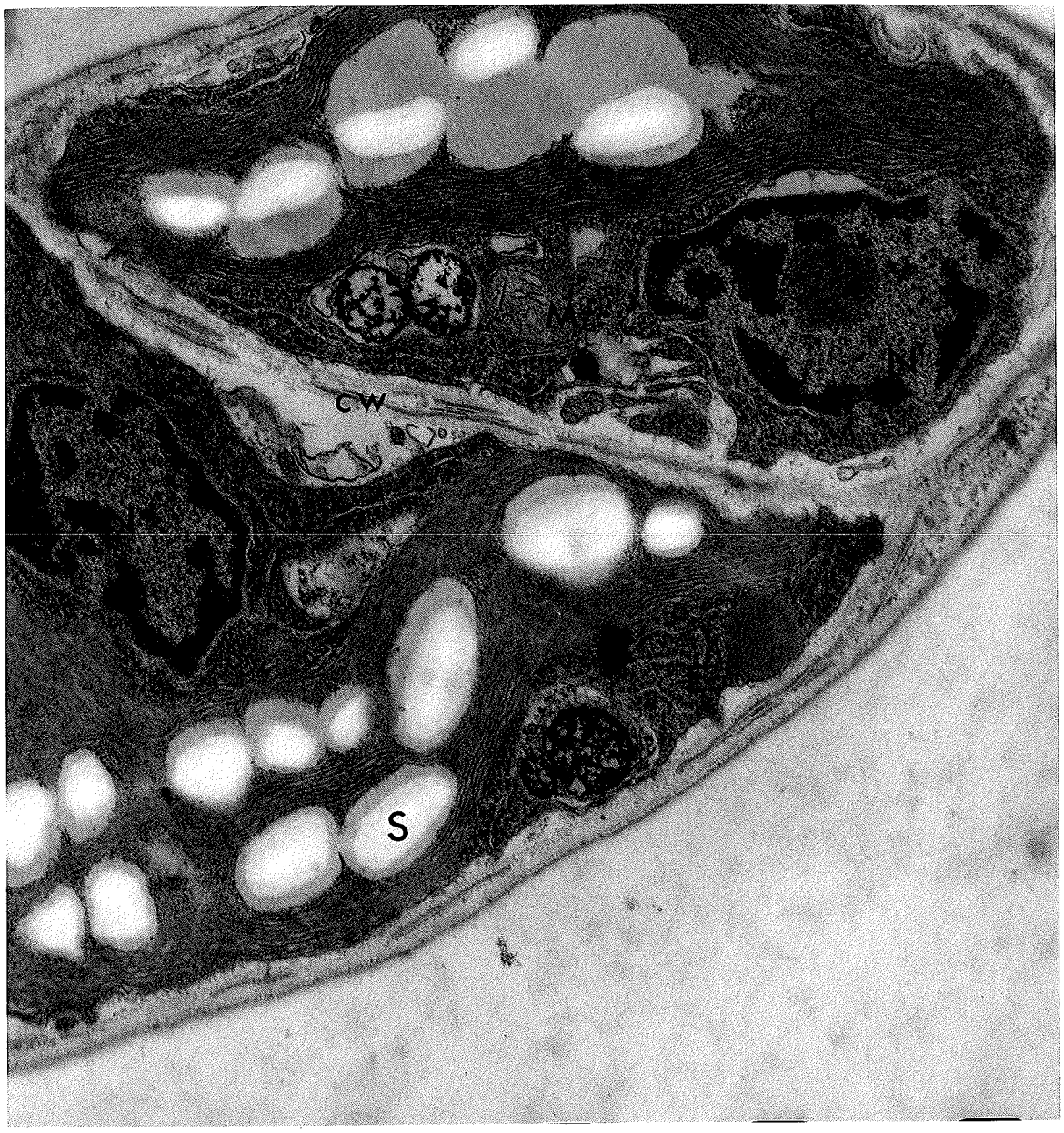
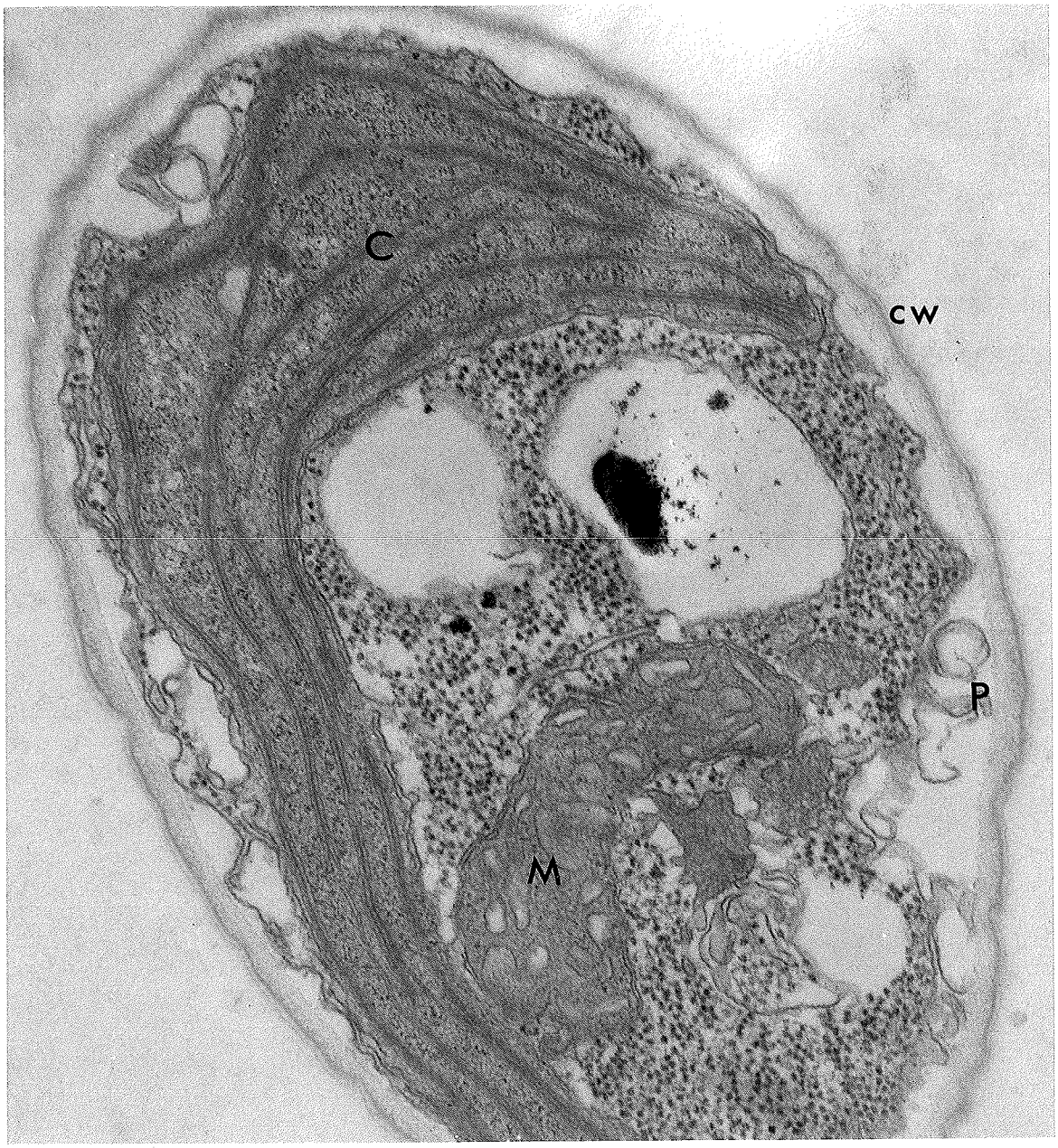

CW
S

PLATE 4-4: Electron micrograph of L4 cell x.s. showing chloroplast (C) with no starch deposits and fewer photosynthetic lamellae, cell wall (CW), mitochondriun (M), highly folded plasmalemma (P) and fewer ribosomes. (x 62,000)


C
CW
P
M

DISCUSSION

The cell cycle of Ankistrodesmus braunii (Naeg.) appears to follow the same pattern as Chlorella ellipsoidea (Tamiya 1964). Starting from the newly released autospores (Plate 4-1, Dn cells), there is a period of rapid growth where the cells are incapable of dark division without glucose or some other suitable organic energy and carbon source. From the Dn to D∼L stage, growth is rapid, starch accumulation high, membrane and catalytic protein synthesis high and endogenous respiration low (Tamiya 1966). Growth slows after the D∼L stage and becomes negligible after the L2 stage (Tamiya 1966; Lorenzen and Hesse 1974). Metabolic emphasis then shifts to nuclear division, cytokinesis and new wall formation (Takeda and Hirokawa 1982). From the L3 to L4 stage, morphogenesis takes place. Photosynthesis is most rapid from the Dn to D∼L stages but after this, oxidative phosphorylation replaces photosynthesis as the major energy source. Carbohydrate reserves are also converted to protein (Pirson and Lorenzen 1966) or utilized as an energy source (Hirokawa et al. 1982).

Absolute synchrony was never attained in the experiments. The closest to absolute synchronous division was 92% but most of the cultures only attained approximately 80%. There are several possibilities why there was never complete synchrony. For example, the light - dark regime of 14L and 10D may not have been sufficient to insure synchrony (Tamiya 1966) Another possibility is that the culture may not have been able to free-run. Although absolute synchrony was never attained, by combining the data from the different experiments, where the percentages of cells

in the L1 to L4 stage were known, it was possible to establish a trend as to the effect of shifts in the cell cycle, on photosynthesis and glucose transport.

The results of the photosynthesis experiments were similar to those reported by others (Pirson and Lorenzen 1966; Tamiya 1966; and Lorenzen and Hesse 1974). When the proportion of dividing cells was less than 25% of the population, the photosynthetic rate was the highest (Table 4-1). As the proportion increased the photosynthetic rate dropped so that when 75% or more cells of the culture were dividing, the photosynthetic rate per cell was less than 30% of the original rate. This would support the argument that at a time when cells are dividing and energy demands are high, the available energy and carbon from photosynthesis is low. This might be a time when heterotrophy could play an important role in cell metabolism.

Transport of U-C^{14} glucose followed a slightly different pattern than photosynthetic rates. While photosynthetic rates dropped to 63.1% (based on cell numbers) or 57.7% (based on cell volume) when the proportion of dividing cells increased to 25-50% of the culture (Table 4-1), the transport rates either dropped slightly (at 1.0 $\mu g.L^{-1}$) or increased by as much as 49% (Table 4-2). When the proportion of dividing cells increased further, the transport rates dropped but never as much as the photosynthetic rates.

These results suggest that heterotrophy may play an increased relative role during this phase of the life cycle. These values are also probably a substantial under estimate of the significance of heterotrophy during the division phases of the cell cycle. Extrapolating the results

to the normal cell cycle and the natural environment, as the proportion of cells dividing increasing, this would mean there were more cells in the L1 to L4 stages. In the natural environment, this would likely occur at night. Since transport is reduced by light (Chapter Two), and since the measurements were done on cells that were free-running in continuous light, the transport rates should be much greater in the dark. Photosynthesis would then be curtailed, but not heterotrophy. This argument can be supported by two points of evidence. Firstly, the cultures which had 25 to 50% of the cells dividing were in the light for only 4 hours. These cells showed very little drop or at some external concentrations, a stimulation of transport. The cultures which had 50 to 100% of the cells dividing were in the light for at least 8 hours and usually longer. By this time, light reduction should have been complete so any drop in transport at this time may have been due to light repression or inactivation of the transport system rather than a phase shift in the life cycle.

Secondly, cells that were grown in continuous light with glucose had a doubling time of 14.03 hours (Chapter One), while those grown in 12 hours light and 12 hours dark had a doubling time of 12.79 hours. This could suggest that while in continuous light, the reduction of transport prevented exploitation of the heterotrophic potential of the cells at a time when photosynthesis was low. The 12 hour dark period allowed heterotrophy to become significant when the cells were not able to photosynthesise and thus resulted in an overall increase in the growth rate.

The ultrastructure of the cell cycle confirms the desirability of

heterotrophy at the later stages of the cell cycle. Plate 4-2 at the Da phase of the cycle show structural evidence of the occurrence of photosynthesis. The extensive and well developed photosynthetic lamellae and the large accumulation of starch would indicate that photosynthesis is occurring rapidly. The dense concentration of ribosomes suggests a high protein synthesis and therefore carbon and energy demand. This is reasonable since, at this time, membranes and wall material would have to be produced to keep pace with the increase in cell size, although Chlorella ellipsoidea demonstrated no net increase in cell wall material (Takeda and Hirokawa 1982) and photosynthetic products were used in respiration and starch accumulation (Hirokawa et al. 1982).

Plate 4-3 is of the alga at the L3 phase of development. At this stage, cytokinesis is nearly complete as is indicated by cross wall formation. The morphogenesis into the characteristic needle shaped autospores has not begun. It is evident from the micrograph, that the chlorplasts have either been duplicated or partitioned and the accumulated starch from the Dn to D~L phases has not yet been utilized. The dense ribosome concentrations could reflect a demand for proteins associated with cytokinesis or organelle replacement. In Chlorella vulgaris most protein and chlorophyll synthesis occurs much earlier in the cell cycle (Malis-Arad and McGowan 1982).

Plate 4-4 is a micrograph of the L4 stage. At this time in the cell cycle, morphogenisis is complete and the autospores have assumed the shape of the parent cells. The micrographs reveal that the photosynthetic apparatus of the chloroplast is now considerably altered. The number of lamellae in the chloroplast is now reduced and the starch

reserves have been utilized. This is consistant with the biochemical evidence of changes in Chlorella ellipsoidea (Hirokawa et al. 1982) If the starch reserves are depleted and the photosynthetic capacity reduced, it is logical to assume that organic transport and heterotrophy would now be of much greater value to the alga. These are also the first micrographs in which the golgi complex is evident. This could be an artifact of sectioning or a need for membrane proteins which are packaged and delivered by vesicles of the golgi complex.

These results support the contention that heterotrophy could play a significant role in the metabolism of A. braunii in the dividing phases of the cell cycle. While much of the evidence for this hypothesis is indirect, none of the results directly refute the hypothesis and the evidence that is available tends to support the arguments. The results could also account for the variability of other experiments if the cultures used were not all in the same phase of the cell cycle and one culture had a greater proportion of dividing cells than another.

SUMMARY

1. The cell cycle of Ankistrodesmus braunii follows the same pattern as Chlorella ellipsoidea (Tamiya 1964).

2. Photosynthesis occurs in the first phases (D_n - D~L stages) of the cell cycle and then is replaced by oxidative phosphorylation.

3. Glucose transport increases at a time in the cell cycle when photosynthesis is declining.

4. By the time the second cytokinetic division is complete the starch reserves of the cells are depleted and heterotrophy would be the only source of cell carbon and energy.

GENERAL SUMMARY

The objectives of this investigation were to determine the heterotrophic capabilities of Ankistrodesmus braunii and to characterize the transport system responsible for the active transport of d-glucose. Chapter One disclosed several important features about the heterotrophic potential of the alga. Firstly, it was demonstrated that A. braunii is capable of dark, heterotrophic growth. The alga was able to double every 22.1 hours when grown on 1.0mM d-glucose and every 31.4 hours on 0.1mM d-glucose. The calculated kinetics for the glucose transport not only accounted for the heterotrophic growth rate, but also revealed that the glucose transport system would be competitive with those transport systems for d-glucose that were reported to occur in natural populations of bacteria. By using methodology outlined by bacteriologists, the uptake rates were found to be as rapid or more rapid than any reported for bacteria. Another observation from this chapter, is that the addition of glucose to cultures grown in continuous light was neither additive or synergistic with regards to the growth rate of the alga.

Chapter Two provided further information on the significance of heterotrophy in the overall metabolism of A. braunii. Determination of photosynthetic rates disclosed the fact that even accounting for excretion, photorespiration and endogenous respiration, the rate of assimilation of fixed organic carbon would result in a projected growth rate 200% of what actually occurred. This could be interpreted as only a 50% efficiency of utilization of carbon fixation. It would then be redundant of the alga to transport glucose in the light. It was found in this

portion of the research, that in fact, the alga reduces its transport of glucose in the light. This would explain why the addition of glucose would not increase growth in the light. In the dark, when excretion and respiration were considered, the transport of d-glucose demonstrated an efficiency of conversion to algal biomass of over 56%. The results of this chapter also revealed that the rate of transport of glucose was strongly dependent on the energy or organic carbon demands of the cells. Although energy availability influenced that rate of uptake, if a cell had an adequate amount of energy, determined from its prehistory, its transport rate would be low. Once the energy demand was present, the cell was capable of utilizing any source of ATP energy to drive the transport system. Another conclusion drawn from this chapter was, that transport was not only less in the light, but that the light itself appeared to repress or inactivate the transport system. Besides periods where light is inadequate for photosynthesis, such as at night, it was found that heterotrophy could also sustain the alga if photosynthesis was reduced due to a CO_2 shortage. Growth in the light under these conditions was less than in the dark but sufficient to double the algal biomass every 32 hours if the d-glucose concentration was 1.0mM. The lower growth rate than seen for dark heterotrophy could be attributed to some light inhibition.

Another objective of the research was to characterize the transport system. The system for transport of d-glucose is believed to be an active (Chapter Three) biphasic (Chapter One) system. It is capable of using any source of ATP (Chapter Two) but it does not appear to involve a phosphorylation of the glucose prior to entering the cell. The system

appears to be partially constitutive and partially inducible. It follows the model of a permease carrier protein.

Finally, electron micrographs disclosed the information that the algal cells would benefit from heterotrophic growth or glucose transport in the later stages of the cell cycle. While it was somewhat inconclusive, the results of the fourth chapter suggest that while photosynthesis drops dramatically in the division phases of the cell cycle, transport rates do not drop as much.

In conclusion, it is apparent that the alga Ankistrodesmus braunii is capable of making significant contributions to its overall metabolism through the use of heterotrophic growth. This may explain the success of this alga in a wide range of habitats, particularly its ability to multiply to bloom proportions in eutrophic waters.

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APPENDICES

APPENDIX I

RATIONALE FOR THE INTERPRETATION OF THE UPTAKE SYSTEMS

In determining the kinetic parameters K_t and V_{max} it was first necessary to establish whether the uptake system was a simple, single transport carrier system or whether it was a biphasic system. It was also necessary to establish which linear transformation of the observed data should be used to best determine the uptake kinetics. Each consideration presented difficulties.

A rectangular hyperbola which reaches an asymptote should be attained when plotting (v) the observed uptake velocity against (s), the substrate concentration if the system is a simple transport carrier. The substrate concentration at which this asymptote is reached is dependent on the number of carrier molecules and the rate of dissociation of the substrate from the carrier to the inside of the cell. This may be somewhat obscured if diffusion occurs, but can be tested with temperature or inhibitor studies. Any of the accepted linear transformations of the data producing the curve should result in a straight line (Fig. 5-1). From this line, the parameters K_t and V_{max} may be calculated.

If a rectangular hyperbola does not describe the line obtained by plotting the (v) against (s) values, then no direct linear transformation can be made. Differently shaped curves may necessitate different linear transformations from which different inferences may be drawn as to the type of uptake. If the transformation produces a line which has two linear portions (Fig. 5-2), it indicates the presence of two uptake

FIGURE 5-1: A hypothetical curve to illustrate a single uptake system. (A) the Michaelis-Menton plot. (B) the Lineweaver-Burke transformation. (C) the Woolf transformation.

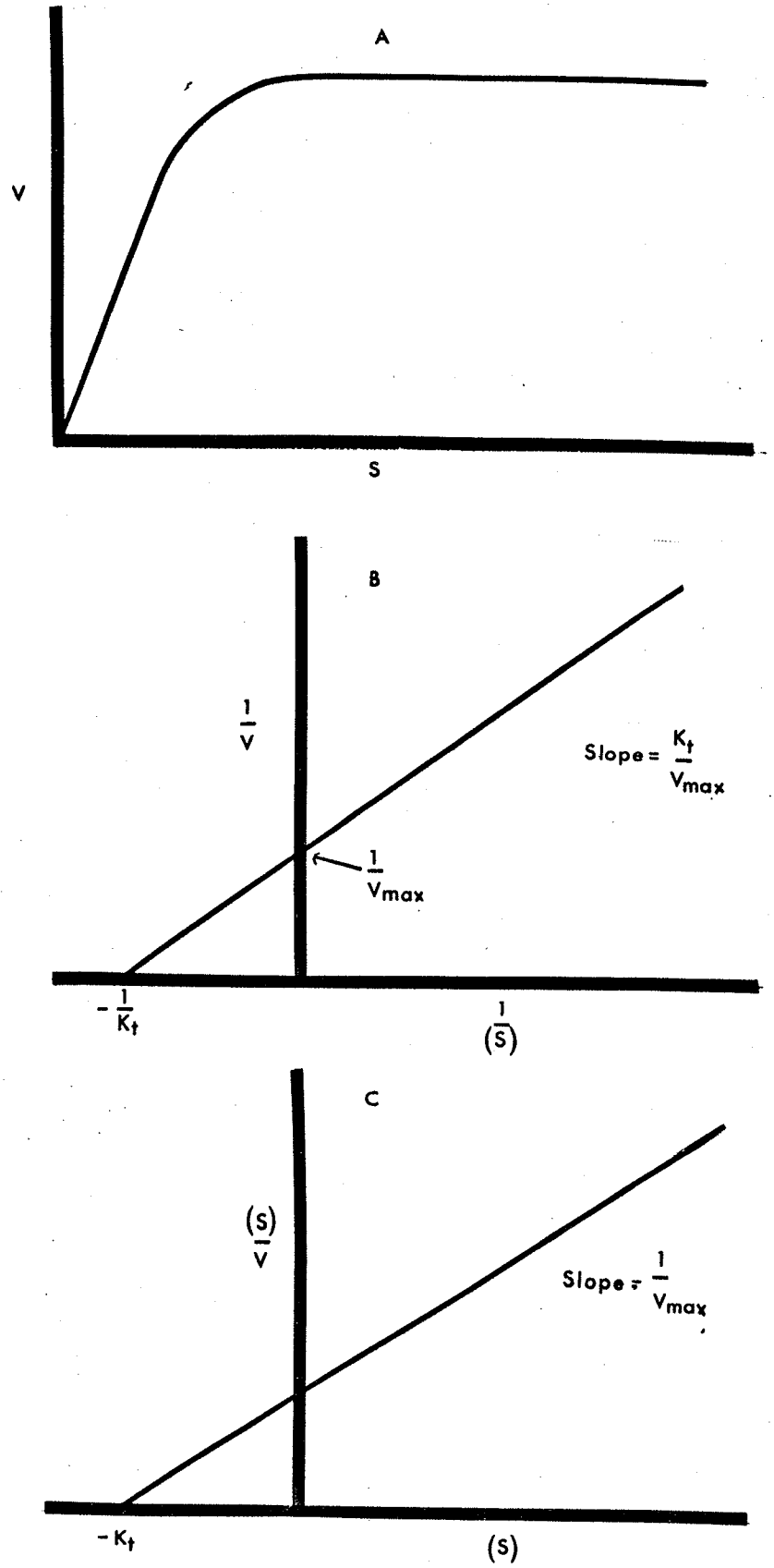
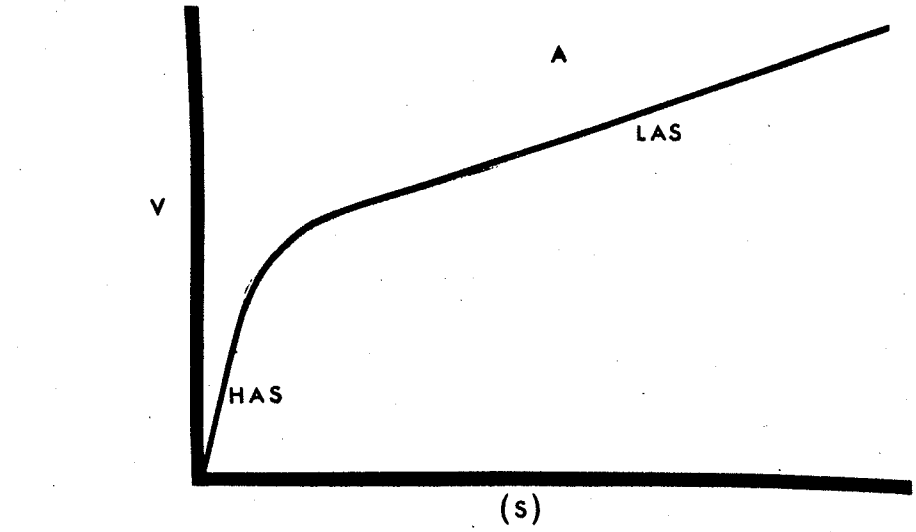
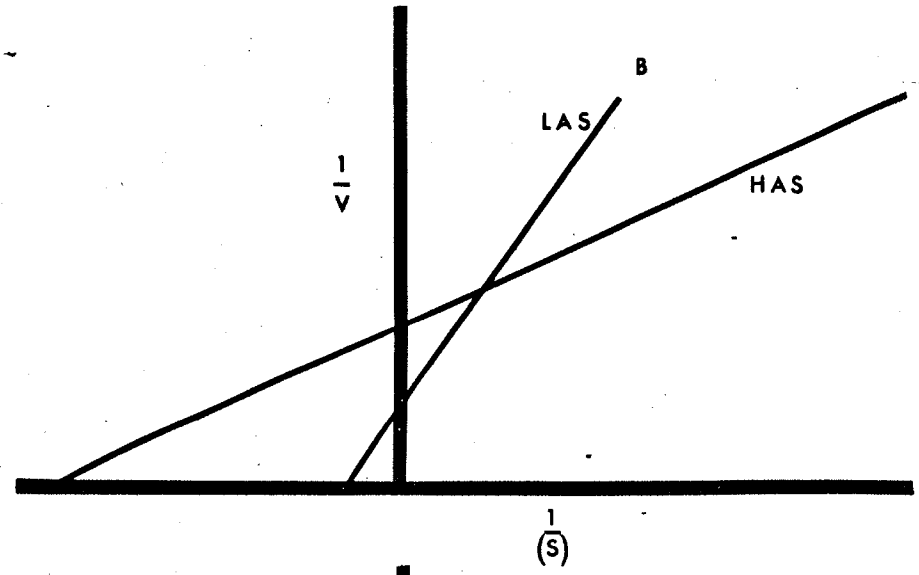
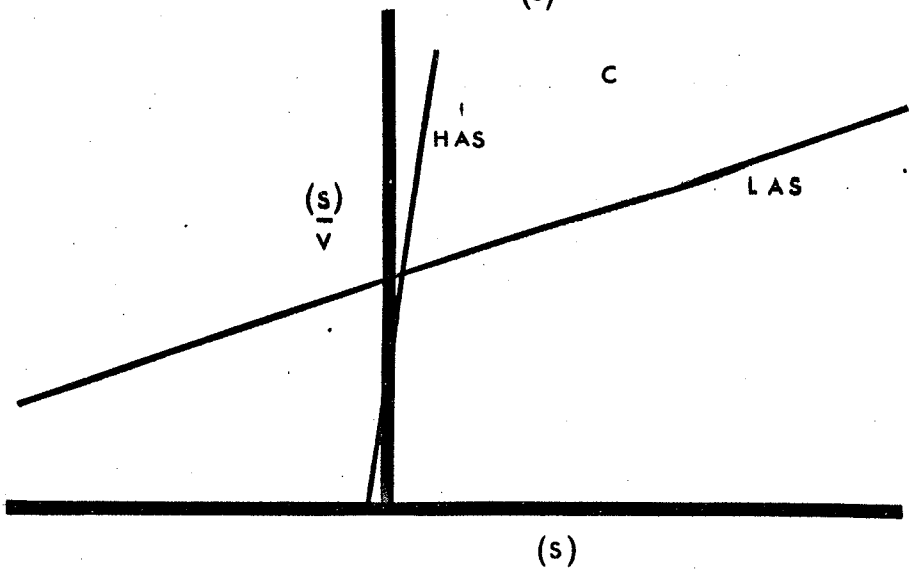

A
V
S
B
$\frac{1}{V}$
Slope = $\frac{K_t}{V_{max}}$
$\frac{1}{V_{max}}$
$-\frac{1}{K_t}$
$\frac{1}{(S)}$
C
$\frac{(S)}{V}$
Slope = $\frac{1}{V_{max}}$
$-K_t$
(S)

FIGURE 5-2: A hypothetical curve to illustrate a biphasic uptake system. (A) the v vs. s plot. (B) the Lineweaver-Burke transformation. (C) the Woolf transformation. HAS = high affinity system, LAS = low affinity system.


A
LAS
V
HAS
(S)


B
LAS
1/V
HAS
1/(S)


C
HAS
(S)/V
LAS
(S)

systems (Akedo and Christensen 1962; Reid et al. 1970; and Hellebust 1976). It is further suggested that each linear portion represents dominant uptake by one of the two systems. The two most widely used linear transformations, the Lineweaver-Burke plot (1/v vs. 1/s) or its modified form, the Woolf or Eadie plot (s/v vs. s) result in the same shaped line with a biphasic or two component uptake system (Fig. 5-2).

The plot of the observed data from Table 5-1, using both of the above transformations, is illustrated in Figs. 5-3 and 5-4. The lines were calculated using the least squares method. Both of these lines are significant at the $P<0.01$ level. If the actual data used to compute these lines (Table 5-2) are examined, it is evident there is a deviation of the observed data from the calculated data. This deviation occurs at glucose concentrations between 0.06 μM and 0.16 μM for both transformations. Because of the wide range of glucose concentrations used (1.22 nM to 12.92 μM), portions of both lines are obscured. The line established in the Lineweaver-Burke conversion is determined by the very low velocities and substrate levels since their reciprocals are so large. The change in slope at the high velocities is then lost in the calculation of the least square line. The Woolf transformation is controlled by the large values of velocity and substrate concentration. This has the effect of obscuring the slope change at the low substrate levels. Graphic illustrations were thus deceptive when trying to describe the uptake systems. The data in Table 5-2 still clearly indicate a break in both lines. The uptake system was therefore considered to be biphasic and all calculations were carried out accordingly.

TABLE 5-1

Uptake velocities calculated by the least squares method. Results of both the Lineweaver-Burke and Woolf conversions compared with the observed velocities.

Glucose Concentration (s) µg/L	Observed[1] Velocities (v)	±%S.E.	L.-B.[2] Calc. (v)	% Deviation[3] From Ob.	Woolf[4] Calc. (v)	% Deviation From Ob.
0.2198	0.002	15.9	0.002	1.4	0.002	26.5
0.5497	0.005	12.4	0.005	1.2	0.004	23.3
1.099	0.009	8.2	0.010	12.7	0.008	10.9
1.645	0.013	7.9	0.015	15.3	0.012	6.1
2.198	0.017	9.3	0.019	14.4	0.016	4.9
5.497	0.039	9.3	0.042	7.1	0.039	0.3
8.243	0.055	8.2	0.058	5.3	0.059	6.9
10.99	0.071	11.1	0.070	0.8	0.078	9.3
16.45	0.101	8.7	0.089	11.1	0.117	13.9
21.98	0.147	12.2	0.105	28.3	0.156	5.6
28.63	0.187	14.9	0.119	36.5	0.202	7.4
57.45	0.385	14.3	0.151	60.6	0.401	4.1
114.50	0.796	14.5	0.176	77.9	0.781	1.9
286.30	1.664	8.3	0.194	88.4	1.825	8.8
574.50	3.129	12.5	0.201	93.6	3.303	5.3
1145.00	5.084	10.5	0.205	95.9	5.513	7.8
2290.00	8.743	8.4	0.207	97.6	8.314	4.9

1. Calculated as the means of 4 separate experiments with 4 replicates each ($\mu g.L^{-1}.min^{-1}$).

2. Calculated from the least squares line 1/v vs. 1/s. ($\mu g/10^8$cells/min) Table 5-2.

3. Percentage difference from the observed velocities.

4. Calculated from the least squares line of s/v vs. s. ($\mu g/10^8$cells/min) Table 5-2.

TABLE 5-2

Linear transformation of uptake velocities using the Lineweaver-Burke and Woolf conversions.

Lineweaver-Burke			Woolf		
1/s observed	1/v observed	1/v calculated	s observed	s/v observed	s/v calculated
4.5496	469.48	478.4	0.2198	103.2	139.92
1.8192	195.31	194.7	0.5497	107.3	139.95
0.9099	133.39	100.3	1.099	124.6	139.98
0.6079	80.00	68.9	1.645	131.6	140.01
0.4550	60.61	53.1	2.198	133.2	140.05
0.1819	25.45	24.7	5.497	139.9	140.24
0.1213	18.32	18.4	8.243	150.8	140.40
0.0910	14.10	15.2	10.99	155.0	140.60
0.1618	9.95	12.1	16.45	163.7	140.90
0.0455	6.81	10.5	21.98	150.7	141.20
0.0349	5.34	9.4	28.63	152.9	141.60
0.0175	2.60	7.59	57.45	149.4	143.30
0.0087	1.255	6.68	114.50	143.8	146.70
0.0035	0.601	6.14	286.30	172.0	156.80
0.0017	0.3195	5.96	574.50	183.6	173.90
0.0009	0.1967	5.87	1145.00	225.2	207.80
0.0004	0.1144	5.83	2290.00	261.9	275.50

$y = 103.52x + 4.796$ ($r = 0.9972$)

$V_{max} = 1.16$ nmol.10^8 cell^{-1}min^{-1}

$K_t = 119.9$ nM

$y = 0.0592x + 139.92$ ($r = 0.8116$)

$V_{max} = 93.9$ nmol.10^8cells^{-1}.min^{-1}

$K_t = 13.13$ μM

FIGURE 5-3: The Lineweaver-Burke plot of the reciprocals of the observed velocities and glucose concentrations. Only the high substrate levels are included to illustrate the deviation of the observed values from the calculated line.

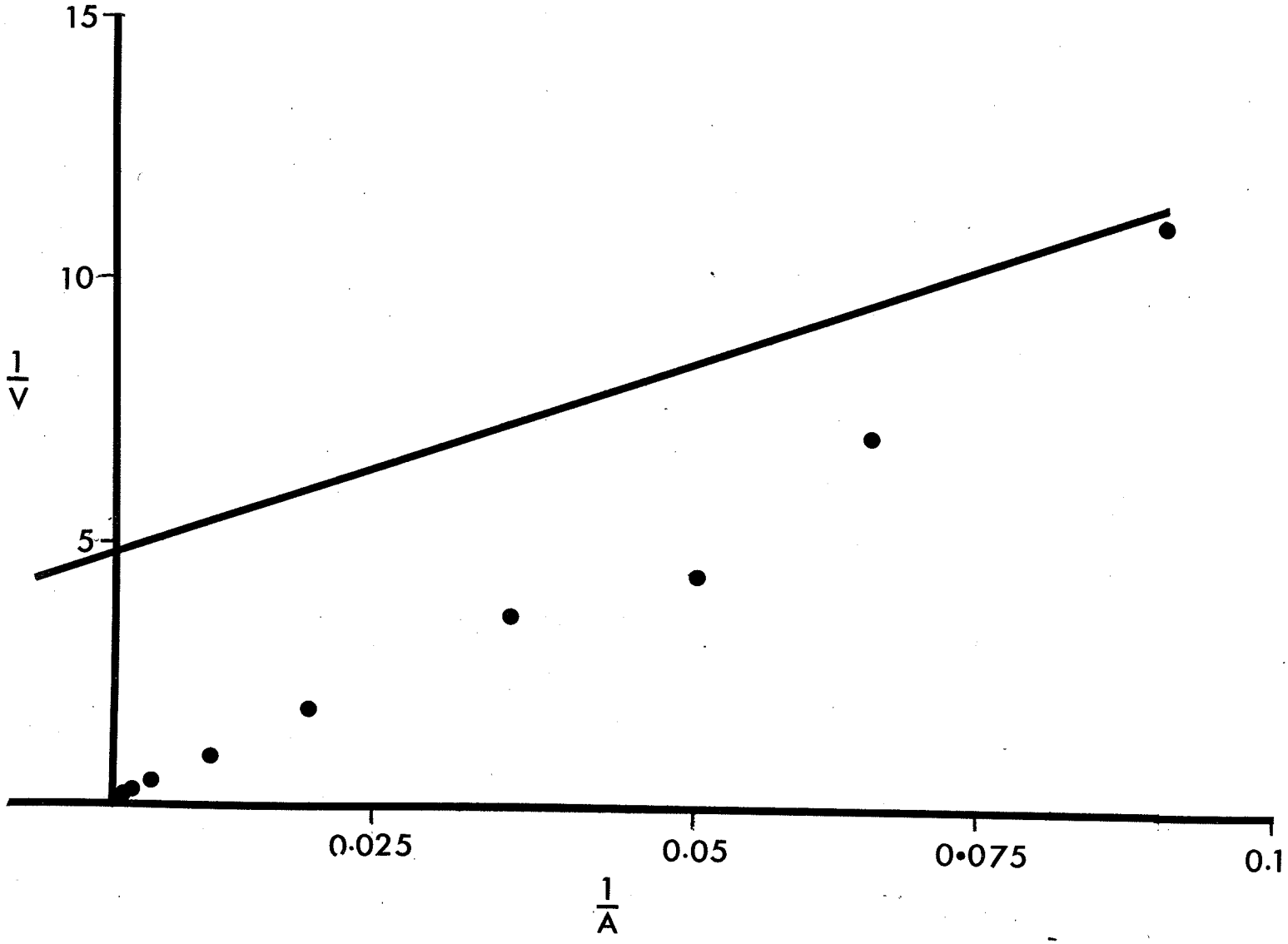
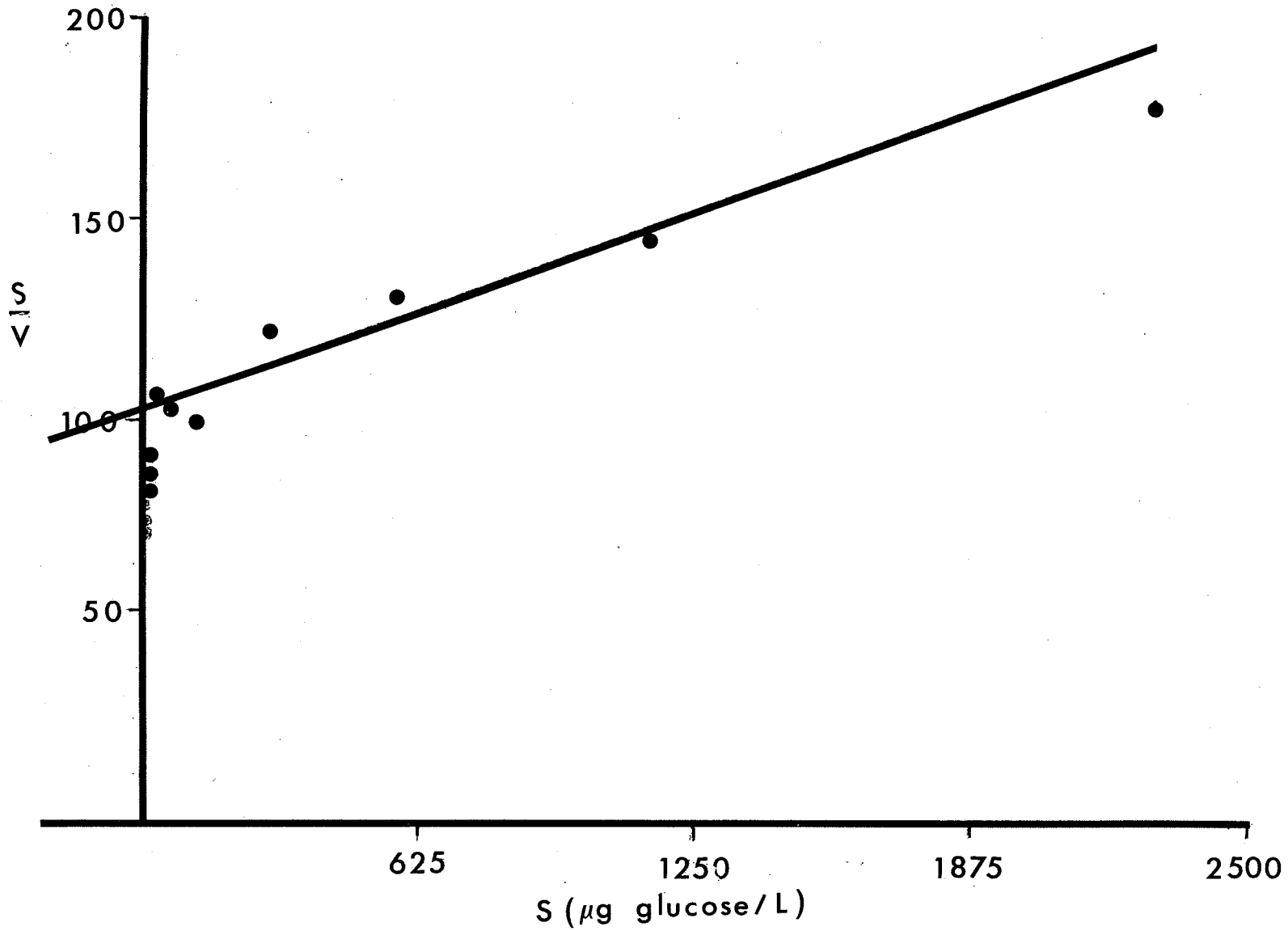

15
10
5
1/V
0·025
0.05
0·075
0.1
1/A

FIGURE 5-4: The Woolf plot of s/v vs. s. Only the low substrate levels are included to illustrate how the observed values deviate from the calculated line.


200
150
100
50
S/V
625
1250
1875
2500
S (μg glucose/L)

The problem of which linear transformation to choose is significant since most phycologists use the Woolf transformation while most bacteriologists use the Lineweaver-Burke conversion. This can result in large discrepancies in the calculated K_t and V_{max} values even when using the same data. it also makes comparison of results from different researchers almost impossible unless the raw data is available. In two studies (Wilkinson 1961; and Dowd and Riggs 1965) it was clearly demonstrated that the Woolf transformation is the superior. This transformation is not as affected by small errors at the low values and results in a more accurate presentation of the observed data (Table 5-1). For this reason the Woolf transformation was the form adopted to present this data.

Calculation of the two different K_t and V_{max} values representing the two uptake systems followed a modification of the method of successive approximations presented by Akedo and Christensen (1962). The substrate concentrations between 0.06 μM (10.99 μg/L) and 0.16 μM (28.63 μg/L) were taken as the point where the relative contribution of the two systems shifted. A least squares line was calculated for the substrate values from 0.16 μM to 12.92 μM (2290 μg/L). This was assumed to include uptake primarily by the low affinity system. This line was then used to calculate the theoretical velocities of uptake at the concentrations from 1.22 nM (0.2198 μg/L) to 0.031 μM (5.497 μg/L). These values were subtracted from the observed velocity values at these concentrations. The resulting values of velocity were taken to represent that portion of the observed velocity resulting from the high affinity system. A second least squares line was calculated for these new high affinity velocities. This line was then employed to determine the portion of the

high velocity values which could be contributed to the high affinity system. These values were then subtracted from the observed velocities and new velocities for the low affinity system were calculated. The entire process was repeated until almost no change was noted in either line. This required eight successive calculations for the data. The resultant lines, one for the low affinity system and one for the high affinity system, were then used to calculate the velocities at each subtrate level contributed by each uptake system. These values were summed and the resultant calculated values were compared with the observed values (Table 5-3). The lines were also used to determine the K_t and V_{max} values for each system (Table 5-3).

TABLE 5-3

Calculated values of uptake velocities using successive subtractions of least squares lines at the low and high substrate levels.

Glucose Concentration (s) µg/L	Calculated (v) High Affinity µg.10^8cells^{-1}min^{-1}		Calculated (v) Low Affinity µg.10^8cell^{-1}min^{-1}		Total Calculated (v)	% Deviation From Observed (v) Table 6-1
0.2198	0.000518	+	0.00145	=	0.00196	7.7
0.5497	0.001073	+	0.00362	=	0.00469	8.3
1.099	0.001671	+	0.00723	=	0.00890	0.9
1.645	0.002050	+	0.01082	=	0.01287	2.9
2.198	0.002320	+	0.01446	=	0.01678	1.6
5.497	0.003015	+	0.03612	=	0.03914	0.4
8.243	0.003230	+	0.05411	=	0.05735	4.7
10.99	0.003350	+	0.07208	=	0.07556	6.7
16.45	0.003480	+	0.10769	=	0.11118	9.6
21.98	0.003550	+	0.14363	=	0.14718	0.2
28.63	0.003600	+	0.18667	=	0.19027	1.5
57.45	0.003680	+	0.37090	=	0.37467	2.5
114.5	0.003730	+	0.72560	=	0.72933	8.4
286.3	0.003760	+	1.71810	=	1.72180	3.3
574.5	0.003770	+	3.16558	=	3.16935	1.3
1145.0	0.003770	+	5.43000	=	5.43377	6.4
2290.0	0.003770	+	8.48665	=	8.49042	2.9

High Affinity System: $y = 264.9x + 366.5$ ($r = 0.9667$ or $P<0.01$)

K_t = 7.7 nM

V_{max} = 0.021 nmol.10^8cells^{-1}. min^{-1}

Low Affinity System: $y = 0.052X + 151.9$ ($r = 0.9773$ or $P<0.01$)

K_t = 16.39 µM

V_{max} = 107.8 nmol.10^8cells^{-1}.min^{-1}

APPENDIX II

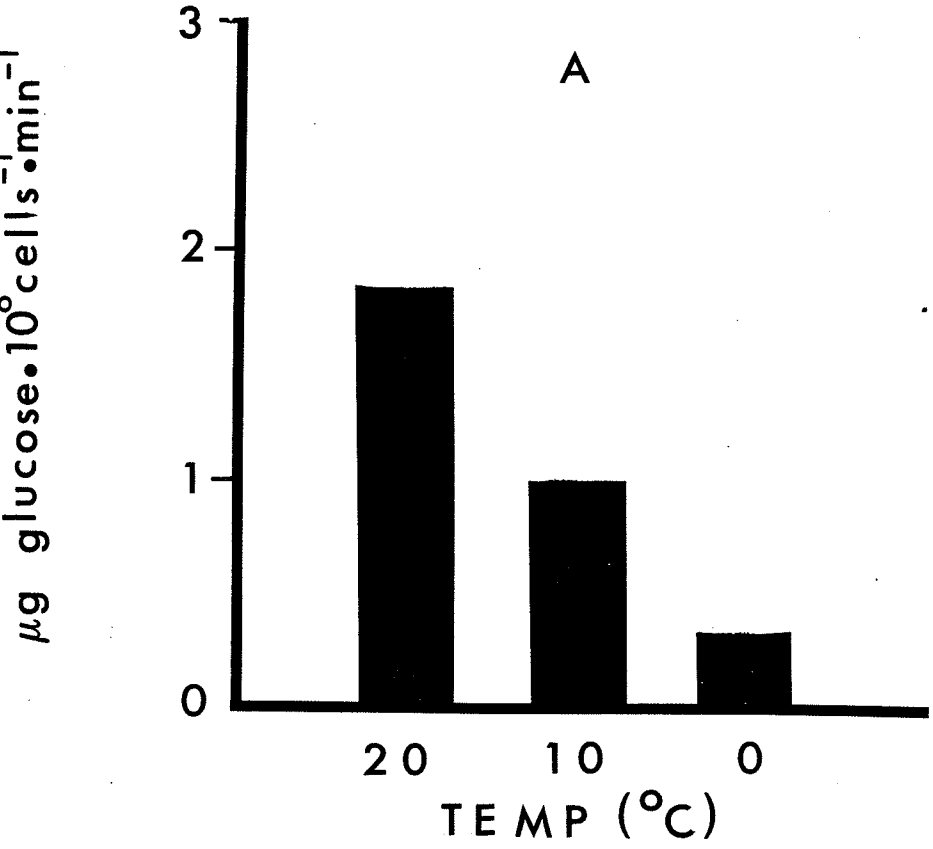
Effect of Temperature on Transport of d-Glucose

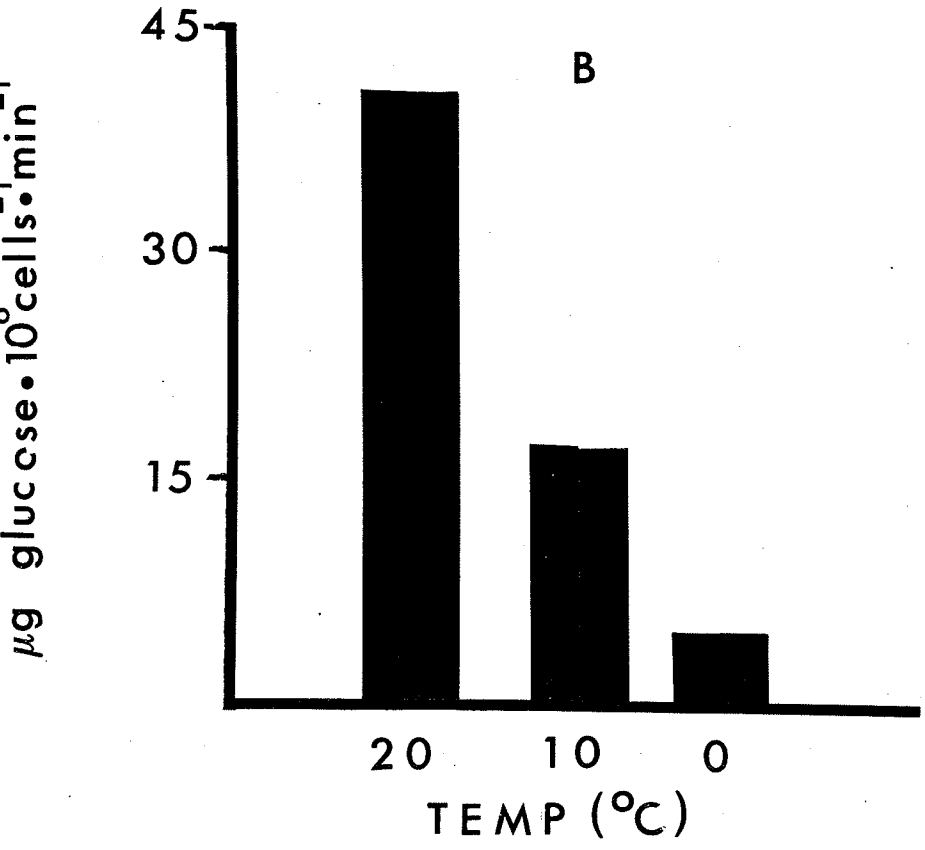
To provide additional evidence as to the nature of the transport system for d-glucose (Chapter Three), uptake was measured under three different temperatures, 20°C, 10°C and 0°C. Cells were prepared as outlined in Chapter One. After dispensing the cells into the experimental tubes, the cell suspensions were cooled to the desired temperature, maintained at this temperature for 20 min. and then supplied with U-C^{14} glucose at 30 $\mu g.L^{-1}$ or 825 $\mu g.L^{-1}$. Uptake was measured after one minute. The transport rates were calculated in the same manner as in Chapter One.

The results of the study show a marked decrease in transport rates as the temperature declined. The Q_{10} (10-20°C) was 1.9 for cells supplied with 30 $\mu g.L^{-1}$ and 2.4 for cells supplied with 825 $\mu g.L^{-1}$ d-glucose. These results indicate an active transport system with a strong temperature dependence. The results are illustrated in Figures 5-5a and 5-5b.

FIGURE 5-5a: Effect of temperature on the transport of d-glucose. The external glucose concentration supplied was 30 $\mu g.L^{-1}$.

FIGURE 5-5b: Effect of temperature on the transport of d-glucose. The external glucose concentration supplied was 825 $\mu g.L^{-1}$.


A
3
2
1
0
μg glucose•10^8cells^-1•min^-1
20
10
0
TEMP (°C)


B
45
30
15
μg glucose•10^8cells^-1•min^-1
20
10
0
TEMP (°C)

APPENDIX III

Inhibitors Used Throughout the Thesis

Table 5-4 is a list of all the inhibitors employed throughout the thesis. The table lists the chemical name of the inhibitor, the abbreviation used and any synonym for the inhibitor. The sites of inhibition are not necessarily the only sites where the inhibitor may exert an influence on the alga but they are the primary sites of action. If more than one site is listed, the primary site, if known, is demarked with an asterisk.

TABLE 5-4: List of inhibitors

Inhibitor	Synonym or Abbreviation	Mol. Weight	Action	Conc. Applied	Reference
Amytal (Na)	amobarbital	248.4	- * inhibits NADH oxidase and energy transfer reactions at the NAD-flavoprotein region	10^{-4}M	Badour, 1978
(Serva)			- inhibits succinic dehydrogenase	10^{-3}M	Dawson et al. 1969
Carbonyl cyanide -m-chlorophenyl hydrazone	CCCP	204.6	- * uncoupler of cyclic and non-cyclic photophosphorylation	10^{-6}M	Raven, 1969
(Aldrich Chem.)			- proton conductor	10^{-5}M	Hoban and Lyric 1976
			- uncouples oxidative phosphorylation		Moreland, 1980
p-Chloromercuri-benzene sulfonate	PCMBS	393.2	- * binds to -SH groups in membrane proteins	10^{-5}M	Dawson et al. 1969
(Sigma)			- inhibits succinate and NADH dehydrogenase	10^{-4}M	Altman and Katz 1976
			- inhibits glucose-6-phosphate dehydrogenase		Harold, 1970

TABLE 5-4: List of inhibitors (continued)

Inhibitor	Synonym or Abbreviation	Mol. Weight		Action	Conc. Applied	Reference
Cycloheximide	actidione	281.4	- *	inhibits DNA synthesis	1.5×10^{-5}M	Dawson et al. 1969
(Sigma)			-	inhibits protein synthesis on cytoplasmic ribosomes	1.5×10^{-6}M	Badour, 1978 Haass and Tanner 1974
3-(3,4-dichloro-phenyl)-1,1-di-methyl urea	DCMU	233.1	- *	inhibits PSII	10^{-6}-10^{-7}M	Raven, 1969
	diuron		-			
(Serva)						
N,N'-dicyclohexyl carbodlimide	DCCD	206.3	- *	inhibits membrane ATPase	10^{-4}M	McCarty and Racker 1967
(J.T. Baker Chem)			-	inhibits phosphorylation reactions		
2,4-dinitrophenol	DNP	184.1	- *	uncouples oxidative phosphorylation	10^{-5}M	Dawson et al. 1969
(Sigma)			-	proton conductor		Harold, 1970
			-	uncouples photophosphorylation	10^{-4}M	Raven, 1969
Gramcidin D.		1141.4	- *	uncouples oxidative phosphorylation by destroying cation gradients	8×10^{-6}M	Dawson, et al. 1969

TABLE 5-4: List of inhibitors (continued)

Inhibitor	Synonym or Abbreviation	Mol. Weight		Action	Conc. Applied	Reference
(Sigma)			-	uncouples photophosphorylation	4×10^{-6}	Olsen and Cox 1979
Imidazole		68.1	- *	uncouples photophosphorylation	10^{-4}M	MacRobbie, 1965
(Sigma)			-	uncouples oxidative phosphorylation		Moreland, 1980
Monensin		692.9	- *	uncoupler, inhibits Na+/H+ exchange	10^{-6}M	Hellebust, 1978
(Sigma)			-	inhibits oxidation of NAD-linked substrates and unmasks ATPases		Hamilton, 1975
Oubain (Serva)	G-strophanthin	728.8	- *	inhibits Na+/K+ dependent ATPase	10^{-6}M	Hochster, et al. 1972,73
Phloretin		273.3	- *	inhibits phosphorylase enzymes	5×10^{-5}M	Dawson, et al. 1969
(Sigma)			-	inhibits erythrocyte glucose uptake		
Phlorizin (Sigma)		472.4	- *	inhibits membrane ATPase and photophosphorylation	10^{-4}M	Hochster, et al. 1972,73
Quercitin		338.3	- *	inhibits transport and mitochondrial ATPase	10^{-3}M	Carpenedo, 1969
(Serva)			-	inhibits pyruvate kinase	10^{-4}M	Grisalia, et al. 1975

TABLE 5-4: List of inhibitors (continued)

Inhibitor	Synonym or Abbreviation	Mol. Weight		Action	Conc. Applied	Reference
Cyanide (K)	KCN	65.1	- *	inhibits cytochrome oxidase	10^{-4}M	Dawson et al. 1969
(Sigma)			-	inhibits photophosphorylation	10^{-3}M	Raven, 1969
Salicylaldoxime		137.1	- *	complexes with heavy metals and inhibits cyclic-photo-phosphorylation	10^{-3}M	Raven, 1969
(Sigma)			-	inhibits cytochromes in electron transport chain	10^{-2}M	Dawson et al. 1969 Raven, 1969
Valinomycin (Sigma)		1111.4	- *	uncouples oxidative and photophosphorylation by destroying proton (K+) gradients	10^{-7}M	Dawson et al. 1969

Names in brackets are the chemical companies where the inhibitors were purchased.

APPENDIX IV

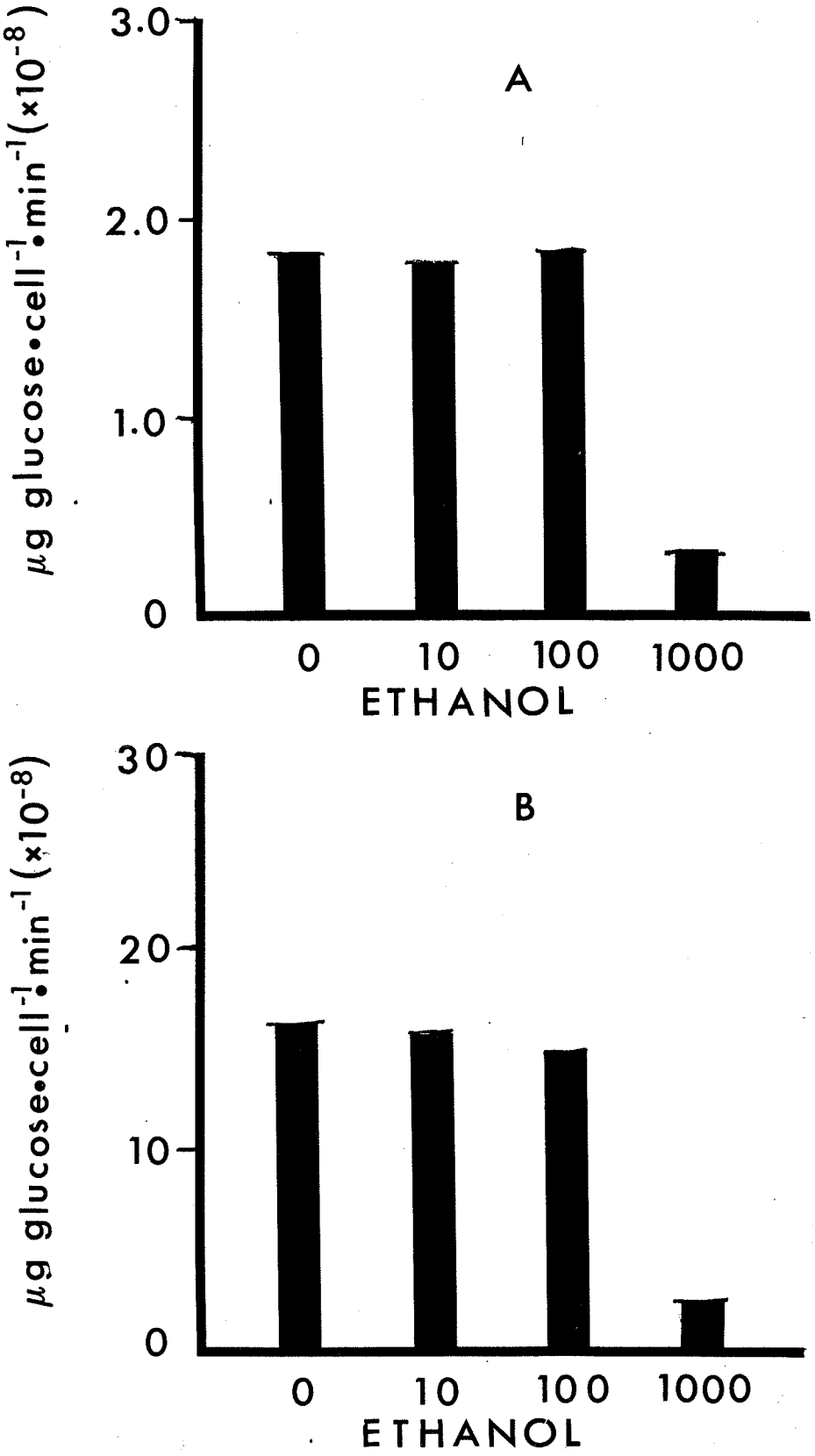
Effect of Ethanol of Transport of d-Glucose

Since several of the inhibitors used in Chapters Two and Three were dissolved in 95% ethanol, this study was done to determine if the ethanol itself would influence the uptake rate. Transport experiments were conducted using two external U-C^{14}d-glucose concentrations (specific activity 291.6 μCi/umol). These were 30 $\mu g.L^{-1}$ and 825 $\mu g.L^{-1}$. The experiments were conducted in the same manner as outlined in Chapter One. The experiments were for one min. and the ethanol concentrations tested were 10 μL, 100 μL and 1000 μL in 15 mL of algal suspension.

The results are illustrated in Figures 5-6a and 5-6b. The 95% ethanol had no effect on the uptake of glucose until a final concentration of 1 mL in 15 mL of culture was applied. No inhibitor-ethanol solution was added at a concentration exceeding 0.1 mL in 15 mL and most were added at a concentration of 0.01 mL in 15 mL. From these results, it was concluded that 95% ethanol did not influence the results of the inhibitor studies.

FIGURE 5-6a: Effect of 95% ethanol on the uptake of d-glucose. The external concentration of glucose was 30 $\mu g.L^{-1}$. The ethanol concentrations were in μL of 95% ethanol in 15 mL medium.

Figure 5-6b: The effect of 95% ethanol on the uptake of d-glucose. External concentration of glucose was 825 $\mu g.L^{-1}$. The ethanol concentrations were in μL of 95% ethanol in 15 ML medium.


A
3.0
2.0
1.0
0
μg glucose•cell^{-1}•min^{-1}(×10^{-8})
0
10
100
1000
ETHANOL
B
30
20
10
0
μg glucose•cell^{-1}•min^{-1}(×10^{-8})
0
10
100
1000
ETHANOL

APPENDIX V

Effect of Cell Concentration on the Transport of d-Glucose

In Chapter Two, two different cell concentrations were used in one of the experiments. These concentrations were 2 x 10^7 cells.L^{-1} and 10^8 cells.L^{-1}. To determine whether this could have an effect on the uptake rates of a cell, three cell concentrations of 2 x 10^7, 10^8 and 10^9 cells.L^{-1} were tested. All experiments were conducted as in Chapter One. The results are listed below in Table 5-5. These results show that there was very little affect, if any, at cell concentrations increasing to 10^8 cells.L^{-1}. At 10^9cells.L^{-1}, there wa a reduction in the uptake per cell of approximately 50%. From these results, it was felt that extrapolations could be made between results at the two concentrations used in Chapter Two.

Table 5-5: Effect of cell concentration on glucose transport. All results are expressed as dpm. The 3 lowest concentrations are with U-C^{14} glucose (specific activity 291.6 μCi/μM, the remaining concentration is with a specific activity of 4.5 μCi/μM.

Cell Concentration	1 μg.L^{-1}	10 μg.L^{-1}	100 μg.L^{-1}	1000 μg.L^{-1}
2 x 10^7	24	147	2139	151
1 x 10^8	83	940	10223	833
1 x 10^9	613	5240	52232	4901

APPENDIX VI

Effect of Preconditioning and Available Energy on Transport

The methods used in this series of experiments are described in Chapter Two. The results are also illustrated in Figs. 2-9, 2-10 and 2-11. In an attempt to analyze these results, a model was conceived which considered the two major areas expected to have an effect on heterotrophy. These areas are the energy demands or requirements of the cell and the available energy to drive the transport system. Using the results of Tables 5-6, 5-7 and 5-8, the model was constructed and tested. By combining 3 sets of variables; light or dark 24h prior to uptake; presence or absence of 0.01mM glucose 24h prior to uptake; and presence or absence of light at the time of uptake, 8 different combinations of conditions could be examined. Each combination was suspected to have a different effect on available energy or the energy demand of the cells.

These combinations were abbreviated and organized into Chart 5-1. A sample abbreviation would be D + L + D. The first letter always represents the light conditions prior to the actual experiments (eg. D = dark 24h prior to experiments). The second letter and sign represents the presence or absence of 0.01mM glucose 24h prior to the experiments (eg. + G = glucose added 24h prior to experiments). The last letter represents the light conditions at the time of uptake (eg. D = dark uptake). Using these symbols, the variables were organized in sequence of projected decreasing uptake rates based on whether energy demand or energy availability was the dominant factor controlling the uptake rate.

Four sets of variables were used (Chart 5-1).

1. Transport rates based entirely on available energy.
2. Transport rates based entirely on energy demand.
3. Transport rates based primarily on available energy but also influenced by energy demand.
4. Transport rates based primarily on energy demands but also influence by available energy.

The model consisted of comparing the actual transport rates in sequence of decreasing rates, with the four sets of 8 combinations of the three variables. The set of variables that most closely matched the actual rates would then be taken as the most representative of the controlling factors in determining the transport rates and therefore heterotrophic potential.

From the comparisons in Chart 5-1, it appears as if the use of heterotrophy is more determined by energy demands than available energy but that the latter factor does have a role in determining the uptake rates. Glucose preconditioning does not appear to have any significance in glucose transport.

TABLE 5-6: The effect of light preconditioning on the transport of U-C^{14} glucose at an external concentration of 10 $\mu g.L^{-1}$.

Experimental Conditions								
Gluc.[1] Prec.	Uptake[2]	Light[3] Prec.	dpm/10^8cells based on vol.	% SE.	Percent[4] Change	dpm/10^8cells	% SE	Percent[4] Change
+	dark	dark	443	+12.7		551	+11.4	
+	dark	light	256	± 8.1	42.0% (+)	355	± 9.8	31.4% (+)
+	light	dark	506	+11.6		574	± 8.8	
+	light	light	274	±11.0	45.9% (+)	400	± 8.4	30.0% (+)
-	dark	dark	395	± 9.0		469	± 7.0	
-	dark	light	313	±12.0	20.5% (+)	405	± 8.1	13.7% (+)
-	light	dark	611	+11.2		680	± 8.1	
-	light	light	378	±11.6	38.1% (+)	451	± 4.1	33.6% (+)

1. Cells preconditioned for 24 h. prior to experiments with 0.01mM d-glucose.
2. Transport measured in the light or dark.
3. Cells preconditioned in continuous light or placed in the dark 24 h. prior to experiments.
4. The percentage change in transports rates dependent on the light preconditioning. The bracketed sign after the percent change in effect of the upper condition.

TABLE 5-7: The effect of glucose preconditioning on the transport of U-C^{14} glucose at an external concentration of 10 $\mu g.L^{-1}$.

Experimental Conditions

Light[1] Prec.	Uptake[2]	Gluc.[3] Prec.	dpm/10^8cells based on vol.	% SE.	Percent[4] Change	dpm/10^8cells	% SE	Percent Change
dark	dark	+	443	±12.7		516	±11.4	
dark	dark	-	395	± 9.0	10.8% (+)	469	± 7.0	9.2% (+)
dark	light	+	506	±11.6		574	± 8.8	
dark	light	-	611	±11.2	17.2% (+)	679	± 8.8	15.5% (+)
light	dark	+	257	± 8.1		354	± 9.8	
light	dark	-	314	±12.0	18.2% (+)	405	± 8.1	12.4% (+)
light	light	+	274	±11.0		400	± 8.4	
light	light	-	378	±11.6	27.6% (+)	451	± 4.1	11.3% (+)

1. Cells precondition in continuous light or in dark for 24 h. prior to experiments.
2. Transport measured in light or dark.
3. Cells preconditioned for 24 h. prior to expeiments with 0.01mM d-glucose.
4. The percentage change in transport rates dependent on the glucose preconditioning. The bracketed sign after the precentage change is the effect on the upper condition.

TABLE 5-8: The effect of available light at the time of experimentation on the transport of U-C^{14} glucose at an external concentration of 10 $\mu g.L^{-1}$.

Experimental Conditions								
Light[1] Prec.	Gluc.[2] Prec.	Uptake[3]	dpm/10^8cells based on vol.	% SE.	Percent[4] Change	dpm/10^8cells	% SE	Percent[4] Change
light	+	light	274	±11.0		400	± 8.4	
light	+	dark	257	± 8.1	6.2% (+)	354	± 9.8	11.4% (+)
light	-	light	378	±11.6		451	± 4.1	
light	-	dark	314	±12.0	16.9% (+)	405	± 8.1	10.2% (+)
dark	+	light	506	±11.6		574	± 8.8	
dark	+	dark	443	±12.7	12.5% (+)	516	±11.4	10.1% (+)
dark	-	light	611	±11.2		679	± 8.1	
dark	-	dark	395	± 9.0	35.4% (+)	469	± 7.0	30.1% (+)

1. Cells precondition in continuous light or in dark for 24 h. prior to experiments.
2. Cells preconditioned for 24 h. prior to experiments with 0.01mM d-glucose.
3. Transport measured in light or dark.
4. The percentage change in transport rates dependent on light or dark uptake. The bracketed sign after the percentage change is the effect of the upper condition.

CHART 5-1: Comparison of projected transport rates based on the influence of energy demands of the cells and available energy to drive the transport system.

1 Available Energy	2 Energy Demands	3 Available Demand	4 Demand Available	5 Actual Rates
L + G + L	D - G + D	L + G + L	D - G + L	D - G + L
L - G + L	D + G + D	L + G + D	D + G + L	D + G + L
L + G + D	D - G + L	L - G + L	D - G + D	D + G + D
L - G + D	D + G + L	L - G + D	D + G + D	D - G + D
D + G + L	L - G + D	D + G + L	L - G + L	L - G + L
D - G + L	L + G + D	D + G + D	L + G + L	L - G + D
D + G + D	L - G + L	D - G + L	L - G + D	L + G + L
D - G + D	L + G + L	D - G + D	L + G + D	L + G + D
No of Matches (0)	(0)	(0)	(6)	

1. Transport rates dependent only on available energy.
2. Transport rates dependent only on energy demands.
3. Transport rates dependent primarily on available energy but also somewhat on energy demand.
4. Transport rates dependent primarily on energy demand but also somewhat on available energy.
5. The actual sequence of decreasing transport rates from Tables 6-5, 6-6 and 6-7.
6. Combinations are listed in projected decreasing transport rates.