THE PHYSIOLOGY OF THE BLUE-GREEN ALGA, *ANACYSTIS NIDULANS* GROWN IN LICHT- AND CARBON DIOXIDE-LIMITED CONTINUOUS-FLOW CULTURE.

by

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A Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Warwick.

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April, 1979.

To my parents Dimitra and Dimitris.

CONTENTS

Page

LIST OF FIGURES

Page

Page

Page

Page

Page

LIST OF TABLES.

Page

Page

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DECLARATION.

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr. J. H. Slater and all sources of information have been specifically acknowledged by means of references.

To my knowledge none of the work contained in this thesis has been previously submitted for examination.

Amalia D. Karagouni.

ABSTRACT

The blue-green alga Anacystis nidulans was grown in closed and open culture. Closed culture studies showed that it was difficult to obtain balanced growth and there were significant variations in μ_{max} values depending on the parameter measured.

Anacystis nidulans was grown in both light- and carbon dioxide-limited chemostat cultures between dilution rates of 0.02 h^{-1} and 0.19 h^{-1}. Under light-limited conditions the culture exhibited a pattern of growth consistent with the model that the biomass concentration was proportional to the reciprocal of the dilution rate. Under carbon dioxide-limited conditions the culture showed normal nutrient-limited kinetics.

Under both limitations the pattern of CO_2 photo-assimilation was similar with a peak at D = 0.10 h^{-1} to D = 0.12 h^{-1}. The rates of CO_2 fixation were 10 fold greater in carbon dioxide-limited organisms compared with light-limited organisms.

The specific activities of a number of enzymes of the reductive and oxidative pentose phosphate and glycolytic pathways and the tricarboxylic acid and glyoxylate cycles were shown to vary as a function of the growth-limiting substrate and organisms growth rate. Ribulose 1,5-bisphosphate carboxylase showed a 15 fold change under carbon dioxide-limited conditions but no change under light-limited conditions. With the exception

of phosphoribulokinase, all enzymes showed an increase in specific activity with decreasing dilution rate. The specific activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, ribulose 1,5-bisphosphate carboxylase and malate dehydrogenase were significantly higher in organisms grown under carbon dioxide-limited conditions than light-limited. Aldolase and phosphoribulckinase specific activities were similar at all growth rates and under both limitations whereas isocitrate lyase was the only enzyme examined which showed higher specific activities under light-limited conditions.

The pattern of growth of Anacystis nidulans during washout from various steady state cultures with either light or carbon dioxide as the growth-limiting nutrient was described. The cell number washout curves for both limitations were biphasic whilst the washout curves by culture absorbance showed no indication of biphasic curve. The mean cell volumes began to increase almost immediately after shift to washout dilution rates in all cases.

PART 1 INTRODUCTION

Investigations into the physiology and biochemistry of the blue-green algae or blue-green bacteria as they are called by Stanier (Stanier, Kunisawa, Mandel and Cohen-Bazire, 1971) or cyanophytes (Brock, 1973), have increased in number since the importance of these organisms in natural blooms was recognized.

The blue-green algae share with the bacteria a type of cellular organization known as prokaryotic, a cellular form which separates them from other algae. The morphology of the unicellular forms is relatively simple and their structure shows little cellular differentiation. The filamentous species of blue-green algae possess a variety of morphological types, differentiated structures and a complexity of life cycle quite without parallel in bacteria (Carr and Bradley, 1973).

Blue-green algae are an ancient group which are believed to have been precursors to the chloroplast organelles of eukaryotic cells evolved during the Precambrian era (Schopf, 1970). The diversity of filamentous types represents a plurality of evolutionary development from a unicellular form (Fritsch, 1945). They were the first oxygen-producing photosynthetic organisms to evolve and were the agents responsible for the initial production of the atmospheric oxygen (Brock, 1973).

Blue-green algae are a cosmopolitan group of organisms, rivalled only by other bacteria in the range of habitats which they occupy. They are distributed in practically all habitats which support life, including hot springs, deserts, icy deserts of Central Asia, mountaintops of Japan, saline lakes, the Antarctic, oil field sump ponds and they also abound in polluted waters. Often blue-green algae are amongst the first organisms to colonise sterile volcanic soils. They occur in static and running water, freshwater and marine habitats. Planktonic freshwater forms belong mostly to the Chroococcales and Nostocales (Fogg, Stewart, Fay and Walsby, 1973; Whitton, 1973). Blue-green algae are abundant in intertidal and supralittoral zones of the seashore and benthic forms have also been reported. Although it is difficult to draw satisfactory taxonomic distinctions between freshwater and marine species, the ability of marine species to grow in sea water relates to a preference for alkaline conditions and tolerance of high salt concentrations (Fogg, 1973).

Blue-green algae have a world-wide distribution in hot springs and they are the only oxygen-evolving photosynthetic organisms found in this environment (Brock, 1967; 1969). They occur in almost every illuminated hot spring which has a pH greater than 5 and a temperature lower than 64^{o}C (Castenholz, 1973). The upper temperature limit may be as high as 72-73^{o}C (Brock, 1973).

A variety of aquatic-terrestrial blue-green algae species, belonging mainly to the genera Nostoc, Schizothrix, Oscillatoria, Lyngbya, Dichothrix, Phormidium and Stigonema, have been recorded (Fogg et al., 1973).

1.1. BASIC PHYSIOLOGY OF BLUE-GREEN ALGAE

Holm-Hansen (1968) concluded that most blue-green algae were obligate phototrophs using light as the only acceptable external energy source, although there are reports that several strains can grow in the dark at the expense of organic compounds. Allen (1952) found that Oscillatoria sp., Lyngbya sp., Phormidium foveolarum and Plectonema notatum could grow very slowly in the dark on media containing glucose and yeast autolysate. Heterotrophic growth by Chlorogloea fritschii has been studied by Fay (1965) who found that there was no growth of this blue-green alga during the first month in the dark and only slow growth during the second and third months. However, Khoja and Whitton (1971) found that 17 out of 24 species of filamentous blue-green algae were able to grow in the dark. Anacystis nidulans was one of those that did not grow in the dark. In all the cases where growth occurred in the dark, the growth rate was considerably lower compared with that in the light. The cultures were incubated in the dark together with 0.01 M sucrose. For Chlorogloea fritschii, the fastest doubling time obtained in the dark was about 5 days whilst the same organism grown with sucrose in the light with an intensity of 500 lux (or 46.5 foot c.) achieved doubling times of one day. Tolypothrix tenuis has also been shown to grow in the dark if supplied with glucose and casamino acids but not if nitrate was the sole nitrogen source (Kiyohara, Fujita, Hattori and Watanabe, 1960; Khoja and Whitton, 1971).

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Stanier et al. (1971) examined the growth of 40 axenic strains of unicellular blue-green algae in the dark in the presence of acetate, succinate, pyruvate, glutamate or glucose. None of the strains examined showed any indication of growth after 1 month. They therefore concluded that these strains were, without exception, obligate phototrophs. More recently Rippka (1972) examined the same strains for photo-heterotrophic capacity, determined by the ability to use glucose (0.5-10% w/v) as a carbon source for growth in the light in the presence of 10^{-5} M 3-(3-4,dichlorophenyl-) 1,1-dimethylurea (DCMU). Six strains of the genus Aphanocapsa and one strain of Chlorogloea proved to be facultative photoheterotrophs. Nostoc muscorum (Allison, Hoover and Morris, 1937) and Nostoc sp. strain Mac (Hoare, Ingram, Thurston and Walkup, 1971; Bottomley and Van Baalen, 1978) were capable of heterotrophic growth in the dark on glucose and fructose.

None of the above workers was able to demonstrate dark heterotrophic growth of Anacystis nidulans. Thus Anacystis nidulans is considered to be one of the blue-green algae which is an obligate phototroph.

All the blue-green algae examined so far have been found to contain thylakoids which are the structural units for the photosynthetic apparatus (Calvin and Lynch, 1952; Fogg et al., 1973; Lang and Whitton, 1973). In Anacystis nidulans and Gloeocapsa sp they are arranged parallel to the cell surface and fill the peripheral part of the cell (Ris and Singh, 1961). Thylakoids contain the photosynthetic

pigments. Allen (1968) reported that in Anacystis nidulans the number of thylakoids was directly proportional to the chlorophyll content and that the thylakoid content and pigment concentration varied inversely with the incident light intensity in organisms grown between 1076 lux (or 100 foot c) and 10,763 lux (or 1000 foot c). Thylakoids also occur, but with some reduction in numbers, in dark grown cells of Nostoc muscorum (Wildon and Mercer, 1963). Evidence from metabolic studies (Biggins, 1969) and electron microscope studies (Bisalputra, Brown and Weier, 1969) have suggested that thylakoids probably have a respiratory function as well as a photosynthetic capability. The idea of the dual role of these membranes in the blue-green algae has been supported by recent investigations of Codd and Sallal (1978). However, comparatively little is known about the existence of various respiratory pathways of blue-green algae. A low respiration rate has been reported in these organisms (Kratz and Myers, 1955b; Biggins, 1969; Leach and Carr, 1968; 1969; 1970) which may be related to their limited ability to utilize organic substrates for growth and for energy production.

On average, blue-green algae have, on a dry weight basis (w/w), 50% protein, 30% carbohydrate, 5% lipid and 15% ash (Collyer and Fogg, 1955). In other studies (Holohan and Moore, 1967; Parsons, Stephens and Strickland, 1961), marine and freshwater blue-green algae cells have been shown to contain, on a dry weight basis (w/w), 20 to 45% protein, 30 to 55% carbohydrate, 15% lipid, 2% RNA, 0.4 to 0.8% DNA and 1.5% total pigment.

Blue-green algae contain chlorophyll a, various phycobiliproteins and carotenoids. Phycobiliproteins are the red and blue bile pigment protein conjugates and they represent 1-10% (w/w) of the dry weight of the cell, although in some cases it may be as much as 24% (w/w) in Anacystis nidulans (Myers and Kratz, 1955).

Nitrogen fixation does not appear to be a universal characteristic of blue-green algae. The ability to reduce molecular N_2 was found in many filamentous forms (Stewart, 1967), but was originally undetected in any unicellular species of the order Chroococcales (Stewart, 1966). Over fifty strains and species of heterocystous, blue-green algae are known to fix nitrogen (Fogg et al., 1973). However, the presence of the nitrogen-fixing enzyme, nitrogenase, in unicellular, blue-green algae was demonstrated in 1969 by Wyatt and Silvey, using a strain of Gloeocapsa.

1.2. METABOLIC PATHWAYS IN BLUE-GREEN ALGAE

Carbon dioxide is the principal, if not the sole, carbon source for the blue-green algae. All the available evidence suggests that the reductive pentose phosphate cycle (RPP cycle or Calvin cycle) is the major assimilatory pathway used during growth of blue-green algae.

Carbon dioxide is added on to a 5-carbon acceptor compound, ribulose bisphosphate (RuBP), which is cleaved to yield 2 molecules of a 3-carbon compound, 3-phosphoglyceric acid (PGA). PGA is reduced to a triose sugar phosphate from

which, by a series of sugar phosphate interconversions, the acceptor for carbon dioxide, RuBP, is regenerated (Figure.1.1). This cycle can be visualized as producing one glucose molecule for every 6 carbon dioxide molecules fed into it but in actual fact the fixed carbon can be taken out in a variety of forms. The energy for the operation of the cycle comes from ATP and $NADPH_2$ supplied by the photochemical reactions.

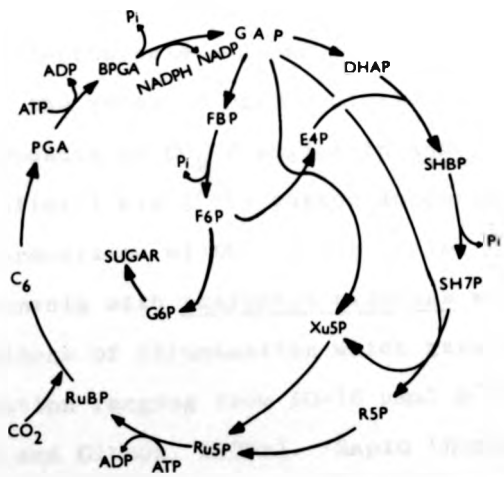
Brief exposure of organisms assimilating carbon dioxide to radioactively labelled carbon dioxide, has been used to elucidate the route of carbon dioxide fixation. Experiments with exposures of only 5 sec to 2 min were carried out by Kandler (1961), using Anacystis nidulans. These showed that after exposure for 5 sec, 3-phosphoglyceric acid (PGA) was the most heavily labelled product and the percentage of the total radioactivity fixed into this compound decreased as the period of exposure to labelled CO_2 increase. This is good evidence that PGA was the primary fixation product. Richter (1961) reported similar results with the same organism.

Kindel and Gibbs (1963) showed that the labelling pattern of [^{14}C]-carbon in the glucose of polysaccharide isolated from Anacystis nidulans was similar to that from Chlorella pyrenoidosa. Most of the label was present in carbon atoms 3 and 4 which is consistent with fixation into PGA followed by the synthesis of glucose.

Figure 1.1.

The reductive pentose phosphate cycle or Calvin cycle (redrawn from Fogg *et al.*, (1973)

RuBP:	ribulose 1,5-bisphosphate
PGA:	3-phosphoglyceric acid
BPGA:	1,3-bisphosphoglyceric acid
GAP:	glyceraldehyde 3-phosphate
DHAP:	dihydroxyacetone phosphate
FBP:	fructose 1,6-bisphosphate
F6P:	fructose 6-phosphate
G6P:	glucose 6-phosphate
E4P:	erythrose 4-phosphate
SHBP:	sedoheptulose 1,7-bisphosphate
SH7P:	sedoheptulose 7-phosphate
Xu5P	xylulose 5-phosphate
R5P:	ribose 5-phosphate
Ru5P:	ribulose 5-phosphate
C_6:	C_6 compound
ATP:	adenosine triphosphate
ADP:	adenosine diphosphate
P_i:	inorganic phosphate
NADPH/NADP:	reduced and oxidized forms of nicotinamide adenine dinucleotide phosphate


Pi
GAP
ADP
BPGA
NADPH
NADP
DHAP
ATP
FBP
E4P
PGA
Pi
SHBP
F6P
Pi
SUGAR
C6
SH7P
G6P
XuSP
RuBP
R5P
CO2
ADP
ATP
RuSP

The detailed studies of Pelroy and Bassham (1972) with Synechococcus 6301 (Anacystis nidulans), Synechococcus 6307, Aphanocapsa 6308 and Aphanocapsa 6714 provided clear evidence that PGA was the first detectable product of carbon dioxide fixation. They examined the kinetics of [^{14}C]-carbon dioxide incorporation into the metabolic pools of these organisms. PGA was the first compound to be labelled and within 30-60 sec radioactivity became detectable in ribulose 1,5-bisphosphate, glucose 6-phosphate, fructose monophosphate and sedulose 7-phosphate. Jansz and Maclean (1973) also investigated the route of carbon dioxide fixation by examining the early products of CO_2 fixation in Anacystis nidulans. They found that after 1 min [^{14}C]-carbon appeared in PGA and related intermediates of the Calvin cycle.

Experiments with Anacystis nidulans were carried out under conditions of illumination which gave rates of carbon dioxide fixation ranging from 10-16 μmol h^{-1} (mg protein)$^{-1}$ (Ihlenfeldt and Gibson, 1975a). Rapid incorporation into phosphorylated intermediates of the reductive pentose phosphate cycle and into amino acids was observed. These findings confirmed the data of Pelroy and Bassham (1972) and Jansz and Maclean (1973).

Cell-free preparations should contain all of the enzymes of the cycle, in amounts consistent with the rate of carbon dioxide fixation by intact organisms. Information is available on the level of the enzymes of the reductive pentose phosphate cycle in blue-green algae (Smith, 1973).

Only one organism, Tolypothrix tenuis, has been shown to contain all the enzymes of the cycle (Latzko and Gibbs, 1969). Ribulose 1,5-bisphosphate carboxylase has been demonstrated in extracts of Anacystis nidulans (Fuller and Gibbs, 1959; Slater, 1975). Fructose bisphosphate aldolase was at one time thought to be absent from blue-green algae (Richter, 1959). However, exposure to [^{14}C]-carbon dioxide was found to give patterns of labelling in blue-green algae which were similar to that obtained in Chlorella pyrenoidosa, an organism known to contain aldolase (Kindel and Gibbs, 1963) and, since the earlier failure, this enzyme has been found in extracts of several blue-green algae (Van Baalen, 1965).

The enzyme glyceraldehyde 3-phosphate dehydrogenase which is responsible for producing a triose sugar from PGA has been detected in crude extracts of several blue-green algae (Hood and Carr, 1967).

A number of investigations with unicellular algae (Pedersæn, Kirk and Bassham, 1966), bacteria (Hart and Gibson, 1971) and blue-green algae (Ihlenfeldt and Gibson, 1975a; Slater, 1975) have suggested that the regulation of CO_2 fixation may occur at several points in the reductive pentose phosphate cycle. These include the ATP consuming steps at the Ru5P kinase and glycerate 3-phosphokinase, the carboxylation step of RuBPCase and the specific phosphatase reactions at fructose 1,6-bisphosphate and sedulose 1,7-bisphosphate. Carr, Hood and Pearce (1969) have presented

evidence suggesting that in Anabaena variabilis the presence of acetate in the growth medium caused a reduction in RuBPCase activity compared with autotrophically grown organisms. However, no alteration of RuBPCase in blue-green algae was found after inclusion of sugars in the phototrophic growth medium (Harrison and Carr, unpublished observations quoted in Carr, 1973). RuBPCase in extracts of photosynthetically grown Chlorogloea fritschii was inhibited by inclusion of glycerophosphate, and less by the inclusion of fructose to the assay mixture (Harrison and Carr, unpublished observations).

It has been shown in Anacystis nidulans that the activity of the partially purified glyceraldehyde 3-phosphate dehydrogenase was 50 times greater if linked with NADPH compared with NADH (Ihlenfeldt and Gibson, 1975a). They suggested that this may be an important enzyme in the regulation of carbon dioxide fixation. The concentration of NADPH characteristic of illumianted cells was not present during prolonged incubation in the dark, suggesting that further fixation may be prevented by lack of reductive capacity.

Pelroy, Rippka and Stanier (1972) determined the specific activities of certain enzymes operative in carbohydrate metabolism in extracts prepared from photo-autotrophically grown organisms of Aphanocapsa 6308 and 6714 and Synechococcus 6301 (Anacystis nidulans). Triose phosphate dehydrogenase and fructose bisphosphate aldolase,

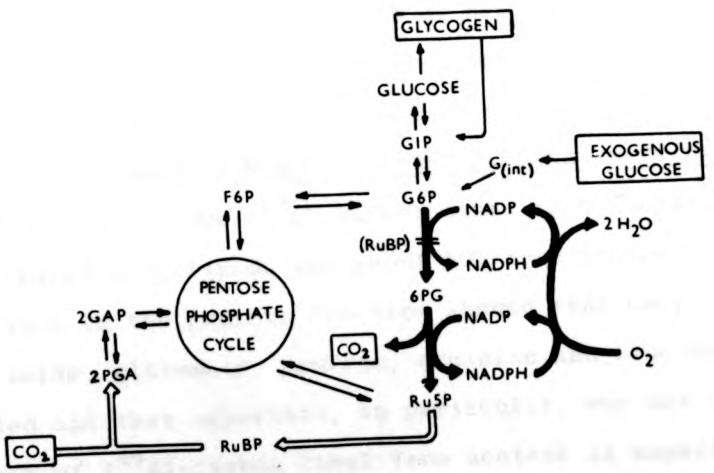
which play an essential role in the Calvin cycle, were found to have specific activities approximately 15 nmol of substrate used min^{-1} (mg protein)$^{-1}$. Glucose 6-phosphate and 6-phosphogluconate dehydrogenases were present in the extracts at comparable levels. They also demonstrated in Synechococcus 6301 and Aphanocapsa 6308 that the glucose 6-phosphate dehydrogenase was allosterically inhibited by ribulose 1,5-bisphosphate. Pelroy and Bassham (1972) found that 6-phosphogluconate appeared in the metabolic pool of unicellular blue-green algae shortly after they were placed in darkness following a period of CO_2 assimilation in the light. Stanier (1973) interpreted the two above observations as follows. When the cells are engaged in the photosynthetic assimilation of CO_2, the pool level of RuBP is sufficiently high to prevent the activity of glucose 6-phosphate dehydrogenase; the pathway remains physiologically inoperative in the light, although high levels of the enzymes for the oxidative pentose phosphate pathway are present. When illumination ceases, the RuBP pool rapidly disappears and the allosteric inhibition of glucose 6-phosphate dehydrogenase is relieved, leading to a rapid conversion of the substrate to 6-phosphogluconate via the uninhibited oxidative pentose phosphate pathway.

Figure 1.2 is a simplified metabolic map proposed by Pelroy et al. (1972) and describes the primary pathways of carbon metabolism in unicellular blue-green algae. This is the synthesis of the available information concerning the primary carbon metabolism of blue-green algae (Rippka, 1972;

Figure 1.2.

A metabolic map which shows the pathways of primary carbon metabolism in unicellular blue-green algae. The substrates and end-products are enclosed in square boxes. Reactions specific to photosynthetic CO_2 assimilation are indicated by heavy white arrows; reactions specific to respiratory metabolism by heavy black arrows. The double line across the arrow connecting glucose 6-phosphate and 6-phosphogluconate indicates the site of allosteric control by ribulose 1,5-bisphosphate. (The figure redrawn from Stanier (1973)).

PGA:	3-phosphoglyceric acid
GAP:	glyceraldehyde 3-phosphate
GIP:	glucose 1-phosphate
G6P:	glucose 6-phosphate
G(int):	intracellular glucose pool
F6P:	fructose 6-phosphate
6PG:	6-phosphogluconic acid
Ru5P:	ribulose 5-phosphate
RuBP:	ribulose 1,5-bisphosphate


GLYCOGEN
GLUCOSE
G1P
G(int)
EXOGENOUS GLUCOSE
F6P
G6P
NADP
2 H2O
(RuBP)
NADPH
PENTOSE PHOSPHATE CYCLE
6PG
2GAP
NADP
CO2
O2
2PGA
NADPH
RuSP
CO2
RuBP

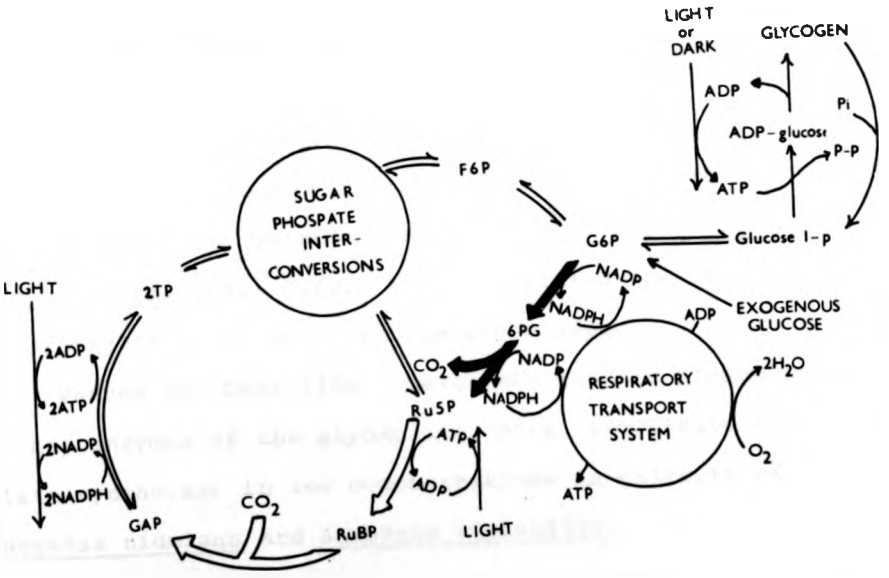
Pelroy and Bassham, 1972; Pelroy et al., (1972). It also includes the findings of Pearce and Carr (1969) with respect to glucose metabolism and of Biggins (1969) with respect to electron transport.

Figure 1.3 shows the metabolic links between photosynthetic and respiratory metabolism in blue-green algae which are close (Schaeffer and Stanier, 1978).

In phototrophic organisms the tricarboxylic acid cycle is necessary for the biosynthesis of oxaloacetate and α-ketoglutarate which are amino acid precursors and of succinyl-CoA which is required for tetrapyrrole biosynthesis. Hoare and Moore (1965) found in Synechococcus sp. (Anacystis nidulans) that about 60% of the [^{14}C]-carbon label from acetate was incorporated into lipids and about 30% into proteins. Hydrolysis in the protein fraction showed that only four amino acids, glutamate, proline, arginine and leucine, were labelled and that aspartate, in particular, was not labelled. The lack of [^{14}C]-carbon label from acetate in aspartate and other amino acids of the aspartate family suggested that the flow of carbon from assimilated acetate proceeded to α-oxoglutarate and hence to glutamate, but further metabolism to succinate was blocked. Smith, London and Stanier (1967) found a similar pattern of acetate assimilation in Coccochloris peniocystis, Gloeothece linearis, Gloeocapsa alpicola and Synechococcus sp. (Anacystis nidulans). In extracts prepared from these blue-green algae, α-oxoglutarate dehydrogenase

Figure 1.3.

The primary pathways of blue-green algae carbon metabolism in the light and in the dark, showing the central role of the reductive pentose phosphate cycle. Reactions specifically operative in the light are indicated by heavy white arrows; those operating at high rates only in the dark, by heavy black arrows. (The figure redrawn from Schaeffer and Stanier (1978)).


LIGHT or DARK
GLYCOGEN
ADP
Pi
ADP-glucose
P-P
ATP
F6P
SUGAR PHOSPATE INTER-CONVERSIONS
G6P
Glucose 1-p
NADP
NADPH
EXOGENOUS GLUCOSE
ADP
LIGHT
2TP
6PG
CO2
NADP
NADPH
RESPIRATORY TRANSPORT SYSTEM
2H2O
O2
ATP
2ADP
2ATP
2NADP
2NADPH
Ru5P
ATP
ADP
GAP
CO2
RuBP
LIGHT

could not be detected. The interruption of the cycle at the point of α-oxoglutarate does not allow the organism a site of substrate level phosphorylation and the formation of reduced pyrimidine nucleotides by oxidation of organic substrates. Therefore they suggested that the absence of this tricarboxylic acid cycle enzyme was one of the principal reasons for the obligate autotrophy of blue-green algae. The presence of most of the tricarboxylic acid cycle enzymes in extracts of Anabaena variabilis has since been demonstrated by Pearce and Carr (1967) and Pearce, Leach and Carr (1969), although here too α-oxoglutarate dehydrogenase and succinyl-CoA synthase could not be detected in Anabaena variabilis or in Anacystis nidulans. Figure 1.4 illustrates the interrupted tricarboxylic acid cycle in blue-green algae.

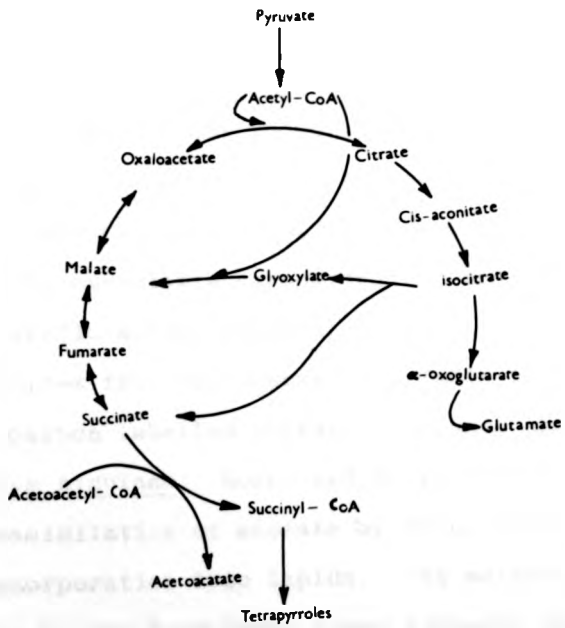
Pearce and Carr (1967) have detected the presence of two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthetase in low concentrations in extracts of Anacystis nidulans and Anabaena variabilis.

1.3. EFFECT OF ORGANIC COMPOUNDS

The metabolism of organic compounds and their influence on growth of several autotrophic prokaryotes has recently been reviewed by Smith and Hoare (1977). There are some organic compounds which stimulate the growth of several blue-green algae under certain growth conditions. One example is the stimulation of growth of Agmenellum quadruplicatum by glucose at a low light intensity (Van

Figure 1.4.

The tricarboxylic acid cycle in blue-green algae (The figure redrawn from Fogg et al., (1973)).


Pyruvate
Acetyl-CoA
Oxaloacetate
Citrate
Cis-aconitate
Malate
Glyoxylate
isocitrate
Fumarate
α-oxoglutarate
Succinate
Glutamate
Acetoacetyl-CoA
Succinyl-CoA
Acetoacatate
Tetrapyrroles

Baalen, Hoare and Brandt, 1971). In general, however, organic compounds do not stimulate the growth of blue-green algae. Furthermore, certain organic compounds in fact inhibit the growth of some phototrophs; for example, such effects have been observed by individual amino acids or mixtures of amino acids (Ingram and Jensen, 1973; Hutber and Smith, unpublished observations quoted in Smith and Hoare, 1977).

The isotopic tracer technique has been used to show the entry of organic compounds into the cell. Growing cultures as well as cell suspensions of several blue-green algae have been shown to assimilate carbohydrates, amino acids, mono- and dicarboxylic acids, purines, pyrimidines, nucleosides and nucleotides into cell material. Table 1.1. summarises the [^{14}C]-carbon labelled organic compounds photoassimilated by Anacystis nidulans. Hoare and Moore (1965) described the photoassimilation of acetate by three blue-green algae and its incorporation into lipids. The metabolism of acetate and glucose by the blue-green algae Anabaena variabilis and Anacystis nidulans was examined by Pearce and Carr (1967, 1969). The incorporation of acetate and glucose on a dry weight basis increased from 7.2 to 16.7% (w/w) and from 33 to 46% (w/w) respectively, when the concentration of carbon dioxide in the gas phase over the cultures was reduced from 5 to 0.05% (v/v).

Table 1.1. [^{14}C]-carbon labelled organic compounds photo-assimilated by Anacystis nidulans (taken from Smith (1973)).

Compound	Reference
CARBOHYDRATES	
Glucose	Carr and Pearce, 1966
	Smith et al., 1967
Fructose	Smith et al., 1967
CARBOXYLIC ACIDS	
Formate	Pigott and Carr, 1971
Acetate	Hoare and Moore, 1965
	Hoare et al., 1967
	Nichols et al., 1965
Propionate	Hoare et al., 1967
Stearate	Nichols et al., 1965
Palmitate	Nichols et al., 1965
Oleate	Nichols et al., 1965
Pyruvate	Smith et al., 1967
Succinate	Smith et al., 1967
Malate	Smith et al., 1967
AMINO ACIDS	
Aspartate	Smith et al., 1967
Glutamate	Smith et al., 1967
Leucine	Smith et al., 1967
Threonine	Maclean et al., 1965
NUCLEIC ACID PRECURSORS	
Uracil	Pigott and Carr, 1971
Thymine	Pigott and Carr, 1971
Orotic acid	Pigott and Carr, 1971
Adenine	Pigott and Carr, 1971
Guanine	Pigott and Carr, 1971
Thymidine	Pigott and Carr, 1971
Guanosine	Pigott and Carr, 1971
Uridine 5-phosphate	Pigott and Carr, 1971

Investigations concerning the photoassimilation of acetate by growing cultures and cell suspensions of blue-green algae were undertaken by Hoare, Hoare and Smith (1969). In Anacystis nidulans the use of the 1-[^{14}C] or 2-[^{14}C]-acetate resulted in radioactivity being detected in four amino acids: glutamate, proline, arginine and leucine, of Anacystis nidulans. Incorporation of arginine into protein by Anabaena variabilis was observed when arginine was added to the growth medium (Hood and Carr, 1971). Fractionation of organisms of Anabaena variabilis indicated that the major part of the U-[^{14}C]-valine was incorporated into the protein fraction (Hood and Carr, 1972).

Synechococcus 6301 (Anacystis nidulans) and Aphanocapsa 6308, both obligate photoautotrophs, converted glucose to CO_2 at extremely low rates which did not increase significantly when glucose was supplied to the organisms at a concentration of 10^{-2} M (Pelroy, Rippka and Stanier, 1972). In contrast, in Aphanocapsa 6714, a facultative photoheterotroph, the rate of glucose metabolism increased with increasing substrate concentration and reached a maximum at approximately 10^{-4} M glucose. Therefore they suggested that the inability of Synechococcus 6301 (Anacystis nidulans) and Aphanocapsa 6308 to grow at the expense of glucose was attributed to their lack of an effective glucose permease and the existence of a specific permeability barrier which prevented effective uptake of glucose from the medium. Pigott and Carr (1971) have described the relative impermeability of Anacystis nidulans to purine and pyrimidine bases. Labelled purine and pyrimidine

bases were included in the growth media of Anacystis nidulans, but only a very small percentage of the available exogenous material was detected in the nucleic acid fraction compared with the Escherichia coli controls. Protoplasts prepared from Anacystis nidulans assimilated purine and pyrimidine bases more readily than did the intact organisms, suggesting an association of the permeability barrier with the cell wall rather than with the cytoplasmic membrane.

1.4. REGULATION OF ENZYME SYNTHESIS IN BLUE-GREEN ALGAE

The work of Carr and his co-workers has led to the conclusion that many blue-green algae apparently lack the ability to regulate enzyme activity through control of the synthesis of the enzymes. Their observations suggested that blue-green algae do not respond to the presence of certain substrates by the usual mechanisms of enzymatic induction and repression.

Table 1.2 shows examples of enzyme activities of glycolytic and pentose phosphate pathways, glyoxylate and tricarboxylic acid cycles, in extracts of Anabaena variabilis grown autotrophically and in the presence of glucose or acetate (Pearce and Carr, 1967; Pearce et al., 1969; Carr, 1973). The activities of these enzymes were not altered by inclusion of glucose or acetate in the growth medium. Similar results were obtained for Anacystis nidulans. Pearce and Carr (1967) reported preliminary results which indicated that RuBPCase, one of the key enzymes of photosynthetic CO_2 fixation, was only slightly altered after growth in the presence of sodium acetate.

Table 1.2. Specific activities of enzymes of intermediary metabolism in extracts of Anabaena variabilis grown autotrophically and in the presence of glucose or acetate. (Table taken from Carr, 1973).

Enzyme	Supplement to growth medium	Activity (nmol min^{-1} mg protein$)^{-1}$) autotrophic growth	autotrophi plus supplement
GLYCOLYTIC AND PENTOSE PHOSPHATE PATHWAY			
Hexokinase	glucose	1.2	1.2
Phosphohexoisomerase	glucose	5.4	5.4
Phosphofructokinase	glucose	8.1	7.8
Fructose bisphosphate aldolase	glucose	3.6	3.6
Triose phosphate dehydrogenase(NAD)	glucose	8.3	7.5
Triose phosphate dehydrogenase (NADPH)	glucose	4.2	3.7
Pyruvate kinase	glucose	13.0	11.0
Glucose 6-phosphate dehydrogenase	glucose	8.7	7.8
6-Phosphogluconate dehydrogenase	glucose	12.0	12.0
Phosphoriboisomerase	glucose	0.35	0.35
GLYOXYLATE CYCLE			
Acetyl-CoA synthetase	acetate	3.1	3.0
Acetate kinase	acetate	4.1	4.5
Phosphotransacetylase	acetate	1.4	1.7
Isocitrate lyase	acetate	0.39	0.38
Malate synthetase	acetate	0,80	0.82
INTERRUPTED TRICARBOXYLIC ACID CYCLE			
Citrate condensing enzyme	acetate	5.7	6.1
Isocitrate dehydrogenase	acetate	4.7	5.0
Succinic dehydrogenase	acetate	0.2	0.2
Fumarase	acetate	3.7	3.5
Malate dehydrogenase	acetate	2.0	2.3
Glutamate dehydrogenase	acetate	2.7	2.4

In Anacystis nidulans certain enzymes of the oxidative pentose phosphate cycle pathway are essential only for dark metabolism. Doolittle and Singer (1974) measured the specific activities of three of these enzymes (glycogen phosphorylase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) in cultures before and after removal of light. Although levels of the third enzyme remained constant, the first two showed reproducible 1.5 to 2.0 fold increases in specific activity. The increase in activity was inhibited by chloramphenicol suggesting that their increase was associated with protein synthesis. These increases occurred over about one-third of a generation time after switching to dark conditions and at a period when overall rates of protein synthesis were rapidly falling. Experiments with incorporation of radioactive leucine into protein in a leucine-requiring mutant of Anacystis nidulans were made by Singer and Doolittle (1975). Label incorporation was usually a direct measure of protein synthesis, when there was no endogenous leucine synthesis in the strain. Rates fell during the first hour of darkness, and remained low until the culture was reilluminated. Several proteins were synthesized in the dark which in the light were either not made or were present in lower relative amounts, suggesting that at least some of these enzymes were specifically required and selectively synthesized in the dark. Although blue-green algae do not respond to most exogenous substrates by changing levels of enzymes responsible for their utilisation or formation, Singer and Doolittle do not accept that these organisms are incapable of regulating enzyme activity at the level of gene expression.

Similarly it has also been suggested that blue-green algae are unable to control their amino acid biosynthetic pathways at the level of the synthesis of the necessary enzymes. Hoare and Hoare (1966) found in extracts of Anacystis nidulans that N-acetylglutamate kinase, which is the second enzyme in the pathway of arginine biosynthesis from glutamate, was inhibited by arginine. The inhibition was shown to be partially competitive since inhibition by a given concentration of arginine was overcome by increasing N-acetylglutamate levels. However, there was no evidence of repression of the enzymes involved in the synthesis of arginine (Hood, Leaver and Carr, 1969). Ornithine transcarbamylase activity in Anabaena variabilis, Anacystis nidulans, Chlorogloea fritschii and Gloeocapsa alpicola were the same after growth in the presence or absence of arginine. Similar results were obtained from Anabaena variabilis from the levels of four other enzymes (N-acetylglutamate phosphokinase, N-acetylomithine transaminase, N-acetylornithine transacetylase and arginosuccinate) catalysing steps from glutamate to arginine (Hood and Carr, 1971). The enzymes (threonine deaminase, α-acetohydroxyacid synthetase, isopropylmalate synthetase and transaminase B) involved in leucine, isoleucine and valine synthesis have been detected in extracts of Anabaena variabilis by Hood and Carr (1972). No repression of threonine deaminase was observed when Anabaena variabilis was grown in the presence of isoleucine or a combination of valine, leucine and

isoleucine, and no repression of transminase B was observed when organisms were grown in the presence of valine, leucine and isoleucine. However, the inclusion of all three branched-chain amino acids, valine, leucine, isoleucine caused up to a 20% decrease of the specific activity of α-acetylhydroxyacid synthetase in Anabaena variabilis (Table 1.3) and a 50% decrease in Anacystis nidulans, whilst in Escherichia coli they caused a 75% reduction.

The lack of transcriptional control of homoserine-O-transsuccinylate, the enzyme of the first step in methionine synthesis by the use of a methionine-requiring mutant of Anacystis nidulans strain 1173 (Delaney, Dickson and Carr, 1973) has been described. The use of this mutant of Anacystis nidulans excluded the possibility that endogenous repression, at least in the case of methionine biosynthesis, was already operating before the addition of an exogenous organic carbon source.

Joset-Espardellier,Astier, Evans and Carr (1978) examined some aspects of sugar metabolism in the facultatively photo-trophic blue-green algae, Aphanocapsa 6714 and Chlorogloea fritschii, after growth under photoautotrophic, photoheterotrophic and heterotrophic conditions. They observed little variation in the specific activities of enzymes involved in sugar and carboxylic acid metabolism. Table 1.4 shows the enzyme activities of Chlorogloea fritschii and Aphanocapsa 6714 after growth under different conditions.

Table 1.3. The effect of inclusion of branched-chain amino acids in the growth medium of *Anabaena variabilis* on the activities of three enzymes involved in their biosynthesis (taken from Hood and Carr (1972)).

Enzyme	Additions to growth medium	Activity (nmol min^{-1} (mg protein)$^{-1}$)	% activity compared with that in absence of added amino acids
Threonine deaminase	None	0.51	100
	+ L-Isoleucine (1.5 mm)	0.48	94.1
	L-Isoleucine, L-valine, L-leucine (mM)	0.57	111.8
α-Acetohydroxy-acid synthetase	None	30	100
	Valine (mM)	27	90
	L-Valine, L-isoleucine, L-leucine (mM)	24	80
Transaminase B	None	69	100
	L-Valine, L-leucine, L-isoleucine (mM)	80	116

Table 1.4. Enzyme activity after growth under different conditions.

Activities expressed are the mean of several determinations. (Joset-Espardellier et al., (1978)).

Enzyme (nmol min^{-1}(mg protein)$^{-1}$)	(a) Chlorogloea fritschii			(b) Aphanocapsa 6714		
Generation time (h)	Growth			Growth		
10%	Photoauto-trophic 30	Photohetero-trophic 25	Hetero-trophic 80	Photoauto-trophic 6	Photohetero-trophic 6	Hetero-trophic 27
Malate dehydrogenase	2.6	2.9	16.6	90	200	260
Isocitrate dehydrogenase	16	10	21	70	60	50
Glucose 6-phosphate dehydrogenase	16	11	52	298	270	250
Gluconate 6-phosphate dehydrogenase	-	-	-	170	100	220
Glyceraldehyde 3-phosphate dehydrogenase	108	171	68	1000	640	230[b]
Fructose 6-phosphate isomerase	-	-	-	180	110	330
Fructose-bisphosphate aldolase	-	-	-	20	20	30
Ribulose bisphosphate carboxylase[a]	2.4×10^3	1.8×10^3	8.0×10^3	1.8×10^5	6×10^4	1.8×10^5
Acetate thiokinase	31	27	73	-	-	-

[a] Expressed as dpm min^{-1}(mg protein)$^{-1}$

[b] This assay was performed only once.

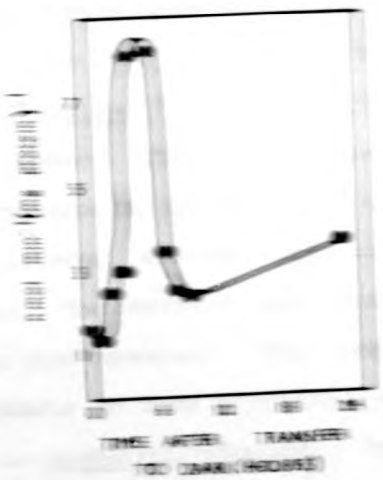
Significant increase in malate dehydrogenase activity in dark grown Chlorogloea fritschii occurred after transfer from photoautotrophic to heterotrophic growth. Furthermore, when Chlorogloea fritschii was transferred from photoheterotrophic to dark heterotrophic conditions, a marked transient in malate dehydrogenase specific activity was observed during the first 6 h after the transfer. The maximum activity during the transition was not maintained, but the final specific activity in the dark was higher than that in the light (Figure 1.5).

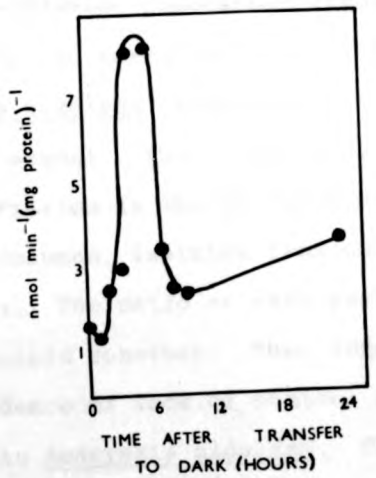
Some other enzymes of blue-green algae, such as phosphatase and nitrogenase, are clearly subject to regulation in response to specific environmental conditions. Ihlenfeldt and Gibson (1975b) reported a 10 to 15 fold increase in alkaline phosphatase specific activity of Anacystis nidulans when the phosphate concentration was reduced to growth-limiting levels. At the start of the period of phosphate-limited growth the enzyme specific activity increased slowly and continued to rise for at least 20 h.

Similar results were obtained by Bone (1971a; 1971b) who investigated the specific activities of alkaline phosphatase and nitrogenase in Anabaena flos-aquae in continuous culture. In organisms grown under phosphate-limited conditions, the alkaline phosphatase activity, examined at dilution rates between 0.015 h^{-1} and 0.03 h^{-1} showed a 20-fold variation. Organisms growing in the presence of high concentration of KNO_3 or $NaNO_3$ had

Figure 1.5.

Variation in malate dehydrogenase followed a light-dark shift. *Chlorogloea fritschii*. (Joset-Espardellier *et al.*, (1978)).


TIME AFTER TRANSFER
TO DARK(HOURS)


nmol min⁻¹(mg protein)⁻¹
7
5
3
1
0
6
12
18
24
TIME AFTER TRANSFER
TO DARK (HOURS)

phosphatase activities 3 to 5-fold greater than when N_2 was the source of nitrogen. Also nitrogenase activity in phosphate-limited organisms was repressed 24-fold by 15 mM KNO_3 with a maximum activity at a dilution rate of 0.025 h^{-1}.

Another method used to investigate transcriptional control in microorganisms is to measure alteration in relative rates of synthesis of different RNA species in cultures with different generation times. This method was used by Mann and Carr (1973) with the obligate phototroph Anacystis nidulans from which selective gene expression appeared to be absent. Their investigation showed no significant variation in the profiles of RNA, analysed on poly-L-lysine columns, isolated from cultures grown at different rates. The ratio of each peak area to the total RNA eluted remained constant. They suggested that this was direct evidence of lack of control at the point of RNA transcription in Anacystis nidulans. The ratio of transfer RNA to ribosomal RNA remained constant although in heterotrophic organisms this ratio varied greatly with growth rate (Kjeldgaard, 1967). The DNA and RNA contents of Anacystis nidulans were examined at different growth rates obtained by using different light intensities (Mann and Carr, 1974). Both increased exponentially with increasing growth rate. However, in this blue-green algae the ratio of DNA to RNA was independent of growth rate.

1.5. THE THEORY OF CONTINUOUS-FLOW CULTURE

Most previous work with blue-green algae has been carried out in batch culture systems. With the introduction of the continuous-flow culture system (Monod, 1950; Novick and Szilard, 1950; Herbert, Elsworth and Telling, 1956), a new type of growth system became available. Chemostat culture is now an essential means for elucidating the relationships between an organism and its environment and the application of continuous-flow culture techniques offers many advantages (Pirt, 1972; Bull, 1974).

The unique features of the chemostat growth may be described as follows:

(1) The system enables the growth rate to be varied whilst maintaining a constant environment. Conversely the environment can be varied whilst the growth rate is kept constant. Thus the continuous-flow system offers the possibility for a more rigorous examination of the effects of individual environmental factors on the physiology of the microorganisms.

(2) The growth rate can be controlled and maintained at a predetermined value for long periods.

(3) The organism can be grown for longer periods under constant conditions. Under these steady state conditions, the biomass concentration can be maintained constant.

(4) It allows substrate-limited growth conditions to be established and enables the study of the physiology of the organisms under different substrate limitations which may be extremely important in terms of the organism's growth in natural environments.

(5) Data obtained from continuous-flow cultures are more reliable and reproducible than those obtained from batch cultures.

(6) Continuous-flow culture systems have a special advantage in enzyme regulation studies. Once a batch culture has been established, there is no further input or output of materials and the whole process is discontinuous because the environment changes continuously as a result of the growth. Thus non-steady states occur in batch cultures. In contrast, in continuous-flow cultures steady state conditions can be obtained while the environment and the biomass concentration remain constant. Therefore critical quantitative changes in enzyme levels can be made in organisms growing under steady state conditions. Many workers have endorsed the special advantages of the continuous-flow culture systems in the study of enzyme regulation (for example, Hamlin, Ng and Dawes, 1967; Clarke and Lilly, 1969; Bull, 1972; Dean, 1972).

Continuous-flow culture techniques have been extensively used to grow heterotrophic organisms as well as phototrophs (Harder and Dijkhuizen, 1976). Slater and Morris (1973a; 1973b) used chemostat cultures to grow Rhodospirillum rubrum. The green alga, Chlorella, has been grown in continuous-flow culture under light- and carbon dioxide-limited conditions (Pipes, 1962; Pipes and Koutsoyannis, 1962). However, to date Anacystis nidulans has been grown successfully only under light-limited conditions (Slater, 1975).

1.5.1. Chemostat continuous-flow culture kinetics

In a continuous-flow culture system, the organism is grown at specific growth rates (μ) which are less than the maximum specific growth rate (μ_{max}). For this purpose, a culture of fixed volume (V) is contained in a growth vessel to which a defined growth medium, designed to contain a single growth-limiting substrate at an initial concentration, (S_R), is pumped accurately at a constant flow rate (F). Within the culture vessel, the incoming fresh medium is mixed into the culture and some of it is used to produce fresh biomass which has a concentration of x. As a result of growth the concentration of the growth-limiting substrate is reduced to s.

1.5.1.1. The dilution rate

In a chemostat culture the concentration of the limiting substrate depends both on the rate at which fresh substrate is supplied and on the dilution factor as the fresh substrate is dispersed throughout the culture vessel. That is, the growth-limiting substrate concentration depends on the ratio of the rate of supply of fresh growth medium to the volume of the culture, a factor which is known as the dilution rate, D:

$$D = \frac{F}{V} \qquad (1.1)$$

The dilution rate has units of reciprocal time, usually h^{-1}, and is a measure of the number (or fraction) of the culture volume changes achieved in unit time. The dilution rate has

the same units as the specific growth rate and under appropriate conditions equals the organism's specific growth rate.

1.5.1.2. The dilution rate and biomass concentration

Within the culture vessel there is a biomass balance such that the rate of change of biomass concentration is equal to the rate of growth minus the rate of biomass washout.

Thus

$$\frac{dx}{dt} = \mu x - Dx$$

$$\frac{dx}{dt} = x(\mu - D) \qquad (1.2)$$

Substituting for μ by equation (1.4) we have:

$$\frac{dx}{dt} = x\left[\frac{\mu_{max} \cdot s}{(K_s + s)} - D\right] \qquad (1.3)$$

With reference to equation (1.2) there are three different situations which need to be considered:

(a) If $\mu > D$, then dx/dt is positive and the biomass concentration in the culture vessel increases since the rate of biomass production exceeds the rate of biomass washout.

(b) If $\mu < D$, then dx/dt is negative and the biomass concentration decreases since the rate of culture washout is greater than biomass synthesis.

(c) If $\mu = D$, then $dx/dt = 0$ and the biomass concentration remains constant. Therefore a steady state culture is obtained.

1.5.1.3. The dilution rate and growth-limiting substrate concentration

Monod (1942) first showed that there was a simple relationship between the specific growth rate (μ) and the concentration of an essential growth substrate (s), μ being proportional to the substrate concentration according to the equation:

$$\mu = \mu_{max} \left[\frac{s}{K_S + s} \right] \qquad (1.4)$$

Similarly a growth-limiting substrate balance equation can be formulated as follows: the rate of change of growth-limiting substrate concentration in the culture vessel is equal to the rate of input of fresh substrate minus the rate of substrate removal (washout) minus the rate of substrate utilization by the organism (growth use).

Thus

$$\frac{ds}{dt} = DS_R - Ds - \frac{\mu x}{Y} \qquad (1.5)$$

where Y is the observed growth yield and is defined as that quantity of biomass produced in unit time as the result of the utilization of unit amount of the limiting nutrient in the same time. Thus for a growing culture in a small time interval:

$$-\frac{dx}{ds} = Y \qquad (1.6)$$

and because $\frac{dx}{dt} = \mu x$

$$\frac{ds}{dt} = \frac{\mu x}{Y} \qquad (1.7)$$

Therefore the final term for the rate of change of growth-limiting substrate concentration in the culture vessel is derived from equation (1.7).

$$\frac{ds}{dt} = D(S_R - s) - \frac{\mu x}{Y} \qquad (1.8)$$

Again, there are three possible situations which need to be considered:

(a) If $\mu > D$, then ds/dt is negative and the growth-limiting substrate concentration decreases. The biomass concentration is increasing in this condition and therefore utilizing more of the available substrate.

(b) If $\mu < D$, then ds/dt is positive and the growth-limiting substrate concentration increases.

(c) Finally, if $\mu = D$, then ds/dt = 0 and the growth-limiting substrate concentration reaches a constant, steady state value at the same time as the biomass concentration. Thus from equation (1.4)

$$0 = \tilde{x}\frac{\mu_{max}\cdot\tilde{s}}{(K_s+\tilde{s})} + D$$

where $\tilde{s}$ and $\tilde{x}$ indicate the steady state growth-limiting substrate and biomass concentrations respectively. Hence:

$$D = \frac{\mu_{max} \cdot \tilde{s}}{(K_s + \tilde{s})}$$

and

$$\tilde{s} = \frac{DK_s}{(\mu_{max} - D)} \qquad (1.9)$$

Also, for equation (1.8): $O = D(S_R - \tilde{s}) - \frac{\mu x}{Y}$ and

$$\tilde{x} = Y(S_R - \tilde{s}) \qquad (1.10)$$

Equations (1.9) and (1.10) enable us to predict the steady state concentrations at any dilution rate provided that the initial growth-limiting substrate concentration and the three basic growth parameters, namely μ_{max}, K_s and Y, are known.

1.5.2. Light-limited growth

Under certain circumstances at some biomass concentration the average light intensity seen by each cell is less than the saturation light intensity and light becomes the factor limiting growth. Pipes and Koutsoyannis (1962) studied theoretically and experimentally the growth of alga Chlorella in light-limited continuous culture and proposed a mathematical model of light-limited growth kinetics. The kinetics of light-limited growth are analysed in the results (section 3.1).

1.5.3. The critical dilution rate and calculation of the maximum specific growth rate from culture washout kinetics

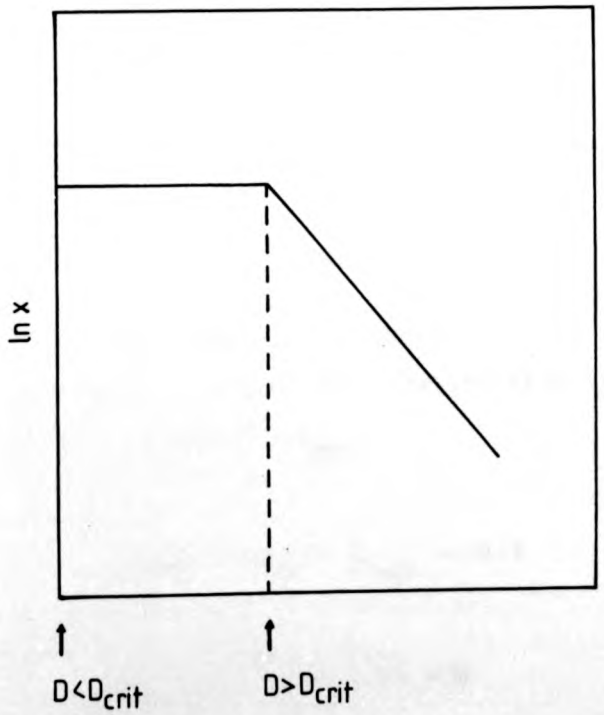
From equation (1.2) it can be seen that there is an upper limit to the dilution rate above which steady state cultures cannot be established. This is because the organism's specific growth rate has a maximum value, μ_{max}, which is genetically determined and therefore cannot be exceeded. Thus, if $D > \mu_{max}$ then dx/dt must be negative, a steady state culture cannot be obtained and the culture is said to washout. There is a unique value of dilution rate which is known as the critical dilution rate, D_{crit}. At dilution rates above D_{crit}, steady state conditions are unobtainable and in theory the organism should grow at μ_{max} during the transient conditions which occur during washout from the growth vessel (Figure 1.6). In fact, D_{crit} is reached when washout from the concentration ($\bar{s}$) of the growth-limiting substrate in the growth vessel has its highest possible value, that is, S_R, which is the initial growth-limiting substrate concentration, and thus from equation (1.4) substituting for s and D and rearranging we have:

$$D_{crit} = \mu_{max} \frac{S_R}{K_s + S_R} \qquad (1.11)$$

Therefore D_{crit} is a good approximation to the maximum specific growth rate, μ_{max}, for the organism under the prevailing conditions provided that the concentration of

Figure 1.6.

Microbial growth during washout. The change of organism concentration $\ln x$ is shown as a function of time.


ln x
D<D_crit
D>D_crit

the growth-limiting substrate in the inflowing medium (S_R), is considerably greater than the organism's saturation constant from the limiting nutrient.

During washout from a culture vessel when $D > \mu_{max}$, the organisms grow at μ_{max} provided that $S_R \gg K_s$ and so the kinetics of washout may be used to calculate μ_{max} values since:

$$\frac{dx}{dt} = x(\mu_{max} - D) \qquad (1.12)$$

where D is a known set value greater than μ_{max}. This has the solution:

$$x_t = x_o.e^{(\mu_{max} - D).t} \qquad (1.13)$$

where x_o is the organism's concentration in the steady state at time t = O when the dilution rate is increased stepwise to a value $D > \mu_{max}$.

Thus,

$$\ln x_t = \ln x_o + (\mu_{max} - D).t \qquad (1.14)$$

and

$$\mu_{max} = \frac{\ln x_t - \ln x_o}{t} + D \qquad (1.15)$$

If x_1 is the initial steady state organism concentration at time t_1 and x_2 is the organism concentration at t_2 after an instantaneous change in the dilution rate to a value $D > \mu_{max}$ at t_1, the equation (1.15) can be rewritten as:

$$\mu_{max} = \frac{\ln x_2 - \ln x_1}{(t_2 - t_1)} + D \qquad (1.16)$$

This method can only be used as long as the organism is capable of growth at μ_{max} immediately after increasing the dilution rate.

Some attention has been paid to the factors which may influence the μ_{max} values determined during washout; for example, wall growth in the culture vessel (Topiwala and Hamer, 1971) may lead to erroneously high values. Moreover the technique depends on the organism's immediate capacity to grow at its maximum rate during washout, and in some cases this does not seem to be true. Jannasch (1969) described some experiments in which the organisms either ceased to grow or washed out at rates in excess of the μ_{max} rate, suggesting that cell lysis and death was responsible for accelerating the rate of culture washout. It has also been proposed that non-growing culture washout could be due to population density effects (Jannasch and Mateles, 1974). It has been shown for Escherichia coli that growth at μ_{max} may not always be physiologically attainable in the first stages of culture washout, particularly for large changes in the dilution rate (Mateles, Ryu and Yasuda, 1965; Koch and Deppe, 1971).

1.6. AIMS OF WORK

This present investigation was concerned with the growth of the blue-green alga Anacystis nidulans in continuous-flow cultures, under light- and carbon dioxide-limited conditions.

Under these conditions, the influence of temperature and dilution rate on growth and carbon dioxide assimilation were studied. The influence of dilution rate on selected enzymes of the reductive pentose phosphate cycle, the oxidative pentose phosphate pathway, the Embden-Meyerhof pathway and tricarboxylic acid and glyoxylate cycles were also examined. Finally, the pattern of growth of Anacystis nidulans during washout from various steady state cultures has been described.

PART 2. MATERIALS AND METHODS

2.1. ORGANISM

The organism used in this study, *Anacystis nidulans* classified as *Synechococcus* PCC 6301 by Herdman, Janvier, Rippka and Stanier (1979) (strain number 625, Indiana University Culture Collection) was kindly provided by Dr N. G. Carr, University of Liverpool.

2.2. CULTURE MAINTENANCE

2.2.1. Maintenance in liquid culture

The organism was maintained in liquid closed cultures using 100 ml Erlenmeyer flasks fitted with a plug of cotton wool and aluminium foil and containing approximately 20 ml of light-limited growth medium (see section 2.3.1), to which 0.05% (w/v) $NaHCO_3$ had been added. The medium was adjusted with 2N HCl to pH 7.5 before autoclaving at 15 lbf in^{-2} for 15 min. The cultures were incubated at 25°C and illuminated by several ordinary tungsten filament light-bulbs giving a total of 670W, placed 60 cm away from the cultures and were subcultured every 15 days.

2.2.2. Maintenance on defined solid medium

Anacystis nidulans was also maintained on agar slopes in 10 ml Pyrex-glass test tubes fitted with polypropylene caps (C.I. Clark and Co.), and also on agar plates. For the preparation of solid medium, 250 ml of sterile double strength basal medium was prepared as described in section 2.3.1. An equal volume of 3.0% (w/v) Difco agar was prepared

and separately sterilised at 15 lbf in^{-2} for 20 min. After cooling to 45°C the basal medium and agar were mixed to give normal strength basal medium solidified with 1.5% (w/v) Difco agar. The cultures on agar slopes and agar plates were incubated at 25°C in the light (see section 2.2.1) and were subcultured every 10 to 15 days.

2.3. MEDIA FOR GROWTH

2.3.1. Light-limited medium

Anacystis nidulans was grown in light-limited culture in a medium C (Kratz and Myers, 1955a) as modified by Slater (1975) containing (g) per litre of glass-distilled water; KNO_3, 1; K_2HPO_4, 1; $Ca(NO_3)_2$, 0.025; $MgSO_4.7H_2O$, 0.25; and 1 ml of Arnon's A5 trace element solution (Allen and Arnon, 1955) containing (g) per litre of glass-distilled water; $MnCl_2.4H_2O$, 1.81; $Na_2MoO_4.2H_2O$, 0.0252; $ZnSO_4.4H_2O$, 0.222; $CuSO_4.5H_2O$, 0.079; H_3BO_3, 2.86; and 1 ml FeEDTA solution, containing (g) per litre of glass-distilled water; NaEDTA, 6.34; $FeSO_4.7H_2O$, 4.98. The medium was adjusted with 2N HCl to pH 7.5 before autoclaving at 12 lbf in^{-2} for 40 min. (section 2.5.3.1).

2.3.2. Carbon dioxide-limited medium

The same basal medium as the light-limited medium (see section 2.3.1) was used for growth under carbon dioxide-limited conditions except that it was supplemented with sodium bicarbonate to give a final concentration of 0.42 g l^{-1} (5 mM). The sodium bicarbonate solution was sterilized (see section 2.5.2) separately from the basal medium. This

medium was used only for growth in continuous-flow culture.

2.3.3. Nutrient agar medium

Oxoid nutrient agar was used at the recommended concentration of 28 g l^{-1} and autoclaved at 15 lbf in^{-2} for 20 min.

2.4. PURIFICATION OF THE CULTURE

The cultures were routinely checked for contamination. On defined solid medium, Anacystis nidulans formed compact, deeply pigmented colonies. Pure cultures were obtained by repeatedly restreaking single colonies in order to eliminate contaminating bacteria (Stanier et al., 1971). The liquid cultures were also routinely tested for heterotrophic bacterial contamination by making transfers to nutrient agar plates (see section 2.3.3) and incubating under the same conditions (see section 2.2.2). The presence of bacterial growth and the number of bacterial colonies showed the amount of contamination.

2.5. AUTOTROPHIC CONTINUOUS-FLOW CULTURE

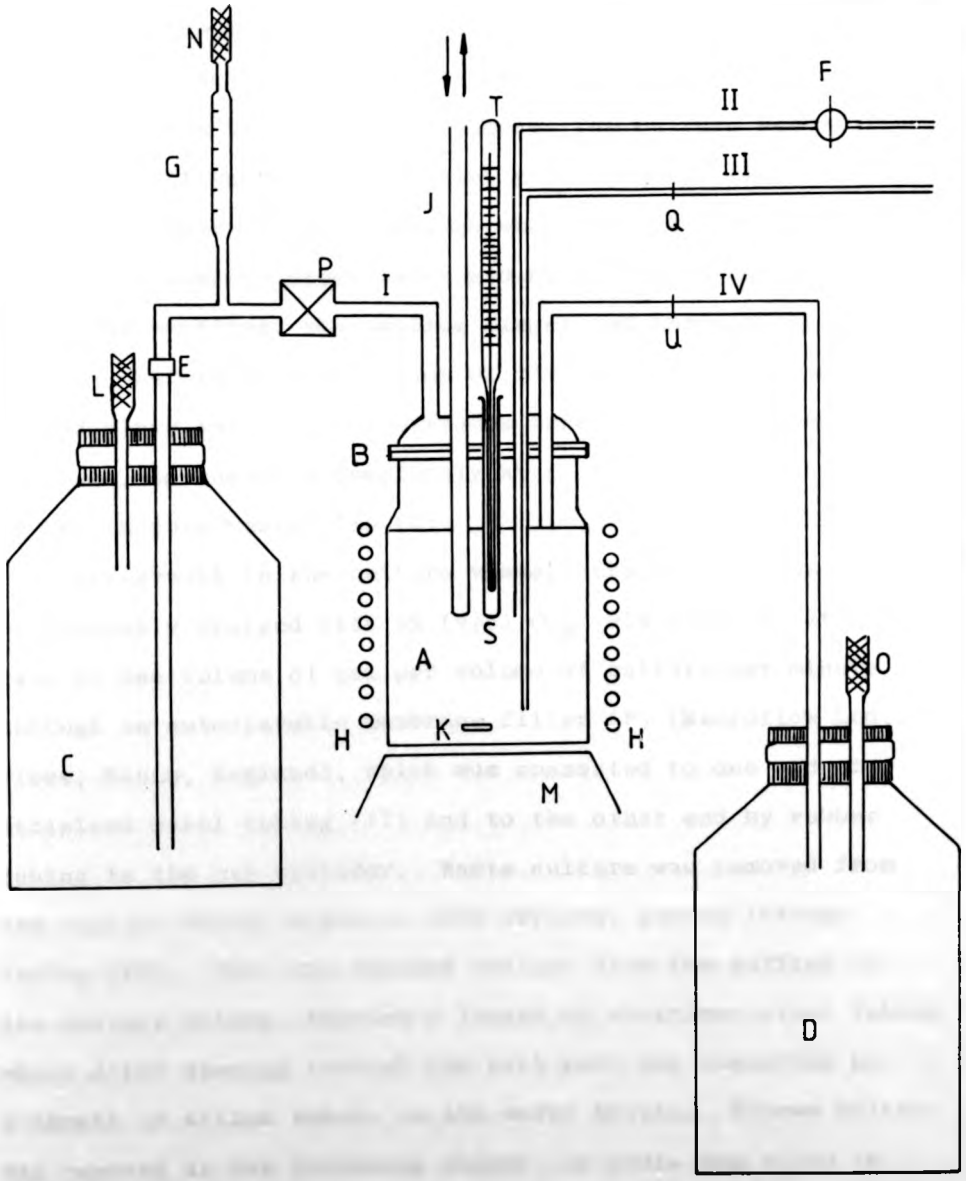
2.5.1. Light-limited continuous-flow culture system

Figure 2.1. illustrates the basic features of the continuous-flow culture system used in this study to provide the controlled autotrophic growth of Anacystis nidulans under light-limited conditions.

Figure 2.1.

A schematic representation of the light-limited chemostat.

A, growth vessel. B, culture vessel lid. C, fresh medium reservoir. D, waste culture reservoir. E, connecting hood. F, membrane filter. G, burette. H, growth lights. J, cooling finger. K, magnetic follower. L,N,O, air glass filters. M, magnetic stirrer/heater P, flow inducer. Q,U, Hoffman clips. S, water pocket. T, thermometer/thermostat. I, fresh medium inlet. II, gas inlet. III, culture harvesting. IV, growth vessel overflow.


N
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D

The growth vessel (A) was a 2.5 1 Pyrex glass vessel (Quickfit, England) with a working culture volume of 2.0 1. The top of the growth vessel was fitted with a glass lid (B) and secured rigidly in position by a ring which was secured on to the neck of the growth vessel. The culture vessel lid (B) carried five inlet and outlet ports and was fitted with silicone rubber stoppers through which passed 2 mm to 5 mm diameter stainless steel inlet and outlet tubes for gas, media and sampling, the cooling finger and thermometer water pocket. Before final location of the rubber stoppers and ground glass inserts, the surfaces were lightly greased with Vaseline, to ensure a completely airtight seal with the parts of the culture vessel lid (B).

For growth in the culture vessel, the culture was continuously sparged with 5% (v/v) CO_2: 95% (v/v) N_2 at a rate of one volume of gas per volume of culture per minute, through an autoclavable membrane filter (F) (Microflow Ltd., Fleet, Hants, England), which was connected to one end of stainless steel tubing (II) and to the other end by rubber tubing to the gas cylinder. Waste culture was removed from the culture vessel together with effluent gasses through tubing (IV). The tube removed culture from the surface of the culture volume, through a length of stainless steel tubing which after passing through the exit part was connected by a length of silica tubing to the waste bottle. Excess culture was removed in the following manner: as media was added to

the culture vessel, the volume increased, covering the end of the effluent tube. Since the culture vessel was continuously gassed, this resulted in a temporary increase in pressure resulting in the removal of an aliquot of culture and then permitting the waste gasses to escape.

The temperature of the growth vessel was controlled by a combination of a cooling finger (J) and the heater element of the combined magnetic stirrer/heater (M) (S 'mag' H, Voss Instruments Ltd.). The cooling finger consisted of a glass 'U'-tube connected to an inlet and outlet part in the culture vessel lid. Tap water at a low flow rate was passed continuously through the finger. The culture temperature was measured by a thermometer/thermostat (T) unit (the Electrical Thermometer Co. Ltd.), positioned in the water pocket (S). This was fitted into a Pyrex glass pocket filled with distilled water and was centrally situated in the culture vessel lid. The thermostat was connected via an electronic switching relay (Gallenkamp, England) to the heater unit of the magnetic stirrer and for most growth experiments the temperature was regulated to 40^{o}C with an accuracy of approximately ± 0.5^{o}C.

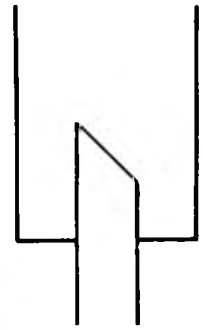
A homogeneous suspension was maintained by stirring with a 5 cm magnetic follower (K) operated by the magnetic stirrer/heater (M). In all growth experiments the same rate of gentle stirring was used, sufficiently rapid to ensure that a homogeneous culture was maintained and to minimise wall growth. The growth vessel was illuminated from two sides by a total of

18 8W Warm White fluorescent tubes placed 10 cm away from the culture vessel (H). Although it was difficult to estimate the light intensity in the centre of the culture because of the cylindrical shape and volume of the culture vessel, the light intensity was in the order of 10,000 lux. The light tubes were regularly replaced with new Warm White fluorescent tubes to ensure that the quality of the light source and the intensity remained constant. In most of the experiments the 18 8W light tubes were used together. However, in some low light intensity washout experiments (section 6.1) 3 or 6 8W light tubes were used. The fresh medium reservoir (C) was a 20 l Pyrex glass vessel with a silicone rubber stopper taking two stainless steel tubes. One end was connected to an air glass filter which consisted of a glass tube packed with glass wool and with a small section of cotton wool (Figure 2.2). Sterile fresh medium from the reservoir was pumped into the growth vessel through an inlet port (I) by a peristaltic flow inducer (P) (MHRE 7, Watson-Marlow Ltd., Falmouth, Cornwall, England). The fresh medium feed line between the fresh medium reservoir and the flow inducer (P) could be disconnected at a connecting hood (E). The connecting hood consisted of a section of stainless steel tubing, 1 cm diameter and 6 cm length, partially surrounded by a shorter length of tubing 3 cm in diameter (Figure 2.2). Between the connecting hood and the flow inducer, one 'T'-piece (Portex, England) was inserted in the

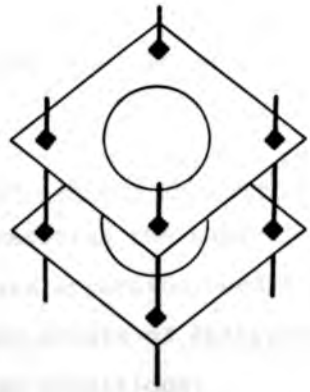
Figure 2.2.

A, a schematic representation of an air glass filter. B, a section of the connecting hood. C, a schematic representation of the aluminium plates. D, a section of the Gamma-12 filter units.

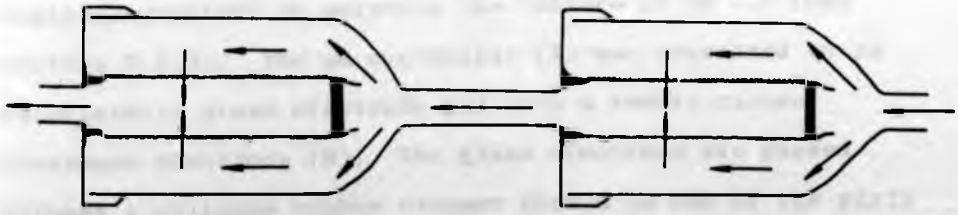
A



B



C



D

fresh medium line. The vertical side of 'T'-piece was connected to a burette which was used for measuring the fresh medium flow rate (see section 2.5.3.4).

The waste culture reservoir (D) was a 20 l Pyrex glass bottle fitted with a rubber stopper taking two stainless steel tubes. One tube (IV) acted as an inlet port which was connected to the overflow from the growth vessel. The second was a gas outlet which was connected to an air glass filter (O). The fresh medium (C) and waste culture (D) reservoirs were fitted with silicone rubber stoppers held in position by two aluminium plates tightly clamped together as shown in Figure 2.2.

2.5.2. Carbon dioxide-limited continuous-flow system

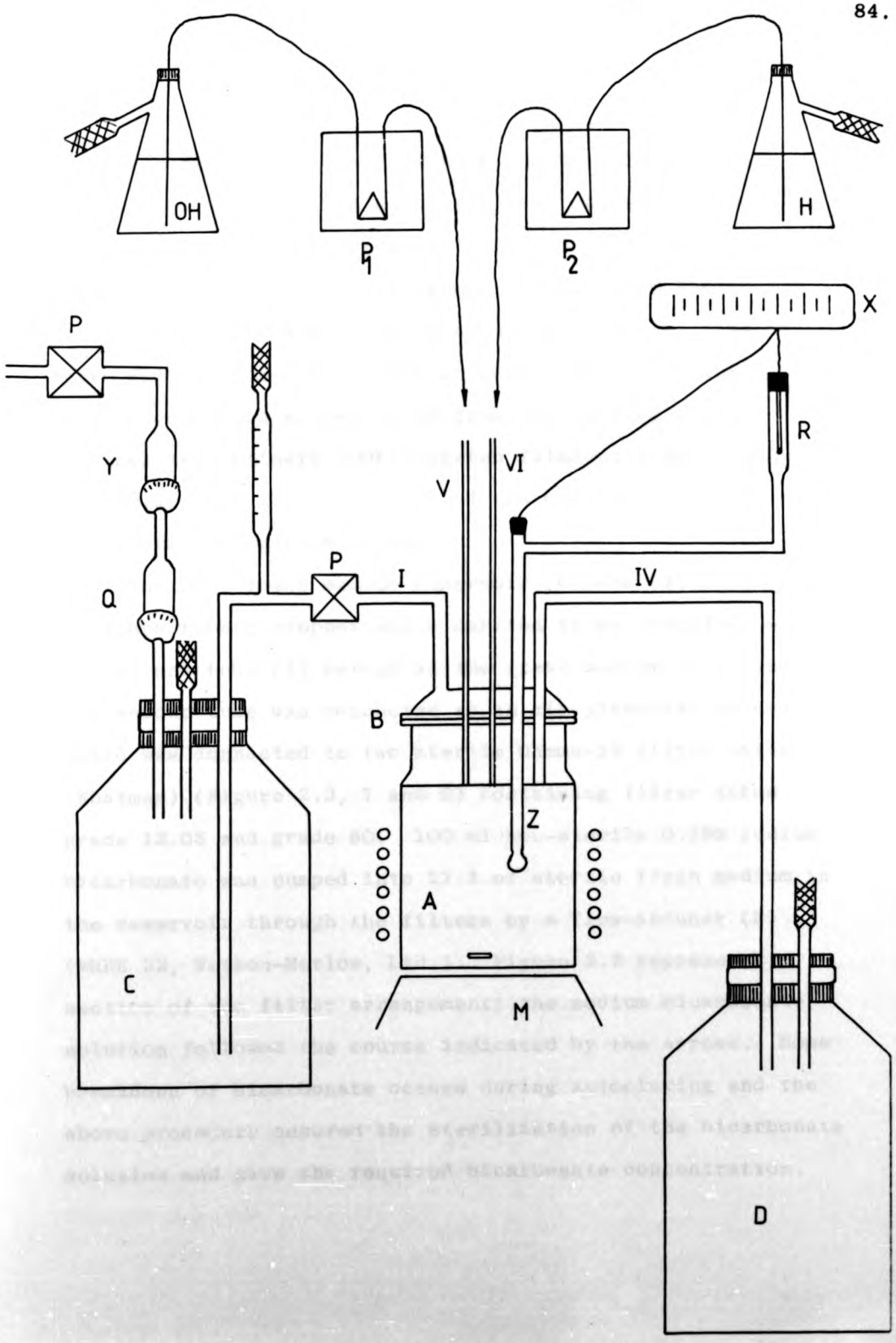
Figure 2.3 illustrates the basic features of the continuous-flow culture apparatus used to provide the controlled autotrophic growth of *Anacystis nidulans* under carbon dioxide-limited conditions.

The basic system was similar to the light-limited chemostat with the addition of pH monitoring and controlling facilities (EIL Model 9150 pH controller, Chertsey, Surrey, England) required to maintain the culture of pH 7.5 (see section 3.2.3). The pH controller (X) was connected to an autoclavable glass electrode (Z) with a remote calomel reference electrode (R). The glass electrode was passed through a silicone rubber stopper fitted to one of the parts of the culture vessel lid (B). Two short lengths of glass (3 mm diameter) tubing (V, VI) were passed through silicone

Figure 2.3.

A schematic representation of the carbon dioxide-limited chemostat.

X, pH controller. Z, glass electrode. R, reference electrode. Y,Q, Gamma-12 filter units. OH, the flask containing 2N NaOH. H, the flask containing HCl. P_1, P_2, the two Shuco triangular flow-inducers. V, inlet port of 2N NaOH. VI, inlet port of 2N HCl. The cooling finger, the thermometer/thermostat with its water pocket, the gas inlet and sampling port, are not shown in the figure.


OH
H
P1
P2
X
P
R
Y
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VI
Q
P
I
IV
B
Z
A
C
M
D

rubber stoppers to supply 2N NaOH and 2N HC1 solutions for pH correction. The solutions were pumped *via* two Shuco triangular flow-inducers (P_1) and (P_2) (Saia, London, England). The arrangement is shown in Figure 2.3.

The culture was illuminated on two sides by five 8W Warm White fluorescent tubes and one 8W Gro-lux fluorescent tube placed 10 cm from the culture vessel. A further two ordinary 100W tungsten filament light bulbs were placed on the third side of the culture vessel 15 cm away from the culture vessel.

The 20 l fresh medium reservoir (C) was fitted with a silicone rubber stopper which carried three stainless steel tubes; one tube (I) served as the fresh medium feed line; the second tube was connected to an air glass-filter and the third was connected to two sterile Gamma-12 filter units (Whatman) (Figure 2.3, Y and Q) containing filter tubes grade 12.03 and grade 80. 100 ml non-sterile 0.85M sodium bicarbonate was pumped into 17 1 of sterile fresh medium in the reservoir through the filters by a flow-inducer (P) (MHRE 22, Watson-Marlow, Ltd.). Figure 2.2 represents a section of the filter arrangement; the sodium bicarbonate solution followed the course indicated by the arrows. Some breakdown of bicarbonate occurs during autoclaving and the above procedure ensured the sterilization of the bicarbonate solution and gave the required bicarbonate concentration.

The culture was continuously sparged with N_2 gas at a rate of one volume of gas per volume of culture per minute through a membrane filter (F) which was connected to one end of stainless steel tubing (not shown in Figure 2.3) and with the second end by rubber tubing to a nitrogen (oxygen-free) gas cylinder.

2.5.3. Continuous-flow culture system operation

2.5.3.1. Autoclaving

The continuous-flow culture systems were autoclaved as two units. The fresh medium reservoir was disconnected from the growth vessel medium at the connecting hood (E). On the growth vessel the thermometer/thermostat was removed and the growth vessel and the 20 l waste bottle were autoclaved empty at 15 lbf in^{-2} for 20 minutes. The 20 l fresh medium bottle was autoclaved at 12 lbf in^{-2} for 40 min; the large volume of fresh medium required more time for autoclaving. After sterilisation, the growth vessel was placed on the magnetic stirrer and positioned by the growth lights. The thermometer/thermostat was located in the water pocket and the fresh medium line was aseptically connected to the fresh medium reservoir. To sterilize the pH glass electrode, it was placed in a 70% (v/v) solution of absolute alcohol for 25 to 30 min.

Before placing in the growth vessel, the pH electrode was washed with approximately 500 ml of sterile glass-distilled water.

2.5.3.2. Inoculation

The growth vessel was inoculated aseptically through inlet (I) with a few colonies taken from stock cultures (section 2.2). Initially the organism was grown as a batch culture with the sparging gas at a low flow rate and with no stirring. Since there was no stirring, the culture settled to the bottom of the culture vessel and this was important in promoting initial culture growth. The continuous flow of fresh medium was started once the culture had reached an absorbance greater than 1.0 at 600 nm.

2.5.3.3. Sampling and waste bottle emptying

Samples were removed from the growth vessel by the following procedure. The Hoffman clip (U) was closed to prevent the sparging gas from leaving the growth vessel and this caused pressure to rise inside the culture vessel. Almost immediately after closing the Hoffman clip (U), the sample tube was opened by opening the Hoffman clip(Q). The rise of the internal pressure in the culture vessel forced the culture down the sample line and was collected in a 25 ml glass universal bottle with a screw top, connected to the sampling tubing.

Waste culture, collected in the waste bottle, was periodically removed by aseptically changing the full waste bottle with a sterile empty waste bottle.

2.5.3.4. Measurements of fresh medium flow-rate

The burette (G) was filled from the medium reservoir and, by appropriate use of Hoffman clips, medium was pumped from the burette rather than the fresh medium reservoir. The time taken to transfer a known volume to the culture vessel was determined and the flow rate calculated in terms of ml h^{-1}.

2.6. ANALYTICAL PROCEDURES FOR CONTINUOUS-FLOW CULTURE

2.6.1. Estimation of culture absorbance

The culture absorbance was measured in a Unicam SP 1700 spectrophotometer at 600 nm and 650 nm, using 3.0 ml glass cuvettes with a 1 cm light path. For some experiments the culture absorbance was measured in a Unicam SP 600 spectrophotometer.

2.6.2. Estimation of culture biomass

The culture biomass was determined by filtering a known volume of culture, usually 40 ml, through predried and preweighted Millipore HAWP filters, 0.45 μ pore size and 22 mm in diameter and drying to constant weight at 105°C for at least 18 hours. The biomass of the culture was expressed in terms of mg dry weight ml^{-1}.

2.6.3. Estimation of cell number

Samples for cell number determination were fixed in an equal volume of 2.5% (v/v) gluteraldehyde solution. The sample was diluted down to 10^{-3} and the cell number determined using a Neubauer counting chamber (Gallenkamp, England). The

values obtained were the mean of three separate determinations and results expressed as numbers of organisms ml^{-1}. For the washout experiments (see part 6) cell number was estimated by means of an electronic particle counter (Model ZBI Coulter Counter). Each culture sample was suitably diluted in Isoton (Isoton II, without azide, C Coulter Electronics Ltd., England). Before use the Isoton solution was filtered. Appropriate dilutions ranging from 1:400 to 1:50 were used, depending on the density of the culture. The results were expressed as numbers of organisms ml^{-1}. The samples in gluteraldehyde could be stored at $4^{o}C$.

2.6.4. Estimation of cell volume

Cell volume was also estimated by the electronic particle counter using the same samples as for the cell number estimation (see section 2.6.3). The distribution display of the Coulter Channelyzer gave a distribution curve of cell size against cell number. Cell size was related to a channel number over the range 1 to 100.

The channel number at the peak of the distribution was determined, and the mean cell volume, V, calculated from the formula:

$$V = \left[\left((\text{channel number}) \times \frac{WW}{100} \right) + BCT \right] \times T_F \qquad (2.1)$$

Where: WW = window width, set to get maximum cell number count (normally setting of 100 used).

BCT = base channel threshold, which determined the smallest particle size to be included in the distribution analysis (normally 10 or 15 used).

T_F = threshold factor was determined from the calibration of the Coulter Channelyzer using a standard particle volume (1.15 μm diameter, Calibration Later, Coulter Electronics Ltd., Herts).

Results expressed as cubic microns. The samples for storage were kept in gluteraldehyde at $4^{o}C$.

2.6.5. Determination of a steady-state culture

To obtain a steady-state culture, the dilution rate was kept constant for a period of time greater than three times the culture doubling time (t_d). After this initial period of time the culture absorbance was measured twice a day, and if the absorbance remained constant a steady-state was established.

2.7. DETERMINATION OF THE MAXIMUM SPECIFIC GROWTH RATE (μ_{max})

The maximum specific growth rate (μ_{max}) was determined under light- and carbon dioxide-limited conditions. All the environmental factors such as temperature, light-intensity, pH, were kept constant. The μ_{max} values depended on the light-intensity but even so growth was exponential (see part 3).

2.7.1. μ_{max} from closed culture growth

Closed culture growth was examined using the culture vessels of the light- and carbon dioxide-limited chemostats starting with a culture volume of 2.0 l and an initial culture absorbance (600 nm) of approximately 0.1. The inoculum was taken from an actively growing stock culture. The temperature of the culture was 40°C and the light intensity exceeded 10,000 lux. The lag and acceleration phase was followed by monitoring culture absorbance and cell number. The values of μ_{max} under both limitations were calculated from the slope of the growth curve (see sections 3.1.2 and 3.2.1) from the equation:

$$\ln x = \ln x_o + \mu_{max} t \qquad (2.2)$$

2.7.2. μ_{max} from chemostat washout

Once a culture had reached a given steady-state (section 2.6.5), culture washout was established by a stepwise increase in the dilution rate to a value greater than the expected maximum specific growth rate. There was no concomitant saturation of the growth-limiting nutrient either by the addition of excess bicarbonate or by an increase of the incident light intensity.

The value of μ_{max} was calculated from the equation (1.16)

$$\mu_{max} = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} + D$$

(see Introduction, 1.5.3).

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$$\mu_{max} = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} + D$$

(see Introduction, 1.5.3).

2.8. MEASUREMENTS OF THE RATE OF CARBON DIOXIDE ASSIMILATION BY INTACT ORGANISMS

The rate of [^{14}C]-carbon dioxide was measured by the following method. Up to 500 ml culture was harvested by centrifugation at 23,000 × g for 15 minutes and the cell pellets resuspended in a bicarbonate-free minimal growth medium (see section 2.3.1) to give an absorbance (600 nm) which was equivalent to the growing culture before harvesting. Some of this cell suspension was diluted with bicarbonate-free minimal growth medium to give an absorbance (600 nm) of approximately 0.1. In most experiments 17.0 ml of cell suspension was dispensed into 100 ml Erlenmeyer flasks. The volume of dispensed cell suspensions was such that after subsequence additions the final volume in all experiments was 20.0 ml. The flasks were fitted with Suba seal vaccine stoppers and illuminated 20 cm away from two 40W Warm White fluorescent tubes at 25^{o}C. Those cell suspensions used to determine the rate of carbon dioxide assimilation in the dark were equilibrated in the same way except that the flasks were covered with aluminium foil. After 10 minutes equilibration, 50 µmol of sodium bicarbonate, with or without 50 µmol glucose or acetate, were added. Each of these solutions was added as 1.0 ml of a 50 mM solution and thus the final concentration was 2.5 mM. For those flasks not requiring glucose or acetate addition, 1.0 ml of distilled water was added instead. The cell suspension was equilibrated for a further 10 minutes and

1.0 ml of a sodium [^{14}C]-bicarbonate solution was added. For most experiments 4 µCi were added to each flask. 1.0 ml samples were removed by syringe through the vaccine seals at suitable time intervals, usually during the first 20 minutes after the addition of sodium [^{14}C]-bicarbonate, to determine the amount of [^{14}C]-carbon incorporated into cellular material. Carbon dioxide assimilation was terminated by injecting the samples into 2.0 ml 95% (v/v) ethanol: 5% (v/v) glacial acetic acid in a glass scintillation vial and the contents of the vials were evaporated to dryness in a stream of air. This process reduced the aqueous content of the sample necessary for the scintillation system used and also ensured the removal of any unassimilated sodium [^{14}C]-bicarbonate. Thus, only that [^{14}C]-carbon assimilated into cellular material remained in the vial and the radioactivity was determined (see section 2.1). The rate of carbon dioxide assimilation was expressed as µmol carbon dioxide assimilated per mg dry weight per hour, or as µmol carbon dioxide assimilated per unit absorbance per hour or as µmol carbon dioxide assimilated per 10^8 organisms per hour.

2.9. COUNTING OF [^{14}C]-RADIOACTIVITY

The radioactivity was determined by liquid scintillation counting, using a Packard 2425 liquid scintillation counter. The vials were filled with 15-16 ml of a scintillation fluid containing 6.0 g 2-(4'tert-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole (Butyl-PBD) in 750 ml sulphur-free toluene and 250 ml methanol. Vials with a known number of

disintegrations per minute were used at regular intervals to check the counting efficiency. The standard vials were prepared from a standard n-hexadecane-1-[^{14}C] with an activity of 1.1 μCi g^{-1} as indicated in Table 2.1. The average efficiency of counting for the Butyl-PBD toluene-methanol based scintillant was 78%. The automatic external standard gave the same values for the exponential vials and it was taken that the efficiency of counting was the same as in the standard vials.

2.10. ENZYME ANALYSIS

2.10.1. Preparation of cell-free extracts

Steady-state cultures (see section 2.6.5) were harvested by centrifugation at 23,000 × g for 15 minutes at 4°C. The cell pellets were washed once with ice-cold 20 mM Tris-HCl buffer, pH 7.5, and resuspended in a small volume of the same buffer, chosen to give approximately the same cell concentration each time. The organisms were passed twice through a French pressure cell (Aminco, Silver Springs, Maryland, U.S.A.) at 8.3×10^7 Pa (12,000 lbf in^{-2}) at 4°C.

The disrupted cell suspension was centrifuged at 38,000 × g for 45 minutes at 4°C to remove unbroken organisms and cell derbis and provide the cell-free extract used for the enzyme assays. Samples were taken before and after disruption for cell number determination and the estimation of cell breakage (see section 2.6.3). Extracts were stored at -20°C for subsequent protein determination.

Table 2.1.

n-Hexadecane 1.1μCi g^{-1} (g)	Expected disintegrations (dpm)	Observed counts (cpm)	Efficiency %	Automatic external standard value
0.102	246840	197884	80.2	0.5683
0.10095	244299	194663	79.7	0.5603
0.01155	27951	21612	77.3	0.5653
0.0706	25652	20313	79.2	0.5663
0.00398	9631	7491	77.8	0.5464
0.00151	3654	2610	71.4	0.5606

2.10.2. Enzyme assays

All the activities were determined in the extract supernatant and only in one case they were determined in the pellets as well (see section 2.10.2.8). All enzymes were assayed at 30°C under conditions of optimum pH (for example Figure 2.4), substrate and cofactor concentration. Where necessary a Unicam SP 1700 recording spectrophotometer was used. The molar extinction coefficient for NADH and NADPH at 340 nm was taken as 6.22×10^3 1 mol^{-1}. The source of chemicals used for the enzyme assays is given in section 2.11.

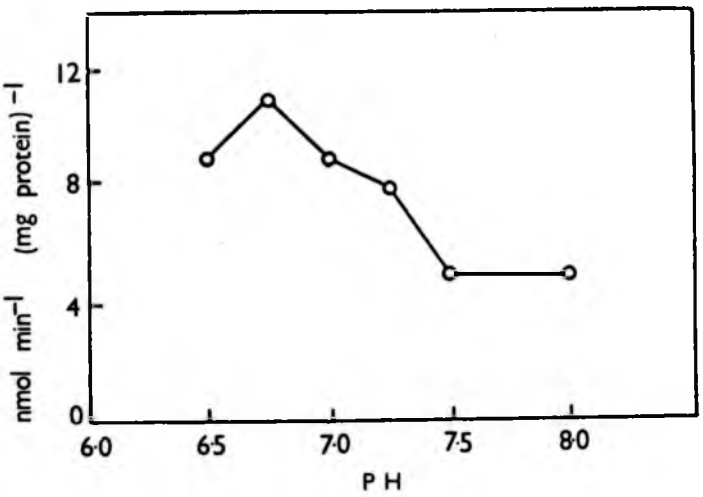
2.10.2.1. Ribulose 1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxylyase (dimerizing) E.C. 4.1.1.39). The enzyme was assayed as described by Slater (1975). 1.2 ml final assay volume contained : 200 µmol Tris-HCl buffer, pH 7.8; 10 µmol $MgCl_2$; 5 µmol reduced glutathione; 25 µmol $NaH^{14}CO_3$ (specific activity, 0.8 µCi μmol^{-1}). The reaction mixture was preincubated with 0.1-0.3 ml crude extract (0.30-5.40 mg protein ml^{-1}) for 10.0 minutes at 30°C in a water bath. The reaction was started by adding 3 µmol ribulose 1,5-bisphosphate. This was prepared from the dibarium salt by adding a small quantity of Dowex 50 ion exchange resin (Sigma Chemical Co., London) and equilibrating for 10 minutes. The water insoluble dibarium salt was converted to the free acid and neutralized to approximately pH 7.0 with 2N sodium hydroxide.

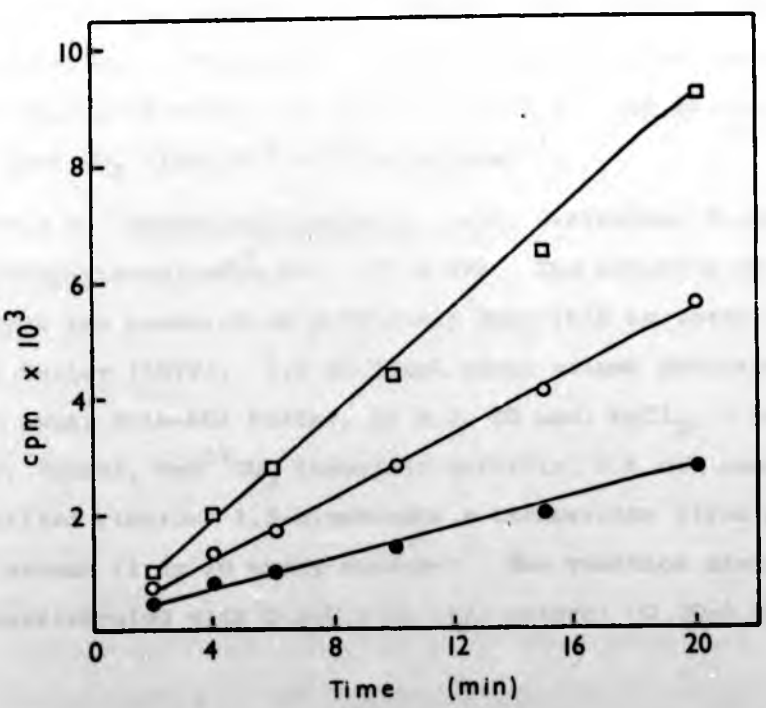
Figure 2.4.

The pH curve of Isocitrate lyase activity. The activity of the enzyme in cell extract measured at different pH values in Tris-HCl buffer.

Figure 2.5.

Incorporation of sodium [^{14}C]-bicarbonate in cell-free extracts; (●), 0.1 ml cell extract (○), 0.2 ml cell extract (□), 0.3 ml cell extract.


nmol min⁻¹ (mg protein)⁻¹
12
8
4
0
6·0
6·5
7·0
7·5
8·0
PH


cpm x 10³
10
8
6
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0
4
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12
16
20
Time (min)

0.1 samples were taken at suitable time intervals over the 20 minutes after the addition of ribulose 1,5-bisphosphate and the reaction terminated in 0.2 ml 95% (v/v) ethanol: 5% (v/v) glacial acetic acid in scintillation vials. The contents of the vials were evaporated to dryness and the [^{14}C]-carbon content of the acid stable products determined. Figure 2.5 shows that the amount of incorporated sodium [^{14}C]-bicarbonate was proportional to the amount of cell-free extracts. The rate of [^{14}C]-carbon dioxide per assay was: with 0.1 ml crude extract, 7,500 cpm h^{-1}; with 0.2 ml crude extract, 16,200 cpm h^{-1}; and with 0.3 ml crude extract, 27,300 cpm h^{-1}. These results represented rates expressed as cpm h^{-1} (ml extract)$^{-1}$ of 903,614; 972,839 and 1,092,000 respectively. Ribulose 1,5-bisphosphate carboxylase activity was expressed either as µmol CO_2 fixed h^{-1} (mg protein)$^{-1}$ or µmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$.

2.10.2.2. Phosphoribulokinase (ATP: D-ribulose 5-phosphate 1-phosphotransferase, E.C. 2.7.1.19). The activity of this enzyme was measured as previously described by Joint, Morris and Fuller (1972). 1.3 ml final assay volume contained: 200 µmol; Tris-HCl buffer, pH 8.3; 20 µmol $MgCl_2$; 1 µmol, ATP; 75µmol, $NaH^{14}CO_3$ (specific activity, 0.8 µCi µmol^{-1}); purified ribulose 1,5-bisphosphate carboxylase (from spinach) in excess (1 µg in assay mixture). The reaction mixture was prequilibrated with 0.1-0.3 ml cell extract (0.30-5.40 mg

protein ml^{-1}) for 10.0 minutes at 30°C in a water bath. The reaction was started by adding 6 µmol ribulose 5-phosphate. The same procedure for sampling and radioactivity determination as for RuBPCase assay was followed. Phosphoribulokinase activity was expressed either as µmol CO_2 fixed h^{-1} (mg protein)$^{-1}$ or per µmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$.

2.10.2.3. 6-Phosphogluconate dehydrogenase (6-Phospho-D-gluconate: NADP oxidoreductase (decarboxylating), E.C. 1.1.1.44). This enzyme was assayed by the procedure of Ng and Dawes (1973). 1 ml assay mixture in silica-glass cuvettes contained: 36 µmol Tris-HCl buffer, pH 9.0; 1.5 µmol 6-phosphogluconate; 1 µmol NADP; 12.5 µmol $MgCl_2$; water and 0.1 ml of cell extract (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same reaction mixture except that the substrate was omitted. The reaction was started by adding the substrate and the increase in absorbance at 340 nm was measured. 6-phosphogluconate dehydrogenase activity was expressed either as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.2.4. Glucose 6-phosphate dehydrogenase (D-Glucose-6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49). This enzyme was assayed by the procedure of Ng and Dawes (1973). 1 ml assay mixture in silica-glass cuvettes contained 36 µmol Tris-HCl buffer, pH 9.0; 6 µmol glucose 6-phosphate; 1.5 µmol NADP; 12.5 µmol, $MgCl_2$; water and 0.1 ml of cell

extract (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same reaction mixture except that the substrate was omitted. The reaction was started by adding the substrate and the increase in absorbance at 340 nm was measured. Glucose 6-phosphate dehydrogenase activity was expressed either as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.2.5. Hexokinase (ATP D-hexose 6-phosphotransferase E.C. 2.7.1.1). This enzyme was assayed by the procedure of Ng and Dawes (1973). 1 ml assay mixture in silica-glass cuvette contained: 36 µmol Tris-HCl buffer, pH 9.0; 100 µmol D-glucose; 12.5 µmol $MgCl_2$; 10.8 µmol ATP; 2 µmol NADP; 0.75 units of glucose 6-phosphate dehydrogenase (from Torula yeast) and 0.1 ml of cell extracts (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same reaction mixture except that the substrate was omitted. The reaction was started by adding the substrate and the increase in absorbance at 340 nm was measured. Hexokinase activity was expressed either as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.2.6. Fructose 1,6-bisphosphate aldolase (Fructose 1,6-bisphosphate D-glyceraldehyde 3-phosphate lyase, E.C. 4.1.2.13) was measured as described by Tabita and Lundgren (1971). 1 ml of reaction mixture contained: 100 µmol

Tris-HCl buffer, pH 8.5; 12.5 µmol fructose 1,6-bisphosphate; α-glycerophosphate dehydrogenase-triosephosphate isomerase (from rabbit muscle) in excess (10 mg ml^{-1}); 0.2 µmol NADH; water and 0.13 ml of cell extract (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same assay mixture except that the substrate was omitted. The reaction was initiated by adding cell extract. Fructose 1,6-bisphosphate aldolase activity was expressed either as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.2.7. 6-Phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) was assayed by the method of Baumann and Baumann (1975) 1.1 ml of reaction mixture contained: 50 µmol, Tris-HCl buffer pH 8.0;1 µmol, dithiothreitol; 1 µmol, ATP; 0.04 µmol, NADH; fructose 1,6-bisphosphate aldolase (from rabbit muscle) and α-glycerophosphate dehydrogenase-triosephosphate isomerase (from rabbit muscle) in excess (10 mg ml^{-1}); water; 1 µmol, fructose 6-phosphate; 40 µmol, KCl; 5 µmol, $MgCl_2$; 10 µmol, NH_4Cl; 0.1 ml of cell extract (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same assay mixture except that the substrate was omitted. The reaction was started by adding the substrate.6-phosphofructokinase activity was expressed either as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.2.8. Fructose 1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, E.C.3.1.3.11) was assayed by the procedure of Johnson and MacElroy (1973) in an assay mixture containing: 40 µmol, Tris-HCl buffer pH 8.5; 5 µmol, fructose bisphosphate; 5 µmol, $MgCl_2$; 0.01 µmol ethylene-diaminetetra acetic acid (EDTA). After initiating the reaction with 0.1-0.3 cell-free extract (1-2.45 mg protein ml^{-1}), 1 ml samples were removed at intervals for 10 minutes and mixed with 1 ml 20% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation for 5 minutes and the supernatant transferred into acid washed pyrex centrifuge tubes, for the phosphate assay. Water was added to make the volume up to 7 ml followed by 0.8 ml perchloric acid, 1 ml amidol reagent, containing (g) per litre of glass-distilled water; 2:4 diaminophenol dihydrochloride (amidol), 8.0; sodium metabisulphite, 160.0; and 1 ml ammonium molybtate, to give a final concentration of 33.2 g l^{-1} . The mixture was incubated for 15 to 60 minutes and the absorbance at 660 nm was determined. The phosphate ion concentration was determined from a standard curve prepared over the range 0 to 150 µg phosphate per assay.

The SP 1700 spectrophotometer was zeroed by reading two blank assays. One contained the same assay mixture and cell extract followed by trichloroacetic acid, except

that fructose bisphosphate was omitted; this was kept in ice until the time for centrifugation. The second blank assay contained the same assay mixture, cell extract followed by additions of trichloroacetic acid and fructose bisphosphate; this was kept in ice until the time for centrifugation.

Fructose 1,6-bisphosphatase activity was expressed as µg phosphate released h^{-1} (mg protein)$^{-1}$.

2.10.1.9. Triose phosphate dehydrogenase (D-Glyceraldehyde 3-phosphate: NAD oxidoreductase (phosphorylating) E.C. 1.2.1.12). 1 ml reaction mixture contained: 60 µmol phosphate buffer pH 8.0; 7.5 µmol, NAD; 125 µmol, $MgCl_2$; water and 0.1 ml of cell extract (0.14-3.16mg protein ml^{-1}). The blank assay contained the same assay mixture except that the substrate was omitted. The reaction was initiated by adding 5 µmol glyceraldehyde 3-phosphate. The decrease in absorbance at 340 nm was measured.

Triose phosphate dehydrogenase activity was expressed either as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.2.10. Isocitrate lyase (L_s-Isocitrate glyoxylate-lyase E.C. 4.1.3.1) was assayed according to the method of Pearce and Carr (1967). The reaction mixture contained in 1.0 ml: 50 µmol, Tris-HCl buffer pH 6.75; 4 µmol, $MgCl_2$; 16 µmol, phenylhydnazine; 4 µmol, cysteine; 6 µmol, DL-isocitrate; water and 0.1 ml of cell extract (0.14-3.16 mg protein ml^{-1}).

The blank assay contained the same assay mixture except that the cell extract was omitted. The reaction was initiated by the addition of cell extract.

Isocitrate lyase activity was expressed either as nmol substrate converted $min^{-1}(mg\ protein)^{-1}$ or nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$. Figure 2.4 shows the pH curve of isocitrate lyase specific activity.

2.10.2.11. Malate dehydrogenase (L-Malate : NAD oxidoreductase E.C. 1.1.1.37). The activity of this enzyme was determined as described by Mehler,Kornberg, Grisolia and Ochoa (1948). The reaction mixture contained in 1.0 ml : 36 µmol, Tris-HCl buffer pH 6.75; 2.5 µmol, NAD; 1.52 µmol, oxalacetate; water. The blank assay did not contain NAD. The reaction was started by adding 0.1 ml of cell extract (0.14-3.16 mg protein ml^{-1}). Malate dehydrogenase activity was expressed either as nmol substrate converted $min^{-1}(mg\ protein)^{-1}$ or nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$.

2.10.2.12. Succinate dehydrogenase (Succinate: (acceptor) oxidoreductase E.C. 1.3.99.1) was assayed as by Arrigoni and Singer (1962) method. The reaction mixture contained in 1.0 ml: 60 µmol, phosphate buffer pH 7.8; 0.016 µmol, dischlorophenolindophenol (DCPIP); 0.22 µmol, phenazine methosulfate (PMS); water and 0.1 ml of cell extract (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same assay mixture except that the cell extract was omitted. The reaction was initiated by adding DCPIP and PMS. Succinate dehydrogenase activity was expressed either

as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.2.13. Glutamate dehydrogenase (L-Glutamate: NADP oxidoreductase (deaminating) E.C. 1.4.1.4) was assayed by the method of Bulen (1956). The reaction mixture contained in 1.0 ml: 162.5 µmol, Tris-HCl buffer pH 8.15; 1.5 µmol, NADH; 10 µmol, potassium 2-oxoglutarate; 75 µmol, $(NH_4)_2SO_4$; 0.1 ml of cell extract (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same assay mixture except that the cell extract was omitted. The reaction started by adding the cell extract. Glutamate dehydrogenase activity was expressed either as nmol substrate converted min^{-1} (mg protein)$^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$.

2.10.3. Protein determination

Protein in cell-free extracts was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

A series of assay tubes were set up containing 0.01-0.4 ml of the cell extract and made up to 0.4 ml with distilled water. 2.0 ml of fresh solution C (50 ml solution A, 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide and 0.5 ml solution B_1, 0.5% (w/v) copper sulphate, and 0.5 ml solution B_2, 0.1% (w/v) sodium tartrate) was added and after mixing incubated for 10 mins. 0.2 ml solution D(Folin and Ciocalteou's (phenol) reagent diluted 1.0 ml with 1.5ml

as nmol substrate converted min^{-1} $(mg\ protein)^{-1}$ or nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$.

2.10.2.13. Glutamate dehydrogenase (L-Glutamate: NADP oxidoreductase (deaminating) E.C. 1.4.1.4) was assayed by the method of Bulen (1956). The reaction mixture contained in 1.0 ml: 162.5 µmol, Tris-HCl buffer pH 8.15; 1.5 µmol, NADH; 10 µmol, potassium 2-oxoglutarate; 75 µmol, $(NH_4)_2SO_4$; 0.1 ml of cell extract (0.14-3.16 mg protein ml^{-1}). The blank assay contained the same assay mixture except that the cell extract was omitted. The reaction started by adding the cell extract. Glutamate dehydrogenase activity was expressed either as nmol substrate converted min^{-1} $(mg\ protein)^{-1}$ or nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$.

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distilled water)was added and mixed. After 30 minutes the absorbance at 700 nm was measured in a Unicam SP 1700 spectrophotometer. A protein standard curve was prepared using concentrations of 0-200 µg bovine serum albumin and from the resulting standard curve the concentration of protein in the extracts was estimated.

2.11. MATERIALS

Most sugars and medium components were obtained from British Drug Houses (Poole, Dorset, U.K) and Fisons Ltd (Loughborough, England). Difco and nutrient agar were obtained from London Analytical and Bacteriological Media Ltd (50 Mank Lane, London). NaOH obtained from a Searle Company, Hopkin and Williams, England. All acids and ethanol, methanol and phenylhydrozine were obtained from Fisons Ltd. Tris bases, Dowex, reduced glutathione, sugar phosphates, 6-phosphogluconate, ribulose 1,5-bisphosphate, ribulose 6-phosphate NAD, NADH, NADP, bovine serum albumin, phenazine methosulphate, dichlorophenolindophenol were obtained from Sigma (London) Chemical Co. (London, U.K); ATP and glyceraldehyde 3-phosphate were obtained from C.F. Boehringer Ltd (Mannheim, Germany); DL-isocitrate (monopotassium) and oxalacetate from Calbiochem Ltd. (San Diego, California, U.S.A). Ribulose 1,5-bisphosphate carboxylase, glucose 6-phosphate dehydrogenase α-glycerophosphate dehydrogenase-triosephosphate isomerase

and fructose 1,6-bisphosphate aldolase were obtained from Sigma (London), Chemical Co. Toluene was obtained from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire,(England).

All the radioactive substrates were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, (England).

PART 3. THE GROWTH OF ANACYSTIS NIDULANS

3.1. GROWTH UNDER LIGHT-LIMITED CONDITIONS

3.1.1. Theory

3.1.1.1. Light-limited growth in closed culture.

Consider a closed (batch) culture of any phototroph growing in an environment in which the supplied mineral resources, including carbon dioxide, are present in excess and therefore are present at concentrations which do not restrict the growth rate of the population. Furthermore, let us assume that all the physicochemical environmental parameters are optimal and remain constant throughout the growth period. The light intensity selected is chosen such that at some point during growth light, rather than a mineral resource, becomes the limiting factor.

Now, the rate of biomass production (or the rate of photosynthesis or the rate of carbon dioxide fixation), dx/dt, is proportional to the initial biomass concentration, x,

Thus:

$$\frac{dx}{dt} \propto x$$

and so

$$\frac{dx}{dt} = \mu x \qquad (3.1)$$

Where μ is a proportionality constant known as the specific growth rate with units, strictly, of g new biomass produced (g existing biomass)$^{-1}h^{-1}$ or simply h^{-1}.

The model developed for light-limited growth from equation (3.1) (and this has been derived in a more complex and circuitous fashion elsewhere by Pipes and Koutsoyannis (1962)) depends on the relationship between the specific growth rate, μ, and the amount of light energy absorbed, I_a, by unit biomass (or per cell) such that:

$$\mu \alpha I_a$$

and so

$$\mu = A \; I_a \tag{3.2}$$

Where A is an undefined proportionality constant. So the organism's specific growth rate is directly proportional to the absorbed light energy. At the beginning of a closed culture growth cycle when the initial biomass concentration, x, is small, and there are no self-shading effects restricting the quantity of light energy absorbed by each cell, the specific growth rate of the organism is proportional to the incident light intensity, I_i. However, as growth occurs and the biomass concentration increases, the absorbed light energy, I_a, must be a function of the incident light intensity and the biomass concentration since mutual shading causes a decrease in the amount of light energy absorbed by each organism.

Thus:

$$I_a \; \alpha \; \frac{I_i}{x} \tag{3.3}$$

and so

$$I_a = B\frac{I_i}{x} \tag{3.4}$$

where B is a proportionality constant.

Thus from equations (3.2) and (3.4):

$$\mu = C \frac{I_i}{x} \qquad (3.5)$$

Where C is a composite proportionality constant of A and B. Since I_i has been defined as a fixed, chosen light intensity with a constant value during the course of the growth cycle, then:

$$\mu \propto \frac{1}{x} \qquad (3.6)$$

Thus this model predicts that for light-limited growth the specific growth rate μ is ultimately proportional to $1/x$.

3.1.1.2. Light-limited growth in an open environment (light-limited chemostat).

For growth in a light-limited chemostat, the rate of change of the biomass concentration in the growth vessel, dx/dt, is given by the equation (1.2) (see Introduction):

The rate of change of biomass concentration in the growth vessel	=	The rate of biomass production (growth)	-	The rate of biomass removal (washout)

$$\frac{dx}{dt} = \mu x - Dx$$

From equation (3.5) we have:

$$\frac{dx}{dt} = CI_i - Dx$$

and in a steady state culture when $\frac{dx}{dt} = 0$ by definition,

$$Dx = CI_i$$

and so:
$$D = \frac{CI_i}{x} \qquad (3.7)$$

and
$$D \propto \frac{1}{x} \qquad (3.8)$$

Thus data from steady state cultures should yield a straight line relationship when D is plotted against $\frac{1}{x}$ and the validity of this model is described and discussed in relation to the experimental results (section 3.1.3).

3.1.2. Growth in closed (batch) culture

A number of closed culture growth curves were obtained as described in section (2.7.1) Anacystis nidulans started growing under conditions where light (and other nutrients) were not growth-limiting but ultimately the growth rate declined because light became the limiting factor before other nutrients were exhausted. Figures 3.1 and 3.3 show the increase in culture absorbance and cell number during growth in a closed culture under these growth conditions. From the figures of ln absorbance and ln cell number against time the maximum growth rate (μ_{max}) for Anacystis nidulans under these growth conditions was calculated (Figures 3.2 and 3.4). The slope of the ln plot curve (Figure 3.2) gave a μ_{max} value equal to 0.16 h^{-1} whereas the slope of the ln cell number curve (Figure 3.4) gave a μ_{max} value equal to 0.27 h^{-1}. The lag phase measured

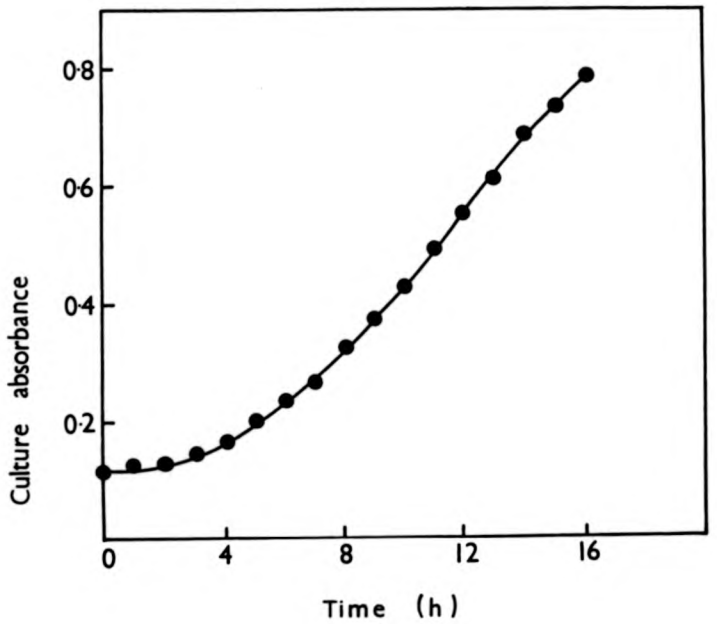
in terms of absorbance was approximately 3 hours whilst the lag phase measured in terms of cell number was approximately 6 hours (Figures 3.1 - 3.4). Apparently the exponential growth phase measured in terms of absorbance was 8 hours (after 3 to 11 hours) compared with a maximum of 3 hours (after 6 to 8 hours) for the exponential growth phase measured in terms of cell number. This may be due to the fact that absorbance measured cell number and was also influenced by cell size. The cell volume increased at the beginning and reached a maximum value of 0.49 μm^3 after 6 hours of growth under these conditions and whilst the organisms were growing exponentially (Figure 3.5). Thus absorbance measured an increase in cell size at the beginning as well as an increase in cell number. Although rapid increase in cell size was observed within the first six hours of growth the increase was less in cell number. At the end of growth the absorbance did not increase so much because cell size decreased and the absorbance measurements tended to work out at an average of cell number and cell size. Clearly for a period of 4 hours after the specific growth rate began to decline, as measured by the cell number determination, the absorbance measurements suggested that exponential growth continued.

Figure 3.1.

The increase in culture absorbance during growth in a closed culture with light limiting growth at higher absorbance values.

Figure 3.2.

Exponential growth in a closed culture with light limiting growth at higher absorbance values. The biomass concentration was expressed as units of absorbance. The data is the same as in Figure 3.1. but with ln scale.


0·8
0·6
0·4
0·2
Culture absorbance
0
4
8
12
16
Time (h)

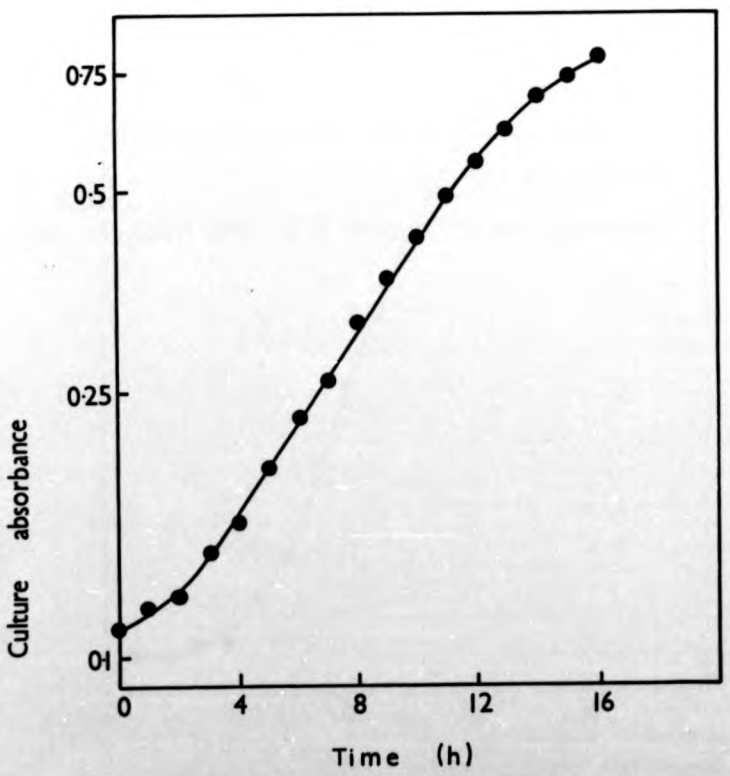
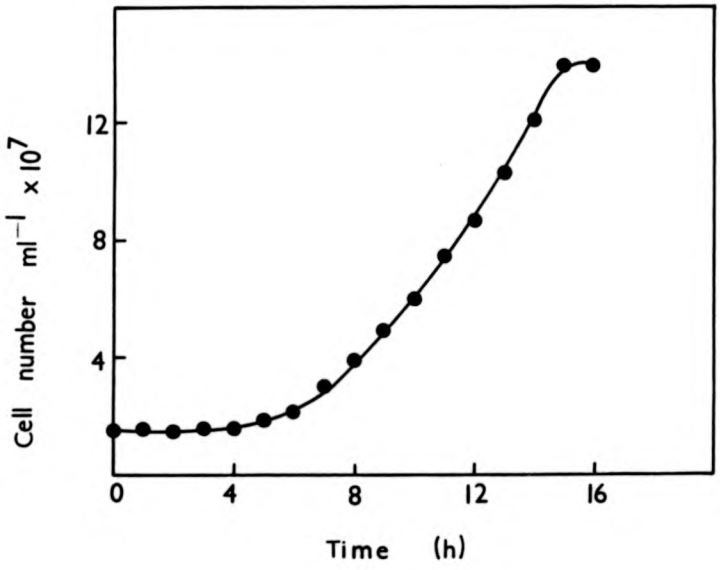

0·75
0·5
0·25
0·1
Culture absorbance
0
4
8
12
16
Time (h)

Figure 3.3.

The increase in cell number during growth in a closed culture with light limiting growth at higher cell number values.

Figure 3.4.

Exponential growth in a closed culture with light limiting growth at higher cell number values. The biomass concentration was expressed as cell number ml^{-1}. The data is the same as in Figure 3.3 but with ln scale.


Cell number ml^{-1} x 10^{7}
12
8
4
0
4
8
12
16
Time (h)

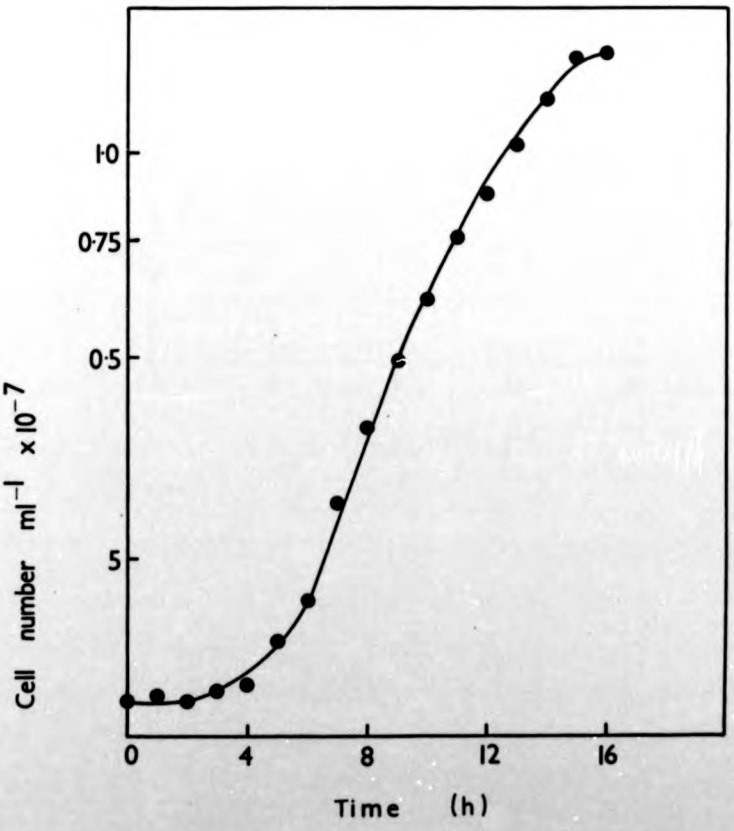
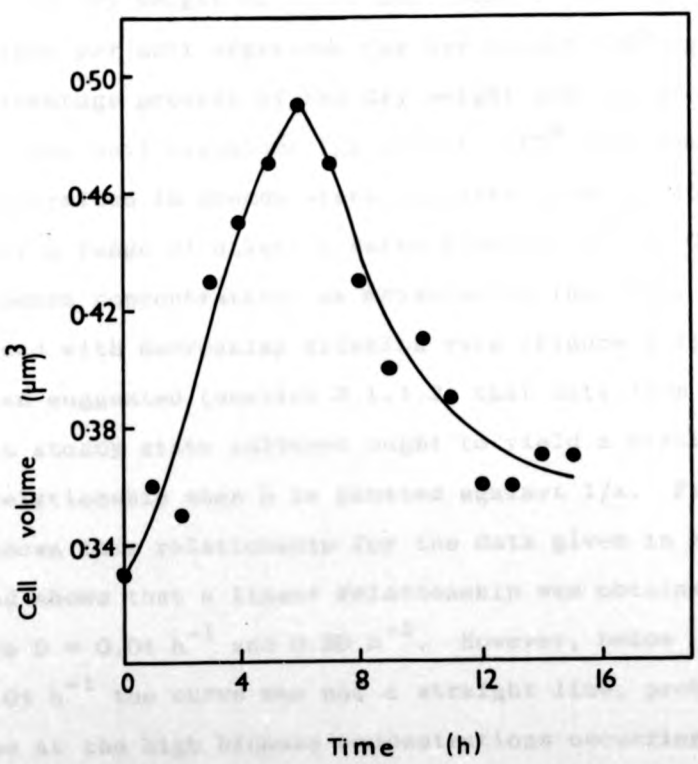

Cell number ml^{-1} x 10^{-7}
1·0
0·75
0·5
5
0
4
8
12
16
Time (h)

Figure 3.5.

The variation of cell volume during growth occurring in a closed culture with light limiting growth at higher culture biomass. Cell volume expressed as cubic microns.


0·50
0·46
0·42
0·38
0·34
Cell volume (μm)3
0
4
8
12
16
Time (h)

3.1.3. Growth in light-limited chemostat culture

Anacystis nidulans was grown in a light-limited chemostat as described in section (2.5.1). The biomass concentration, expressed in terms of absorbance or dry weight (μg dry weight ml^{-1}) or cell number (ml^{-1}), the dry weight per unit organisms (μg dry weight (10^8organisms)$^{-1}$), the percentage protein of the dry weight and the protein content per unit organisms (μg protein (10^8 organisms)$^{-1}$), were determined in steady state cultures grown at 40°C and over a range of dilution rates from 0.02 h^{-1} to 0.19 h^{-1}. The biomass concentration, as measured by the three methods, increased with decreasing dilution rate (Figure 3.6). It has been suggested (section 3.1.1.2) that data from light-limited steady state cultures ought to yield a straight line relationship when D is plotted against 1/x. Figure 3.7 shows this relationship for the data given in Figure 3.6 and shows that a linear relationship was obtained between D = 0.04 h^{-1} and 0.20 h^{-1}. However, below D = 0.04 h^{-1} the curve was not a straight line, probably because at the high biomass concentrations occurring at low dilution rates, a second nutrient began to limit biomass production, in addition to light. It is likely, but has not been conclusively demonstrated, that in this case NO_3^- was the limiting substrate.

There was an approximately 4 fold increase in the dry weight per unit organisms (μg dry weight (10^8 organisms)$^{-1}$) as the dilution rate increased from 0.02 h^{-1} to 0.19 h^{-1}

Figure 3.6.

The influence of dilution rate on the steady state biomass concentration for Anacystis nidulans grown in light-limited chemostat culture. The biomass concentration was determined as absorbance (O), dry weight expressed as μg dry weight ml^{-1} (●) and cell number ml^{-1} (□).

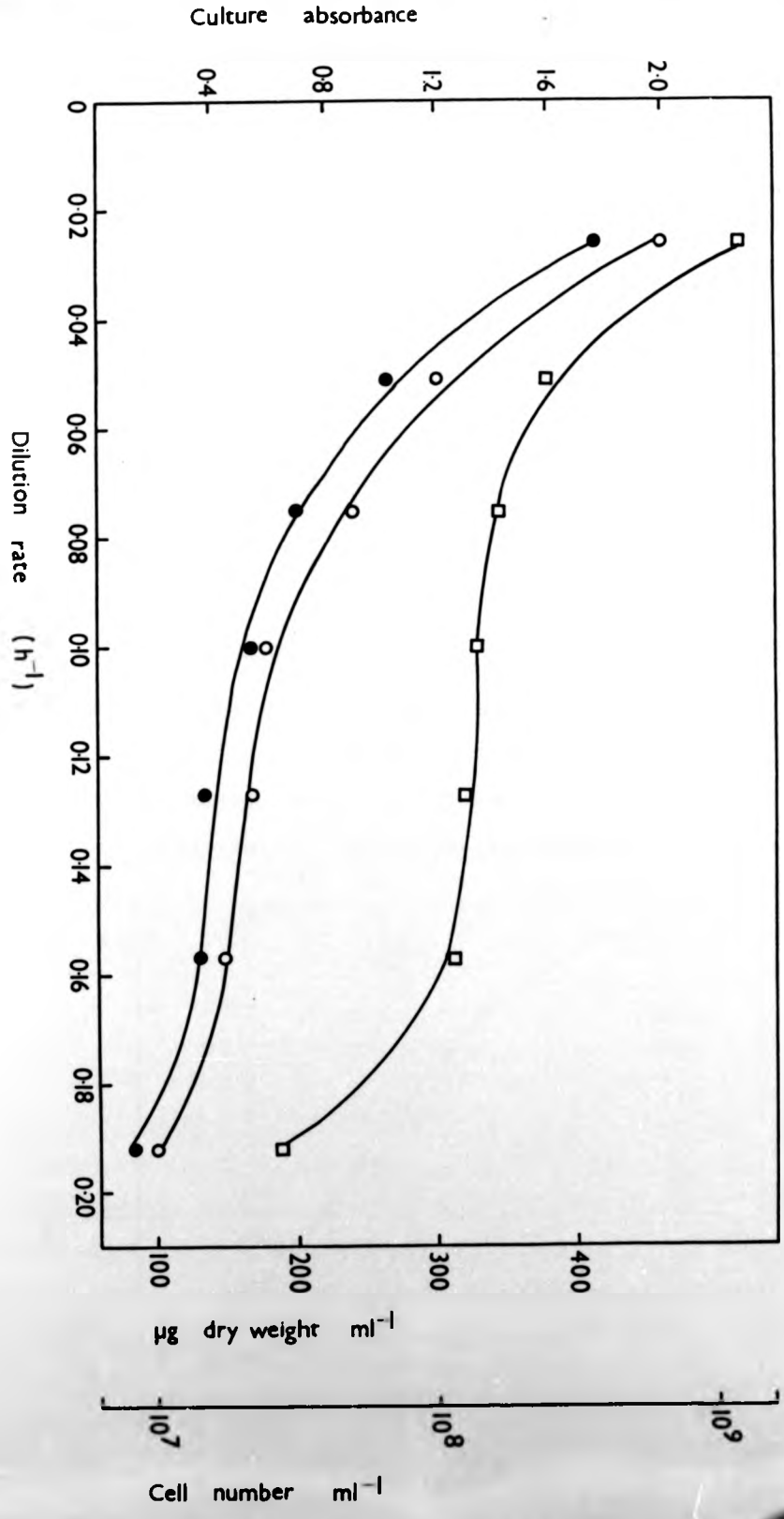
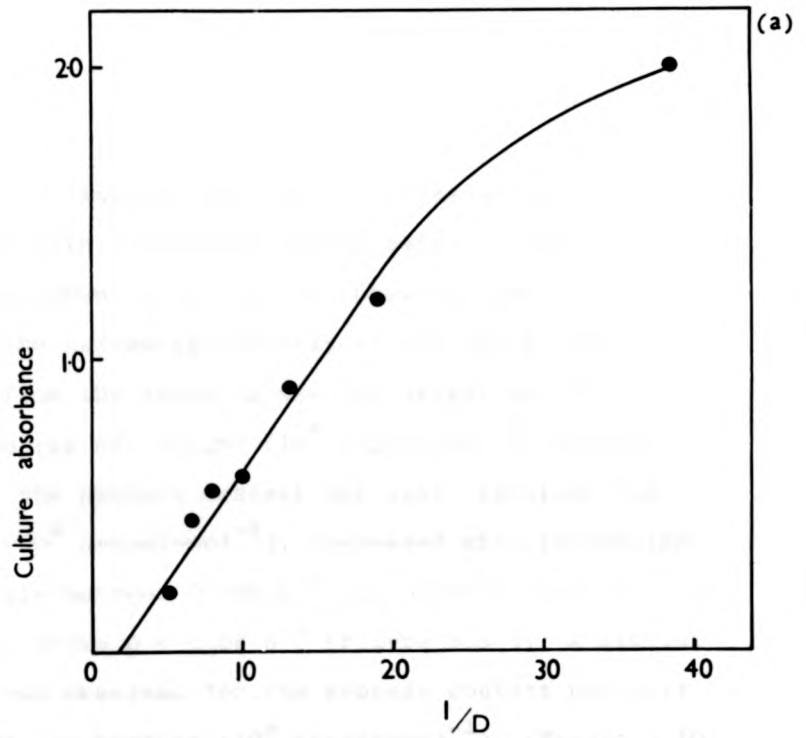
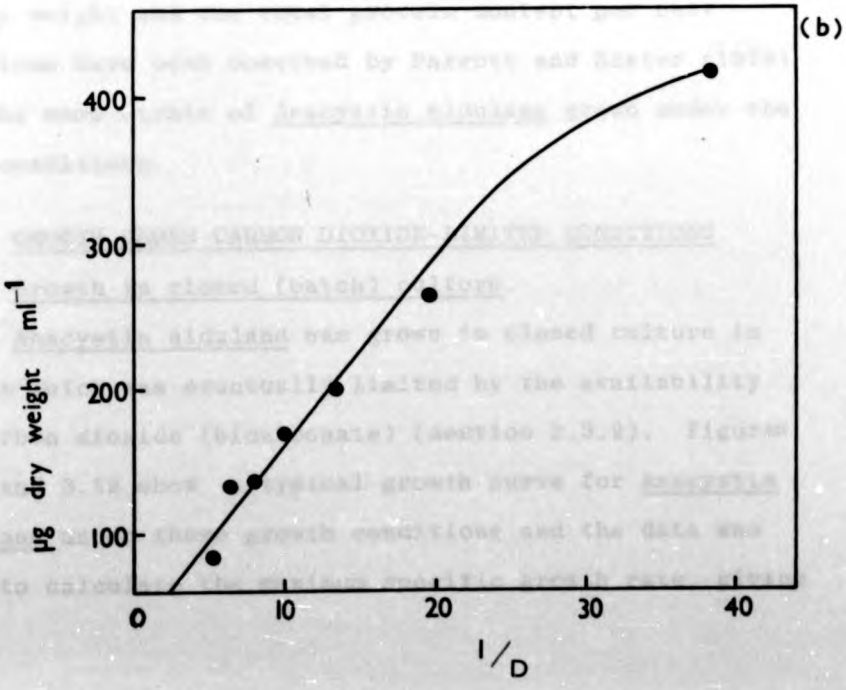

Culture absorbance
0·4
0·8
1·2
1·6
2·0
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)
100
200
300
400
µg dry weight ml⁻¹
10⁷
10⁸
10⁹
Cell number ml⁻¹

Figure 3.7.

The relationship between the biomass concentration and the reciprocal of the dilution rate under light-limited conditions. Biomass concentration is expressed (a) in terms of absorbance and (b) in terms of dry weight (μg dry weight ml^{-1}).


(a)
20
1·0
Culture absorbance
0
10
20
30
40
1/D


(b)
400
300
200
100
µg dry weight ml⁻¹
0
10
20
30
40
1/D

(Figure 3.8) indicating that there was an increase in cell size with increasing growth rate. These results are in agreement with previous observations (Slater, 1975). The percentage protein of the dry weight, calculated from the value of the dry weight per unit organisms (μg dry weight $(10^8$ organisms$)^{-1}$) and the value of the protein content per unit organisms (μg protein $(10^8$ organisms$)^{-1}$), increased with increasing growth rate between 0.026 h^{-1} and 0.08 h^{-1} and declined gradually above D = 0.08 h^{-1} (Figure 3.9). A similar pattern was observed for the protein content per unit organisms (μg protein $(10^8$ organisms$)^{-1}$) (Figure 3.10) and gave a maximum value of 48 μg protein $(10^8$ organisms$)^{-1}$ at D = 0.08 h^{-1}. Similar results for the percentage protein in dry weight and the total protein content per unit organisms have been observed by Parrott and Slater (1979) for the same strain of Anacystis nidulans grown under the same conditions.

3.2. GROWTH UNDER CARBON DIOXIDE-LIMITED CONDITIONS

3.2.1 Growth in closed (batch) culture.

Anacystis nidulans was grown in closed culture in medium which was eventually limited by the availability of carbon dioxide (bicarbonate) (section 2.3.2). Figures 3.11 and 3.12 show a typical growth curve for Anacystis nidulans under these growth conditions and the data was used to calculate the maximum specific growth rate, giving

Figure 3.8.

The influence of dilution rate on the dry weight per unit organisms (μg dry weight $(10^8 \text{ organisms})^{-1}$) for light-limited chemostat cultures.

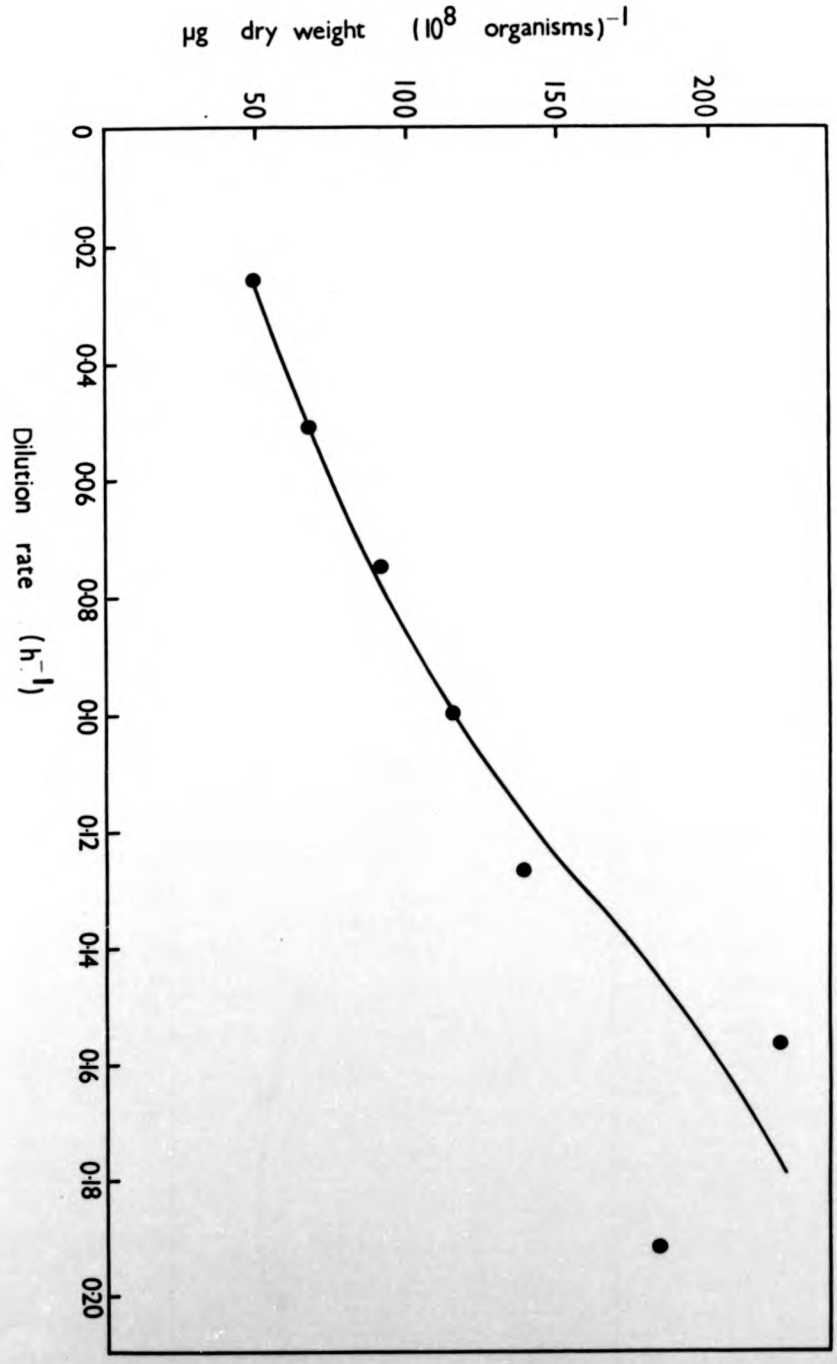
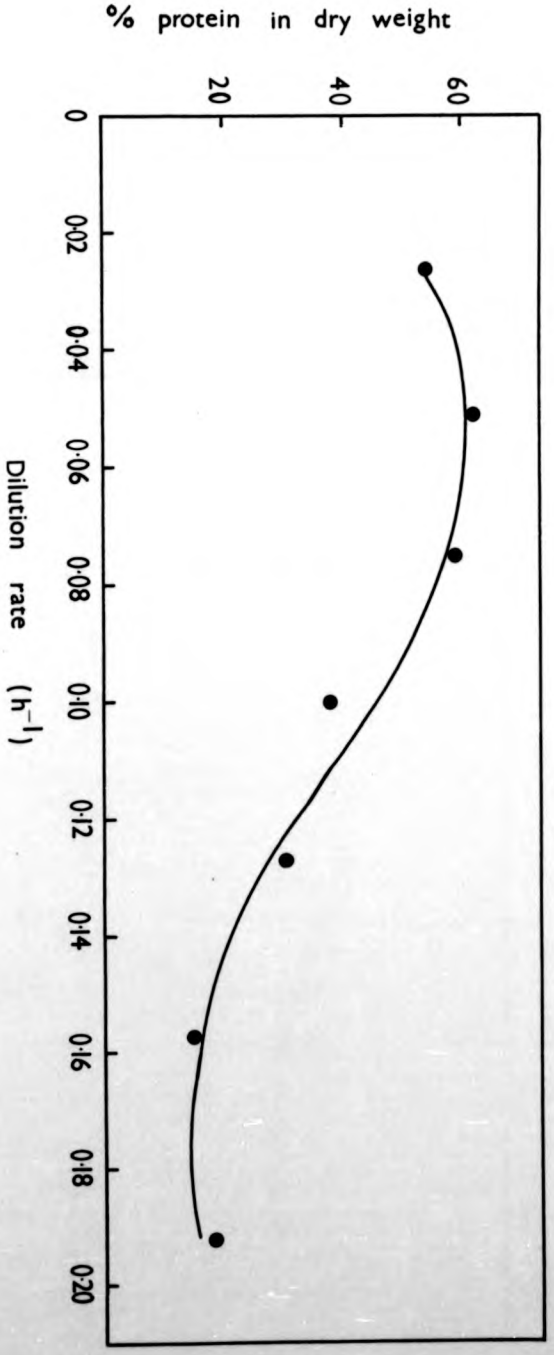

µg dry weight (10^8 organisms)^-1
50
100
150
200
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^-1)

Figure 3.9.

The influence of dilution rate on the percentage of protein in the dry weight under light-limited conditions.


% protein in dry weight
20
40
60
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)

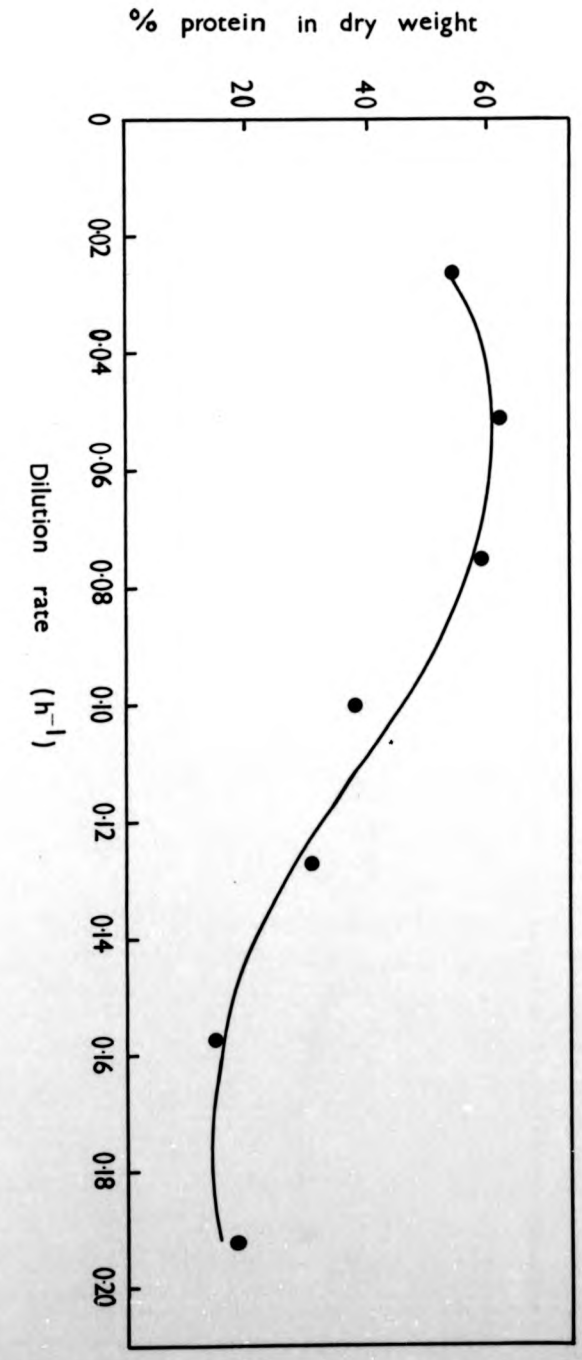

% protein in dry weight
0
20
40
60
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})

Figure 3.9.

The influence of dilution rate on the percentage of protein in the dry weight under light-limited conditions.

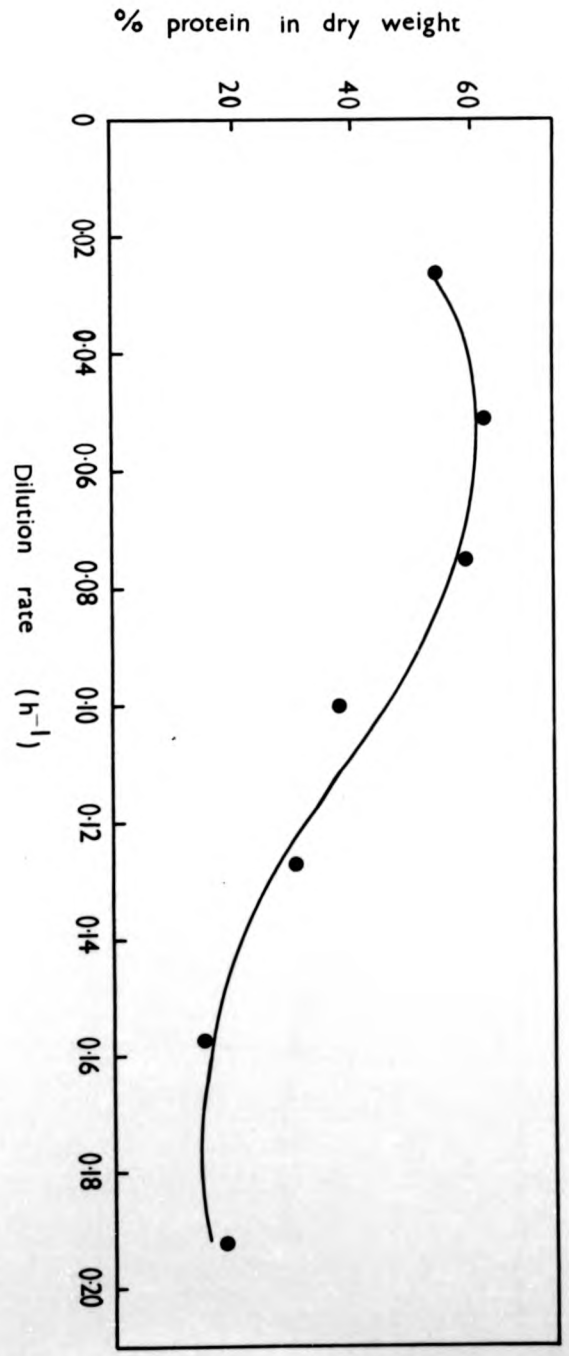

% protein in dry weight
20
40
60
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)

Figure 3.10.

The influence of the dilution rate on the total protein content per unit organisms (μg protein (10^8 organisms)$^{-1}$) for light-limited chemostat cultures.

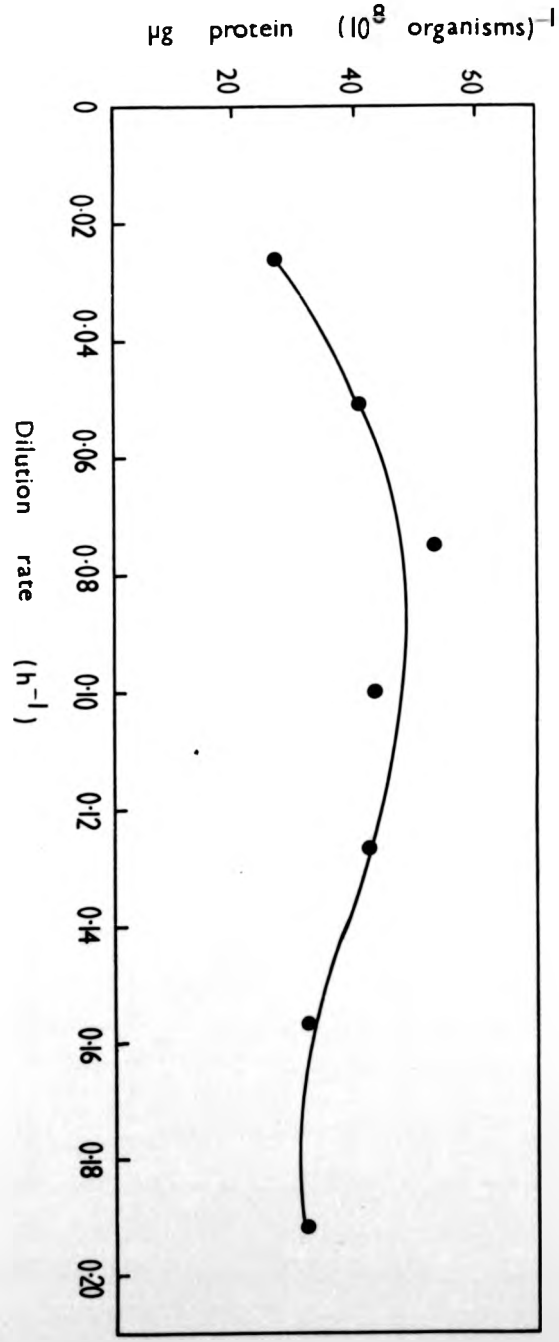
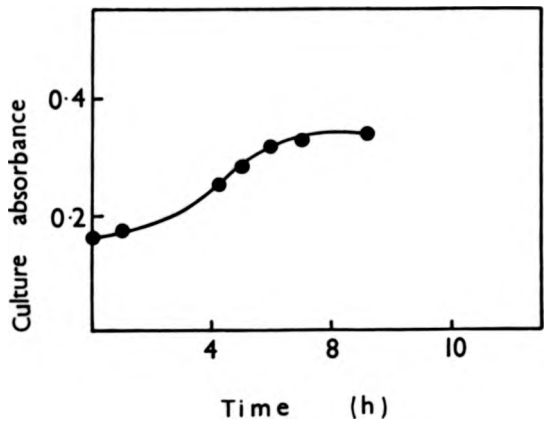

µg protein (10⁸ organisms)⁻¹
20
40
50
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)

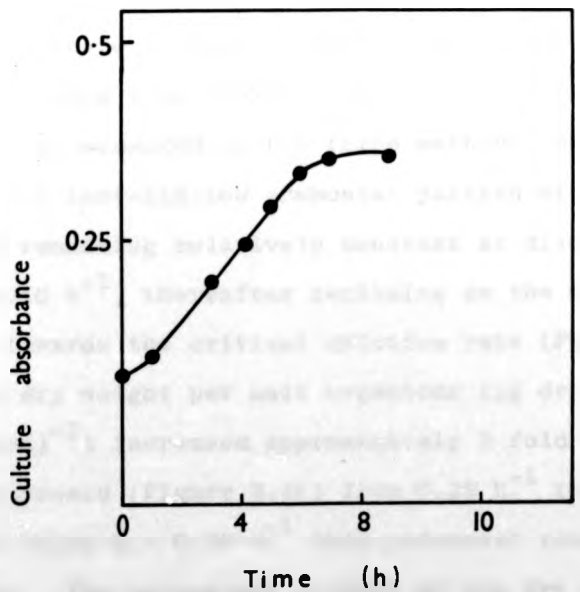
Figure 3.11.

The increase in culture absorbance during growth in a closed culture with carbon dioxide limiting growth at higher absorbance values.

Figure 3.12.

Exponential growth in a closed culture with carbon dioxide eventually limiting the growth at higher biomass concentrations. The biomass concentration was expressed as units absorbance. The data is the same as in Figure 3.11 but with ln scale.


Culture absorbance
0·4
0·2
4
8
10
Time (h)


Culture absorbance
0·5
0·25
0
4
8
10
Time (h)

a value of μ_{max} = 0.13 h^{-1}. The μ_{max} value was found to be lower under these conditions than that observed when light limited the growth of a closed culture (section 3.1.2). The exponential phase measured in terms of absorbance under light-limited conditions was two times longer compared with that under carbon dioxide-limited conditions.

3.2.2. Growth in carbon dioxide-limited chemostat culture

The biomass concentration, expressed in terms of absorbance or dry weight (µg dry weight ml^{-1}) or cell number (ml^{-1}), the dry weight per unit organisms (µg dry weight (10^8 organisms)$^{-1}$), the percentage protein of the dry weight and the protein content per unit organisms (µg protein (10^8 organisms)$^{-1}$) were determined in steady state cultures grown at 40°C, over a range of dilution rates from 0.02 h^{-1} to 0.19 h^{-1}. The culture biomass, as measured by the three methods, showed the normal nutrient-limited chemostat pattern with the culture density remaining relatively constant at dilution rates up to 0.10 h^{-1}, thereafter declining as the dilution rate tended towards the critical dilution rate (Figure 3.13).

The dry weight per unit organisms (µg dry weight (10^8 organisms)$^{-1}$) increased approximately 3 fold as the dilution rate decreased (Figure 3.14) from 0.19 h^{-1} to 0.08 h^{-1}. However below D = 0.08 h^{-1} this parameter remained relatively constant. The percentage protein of the dry weight is shown in Figure 3.15 which was approximately 30% to 60% lower between 0.02 h^{-1} and 0.08 h^{-1} dilution rates compared with

Figure 3.13.

The influence of dilution rate on the steady state biomass concentration for Anacystis nidulans grown in carbon dioxide-limited chemostat culture. The biomass concentration was determined as absorbance (O), dry weight expressed as μg dry weight ml^{-1} (●) and cell number ml^{-1} (□).

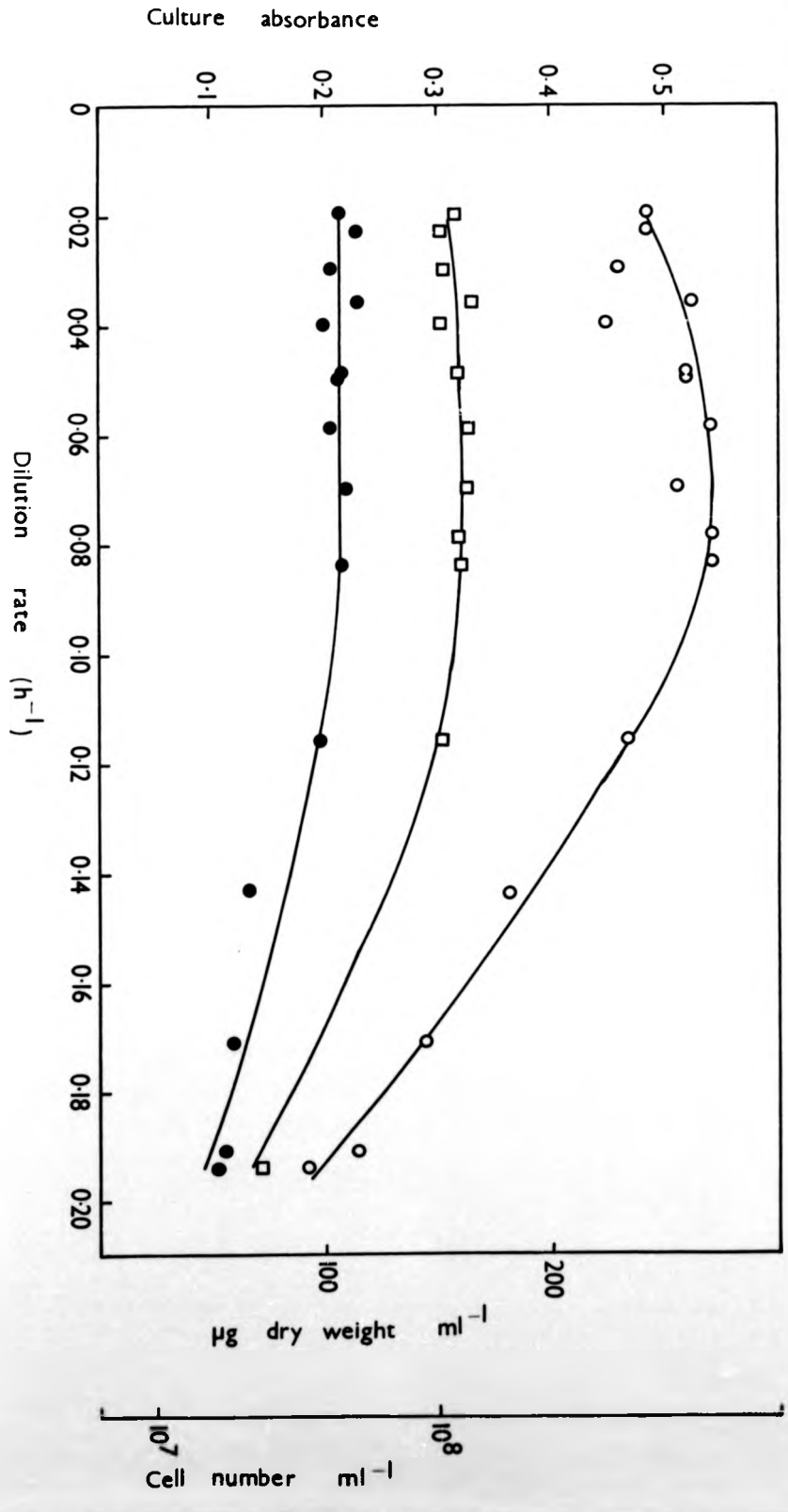

Culture absorbance
0·1
0·2
0·3
0·4
0·5
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
100
200
µg dry weight ml^{-1}
10^7
10^8
Cell number ml^{-1}

Figure 3.14.

The influence of the dilution rate on the dry weight per unit organisms (μg dry weight $(10^8$ organisms$)^{-1}$) for carbon dioxide-limited chemostat cultures.

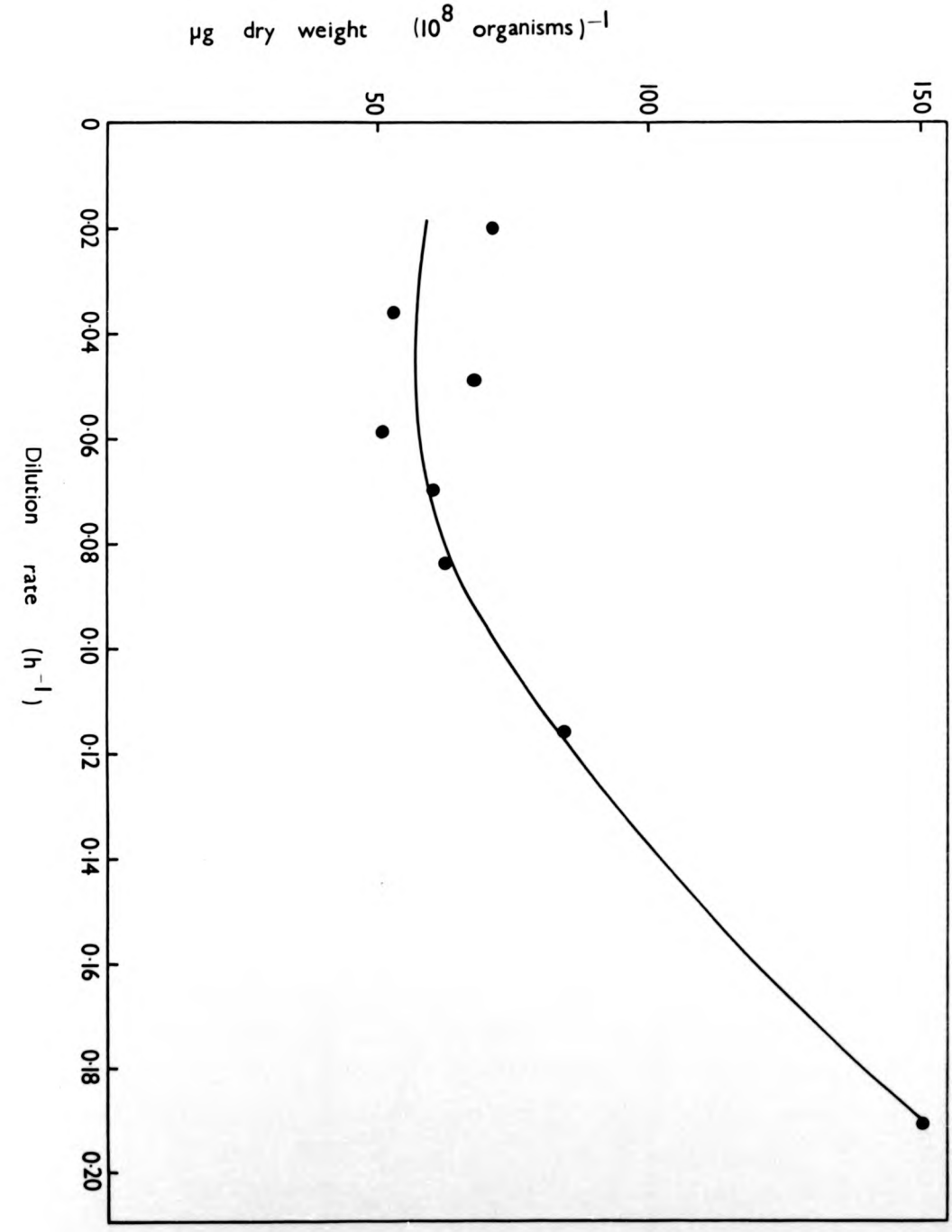
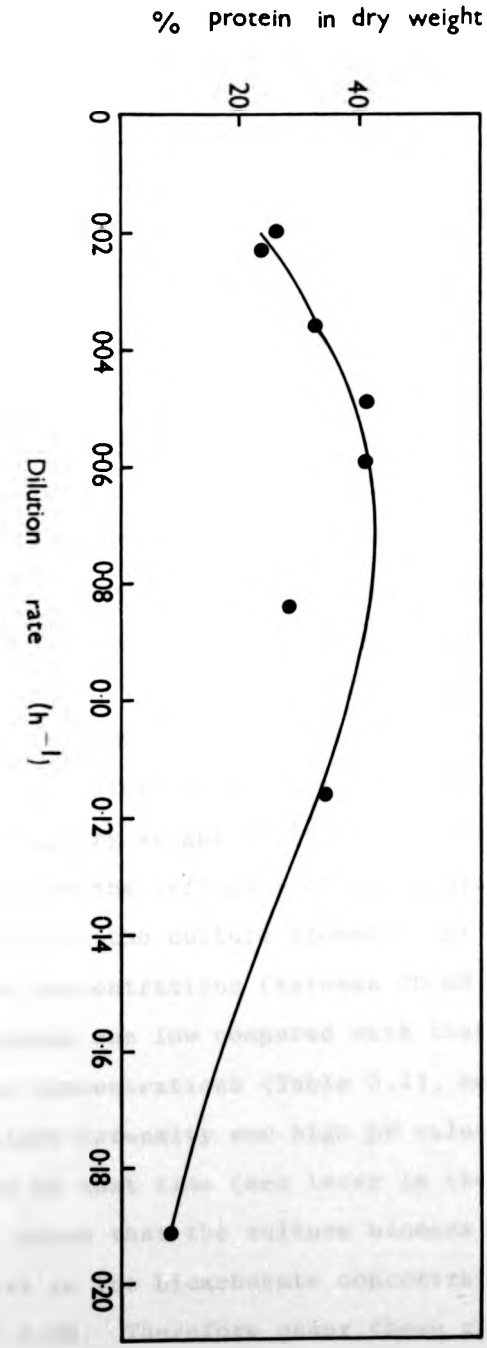

µg dry weight (10^8 organisms)^-1
50
100
150
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^-1)

Figure 3.15.

The influence of dilution rate on the percentage of protein in the dry weight under carbon dioxide-limited conditions.


% protein in dry weight
20
40
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)

the percentage protein under light-limited conditions. The pattern of the protein content per unit organisms (μg protein (10^8 organisms)$^{-1}$) was similar to that under light-limited conditions showing a peak at dilution rates between 0.06 h^{-1} and 0.08 h^{-1} (Figure 3.1 6).

3.2.3. Establishing a carbon dioxide-limited chemostat culture

Growth of Anacystis nidulans in a series of open cultures (section 2.3.2) differing in the sodium bicarbonate concentrations in the fresh inflowing medium and ranging between 2 mM to 60 mM were compared. Once a well growing culture had been established as a result of closed culture growth in the chemostat growth vessel at 40°C, fresh medium with a certain sodium bicarbonate concentration was pumped into the growing culture with a dilution rate of 0.1 h^{-1}. Growth under steady state conditions was monitored by measuring the culture biomass in terms of absorbance, or dry weight (μg dry weight ml^{-1}) or cell number (ml^{-1}). Table 3.1 shows the influence of the sodium bicarbonate concentration on the culture biomass. At high sodium bicarbonate concentrations (between 60 mM and 15 mM) the culture biomass was low compared with that at lower bicarbonate concentrations (Table 3.1), because of the low incident light-intensity and high pH values in the growth vessel used at that time (see later in the section 3.2.3). Figure 3.17 shows that the culture biomass was directly proportional to the bicarbonate concentration between 2 mM and 7.5 mM. Therefore under these growth conditions the only limiting nutrient was the bicarbonate.

Figure 3.16.

The influence of the dilution rate on the total protein content per unit organisms (μg protein $(10^8$ organisms$)^{-1}$) for carbon dioxide-limited chemostat cultures.

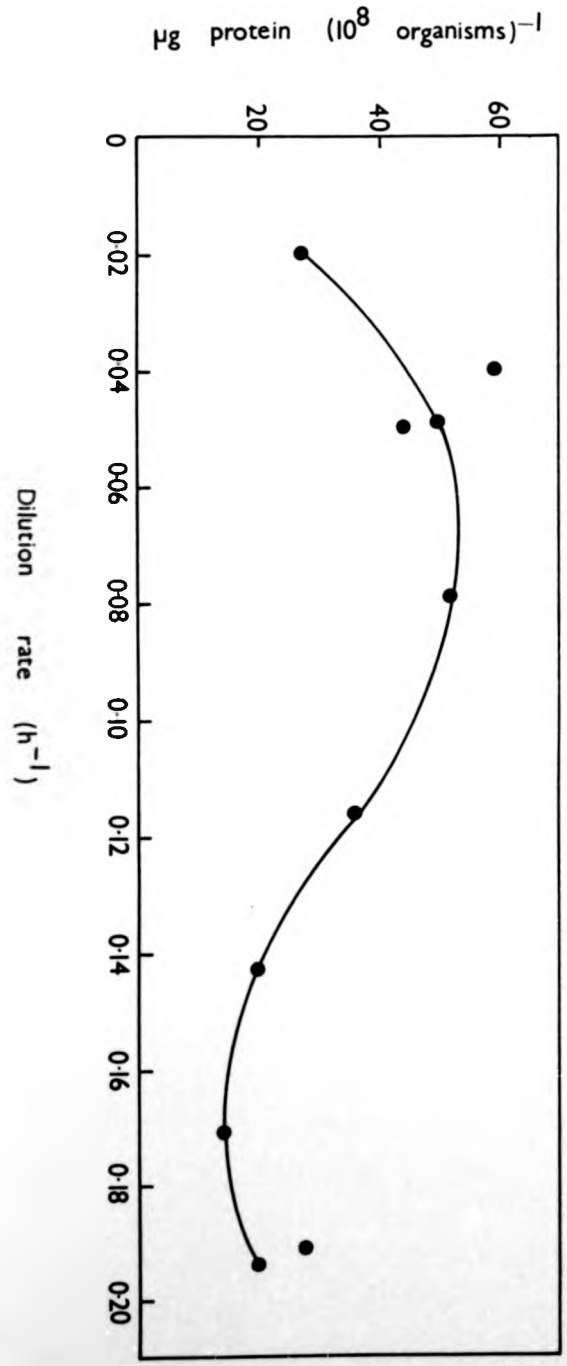
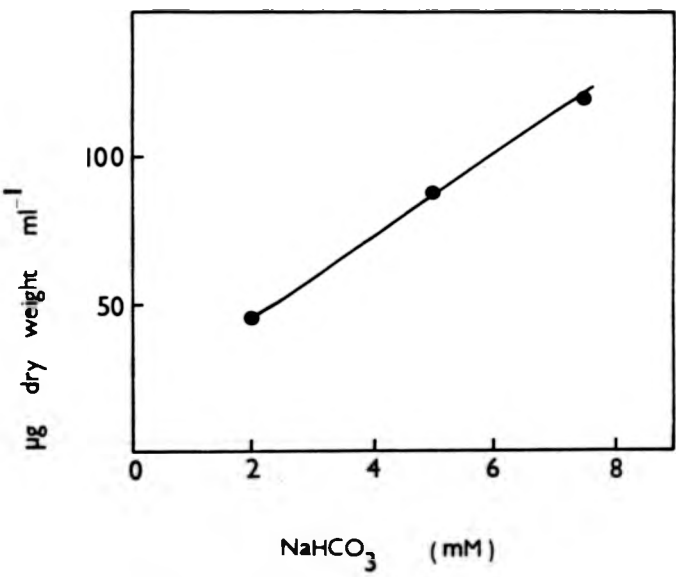

μg protein (10^8 organisms)^-1
20
40
60
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^-1)

Figure 3.17.

The effect of the concentration of sodium bicarbonate on the culture biomass. The culture biomass was expressed as μg dry weight ml^{-1}.


100
50
0
2
4
6
8
µg dry weight ml⁻¹
NaHCO3 (mM)

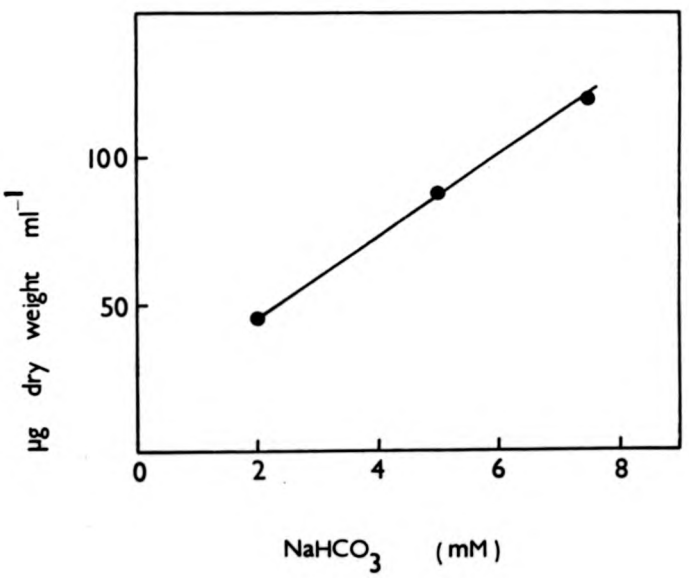

100
50
0
2
4
6
8
µg dry weight ml⁻¹
NaHCO3 (mM)

Table 3.1. The effect of sodium bicarbonate concentration on the culture biomass at a constant dilution rate D = 0.10 h^{-1}.

$NaHCO_3$ (mM)	Absorbance (600 nm)	Dry Weight ($\mu g\ ml^{-1}$)	Cell number ($ml^{-1} \times 10^8$)	Y
60	0.544	193	2.09	
30	0.404	63	1.04	
15	0.446	85	1.01	
7.5	0.378	72	1.00	
7.5	0.705	145	1.20	0.77
5	0.563	92	0.88	0.73
2	0.187	35	0.44	0.69

The concentration of 5 mM of sodium bicarbonate was used for all the experiments, giving an absorbance of approximately 0.5 units at 600 nm and the expected substrate-limited pattern of growth (section 3.2.2).

Difficulties always occured in establishing cultures under carbon dioxide-limited conditions. It was necessary to add approximately 200 ml of an exponentially growing inoculum in the growing vessel and to first establish growth with a gas phase of 5% (v/v) CO_2: 95% (v/v) N_2 instead of nitrogen alone. This continued until a well growing culture had been established. Thereafter the 5% (v/v)CO_2: 95% (v/v)N_2 gas phase was changed to N_2 and after 24 hours the fresh media flow was started.

Another difficulty was that the pH of the fresh medium after autoclaving and the addition of the sodium bicarbonate solution (section 2.5.2) rose to a value between pH 9.0 and pH 10.0. Under these pH conditions Anacystis nidulans could not grow. Therefore a pH controller was used in order to monitor the pH in the culture vessel and to maintain a value of pH 7.5 ± 0.1.

3.3. INFLUENCE OF TEMPERATURE ON GROWTH

3.3.1. Light-limited growth

Using light-limited chemostat cultures the effect of temperature on the growth of steady state cultures was examined at a dilution rate of 0.1 h^{-1}. The temperature was progressively increased from 40°C to 44°C and decreased from 40°C to 25°C in increments of 1°C to 2°C. This was

important because the organisms subjected to larger, instantaneous changes in temperature ceased to grow within 2 to 3 hours.

Figure 3.18 shows the culture absorbance as a function of temperature and Figures 3.19 and 3.20 show the changes in dry weight and cell number between 25°C and 44°C respectively. These results show that under light-limited continuous culture conditions the maximum biomass concentration occured at 40°C, a result which was in agreement with that reported for Anacystis nidulans under different growth conditions by Kratz and Myers (1955a). Above 40°C the culture biomass began to fall rapidly with increasing temperature. Below 25°C and above 44°C it was not possible to obtain a steady state culture at a dilution rate of 0.1 h^{-1}. Table 3.2 shows a comparison of the biomass concentrations at the optimum temperature 40°C and at the minimum achieved temperature 25°C for different growth rates under light-and carbon dioxide-limited conditions. Under light-limited conditions the culture biomass at 25°C was considerably lower compared with the culture biomass at 40°C at similar growth rates. The difference was approximately 66% greater at high growth rates. The maximum growth rate achieved at 25°C under these conditions was 0.16 h^{-1}. Similar results were obtained under carbon dioxide-limited conditions. It was not possible for the organisms to achieve a steady state at growth rates higher than 0.05 h^{-1} because these growth rates were above the organism's D_{crit} value at 25°C.

Figure 3.18.

The effect of temperature on culture absorbance under light-limited conditions at a constant dilution rate, $D = 0.1\ h^{-1}$.

Figure 3.19.

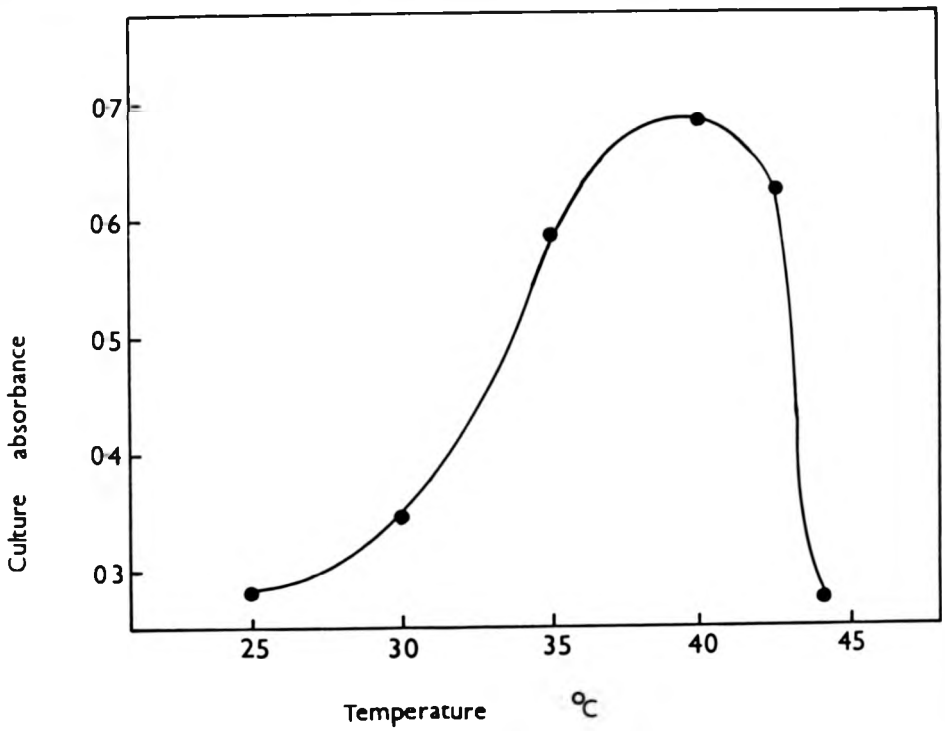
The effect of temperature on dry weight (µg dry weight ml^{-1}) under light-limited conditions at a constant dilution rate, $D = 0.1\ h^{-1}$.

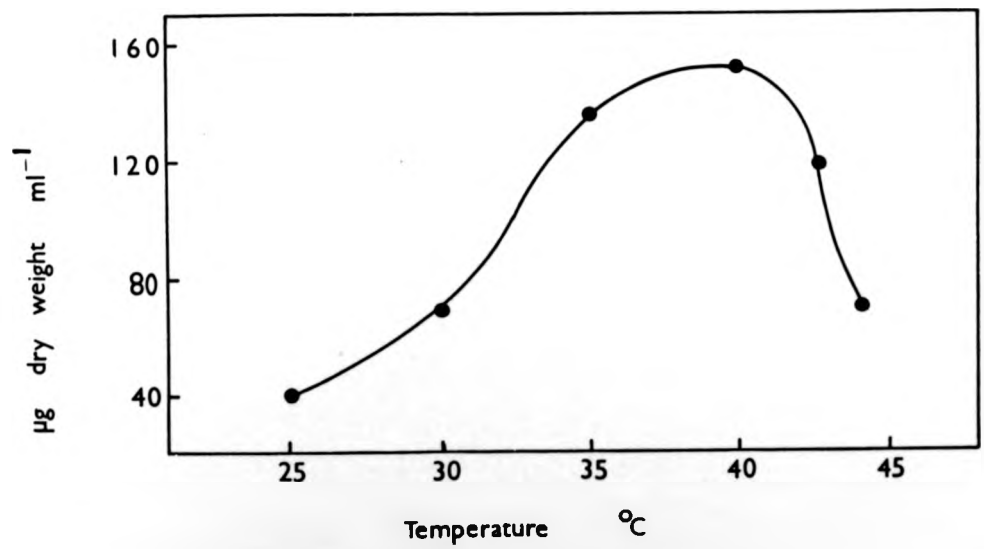
sorbance

stant

(µg dry

ions at





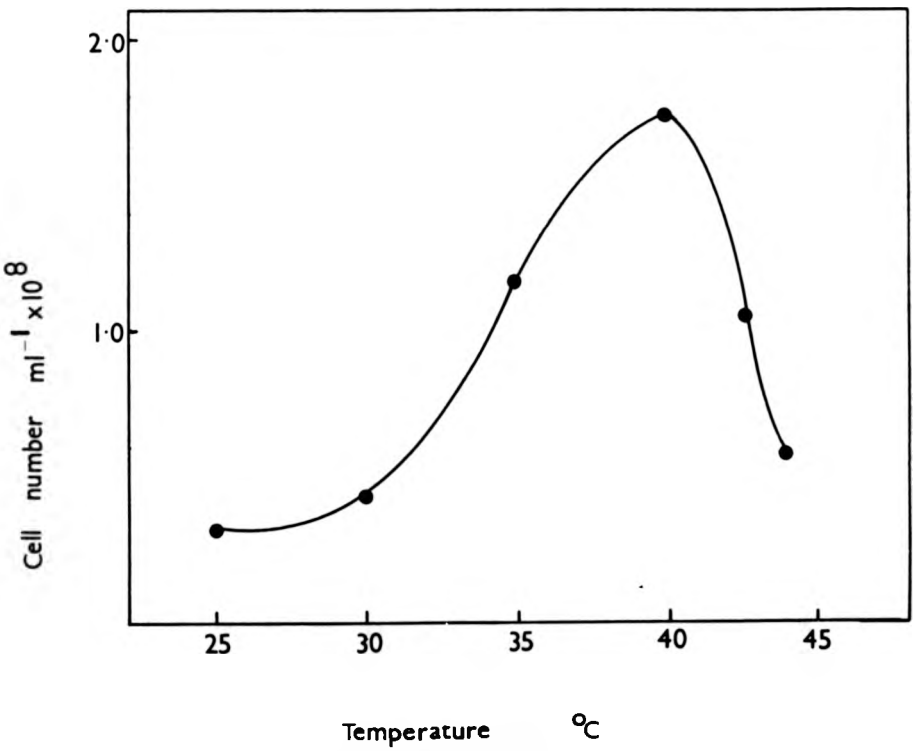

2·0
1·0
Cell number ml⁻¹ x10⁸
25
30
35
40
45
Temperature °C

Table 3.2. A comparison of the effect of the dilution rate on the culture biomass under light-limited and carbon dioxide-limited conditions at 25°C and 40°C. np = not possible to obtain steady state cultures at higher dilution rates at 25°C.-, not determined.

	Temperature							
	25°C				40°			
	D (h^{-1})	Absorbance (600 nm)	Dry Weight ($\mu g\ ml^{-1}$)	Cell number ($ml^{-1} \times 10^8$)	D (h^{-1})	Absorbance (600 nm)	Dry Weight ($\mu g\ ml^{-1}$)	Cell number ($ml^{-1} \times 10^8$)
light-limited	0.020	1.530	436	-	0.026	2.000	438	8.42
	0.060	0.661	196	2.33	0.060	0.910	240	3.60
	0.100	0.428	121	1.62	0.100	0.600	164	1.50
	0.125	0.310	75	1.20	0.126	0.540	124	1.00
	0.160	0.115	38	-	0.157	0.450	116	0.60
	np	np	np	np	0.192	0.200	82	0.45
CO_2-limited	0.020	0.332	100	-	0.020	0.486	110	1.57
	0.050	0.186	73	-	0.050	0.521	110	1.69
	np	np	np	np	0.120	0.466	100	1.14
	np	np	np	np	0.190	0.225	60	0.37

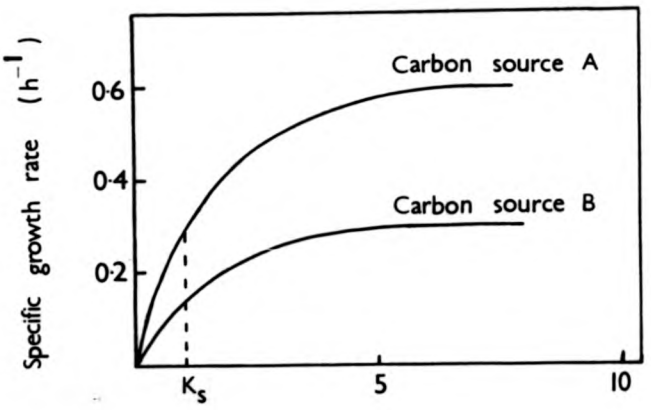
3.3.2. The concept of "relative" growth rate in light-limited chemostat cultures.

3.3.2.1 Theory

Tempest (1976) has suggested a concept of a "relative" growth rate which is the ratio of the imposed growth rate to the maximum growth rate (μ/μ_{max}). The "relative" growth rate can be used as a basis for comparison of two chemostat cultures of organisms growing at the same fixed growth rate when the steady state concentrations of growth-limiting nutrient are not equal. This is, in cases where for any given conditions (temperature, pH value, medium composition) some secondary change is imposed on the culture such that the value of μ_{max} changes (K_s remaining constant) then s must also change because $s = K_s \left| \frac{\mu}{\mu_{max}-\mu} \right|$ (see Introduction, equation 1.4). This situation is represented graphically in Figure 3.21 which shows that in order to maintain a steady state extracellular concentration of a growth-limiting substrate (say, phosphate) at a value close to K_s, when the medium carbon source is changed from substance A to substance B, the dilution rate must be decreased from 0.3 to 0.15 h^{-1}. However the "relative" growth rate (μ/μ_{max}) will remain constant since K_s defines a relative growth rate value, that is $\mu/\mu_{max} = 0.5$. The above theory is realistic assuming that the factors which affect the maximum growth rate do not simultaneously affect the saturation constant.

Figure 3.21.

Effect of growth rate on the steady state extracellular phosphate concentration in the presence of alternative carbon sources. (The figure redrawn from Tempest (1976)).


Specific growth rate (h^{-1})
0·6
0·4
0·2
Carbon source A
Carbon source B
K_s
5
10
Steady state extracellular phosphate concen. (μM)

3.3.2.2. Application.

One application to the "relative" growth rate is a comparison of light-limited cultures of Anacystis nidulans grown at two different temperatures and at the same fixed dilution rate. Figure 3.22 shows the effect of the dilution rate on the culture absorbance of light-limited cultures grown at 25°C and 40°C. It can be seen that the steady state culture biomass was greater when the organisms grown at 40°C compared with 25°C. However, the maximum growth rate of organisms grown at 25°C was only about 75% of that of organisms grown at 40°C. Clearly, considering the plot of the steady state culture biomass against the "relative" growth rate, the two culture biomass concentrations at 25°C and 40°C were not greatly different (Figure 3.23).

3.4. ELEMENTAL ANALYSIS

It has been ascertained from elemental analysis that organisms of Anacystis nidulans grown under either light-limited or carbon dioxide-limited conditions contained on a dry weight basis (w/w) 47.6% carbon, 11.1% nitrogen and 6.9% hydrogen.

3.5. CALCULATION OF THE SATURATION CONSTANT, K_s, VALUE FOR CARBON DIOXIDE (BICARBONATE)

Considering the data in Figure 3.13 and assuming that all the available carbon dioxide (bicarbonate) was converted into culture biomass at the lowest dilution rate,

Figure 3.22.

The effect of dilution rate on the culture absorbance of light-limited steady state cultures grown at 25°C (●) and 40°C (○).

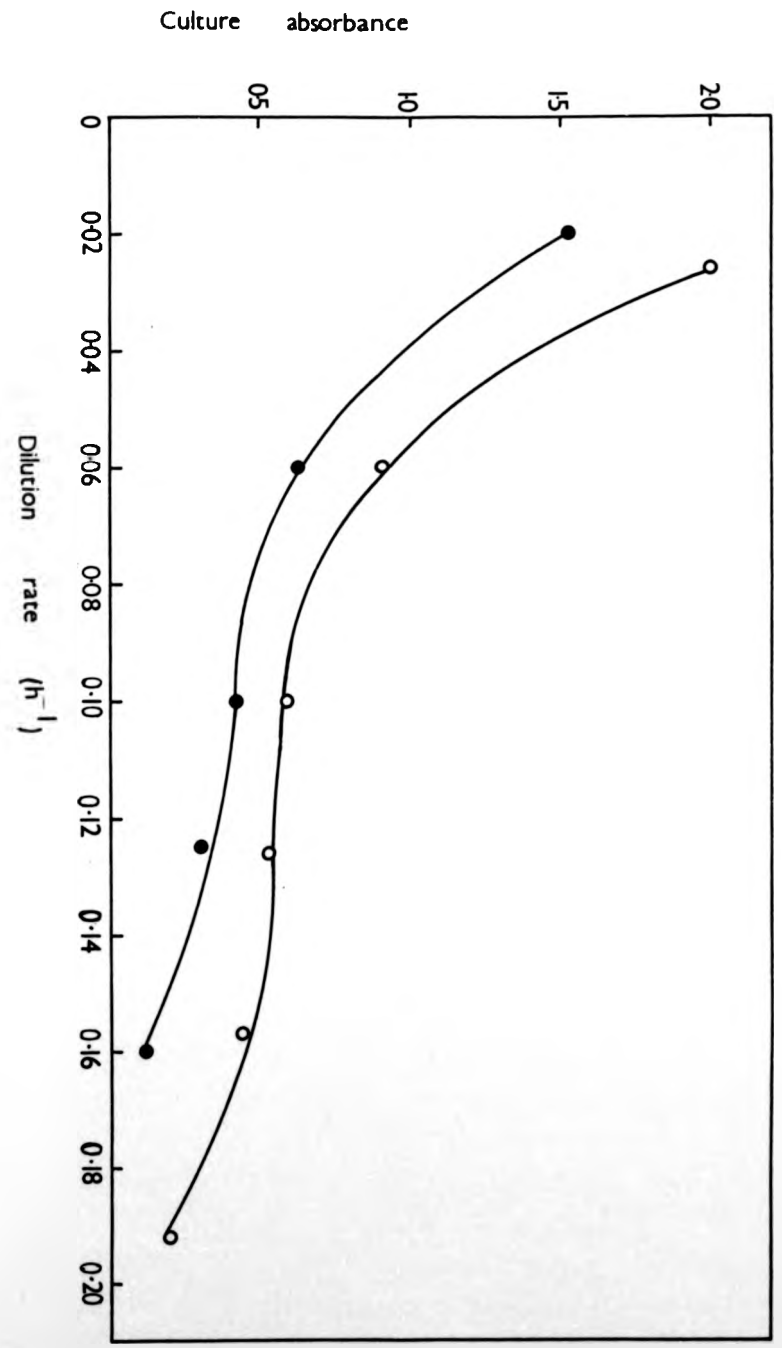
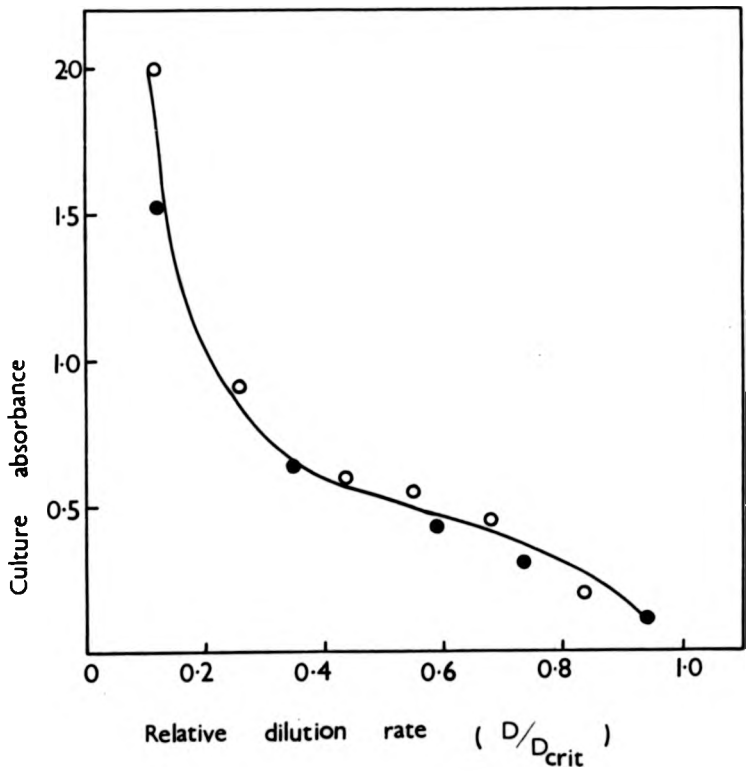

Culture absorbance
0·5
1·0
1·5
2·0
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})

Figure 3.23

Effect of the "relative" dilution rate on the culture absorbance of light-limited steady state cultures grown at 25°C (●) and 40°C (○).


Culture absorbance
2·0
1·5
1·0
0·5
0
0·2
0·4
0·6
0·8
1·0
Relative dilution rate (D/Dcrit)

the

,

(o).

then it is possible to calculate the $\bar{s}$ value between $D = 0.02\ h^{-1}$ and $D = 0.194\ h^{-1}$, given that S_R = 0.42 g bicarbonate l^{-1} or 0.06 g carbon l^{-1} and also given that 47% of the dry weight is carbon (section 3.4).

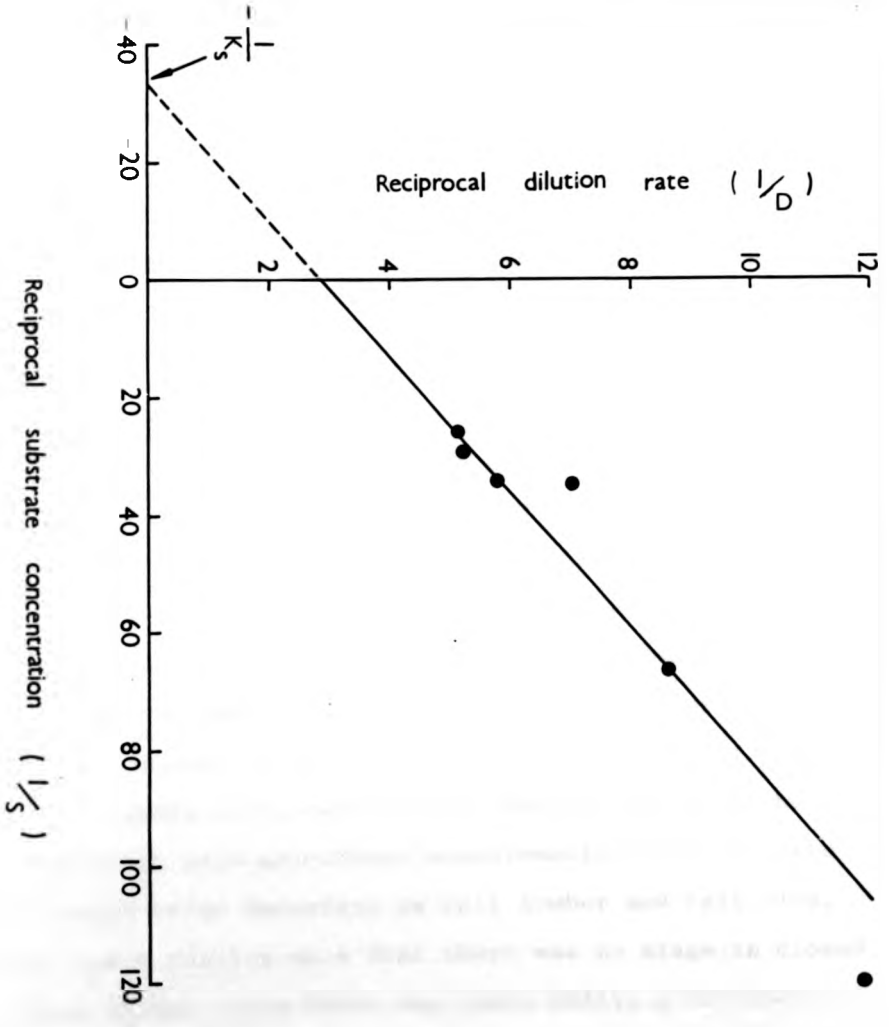
Table 3.3 gives the calculated values for 1/D and 1/s which are used in Figure 3.24. The K_s value for bicarbonate given from this plot was equal to 2.5 mM (0.212 g sodium bicarbonate l^{-1}).

Table 3.3. Calculated values for the reciprocal dilution rate and the reciprocal $\bar{s}$ value. (Data taken from Figure 3.13).

D (h^{-1})	1/D	$\bar{x}$ ($\mu g\ ml^{-1}$)	μg C in organisms (ml^{-1})	$\bar{s}$ ($\mu g\ C\ ml^{-1}$)	1/s
0.02	50	110	51.7	8.3	120
0.023	43.5	120	56.4	3.6	280
0.03	33.3	100	47.0	13.0	80
0.036	27.7	120	56.4	3.6	280
0.04	25	98	46.06	13.94	70
0.049	20.4	115	54.05	5.95	170
0.05	20	110	51.7	8.3	120
0.059	16.9	97	45.6	14.41	70
0.07	14.3	116	54.52	5.48	180
0.084	11.9	110	51.7	8.3	120.5
0.116	8.6	96	45.12	14.88	67.2
0.143	7.0	67	31.49	28.51	35.1
0.171	5.8	66	31.02	28.9	34.6
0.191	5.2	56	26.32	33.68	29.7
0.194	5.15	48	22.56	37.44	26.7

Figure 3.24.

The linear form (Lineweaver-Burke plot) of the reciprocal dilution rate as a function of the reciprocal substrate concentration.


Reciprocal dilution rate (1/D)
Reciprocal substrate concentration (1/S)
-1/Ks
-40
-20
0
2
4
6
8
10
12
20
40
60
80
100
120

the

the

DISCUSSION

The results reported here for the growth of Anacystis nidulans under conditions where light was limiting at higher biomass concentrations showed that there was a difference between the lag phase as measured in terms of absorbance or cell number. Absorbance measures both the increase in cell size as well as the increase in cell number, thus the difference observed between the lag phases may be due to the complex relationship between absorbance and cell number and cell size changes. Furthermore the μ_{max} values obtained depended on what parameter was measured. When the absorbance data were calculated the value of μ_{max} was 0.16 h^{-1} whilst when the cell number values were used, μ_{max} was 0.27 h^{-1}. This may be due to the fact that although the exponential phase measured in terms of absorbance lasted longer than that measured in terms of cell number, absorbance values increased less than cell number values respectively at the end of the growth because cells were getting smaller and as it has already been said absorbance measurements tended to give an average value dependant on cell number and cell size. Thus, these results show that there was no stage in closed culture growth cycle where one could obtain a balanced exponential growth with absorbance, cell number and cell size all increasing at the same specific growth rate.

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Once a closed culture has been established the whole process is discontinuous and we always have transient conditions due to environment changes resulting in non steady state conditions. Thus it is not possible to be certain about parameters such as enzyme activities and the influence of the growth environment on these parameters. Critical, quantitative changes in enzyme levels can be examined only as organisms growing under steady state conditions, whereas closed (batch) cultures can be used quite adequately to follow qualitative variations (Bull, 1972; Dean, 1972; Bull, 1974). The difficulty of obtaining balanced growth in closed culture emphasizes the importance of using continuous-flow culture growth systems where steady state conditions can be obtained enabling a more rigorous study of the physiology of the organisms and where a much more delicate control over growth rate can be made simply by altering the concentration of the limiting substrate which in practice it can be achieved by varying the dilution rate.

The kinetic theory of a light-limited chemostat culture of Anacystis nidulans proposed here should be of some value in interpreting the results obtained in laboratory cultures of Anacystis nidulans. Equation 3.8 indicates that a plot of the biomass concentration (x) against the reciprocal of the dilution rate (1/D) is a straight line. The results obtained from the continuous-flow chemostat culture designed to be light-limited showed that the above relationship

between the biomass concentration (x) and the reciprocal of the dilution rate (1/D) was realistic at least at dilution rates higher than D = 0.04 h^{-1}. At dilution rates below D = 0.04 h^{-1} with the high biomass concentration obtained, a second nutrient began to limit growth and the above relationship was not valid. Below D = 0.04 h^{-1} the biomass concentration obtained was lower than the expected, the curve was not a straight line and in addition to light probably NO_3^- was a second limiting substrate, resulting in the lower steady state biomass concentration.

In addition other experiments indicate that light actually was the limiting factor since there was a rapid increase in culture biomass concentration in all cases when the incident light intensity was increased. Two experiments were undertaken in steady state light-limited chemostat cultures grown at D = 0.03 h^{-1} and D = 0.10 h^{-1}, where at t = 0 the incident light intensity increased 12 or 8 fold respectively. The culture absorbance started to increase within the first hour after the additional illumination (see section 6.1).

There were considerable difficulties in establishing a culture of Anacystis nidulans growing under carbon dioxide-limited conditions. The pH value of the fresh medium was an important factor for the growth of Anacystis nidulans under these conditions. In all cases where attempts were made to obtain growth curves in a closed culture with medium

which was eventually limited growth by the availability of carbon dioxide (bicarbonate), slow growth with a small increase in culture absorbance occurred, although all nutrients were in excess. The value of μ_{max} obtained under these growth conditions measured in terms of absorbance was 0.13 h^{-1}. However, in a chemostat culture the biomass concentration at a particular growth rate increased with increasing the carbon dioxide (bicarbonate) concentration under these growth conditions shown that open cultures were carbon dioxide-limited. All nutrients were in excess except the carbon dioxide (bicarbonate) and therefore the culture biomass showed the expected substrate-limited chemostat pattern.

The calculated saturation constant, K_s, value for sodium bicarbonate was found to be high (K_s = 2.5 mM) compared with the K_s value for other bacteria, for example Klebsiella has a K_s = 0.009 mM for carbon dioxide according to Pirt (1975). This may suggest that Anacystis nidulans has a low affinity for bicarbonate.

Organisms grown in carbon dioxide-limited chemostat contained 30% to 60% less protein in dry weight than organisms grown in light-limited chemostat between 0.02 h^{-1} and 0.19 h^{-1} dilution rates. Eley (1971) reported approximately 50% decrease in total pigment content when organisms of Anacystis nidulans grown with 0.03% CO_2 in air than 1% CO_2. Recently Parrott and Slater (1979) demonstrated that at lower dilution rates where the

bicarbonate availability was less, Anacystis nidulans grown in carbon dioxide-limited chemostat cultures contained only one third of the amount of phycocyanin which was contained in light-limited organisms. Therefore the lower total protein content in carbon dioxide-limited organisms may be due to the lower amount of phycocyanin. This may be important in the generally greater increase in enzyme specific activities under carbon dioxide-limited conditions compared with those under light-limited conditions (see part 5).

Temperature was an important parameter influencing the growth of Anacystis nidulans. The optimal temperature was 40°C ± 0.5 under both limitation as measured by biomass concentration. The organism's D_{crit} value at the lower temperature, 25°C, examined was above D = 0.05 h^{-1} but less than D = 0.08 h^{-1} for carbon dioxide-limited organisms whilst for light-limited organisms the D_{crit} value at 25°C was above D = 0.16 h^{-1} but less than D = 0.18 h^{-1} and this is due to the effects of temperature on the rates of cell reactions, the nature of metabolism, the nutritional requirements and the biomass concentration (Pirt, 1975). For example chemical reaction rates are related to temperature by the Arrhenius equation

$$K = Ae^{-E/RT} \qquad (3.9)$$

where K is the reaction rate, R is the gas constant, T the absolute temperature, A is a constant dependent on

the frequency of formation of activated complexes of the reactants and E is a constant known as the 'activation energy' or 'temperature characteristic'. Evidence for the effects of temperature on metabolic regulation was presented by Ng, Ingraham and Marr (1962) who found that in E. coli the repression of β-galactosidase by glucose began to fail below 25°C and disappeared entirely during growth at 10°C. Also the pathway of metabolism of the carbon and energy source can be temperature sensitive. An example was Lactobacillus brevis which at 24°C fermented glucose by the heterolactic pathway whereas at 37°C required fructose as a hydrogen acceptor (forming mannitol) for glucose fermentation (De Ley, 1962). Also Tempest (1969) reported that at a given growth rate, the growth yielded from magnesium, potassium and phosphate decreased on lowering the temperature, which reflected the increase in the RNA content of the biomass.

The culture biomass of steady state light-limited cultures was greater when the organisms grown at 40°C compared with 25°C. Although the steady state culture biomass concentrations were very similar irrespective of the temperature when plotted against the 'relative' dilution rates. An interpretation of these data is that the greater synthesis of biomass by cultures growing at 40°C reflects at each particular growth rate a more efficient light absorbsion by the organisms.

The fundamental assumption made in developing the theory of the concept of 'relative' growth rate was that factors affecting the maximum growth rate, for example temperature in this case, do not simultaneously affect the saturation constant (Tempest, 1976). However, temperature may well be one of the factors which affect simultaneously both μ_{max} and K_s, in other words when at 40°C temperature the μ_{max} increased the affinity of the organisms for the growth-limiting substrate also increased . Although it seems unlikely that such circumstances will lead to a simultaneously decreased μ_{max} and increased affinity of the organisms for the growth-limiting substrate.

PART 4. INFLUENCE OF DILUTION RATE AND TEMPERATURE ON CARBON DIOXIDE ASSIMILATION

4.1. INFLUENCE OF DILUTION RATE ON CARBON DIOXIDE ASSIMILATION

4.1.1. Light-limited conditions

The rate of carbon dioxide assimilation by intact organisms was measured in steady state cultures under optimal experimental conditions over a range of dilution rates from 0.025 h^{-1} to 0.19 h^{-1}. Over this growth rate range light was the sole growth-limiting nutrient (see section 3.1) and so any changes in carbon dioxide assimilation observed in different steady state cultures could be attributed to alterations in the organism's growth rate alone. Although all the experiments took place outside the chemostat under optimum conditions and the rate of carbon dioxide fixation by organisms taken from a particular steady state culture was the maximum rate possible, but the observed rates of carbon dioxide fixation may not be those of the organisms growing in the chemostat.

However, under light-limited conditions the rate of carbon dioxide assimilation increased with increasing growth rate up to D = 0.10 h^{-1} (Figure 4.1) and showed a peak of activity (0.14 µmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$ or 0.62 µmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$) between D = 0.10 h^{-1} and D = 0.11 h^{-1}. Between D = 0.025 h^{-1} and D = 0.11 h^{-1} there was an approximately 5 fold increase in rate of carbon dioxide fixation per unit absorbance and

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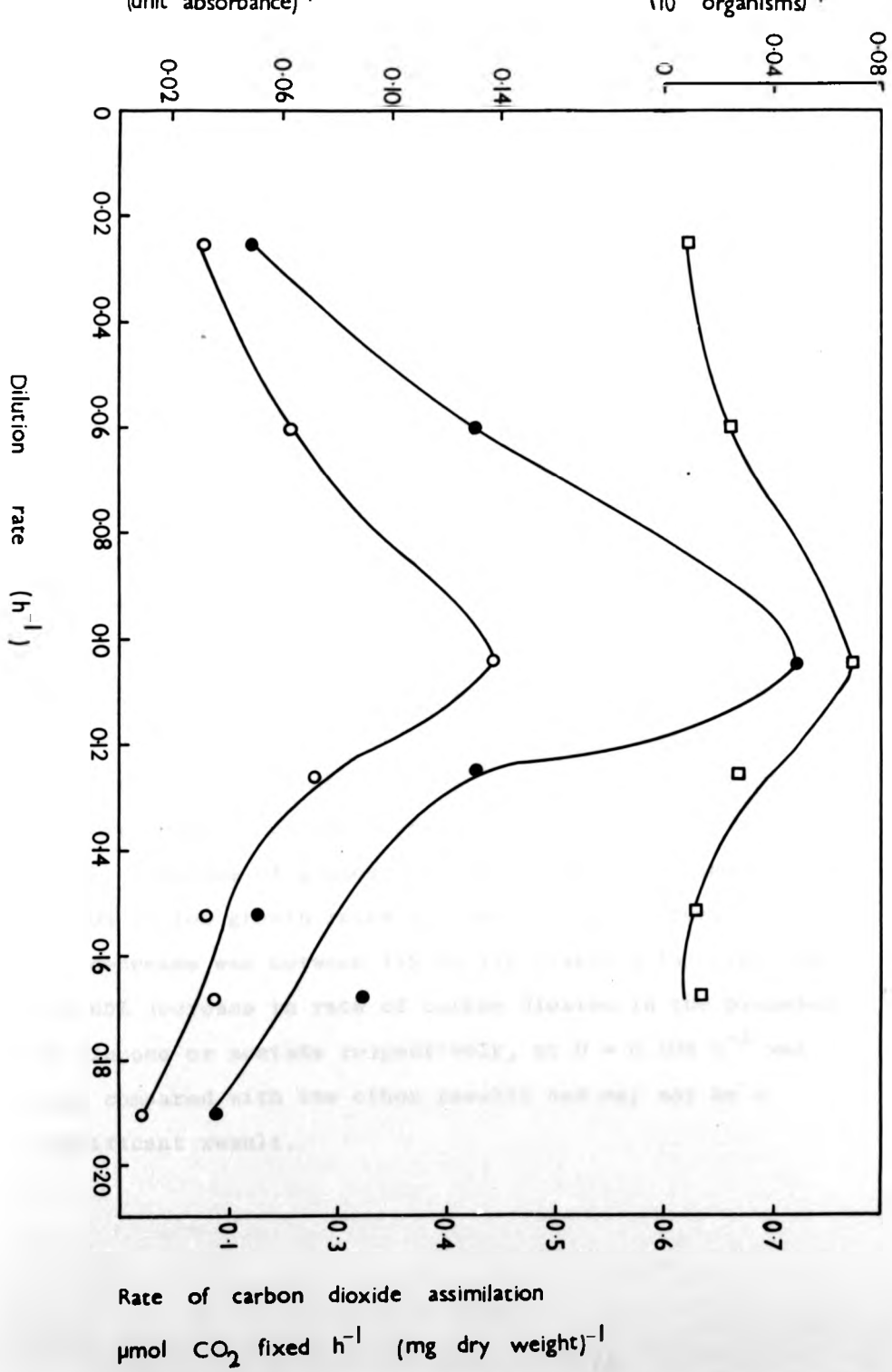
Figure 4.1.

The influence of dilution rate on the rate of carbon dioxide photoassimilation by intact organisms grown under light-limited conditions. The rate is expressed either as μmol CO_2 fixed h^{-1}(unit absorbance)$^{-1}$(O), or as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$(●), or as μmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$ (□).


Rate of carbon dioxide assimilation
µmol CO_2 fixed h^{-1}
(unit absorbance)$^{-1}$
µmol CO_2 fixed h^{-1}
(10^8 organisms)$^{-1}$
0·02
0·06
0·10
0·14
0
0·04
0·08
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
0·1
0·3
0·4
0·5
0·6
0·7
Rate of carbon dioxide assimilation
µmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$

per unit dry weight whereas on a per cell basis the rate increased 7 fold with a maximum of 0.07 μmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$. Above D = 0.11 h^{-1} the rate of carbon dioxide fixation decreased with increasing growth rate approximately 14 fold on the unit absorbance basis and 7 fold on the unit dry weight basis and on the cell number basis.

In the dark there was no measurable fixation of [^{14}C]-carbon dioxide, at least during the 20 minutes incubation period of the experiment.

The rate of carbon dioxide fixation in the presence of 50 mM glucose or 50 mM acetate was also measured. Figures 4.2 and 4.3 show the effect of glucose and acetate on the rate of carbon dioxide assimilation at dilution rates between 0.025 h^{-1} and 0.17 h^{-1} expressed either as μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$ or as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$. The decrease in rate of carbon dioxide in the presence of glucose or acetate was approximately 2% to 10% at low growth rates whereas at higher growth rates the decrease was between 11% to 17% (Table 4.1). The 50% and 65% decrease in rate of carbon dioxide in the presence of glucose or acetate respectively, at D = 0.104 h^{-1} was high compared with the other results and may not be a significant result.

Figure 4.2.

The influence of dilution rate on the rate of carbon dioxide photoassimilation by intact organisms in the presence of 50 mM glucose: the rate expressed either as μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$(○) or as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$ (●).

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anisms in the
ressed either
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).

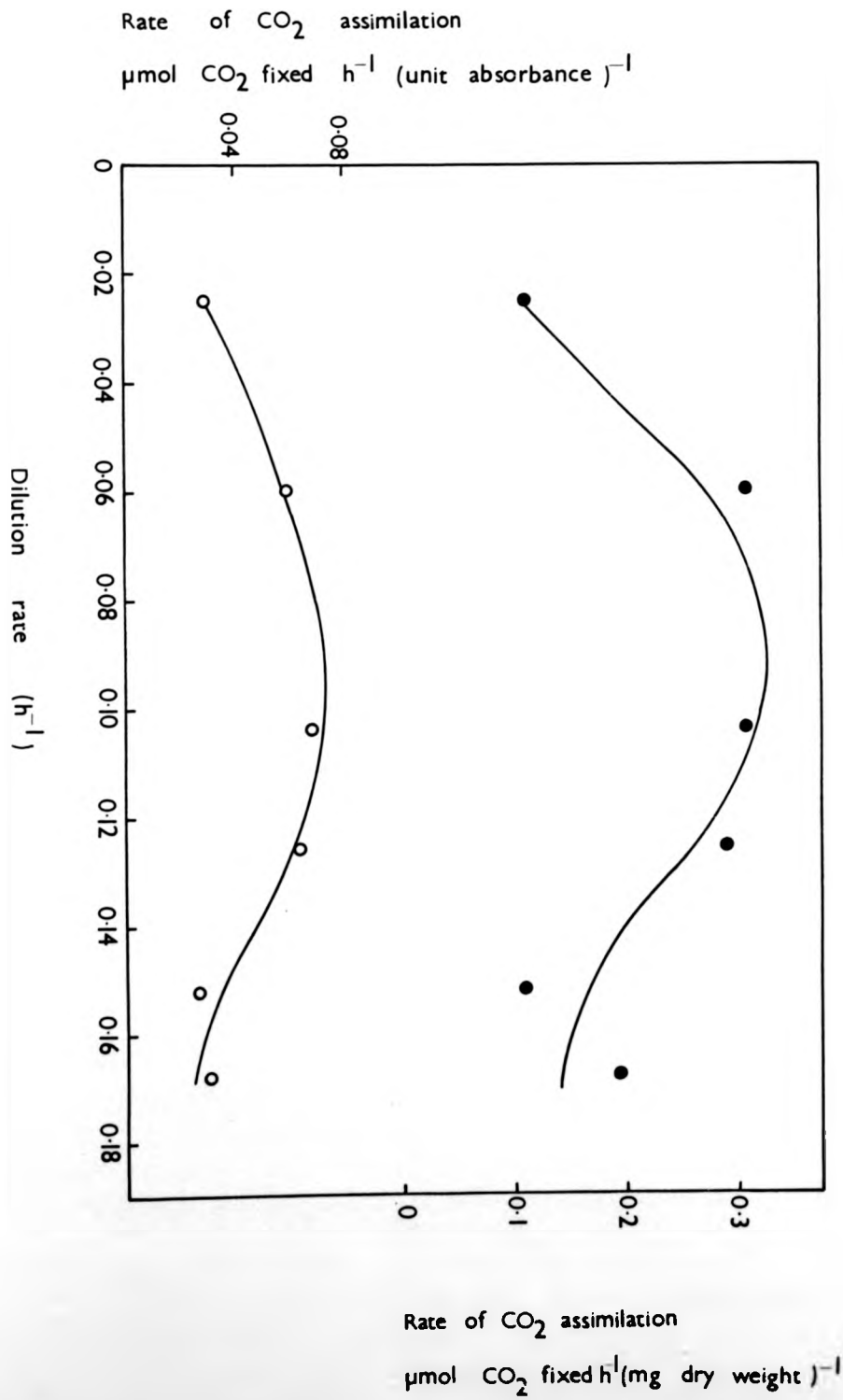


Figure 4.3

The influence of dilution rate on the rate of carbon dioxide photoassimilation by intact organisms in the presence of 50 mM acetate: the rate expressed as μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$ (○) or as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$ (●).

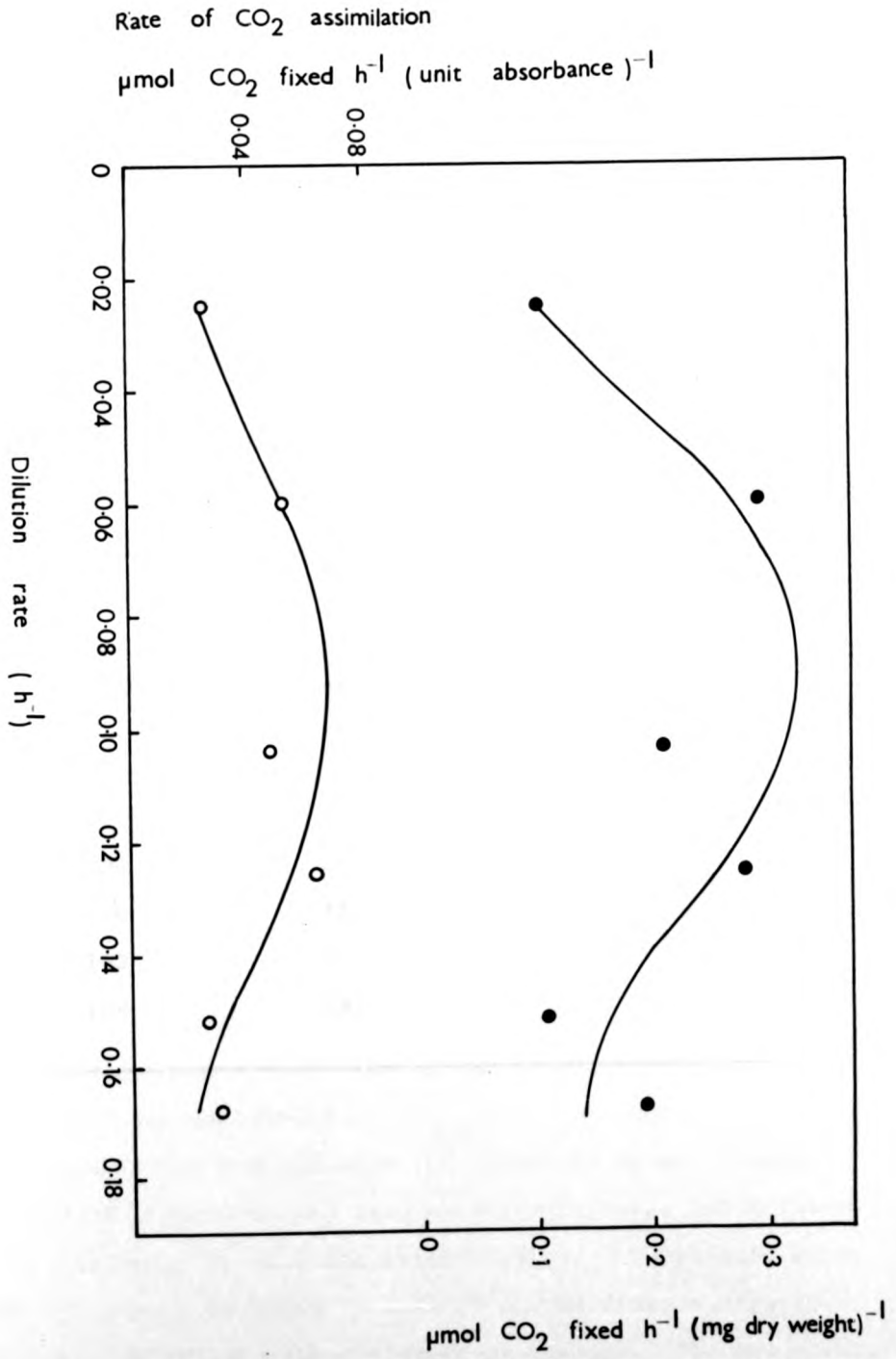

Rate of CO_2 assimilation
µmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$
0·04
0·08
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
Dilution rate (h^{-1})
0
0·1
0·2
0·3
µmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$

Table 4.1. The percentage decrease in rate of carbon dioxide fixation in the presence of 50 mM glucose or 50 mM acetate (compared with the rate in the absence of either compound) in organisms taken from steady state light-limited cultures.

D (h^{-1})	% decrease in rate of CO_2-fixation in presence of glucose or acetate	
	Glucose	Acetate
0.025	2	10
0.060	5	10
0.104	50	65
0.126	11	11
0.152	17	17
0.168	12	12

4.1.2. Carbon dioxide-limited conditions

The pattern of carbon dioxide fixation was measured in intact organisms under optimal experimental conditions over a range of dilution rates from 0.02 h^{-1} to 0.194 h^{-1} and was found to be similar to the pattern of carbon dioxide fixation obtained under light-limited conditions. That is, a peak of assimilation was found at D = 0.11 h^{-1} to 0.12 h^{-1} (Figure 4.4). Between D = 0.02 h^{-1} and D = 0.12 h^{-1} the rate of carbon dioxide fixation per unit absorbance and per unit dry weight increased approximately 14 fold whereas the rate on a per cell basis increased only 9 fold. The rates of carbon assimilation between 0.02 h^{-1} and 0.10 h^{-1} dilution rates under carbon dioxide-limited conditions were approximately 10 fold greater than the rates under light-limited conditions (Table 4.2). Above D = 0.10 h^{-1} the rate of carbon dioxide fixation decreased with increasing growth rate approximately 2.6 fold on the unit absorbance basis, 3 fold on the unit dry weight basis and 1.3 fold, on the cell number basis.

In the dark there was no measurable fixation of[^{14}C]-carbon dioxide, at least during the 20 minutes incubation period of the experiment.

Figures 4.5 and 4.6 show the effect of 50 mM glucose and 50 mM acetate on the rate of carbon dioxide assimilation over the range of dilution rates examined. Table 4.3 gives the percentage decrease in rate of carbon dioxide fixation in the presence of either glucose or acetate. The percentage

Figure 4.4.

The influence of dilution rate on the rate of carbon dioxide photoassimilation by intact organisms grown under carbon dioxide limited conditions. The rate expressed either as μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$ (O), or as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$(□), or as μmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$ (●).

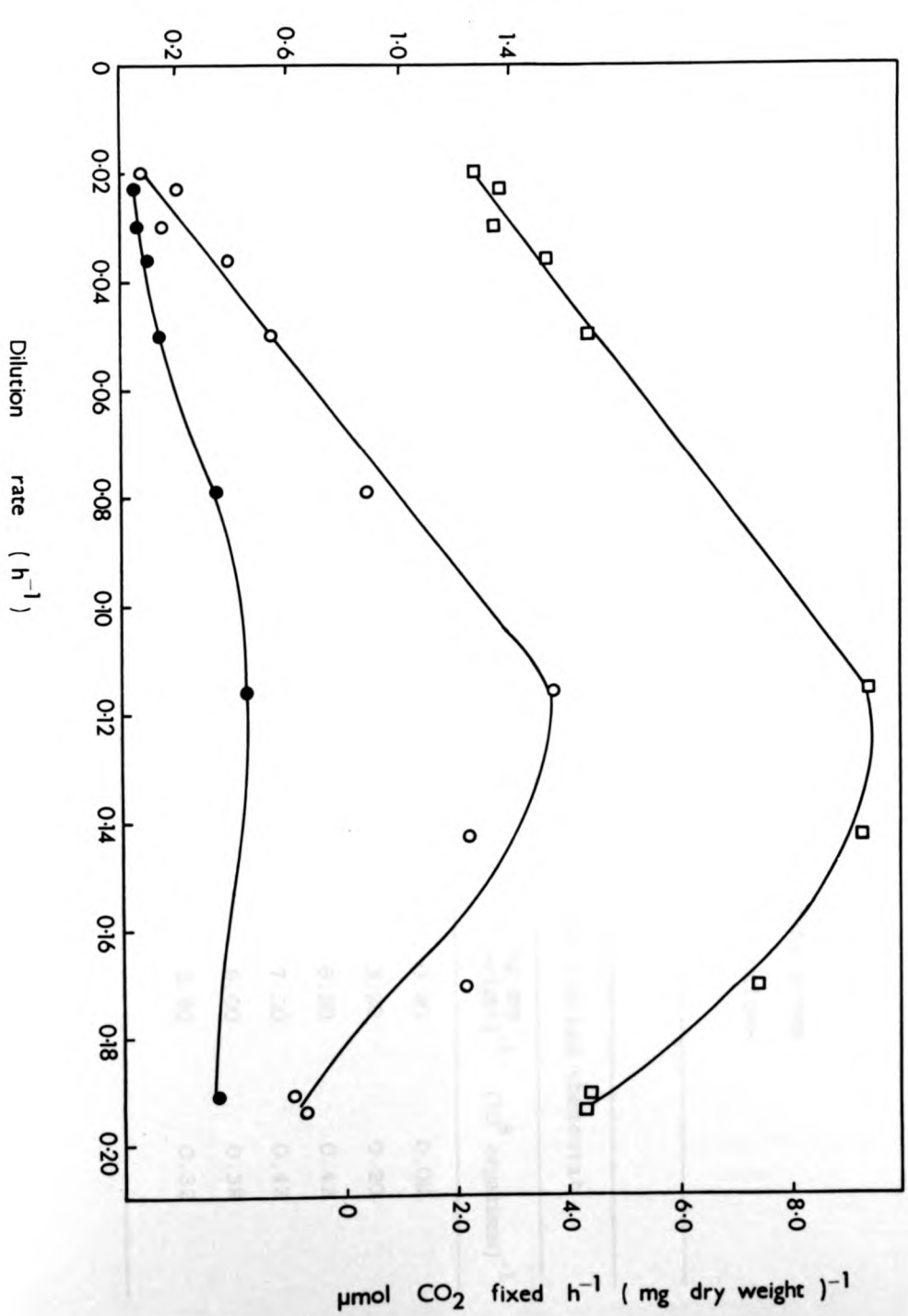

Rate of CO_2 assimilation
µmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$
" " " " (10^8 organisms)$^{-1}$
0·2
0·6
1·0
1·4
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
0
2·0
4·0
6·0
8·0
µmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$

Table 4.2. The rate of carbon dioxide assimilation by intact organisms grown at various dilution rates under light-limited and carbon dioxide-limited conditions.-, not determined.

	Rate of CO_2 fixation, μmol CO_2 fixed h^{-1}					
D	Light-limited chemostat			Carbon dioxide-limited chemostat		
(h^{-1})	(unit absorbance)$^{-1}$	(mg dry weight)$^{-1}$	(10^8 organisms)$^{-1}$	(unit absorbance)$^{-1}$	(mg dry weight)$^{-1}$	(10^8 organisms)$^{-1}$
0.02	0.03	0.12	0.01	0.20	0.80	0.06
0.06	0.06	0.32	0.02	0.70	3.20	0.20
0.10	0.13	0.60	0.07	1.30	6.20	0.42
0.13	0.07	0.30	0.03	1.50	7.50	0.42
0.16	0.03	0.18	0.01	1.16	6.00	0.38
0.19	0.01	0.08	-	0.66	2.80	0.32

Figure 4.5.

The influence of dilution rate on the rate of carbon dioxide photoassimilation by intact organisms grown under carbon dioxide-limited conditions in the presence of glucose: the rate expressed either as μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$(●) or as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$ (○).

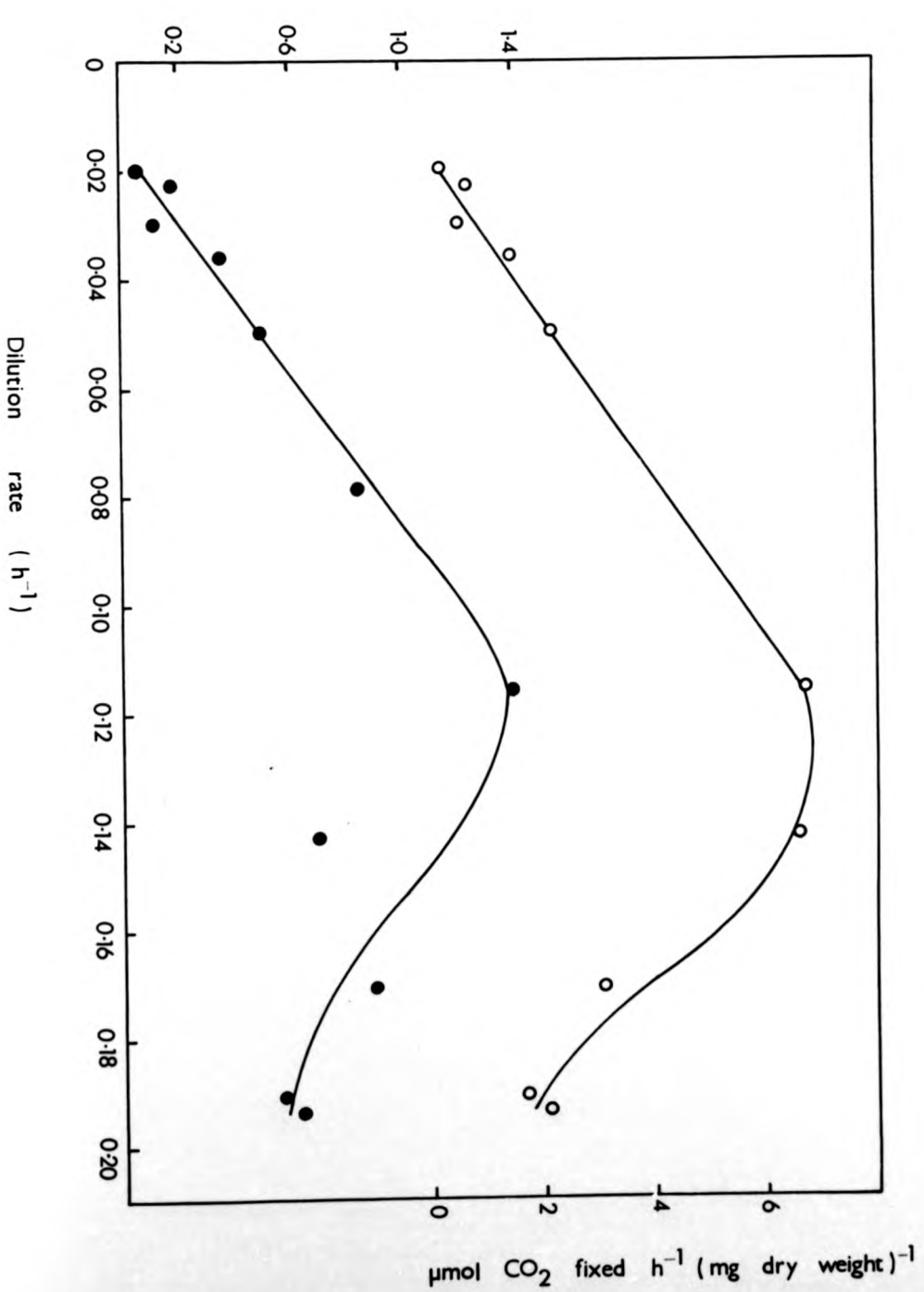

Rate of CO_2 assimilation
μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$
0·2
0·6
1·0
1·4
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
0
2
4
6
μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$

Figure 4.5.

The influence of dilution rate on the rate of carbon dioxide photoassimilation by intact organisms grown under carbon dioxide-limited conditions in the presence of glucose: the rate expressed either as μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$(●) or as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$ (○).

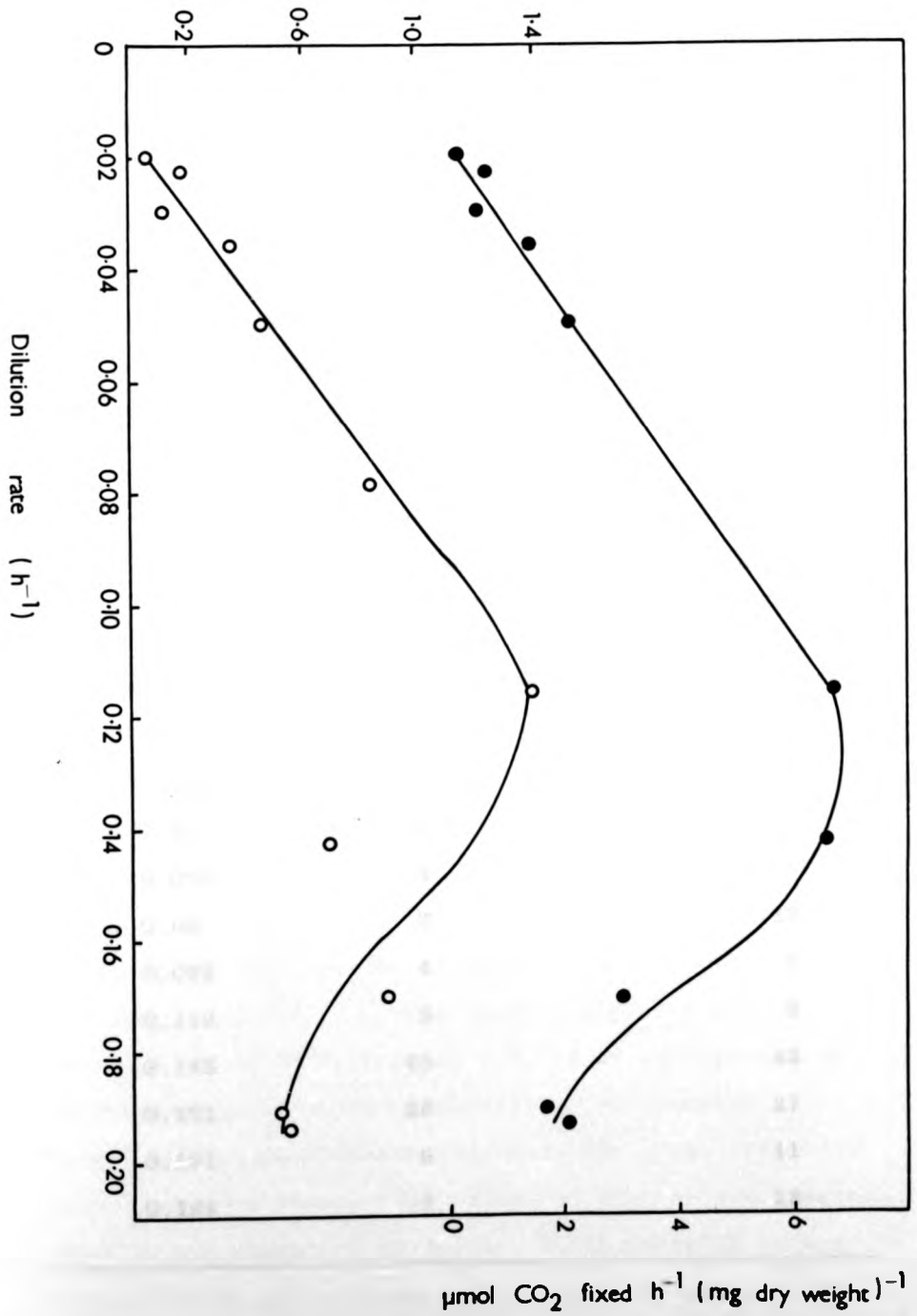

Rate of CO_2 assimilation
µmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$
0·2
0·6
1·0
1·4
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
0
2
4
6
µmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$

Table 4.3. The percentage decrease in rate of carbon dioxide fixation in the presence of 50 mM glucose or 50 mM acetate (compared with the rate in the absence of either compound) in organisms taken from steady state carbon dioxide-limited cultures.

D (h^{-1})	% decrease in rate of CO_2-fixation in presence of glucose or acetate	
	Glucose	Acetate
0.02	20	32
0.023	6	11
0.03	16	20
0.036	4	8
0.05	7	13
0.079	4	4
0.116	9	9
0.143	43	43
0.171	26	27
0.191	6	11
0.194	7	13

decrease in rate of carbon dioxide fixation was slightly higher in the presence of acetate rather than glucose, although the pattern of changing rates expressed in percentage decrease in the presence of either compound as a function of dilution rate was not regular. The 43% decrease in rate of carbon dioxide in the presence of glucose or acetate at D = 0.143 h^{-1} seemed high compared with the other results.

4.2. EFFECT OF TEMPERATURE ON CARBON DIOXIDE ASSIMILATION

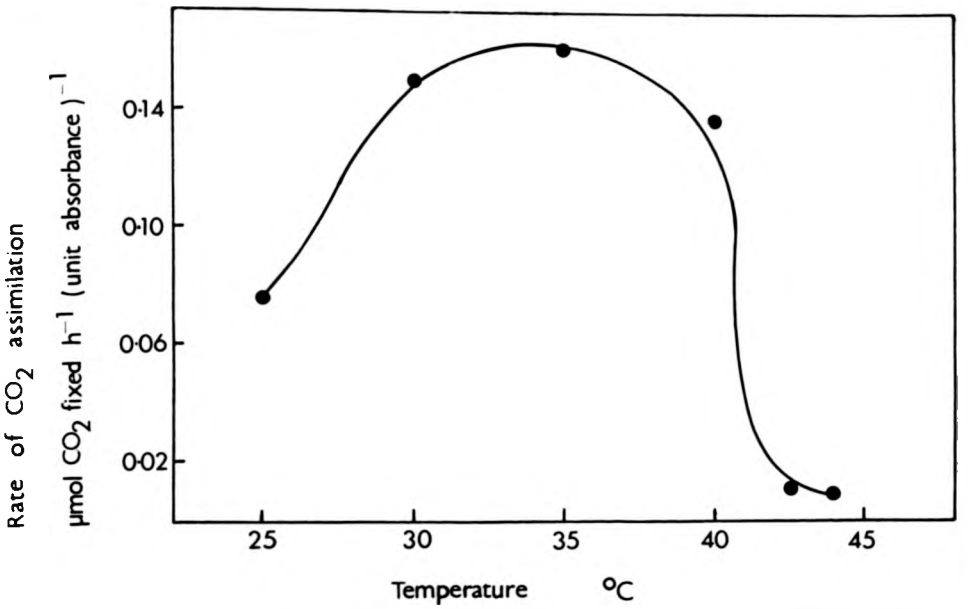
The rate of carbon dioxide assimilation by intact organisms was measured, in steady state cultures taken from light-limited chemostat culture growing at a dilution rate of 0.10 h^{-1} and at various growth temperatures. The temperature was progressively increased from 40°C to 44°C and decreased from 40°C to 25°C in increments of 1°C to 2°C. This was important because the organisms subjected to larger, instantaneous changes in temperature ceased to grow within 2 to 3 hours. The pattern of carbon dioxide fixation expressed either as µmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$ or µmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$ or µmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$ showed a peak of assimilation at 35°C (Figures 4.7 - 4.9) and apart from the maximum point showed a similar to the pattern of change of biomass concentration over the range of growth temperatures examined. The effect of glucose and acetate, added to the experimental flasks, on the rate of carbon dioxide assimilation at different temperatures examined.

Figure 4.7.

The effect of growth temperature on the rate of carbon dioxide fixation at dilution rate of 0.10 h^{-1} expressed as μmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$.

Figure 4.8.

The effect of growth temperature on the rate of carbon dioxide fixation at dilution rate of 0.10 h^{-1} expressed as μmol CO_2 fixed h^{-1} (mg dry weight)$^{-1}$.


Rate of CO_2 assimilation
µmol CO_2 fixed h^{-1} (unit absorbance)$^{-1}$
0·14
0·10
0·06
0·02
25
30
35
40
45
Temperature °C

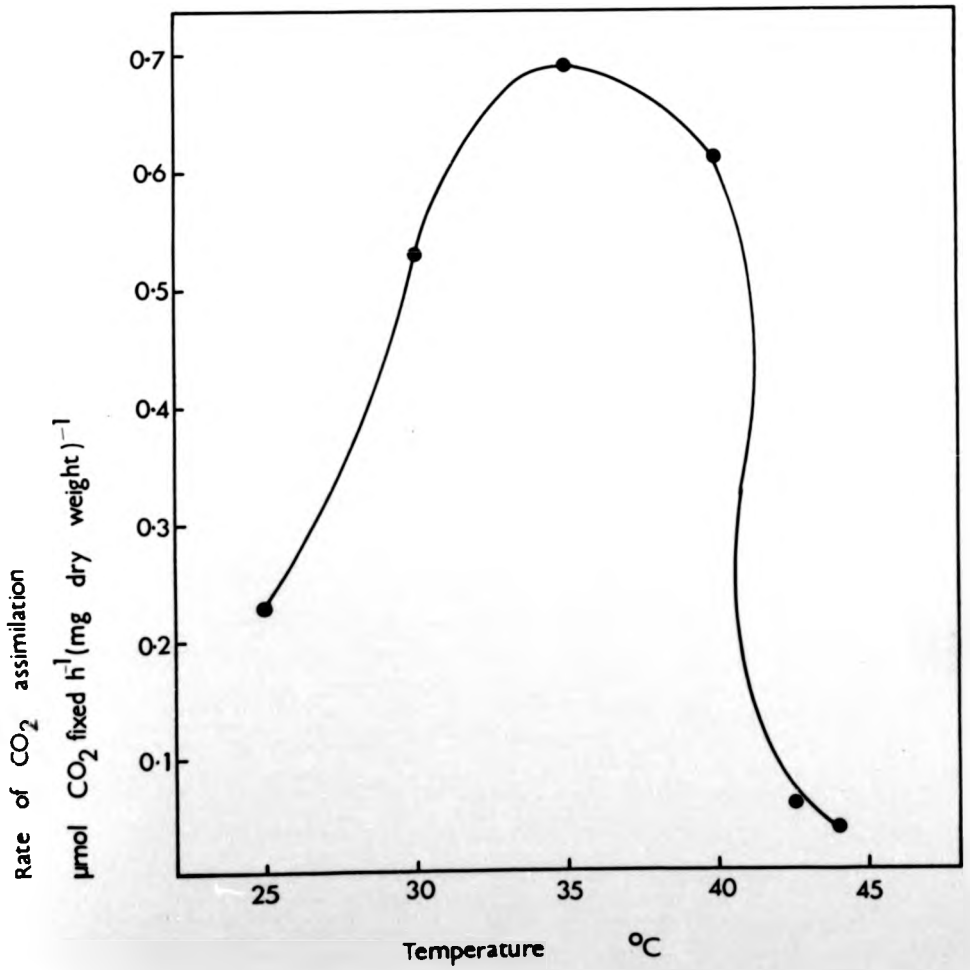

Rate of CO_2 assimilation
µmol CO_2 fixed h^{-1}(mg dry weight)$^{-1}$
0·7
0·6
0·5
0·4
0·3
0·2
0·1
25
30
35
40
45
Temperature °C

Figure 4.9.

The effect of growth temperature on the rate of carbon dioxide fixation at dilution rate of 0.10 h^{-1} expressed as µmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$.

rate of
of
(10^8

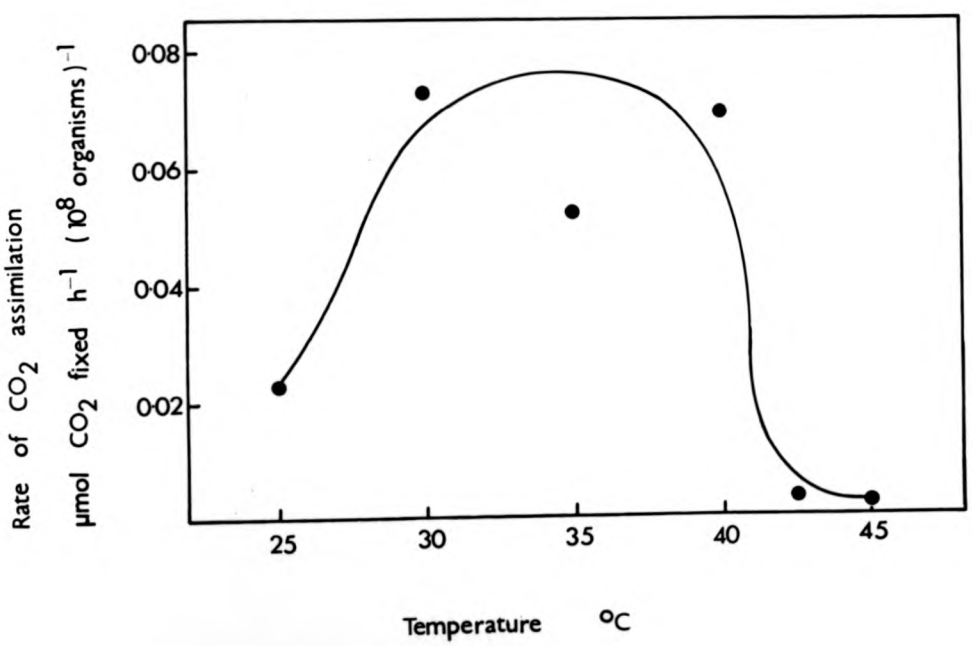


Table 4.4 gives the percentage decrease in rate of carbon dioxide assimilation in the presence of glucose or acetate at temperatures between 25°C and 44°C and at dilution rate of D = 0.10 h^{-1}. The presence of acetate caused a slightly higher decrease in rate of carbon dioxide fixation than the presence of glucose, although the percentage decrease in rate of carbon dioxide assimilation in the presence of either compound was similar in organisms taken from steady state light-limited cultures grown at 40°C and at various dilution rates (Table 4.1).

Table 4.4. The percentage decrease in rate of carbon dioxide fixation in the presence of 50 mM glucose or 50 mM acetate (compared with the rate in the absence of either compound) in organisms taken from steady state light-limited chemostat cultures grown at D = 0.10 h^{-1} and at various growth temperatures.

Temperature °C	% decrease in rate of CO_2-fixation in presence of glucose or acetate	
	Glucose	Acetate
25	17	21
30	24	35
35	30	53
40	50	65
42.5	0	18
44	18	23

DISCUSSION

The observed rates of carbon dioxide assimilation in organisms grown under carbon dioxide-limited conditions were considerably higher than the rates of carbon dioxide assimilation in organisms grown under light-limited conditions. The 10 fold observed difference in rates of carbon dioxide assimilation between the two limitations was in keeping with the higher enzyme specific activities in organisms grown under carbon dioxide-limited conditions compared with those under light-limited conditions at least at the lower dilution rates (see part 5). However, the rates of carbon dioxide assimilation observed under both growth conditions were not great enough to account for the required growth rates of the organism in steady state culture. Table 4.5 shows the required rate of carbon assimilation and the observed rate of carbon assimilation by intact organisms under light- and carbon dioxide-limited conditions.

Under light-limited conditions the observed rate of carbon dioxide fixation was only 1-2% of the required rate of CO_2 fixation for growth at high dilution rates. At low and medium dilution rates the observed rate of CO_2 fixation was only 10% of the required rate of CO_2 fixation for growth (Table 4.5). Under carbon dioxide-limited conditions the observed rate of carbon dioxide assimilation was very similar to the required rate of CO_2 fixation for growth at dilution rates between 0.02 h^{-1} and

Table 4.5. A comparison of observed values of carbon dioxide fixation with the required values to sustain a given growth rate.

D (h^{-1})	t_d (h)	Required rate of C assimilation (μmol C fixed h^{-1} (mg dry weight)$^{-1}$)	Intact organisms: Measured rate of C assimilation (μ mol C fixed h^{-1} (mg dry weight)$^{-1}$)	Intact organisms: (Low values of rate of C assimilation due to possible loss of [^{14}C]-solution activity during preparation)
			CO_2-limited	Light-limited
0.02	39.65	1.20	0.35	0.120
0.04	17.33	2.40	1.70	0.200
0.06	11.55	3.61	3.20	0.325
0.08	8.66	4.81	4.70	0.485
0.10	6.93	6.02	6.10	0.610
0.12	5.78	7.22	7.50	0.475
0.14	4.95	8.42	7.20	0.245
0.16	4.33	9.63	6.00	0.180
0.18	3.85	10.83	4.00	0.115
0.20	3.47	12.03	1.50	0.050

0.12 h^{-1}. Above D = 0.12 h^{-1} the observed rate of carbon dioxide assimilation was 40-80% of the required rate of CO_2 assimiltion between dilution rates 0.14 h^{-1} and 0.18 h^{-1} whilst at D = 0.20 h^{-1} the observed rate of CO_2 fixation was only 13% of the required rate of CO_2 fixation for the growth of the organisms (Table 4.5). These observations most probably reflect problems in measuring the rate of CO_2 fixation in intact organisms. Firstly the rate measurements were made under optimal experimental conditions, namely light in excess and complete carbon dioxide (bicarbonate) saturation, but the experimental temperature used was 25°C compared with the growth temperature at 40°C and this lower temperature would have had an effect on the rate of carbon dioxide assimilation. As it has been already noted chemical reaction rates are related to temperature by the Arrhenius equation (see part 3). From equation (3.9) if K is the rate of carbon dioxide assimilation we have

$$\log K = \log A - E/2.30\ RT \qquad (3.10)$$

Hence a plot of log K against 1/T should be a straight line. Thus the lower experimental temperature used may have resulted in lower rates of carbon dioxide assimilation. Secondly the determination of the assimilated [^{14}C]-radioactivity may have been inaccurate. The determination did not take into account the efficiency of the counting as a result of quenching of counts by the presence of the cell material, particularly pigments.

Thirdly, the observed rates of carbon dioxide assimilation may not be those of the organisms growing in the experimental cell suspensions. This is because growing cultures were taken out of the chemostat, harvested by centrifugation, washed with buffer, resuspended in fresh medium and the rate of carbon dioxide assimilation was then measured approximately 2 hours after the cultures were removed from the chemostat. Fourthly effects of dilution of the labelled carbon dioxide caused by the intracellular carbon dioxide should take into consideration because the amount of the intracellularly produced carbon dioxide may have affect and diluted the known amount of the labelled carbon dioxide added. Thus the calculated rates of carbon dioxide assimilation may be lower than the actual rate of CO_2 fixation. Nevertheless a comparison of the two limitations shows that under carbon dioxide-limited conditions there was greater potentiality for carbon dioxide fixation as it was expected because in environments where growth was limited by the availability of carbon dioxide (bicarbonate) the rate of carbon dioxide assimilation depended on the concentration of this substrate; hence in environments with low carbon dioxide concentration the rate of fixation should increase.

The study of carbon dioxide assimilation in organisms grown under both limitations resulted in patterns of photo-assimilation which showed a peak at dilution rates between 0.10 h^{-1} and 0.12 h^{-1}. The increase of carbon dioxide assimilation between 0.02 h^{-1} and 0.10 h^{-1} dilution rates was approximately 50% greater in organisms grown under carbon dioxide-limited

conditions than that in organisms grown under light-limited conditions whilst the decrease of carbon dioxide assimilation in organisms grown under carbon dioxide-limited conditions between 0.10 h^{-1} and 0.19 h^{-1} dilution rates was only 20% of the decrease in carbon dioxide assimilation under light-limited conditions. The decrease in the rate of carbon dioxide assimilation observed under these growth conditions is not fully understood and it cannot be explained at this stage. It was expected that the rate of carbon dioxide assimilation would have increased with increasing growth rate, since at higher growth rates with a greater requirement to generate cellular carbon than in slower growing organisms the capability to assimilate carbon dioxide should have been greater.

The growth temperature had a clear effect on the rates of carbon dioxide assimilation which was depressed at temperatures above and below the optimum growth temperature. This effect of temperature on the rate of carbon dioxide assimilation can be explained in terms of the temperature coefficients of reaction rates which are a function of the temperature since from the Arrhenius equation (3.9), which in turn depend on the activation energies of the reactions, (Pirt, 1975). The activation energy is a valuable constant and changes in the activation energy indicate that differences occur in the rates of reactions.

An examination of whether or not the RuBPCase activities under both limitations influenced the rate of carbon dioxide fixation by intact organisms (see part 5) suggested that some

other factors limited the rate of carbon assimilation than the RuBPCase levels and reflected the problems of correlating the enzyme's activity with the rate of carbon dioxide assimilation and the behaviour of the intact organisms. Slater and Morris (1973a) reported no correlation between the carbon dioxide photoassimilation and the changes in levels of RuBPCase activity in organisms of Rhodospirillum rubrum grown in chemostat cultures. They recognized three conditions during autotrophic growth where there were relatively high rates of carbon dioxide fixation and high activities of RuBPCase, during malate-limited growth in a chemostat relatively high rates of carbon dioxide fixation were accompanied by relatively low rates of RuBPCase and during rapid growth in batch culture and in continuous-flow turbidostat cultures the lower level of RuBPCase (compared with autotrophic cultures) was paralleled by a lower rate of carbon dioxide assimilation. These observations are not in agreement with the hypothesis that the first enzyme of the reductive pentose phosphate cycle to regulate the carbon dioxide fixation.

The activity of phosphoenolpyruvate (PEP) carboxylase in three blue-green algae, Anabaena flos-aquae, Anacystis nidulans and Oscillatoria sp was examined by Colman, Cheng and Ingle (1976) when the cell extracts were prepared by lysis, in phosphate buffer, of spheroplasts of these cells. The results obtained

showed 1.5 to 5 fold higher PEPCase activity than that of RuBPCase suggesting that the three blue-green algae have the enzymatic capability of carbon dioxide fixation by a C_4 pathway. This study has not examined the influence of other carboxylases and it is unknown whether or not any of these enzymes markedly affect carbon dioxide fixation in cultures of Anacystis nidulans grown under light- or carbon dioxide-limited conditions.

PART 5. ENZYMES OF THE REDUCTIVE PENTOSE PHOSPHATE CYCLE AND INTERMEDIARY METABOLISM IN ANACYSTIS NIDULANS GROWN IN CHEMOSTAT CULTURE.

The activity of selected enzymes was examined in steady state cultures grown under carbon dioxide-limited and light-limited conditions over a range of dilution rates from 0.02 h^{-1} to 0.19 h^{-1}. A number of different patterns of changing enzyme specific activity with dilution rate were observed in the cultures grown under these two different conditions.

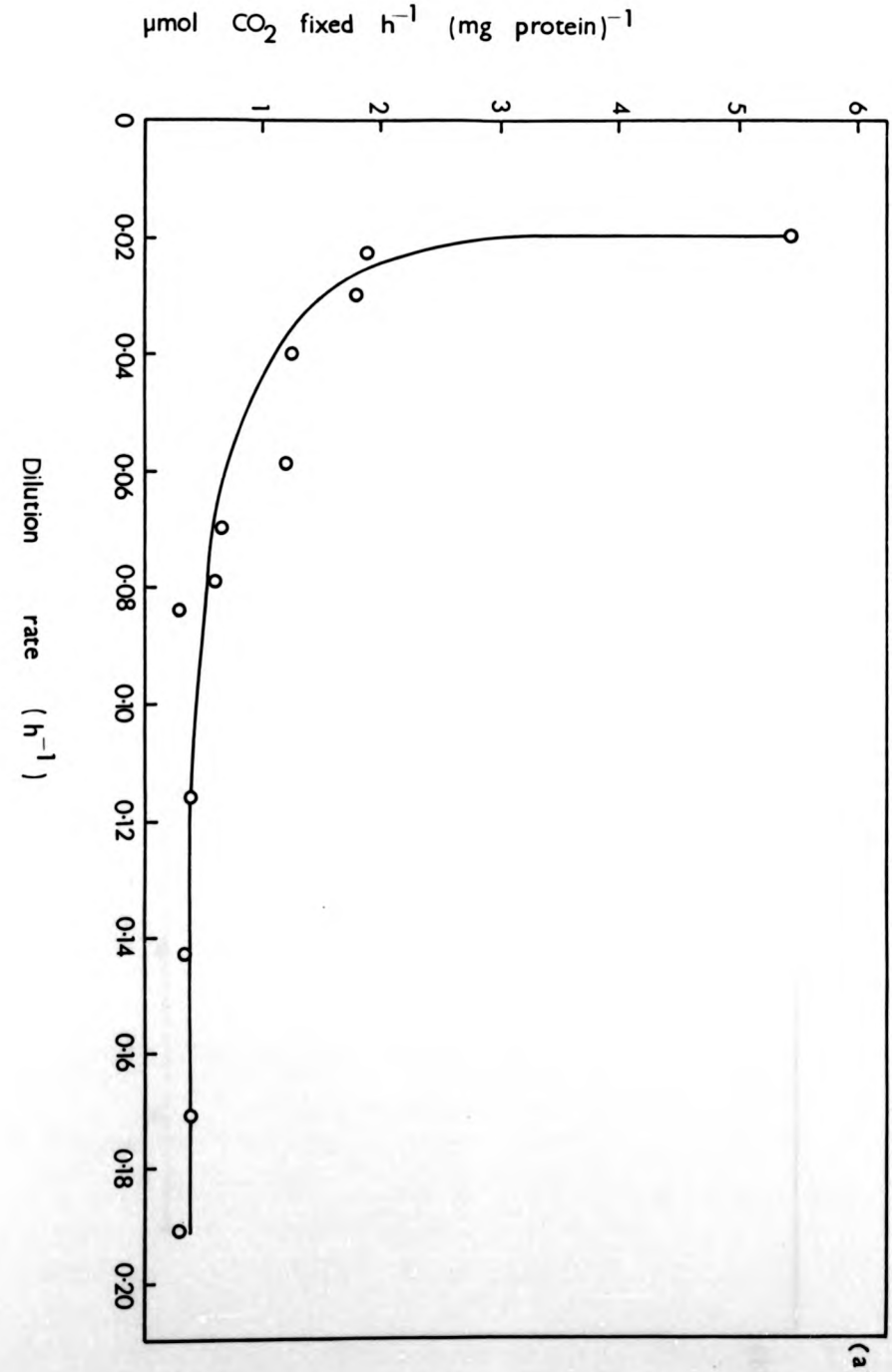
5.1. REDUCTIVE PENTOSE PHOSPHATE CYCLE ENZYMES.

The specific activity of ribulose 1,5-bisphosphate carboxylase (3-phospho D-glycerate carboxylyase (dimerizing), E.C. 4.1.1.39), both in terms of activity per unit protein or per unit cell number, increased 15 fold with decreasing dilution rate under carbon dioxide-limited conditions (Figures 5.1a and 5.1b). At dilution rates above D = 0.10 h^{-1}, the RuBPCase specific activity was constant, showing the lowest activity of 0.40 µmol CO_2 fixed h^{-1} (mg protein)$^{-1}$, only increasing once the dilution rate was lowered beyond D = 0.10 h^{-1} (Figure 5.1a and 5.1b).

Under light-limited conditions over the same dilution rate range, RuBPCase specific activity remained constant (Figure 5.2) with an activity, 0.45 µmol CO_2 fixed h^{-1} (mg protein)$^{-1}$, which was similar to the lowest specific activity observed in high dilution rate carbon dioxide-limited cultures (0.40 µmol CO_2 fixed h^{-1} (mg protein)$^{-1}$).

Figure 5.1.

The influence of dilution rate on the steady state activities of ribulose 1,5-bisphosphate carboxylase in organisms grown in carbon dioxide-limited cultures. RuBPCase specific activity (a) in terms of μmol CO_2 fixed h^{-1} (mg protein)$^{-1}$ (○) and (b) in terms of μmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$ (●).


µmol CO_2 fixed h^{-1} (mg protein)$^{-1}$
0
1
2
3
4
5
6
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
(a)

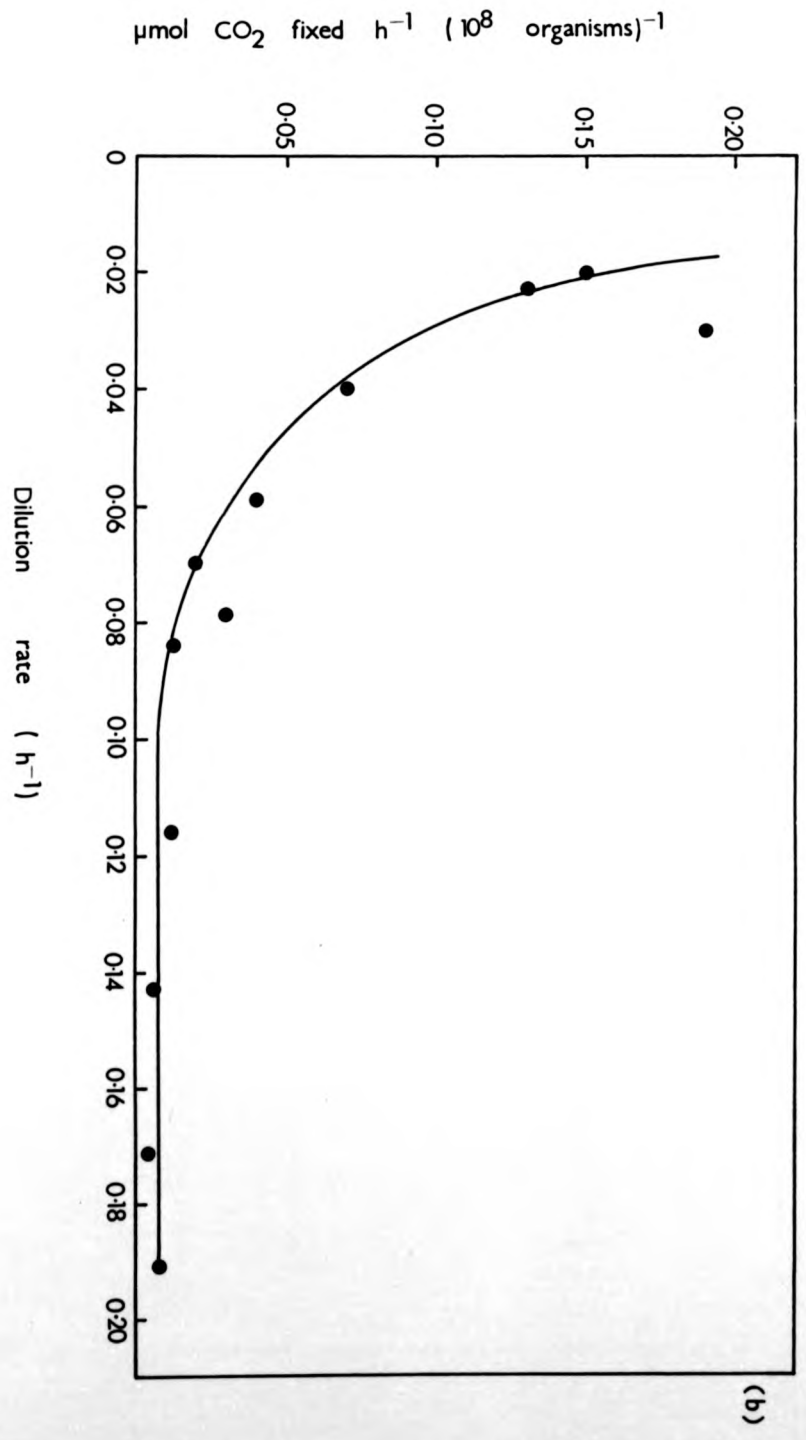

μmol CO2 fixed h−1 (10^8 organisms)−1
0·05
0·10
0·15
0·20
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h−1)
(b)

Figure 5.2.

The influence of dilution rate on the steady state activity of ribulose 1,5-bisphosphate carboxylase in terms of μmol CO_2 fixed h^{-1} (mg protein)$^{-1}$ (○) and μmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$ (●), in organisms grown in light-limited chemostat cultures.

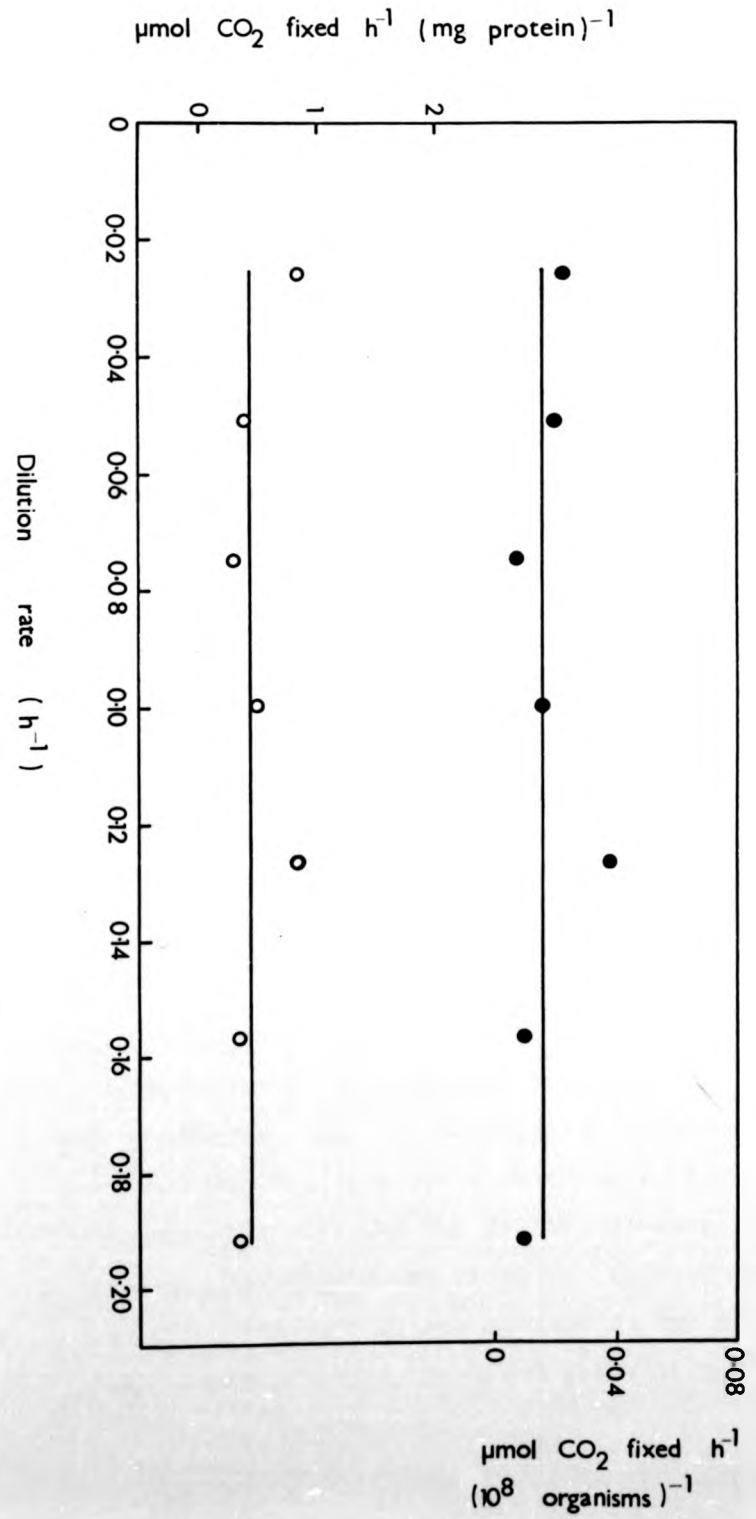

µmol CO2 fixed h-1 (mg protein)-1
0
1
2
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h-1)
0
0·04
0·08
µmol CO2 fixed h-1
(10^8 organisms)-1

Table 5.1. RuBPCase specific activity in cultures grown at 25°C (nd, not determined).

D (h^{-1})	CO_2-limited cultures (μmol CO_2 fixed h^{-1} (mg protein)$^{-1}$)	Light-limited cultures (μmol CO_2 fixed h^{-1} (mg protein)$^{-1}$)
0.02	8.98	1.72
0.05	5.71	nd
0.10	-	3.52
0.17	-	5.75

The specific activity of RuBPCase was also examined at different dilution rates under carbon dioxide- and light-limited conditions at a growth temperature of 25°C (Table 5.1). In carbon dioxide-limited cultures grown at 25°C, two steady state cultures were examined and these showed a 2 to 5 fold increase in activity compared with organisms grown at the same dilution rates at 40°C. It was not possible to obtain steady state cultures above D = 0.05 h^{-1} at 25°C under carbon dioxide-limited conditions since these were above the critical dilution rate under these conditions and so culture washout occurred.

Under light-limited conditions temperature also had an effect on the specific activity of RuBPCase in terms of activity per unit protein. Firstly the lowest specific activity, 1.72 µmol CO_2 h^{-1} (mg protein)$^{-1}$, observed in organisms grown at D = 0.02 h^{-1} was 4 fold greater than the constant specific activity found in organisms grown at a temperature of 40°C. Secondly, at 25°C the RuBPCase specific activity was not constant with changing dilution rate as was the activity in organisms grown at 40°C (Table 5.1 and Figure 5.2). At the highest dilution rate, D = 0.17 h^{-1} for which steady state cultures were obtained at 25°C, the specific activity was 13 fold greater than that in Anacystis nidulans grown at 40°C. Furthermore the activity at this dilution rate was similar to the maximum specific activity recorded in organisms grown at low dilution

rates at 40°C under carbon dioxide-limited conditions (Figure 5.1a and 5.1b).

The second reductive pentose phosphate cycle enzyme examined, phosphoribulokinase, (ATP: D-ribulose 5-phosphate 1-phosphotransferase, E.C. 2.7.1.19), showed a different pattern with a slight increase in activity, approximately 2 fold, in organisms grown at higher dilution rates in carbon dioxide-limited cultures (Figure 5.3). The specific activity of phosphoribulokinase in light-limited cultures was not measured.

Attempts to examine the specific activity of fructose 1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, E.C. 3.1.3.11) over the same range of dilution rates were made only in light-limited cultures. The specific activity of FBPase was very low and the only measurable FBPase activity observed at D = 0.10 h^{-1} was 0.262 µg $PO_4^{=}$ h^{-1} (mg protein)$^{-1}$.

5.2. OXIDATIVE PENTOSE PHOSPHATE PATHWAY ENZYMES

The two initial enzymes of this pathway, 6-phosphogluconate dehydrogenase (6-phospho D-gluconate: NADP oxidoreductase (decarboxylating) E.C. 1.1.1.44) and glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49), were examined (Figure 5.4. - 5.7).

Although 6PGDase increased approximately 2 fold in both carbon dioxide-limited and light-limited cultures, in terms of activity per unit protein, the minimum 6PGDase activity

Figure 5.3.

The influence of dilution rate on the steady state activities of phosphoribulokinase in organisms grown in carbon dioxide-limited culture in terms of μmol CO_2 fixed h^{-1} (mg protein)$^{-1}$ (○) and in terms of μmol CO_2 fixed h^{-1} (10^8 organisms)$^{-1}$ (●).

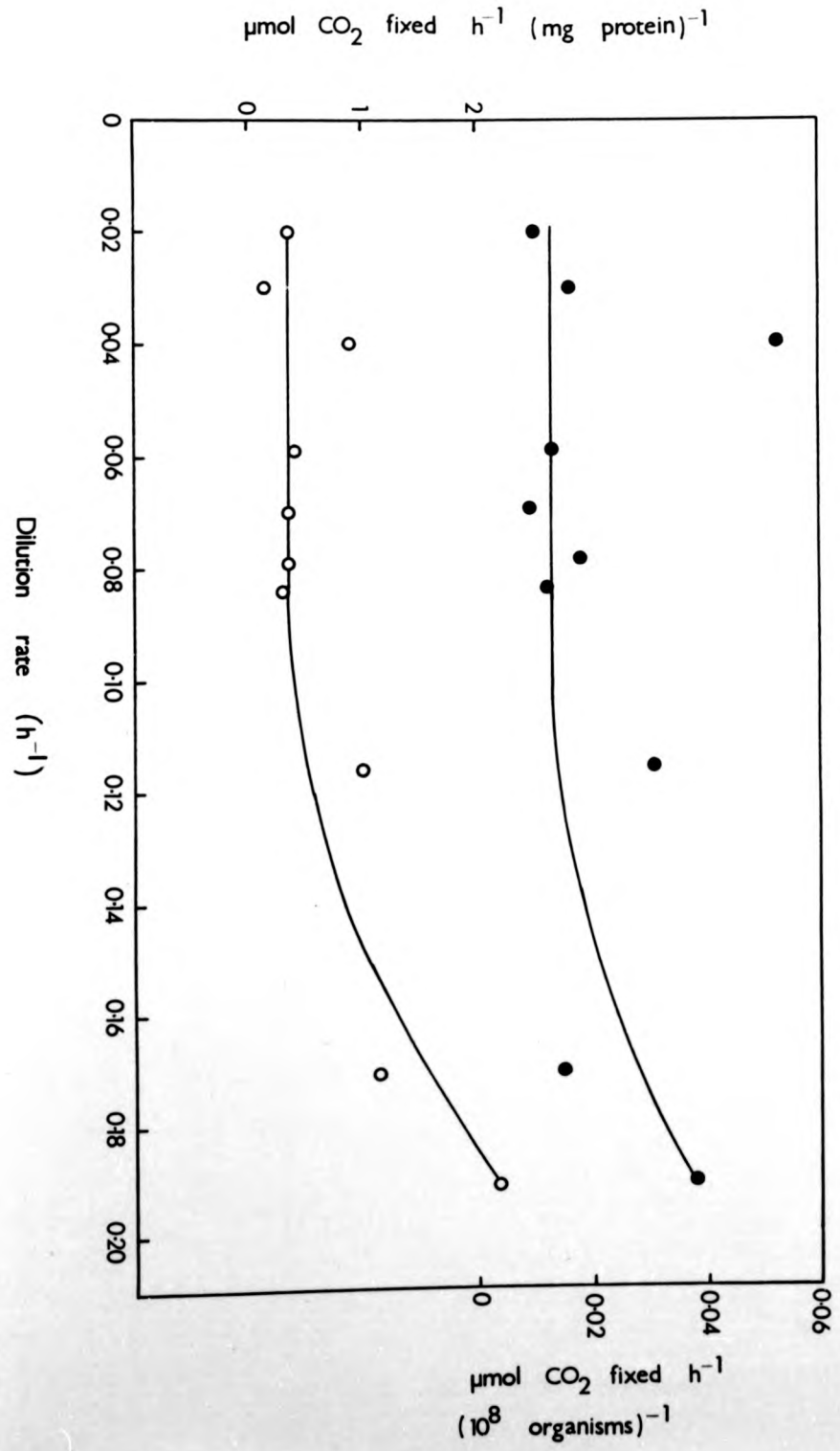

µmol CO2 fixed h-1 (mg protein)-1
0
1
2
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h-1)
0
0·02
0·04
0·06
µmol CO2 fixed h-1
(10^8 organisms)-1

Figure 5.4.

The influence of dilution rate on the steady state activities of 6-phosphogluconate dehydrogenase in terms of nmol substrate converted min^{-1} (mg protein)$^{-1}$ (○) and in terms of nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ (●), in organisms grown in carbon dioxide-limited chemostat culture.

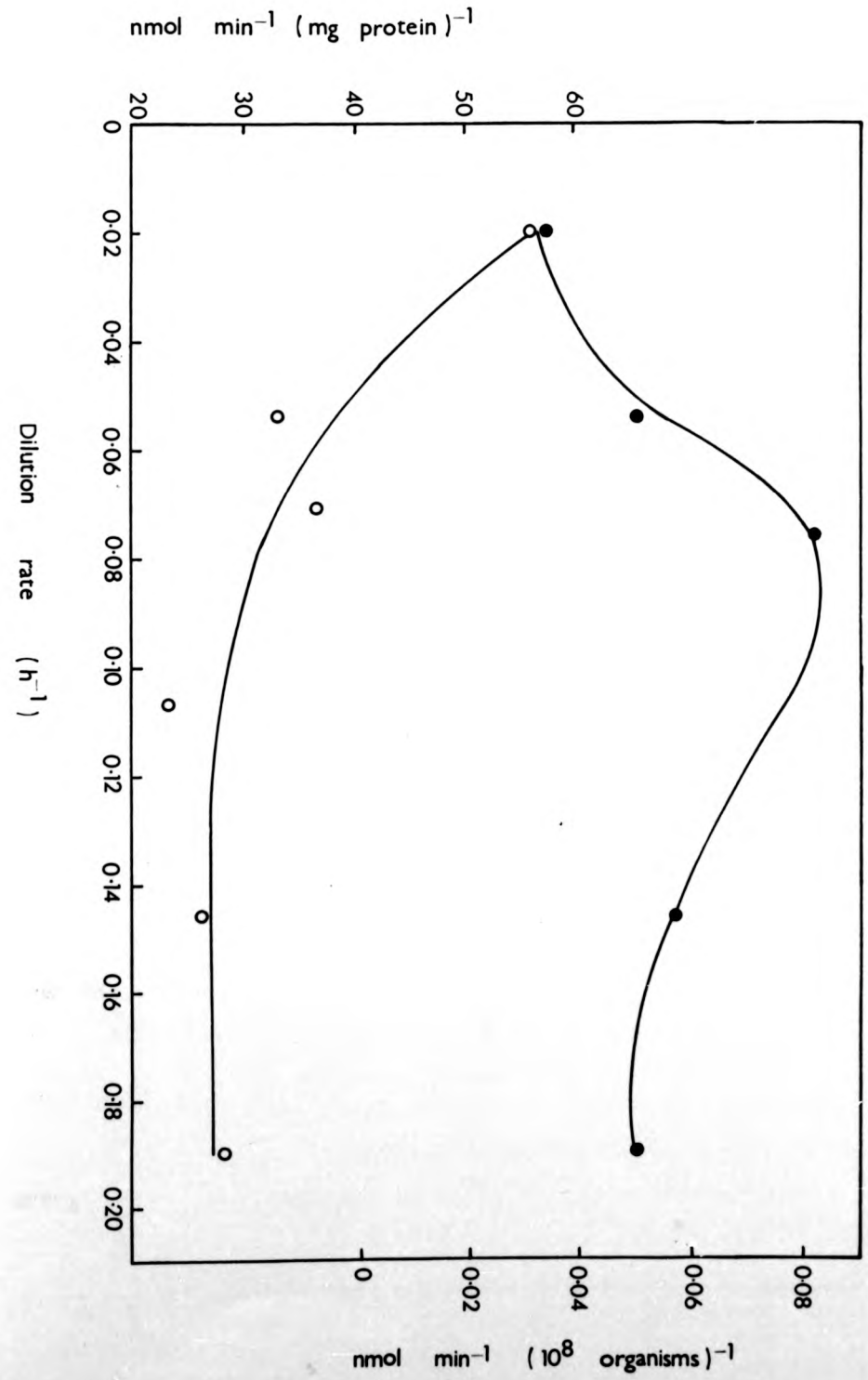
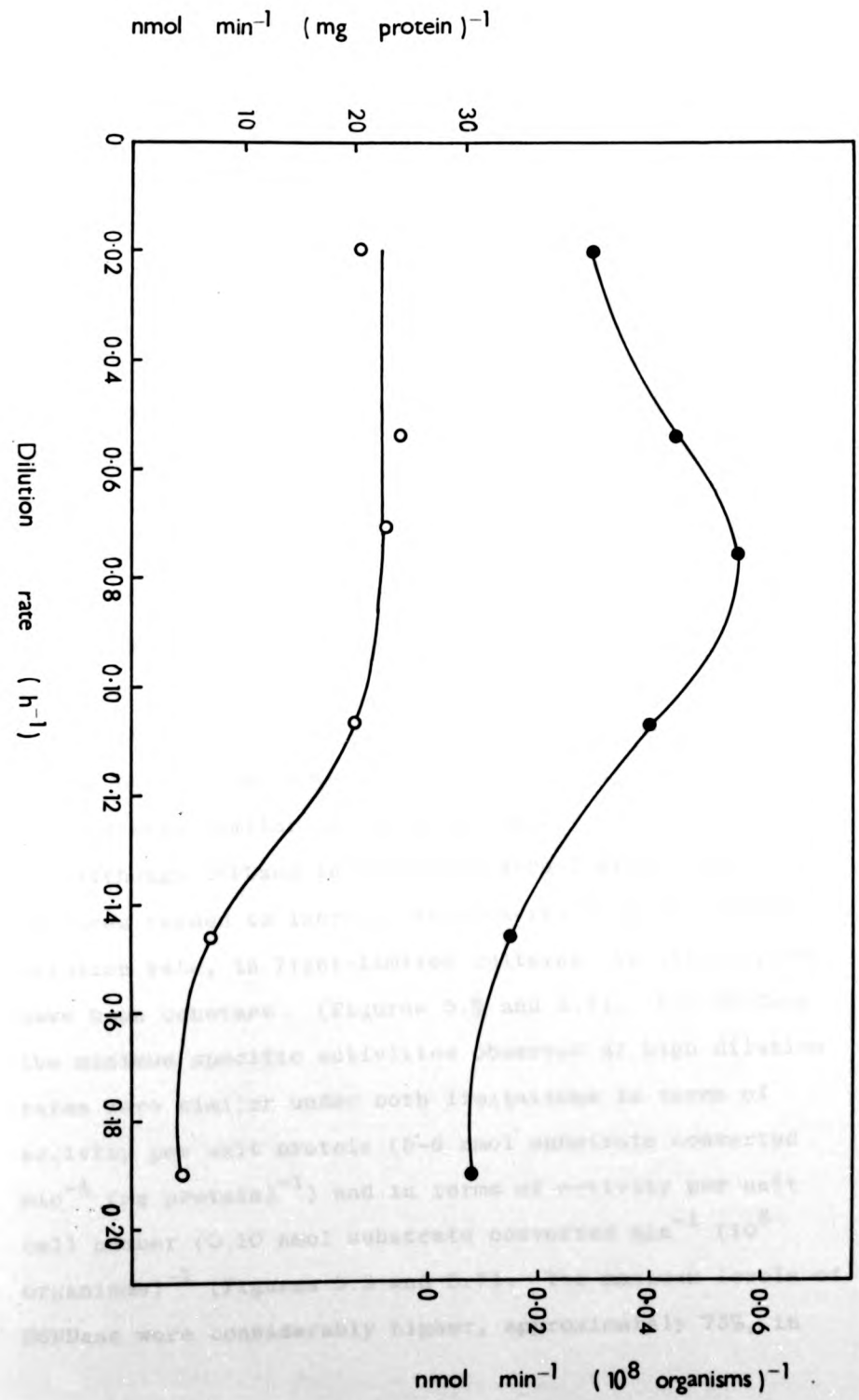

nmol min^{-1} (mg protein)$^{-1}$
20
30
40
50
60
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
0
0·02
0·04
0·06
0·08
nmol min^{-1} (10^8 organisms)$^{-1}$

Figure 5.5.

The influence of dilution rate on the steady state activities of glucose 6-phosphate dehydrogenase in organisms grown in carbon dioxide-limited chemostat culture. G6PDase specific activity expressed in terms of nmol substrate converted min^{-1} (mg protein)$^{-1}$ (○) and in terms of nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ (●).


nmol min⁻¹ (mg protein)⁻¹
0
10
20
30
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)
0
0·02
0·04
0·06
nmol min⁻¹ (10⁸ organisms)⁻¹

in carbon dioxide-limited cultures (25 nmol substrate converted min^{-1} (mg protein)$^{-1}$) was 2.5 times greater than the minimum level in light-limited cultures (10 nmol substrate converted min^{-1} (mg protein)$^{-1}$) (Figures 5.4 and 5.6). The pattern of change in enzyme activities in relation to unit number of organisms under carbon dioxide-limited conditions for both enzymes of the oxidative pentose phosphate pathway was unexpectedly different to the pattern of change in enzyme activities in terms per unit protein showing a peak of activity, 0.083 nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ for 6PGDase (Figure 5.4) and 0.056 nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ for G6PDase (Figure 5.5), between 0.075 h^{-1} and 0.095 h^{-1} dilution rates.

Under light-limited conditions the pattern of change in enzyme activities in terms of unit protein and unit cell numbers were similar for both enzymes.

Although G6PDase in carbon dioxide-limited chemostat cultures tended to increase in activity with decreasing dilution rate, in light-limited cultures its activity may have been constant (Figures 5.5 and 5.7). For G6PDase the minimum specific activities observed at high dilution rates were similar under both limitations in terms of activity per unit protein (5-6 nmol substrate converted min^{-1} (mg protein)$^{-1}$) and in terms of activity per unit cell number (0.10 nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ (Figures 5.5 and 5.7). The maximum levels of G6PDase were considerably higher, approximately 75%, in

Figure 5.6.

The influence of dilution rate on the steady state activities of 6-phosphogluconate dehydrogenase in organisms grown in light-limited chemostat culture. 6PGDase specific activity expressed in terms of nmol substrate converted min^{-1} $(mg\ protein)^{-1}$ (○) and in terms of nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$ (●).

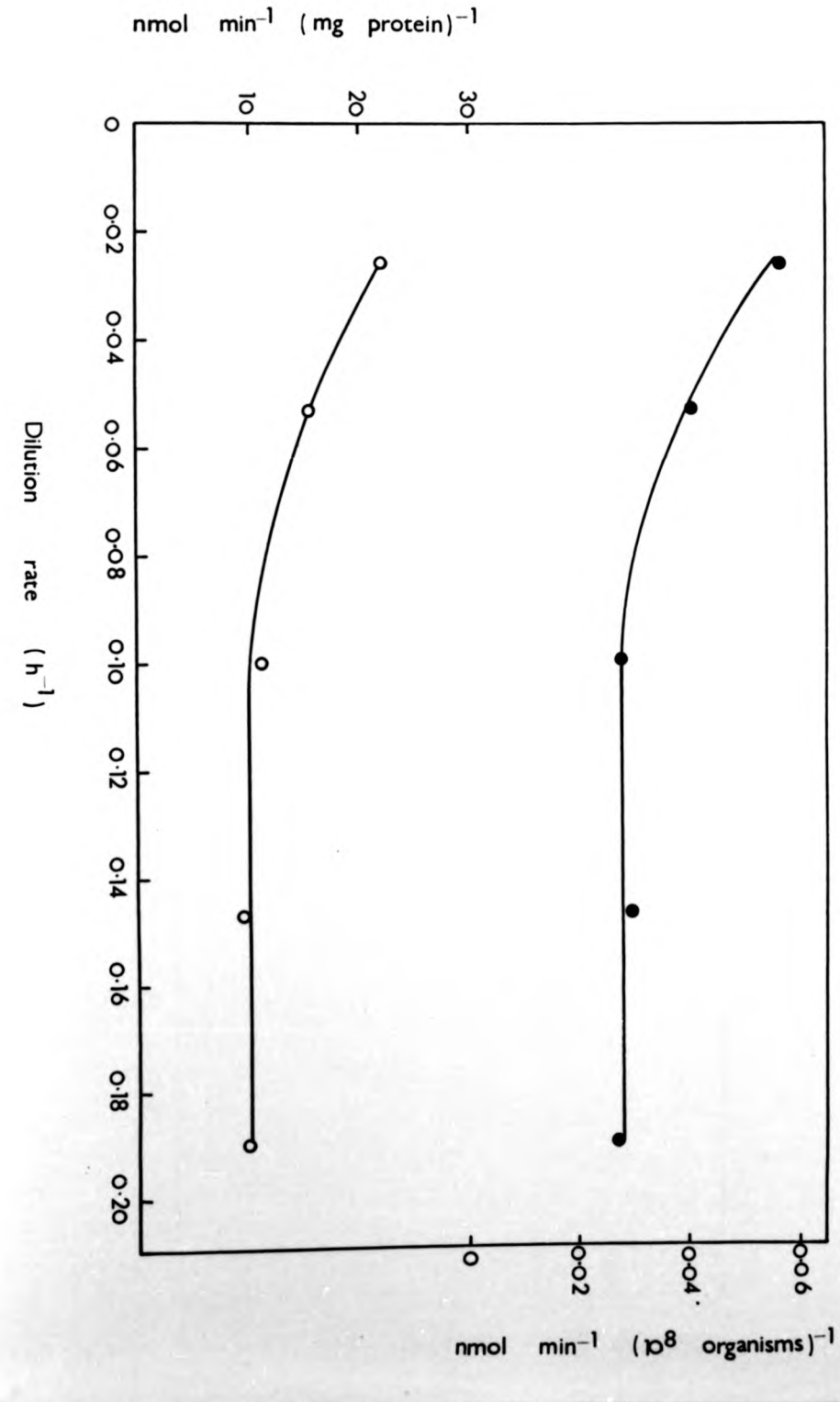


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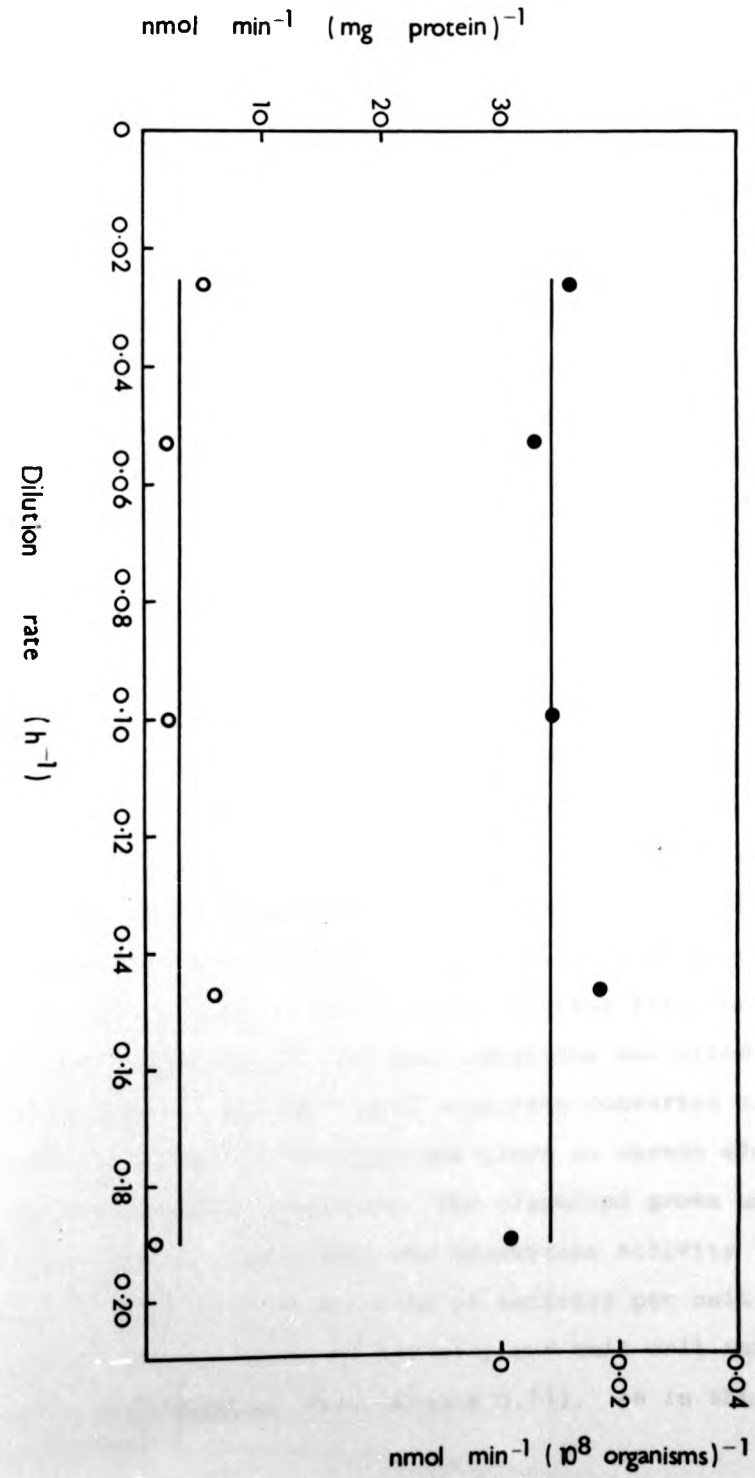
Figure 5.7.

The influence of dilution rate on the steady state activities of glucose 6-phosphate dehydrogenase in terms of nmol substrate converted min^{-1} (mg protein)$^{-1}$ (○) and in terms of nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ (●) in organisms grown in light-limited chemostat culture.


nmol min^{-1} (mg protein)$^{-1}$
0
10
20
30
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})
0
0·02
0·04
nmol min^{-1} (10^8 organisms)$^{-1}$

carbon dioxide-limited cultures than in equivalent dilution rate in light-limited cultures (Figures 5.5. and 5.7).

5.3. GLYCOLYTIC PATHWAY ENZYMES

Hexokinase (ATP D-hexose 6-phosphotransferase E.C. 2.7.1.1) and fructose 1,6-bisphosphate aldolase (Fructose 1,6-bisphosphate D-gluceraldehyde 3-phosphate lyase, E.C. 4.1.2.13) activities, as representatives of the glycolytic pathway, although FBAase is an enzyme with a function in the reductive pentose phosphate cycle as well as the glycolytic pathway, altered in a similar fashion whether the specific activities were calculated on basis of unit total protein or unit cell number. The hexokinase specific activity showed a sharp increase below D = 0.10 h^{-1} representing an approximately 4 fold increase in terms of activity per unit protein (Figure 5.8) and a 3.5 fold increase in terms of activity per unit cell number (Figure 5.9) with a peak of activity at dilution rates between 0.05 h^{-1} and 0.06 h^{-1} (95 nmol substrate converted min^{-1} $(mg\ protein)^{-1}$ or 0.176 nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$) in organisms grown in carbon dioxide-limited chemostat cultures. For organisms grown under light-limited conditions, the hexokinase activity increased approximately 3 fold in terms of activity per unit protein and 2.5 fold in terms of activity per unit cell number, with decreasing dilution rate (Figure 5.11). As in the case of

Figure 5.8.

The influence of dilution rate on the steady state activities of hexokinase in terms of nmol substrate converted min^{-1} (mg protein)$^{-1}$ (O), in organisms grown in carbon dioxide-limited chemostat culture.

Dilution rate (h^{-1})

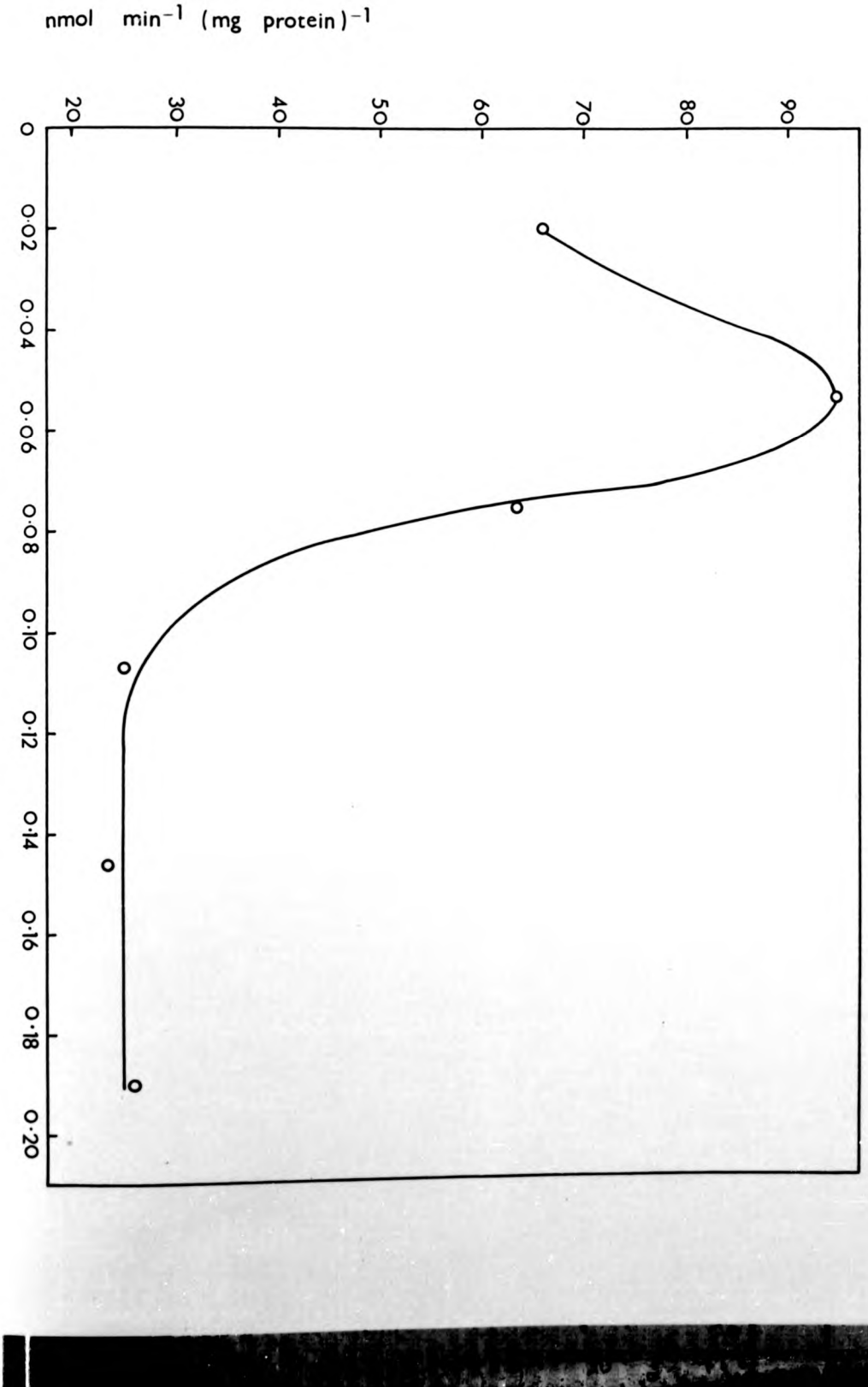

nmol min⁻¹ (mg protein)⁻¹
20
30
40
50
60
70
80
90
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)

Figure 5.9 .

The influence of dilution rate on the steady state activities of hexokinase in terms of nmol substrate converted min^{-1} $(10^8$ organisms$)^{-1}$, in organisms grown in carbon dioxide-limited chemostat culture.

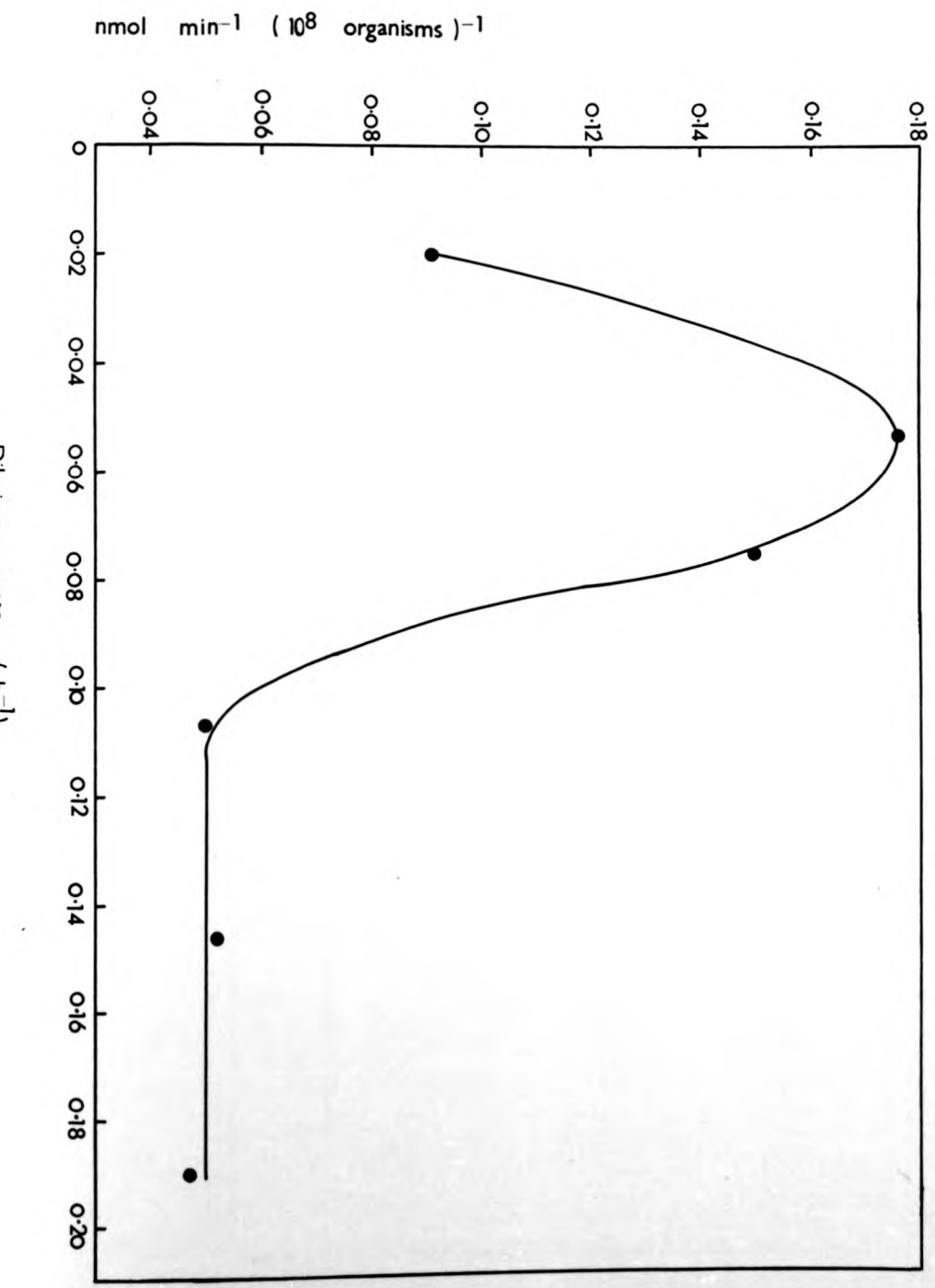

nmol min^{-1} (10^8 organisms)$^{-1}$
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h^{-1})

Figure 5.10

The influence of dilution rate on the steady state activities of fructose 1,6-bisphosphate aldolase in terms of nmol substrate converted min^{-1} (mg protein)$^{-1}$ (○) and in terms of nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ (●) in organisms grown in carbon dioxide-limited chemostat culture.

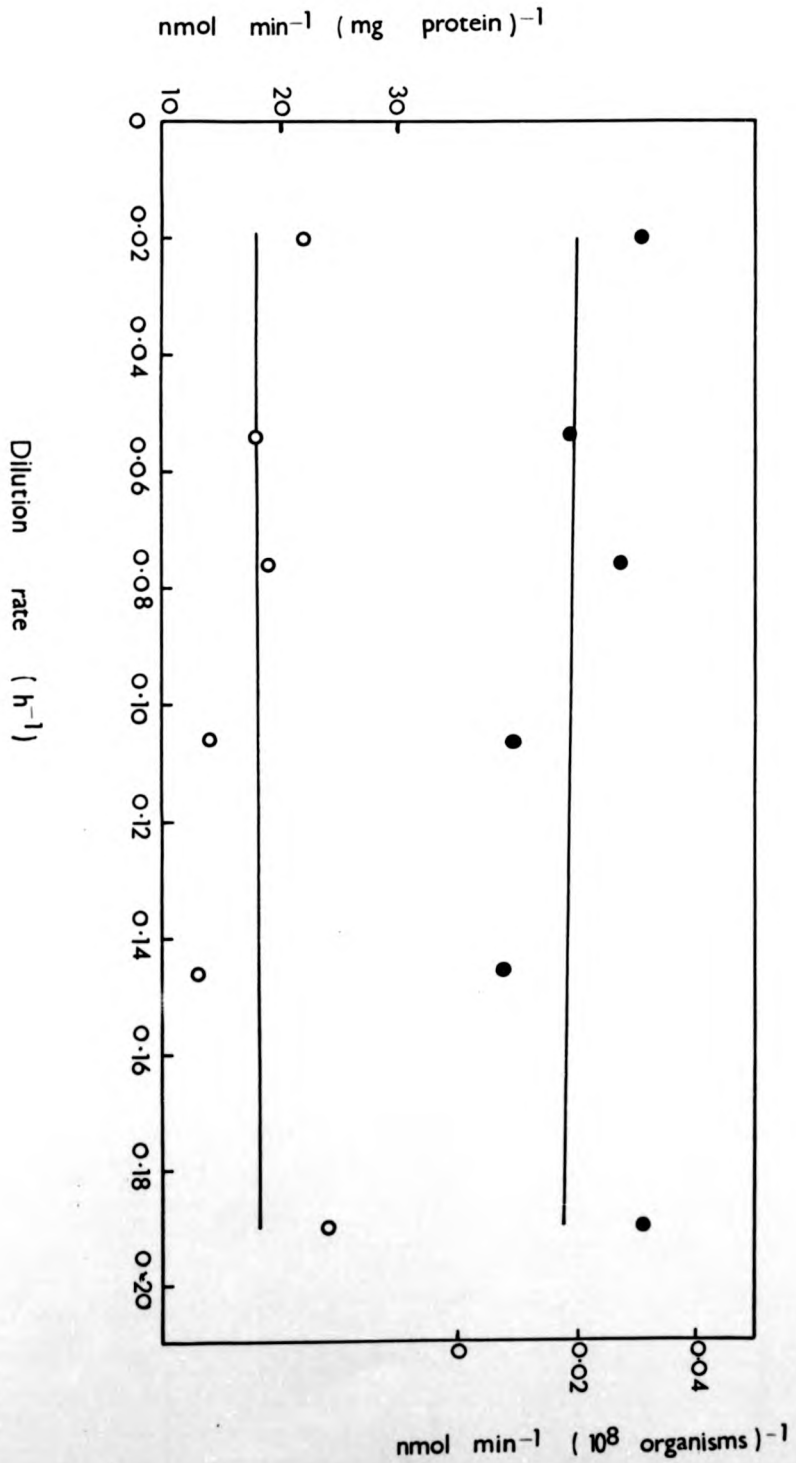
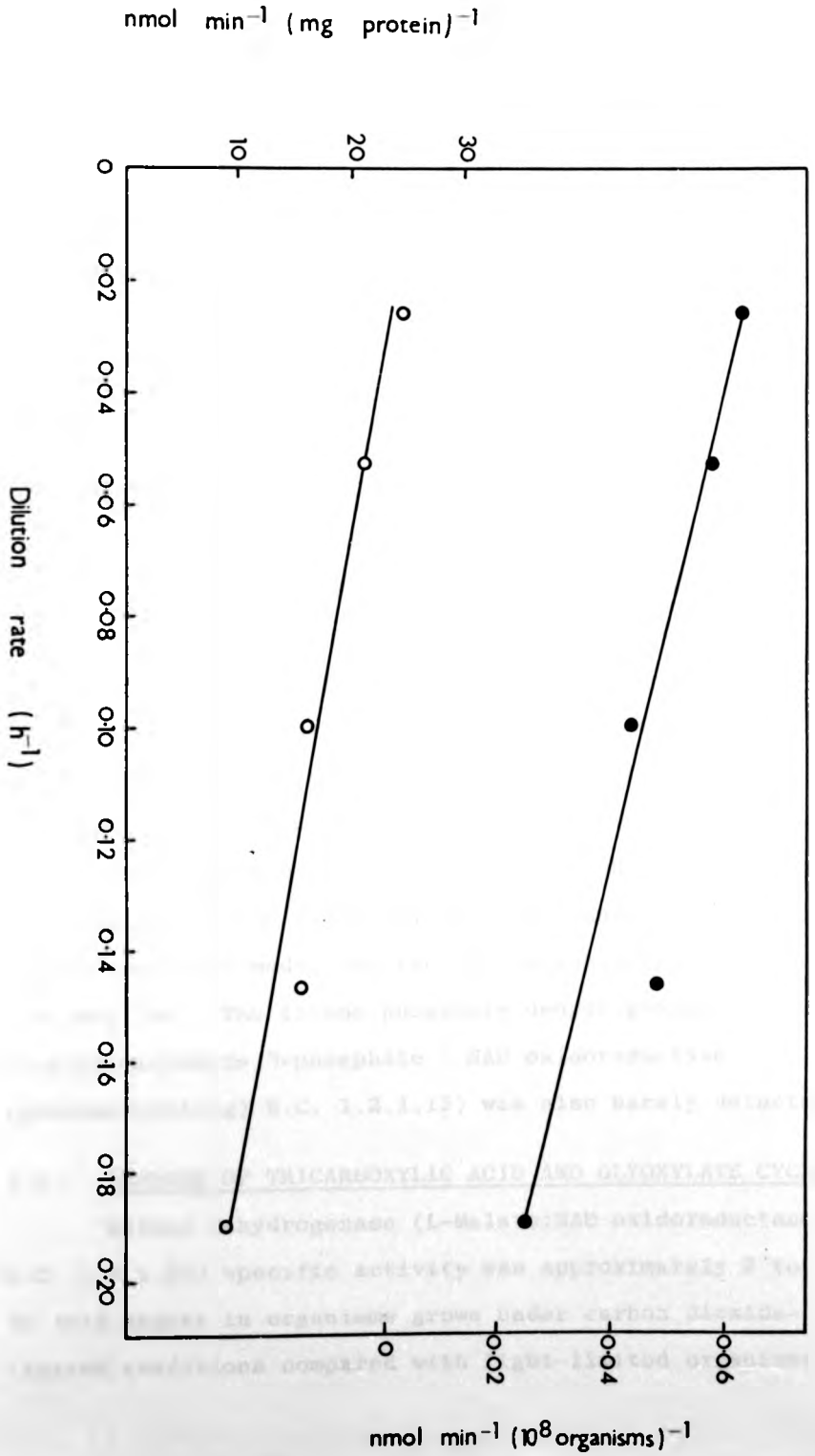

nmol min⁻¹ (mg protein)⁻¹
10
20
30
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)
0
0·02
0·04
nmol min⁻¹ (10⁸ organisms)⁻¹

Figure 5.11.

The influence of dilution rate on the steady state activities of hexokinase in terms of nmol substrate converted min^{-1} (mg protein)$^{-1}$ (○) and in terms of nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ (●), in organisms grown in light-limited chemostat culture.


nmol min⁻¹ (mg protein)⁻¹
10
20
30
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
Dilution rate (h⁻¹)
0
0·02
0·04
0·06
nmol min⁻¹ (10⁸ organisms)⁻¹

6PGDase, however, the minimum specific activity observed under carbon dioxide-limited conditions was considerably greater than that under light-limited conditions. Indeed the minimum specific activity under high dilution rate carbon dioxide-limited conditions (24.5 nmol substrate converted min^{-1} $(mg\ protein)^{-1}$) was found to be the same as the maximum activity under low dilution rate light-limited conditions.

Fructose 1,6-bisphosphate aldolase was found to have specific activities which did not vary with dilution rate and moreover carbon dioxide-limited and light-limited organisms showed similar levels (7-8 nmol substrate converted $min^{-1}(10^8\ organisms)^{-1}$) (Figures 5.10 and 5.12).

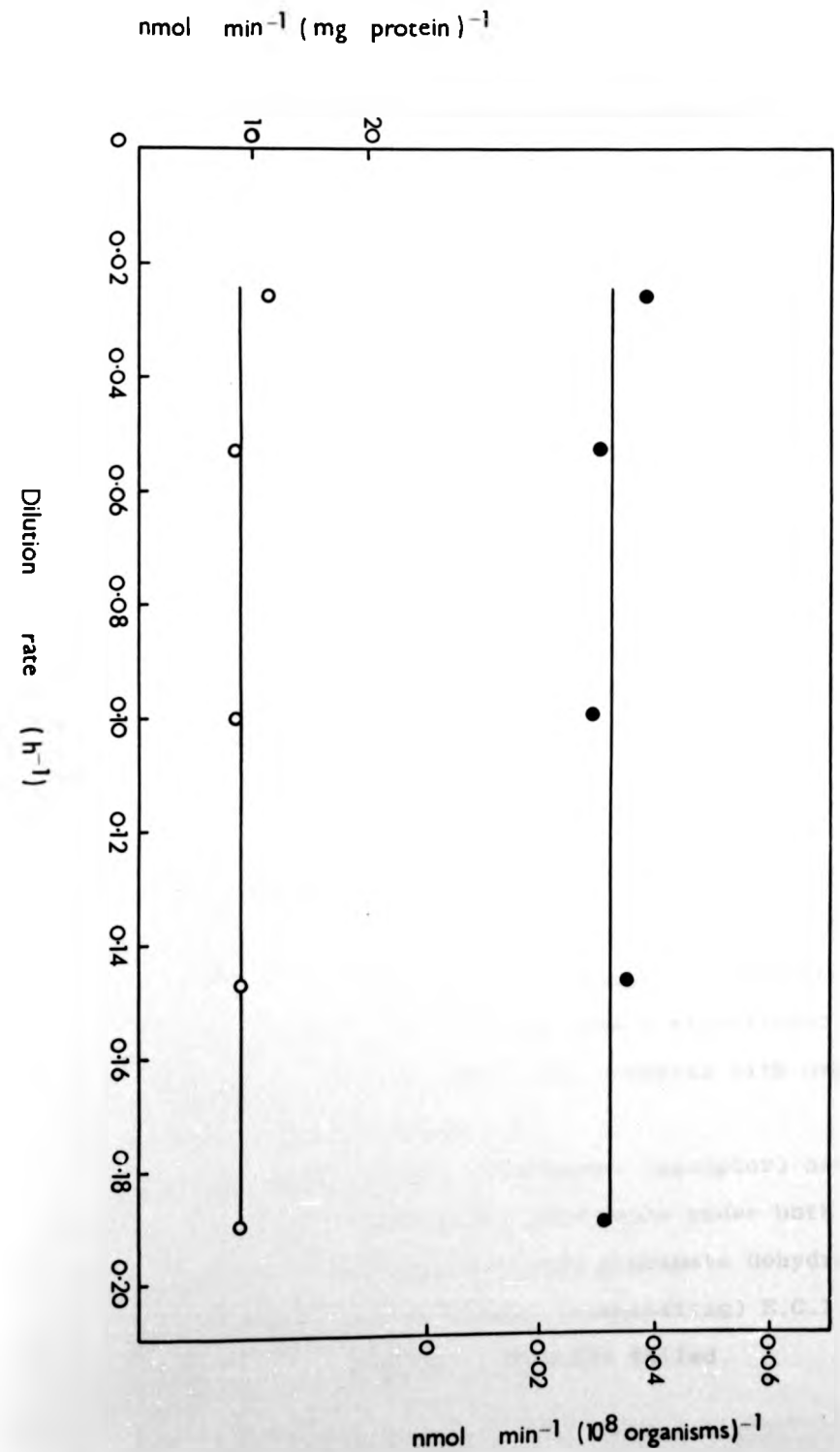
Attempts to measure the specific activity of 6-phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, E.C. 2.7.1.11), which also operates specifically in the glycolytic pathway, under both limitations were made, but the enzyme activity measured was very low. The triose phosphate dehydrogenase (D-glyceraldehyde 3-phosphate : NAD oxidoreductase (phosphorylating) E.C. 1.2.1.12) was also barely detectable.

5.4. ENZYMES OF TRICARBOXYLIC ACID AND GLYOXYLATE CYCLES

Malate dehydrogenase (L-Malate:NAD oxidoreductase E.C. 1.1.1.37) specific activity was approximately 2 to 15 fold higher in organisms grown under carbon dioxide-limited conditions compared with light-limited organisms

Figure 5.12.

The influence of dilution rate on the steady state activities of fructose 1,6-bisphosphate aldolase in terms of nmol substrate converted min^{-1} (mg protein)$^{-1}$ (○) and in terms of nmol substrate converted min^{-1} (10^8 organisms)$^{-1}$ (●), in organisms grown in light-limited chemostat culture.



ady state
ldolase
1 (mg
rate
organisms

(Table 5.2) although the pattern of changing activities as a function of dilution rate was not regular. Isocitrate lyase (Ls-Isocitrate glyoxylate lyase E.C. 4.1.3.1) was the only enzyme examined which showed considerably elevated specific activities in light-limited organisms (average value of 74 nmol substrate converted min^{-1} $(mg\ protein)^{-1}$ or an average value of 0.19 nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$) between 0.05 h^{-1} and 0.19 h^{-1} dilution rates compared with carbon dioxide-limited grown organisms (average value of 13 nmol substrate converted min^{-1} $(mg\ protein)^{-1}$ or an average value of 0.03 nmol substrate converted min^{-1} $(10^8\ organisms)^{-1}$) (Table 5.2). However, isocitrate lyase specific activity in carbon dioxide-limited organisms increased 8.5 fold in terms of activity per unit total protein and 4 fold in terms of activity per unit cell number once the dilution rate was lowered beyond D = 0.05 h^{-1}. The high isocitrate lyase activity at D = 0.02 h^{-1} (110 nmol substrate converted h^{-1} $(mg\ protein)^{-1}$) was the average result of four separate steady state cultures all of which showed significantly greater levels at this dilution rate compared with other steady state cultures (Table 5.2).

Succinate dehydrogenase (Succinate: (acceptor) oxidoreductase E.C. 1.3.99.1) was barely detectable under both limitations whilst attempts to assay glutamate dehydrogenase (L-Glutamate: NADP oxidoreductase (deaminating) E.C.1.4.1.4) by the method used (section 2.10.2.13) failed.

Table 5.2. Changes in the specific activities of malate dehydrogenase and isocitrate lyase in response to dilution rate and growth-limiting substrate.

Enzyme Activity (nmol min^{-1} (mg protein)$^{-1}$)	Light-limited chemostat dilution rate, (h^{-1})					Carbon dioxide-limited chemostat dilution rate, (h^{-1})					
	0.03	0.05	0.10	0.15	0.19	0.02	0.05	0.08	0.11	0.15	0.19
Malate dehydrogenase	1.0	1.4	3.2	5.7	2.8	14.7	9.3	7.8	9.5	6.7	6.7
Isocitrate lyase	77.5	83.0	90.1	54.0	63.7	110.0	6.7	23.5	8.2	10.1	14.5
(nmol min^{-1} (10^8 organisms)$^{-1}$)											
Malate dehydrogenase	0.003	0.004	0.008	0.020	0.008	0.02	0.02	0.02	0.02	0.02	0.012
Isocitrate lyase	0.20	0.22	0.17	0.17	0.18	0.13	0.01	0.06	0.02	0.02	0.03

FBPase, 6PFKase, triose phosphate dehydrogenase, succinic dehydrogenase and glutamate dehydrogenase were not satisfactory nor adequately assayed. The methods (section 2.10.2) used did not give the expected proportionality between the substrate concentration and the amount of cell extract.

DISCUSSION

The study of the activity of selected enzymes which were examined in steady state cultures grown under either light-limited or carbon dioxide (bicarbonate)-limited conditions has shown that under appropriate growth conditions the blue-green alga Anacystis nidulans was capable of altering the specific activities of a number of enzymes involved in intermediary carbon metabolism. The variations between different enzymes showed that the changes in specific activities were not associated with a general phenomenon, such as an alteration in the total protein content of the organisms, since such changes would have been expected to alter all the activities in a similar fashion. Furthermore, in some cases the same enzyme behaved differently between light-limited and carbon dioxide-limited conditions indicating that differences due to different environmental conditions, in contrast to changes in the organisms' specific growth rate only, were important in the expression of enzyme activity. These results suggest that Anacystis nidulans was capable of a certain degree of selective expression of different enzymes as a result of the regulation of synthesis at the transcriptional level, although these data cannot exclude completely the possibility of regulation at the translational level or control of enzyme levels due

to activation or rates of enzyme turnover.

In one case the magnitude of the change in specific activity was similar to those reported for enzymes known to be under transcriptional control (Bone, 1971a, and b; Ihlenfeldt and Gibson, 1975b). The enzyme in question, RuBPCase, showed a wide range of activities under growth conditions where it might be expected to show such changes; that is, an increase in specific activity in organisms grown in environments where growth was limited by the availability of one of the enzyme's substrates, namely carbon dioxide (bicarbonate). The rate of carbon dioxide assimilation depended on the availability and concentration of this substrate (see part 4) and hence in environments with low carbon dioxide concentrations an increase in the level of RuBPCase could increase the rate of fixation. The kinetics of conventional nutrient-limited chemostat culture predict that the carbon dioxide concentration would decrease with decreasing dilution rate and account for the greater RuBPCase specific activity with decreasing dilution rate.

The higher the RuBPCase levels, then the greater the capacity to scavenge for and assimilate low, growth-limiting concentrations of the sole carbon source. The above explanation also applies to the increase of RuBPCase levels at growth temperatures lower than the optimal (see Table 5.1 and Figure 5.2) since at these low growth temperatures

the rate of carbon dioxide assimilation was depressed (see section 4.2). Furthermore the RuBPCase activity for organisms grown under light-limited conditions at 25^{o}C, was for an unknown reason the only enzyme which increased in activity with increasing growth rate under light-limited conditions, but again demonstrated that with certain changes in environmental conditions, Anacystis nidulans was able to alter selectively the specific activity of RuBPCase.

The principle of elevated activities of an initial enzyme involved in the metabolisms of a growth-limiting nutrient is well established (see Dean, 1972); for example, heterotrophic bacteria synthesise elevated levels of β-galactosidase when growing on lactose as the limiting nutrient (Horiuchi, Tomizawa and Novick, 1962). However, it is interesting to note that the levels of RuBPCase, or any of the other enzymes determined, did not show the wide range of activities exhibited by the catabolic enzymes of heterotrophic bacteria (see, for example Clark, Houldsworth and Lilly, 1968; Clarke and Lilly, 1969). From the results reported here it seems that the alteration in the activity of RuBPCase was in response to changes in the external carbon dioxide concentration and not different growth rates, since under light-limited conditions with carbon dioxide in excess at all dilution rates examined there was no

change in the specific activity of RuBPCase with growth rate.

The RuBPCase activity *in vivo* in carbon dioxide-limited cultures accounted for only 10% (Table 5.3) of the *in vitro* rate of carbon dioxide assimilation (Table 4.5) required for the growth of the organisms at high dilution rates whilst the RuBPCase activity *in vivo* in light-limited cultures accounted for 0.6% of the *in vitro* rate of carbon dioxide assimilation (Tables 5.3 and 4.5) or in other words the rate of carbon dioxide assimilation *in vitro* was not high enough to support growth. For the growth of the organisms at medium dilution rates the RuBPCase activity *in vivo* accounted for 50% of the required *in vitro* rate of carbon dioxide assimilation (Table 5.3 and 4.5) for organisms grown under carbon dioxide-limited whilst this percentage was as low as 4% for organisms grown under light-limited conditions (Table 5.3 and 4.5).

This reflects the problems of correlating this enzyme's activity with the required activity for the necessary rate of CO_2 fixation by intact organisms at a given growth rate.

In neither carbon dioxide-limited nor light-limited cultures was the changing pattern of carbon dioxide fixation by intact organisms as a function of dilution rate, correlated with the observed RuBPCase levels. This suggests that RuBPCase may not have been the only enzyme involved in regulating the rate of carbon dioxide fixation by intact organisms,(see part 4 for carbon dioxide assimilation results).

Table 5.3. A comparison of observed values of RuBPCase specific activity with the required values to sustain a given growth rate.

D (h^{-1})	t_d (h)	RuBPCase: Required specific activity of C assimilation µmol C h^{-1} (mg protein)$^{-1}$	RuBPCase: Observed specific activity of C assimilation µmol C h^{-1} (mg protein)$^{-1}$	
			CO_2-limited	Light-limited
0.02	34.65	2.41	5.45	0.45
0.04	17.33	4.81	1.20	0.45
0.06	11.55	7.22	0.75	0.45
0.08	8.66	9.62	0.55	0.45
0.10	6.93	12.03	0.45	0.45
0.12	5.78	14.44	0.40	0.45
0.14	4.95	16.84	0.40	0.45
0.16	4.33	19.25	0.40	0.45
0.18	3.85	21.65	0.40	0.45
0.20	3.47	24.06	0.40	0.45

It has been reported that the methods used so far for assaying RuBPCase specific activity only the 10% of the enzyme's activity was measured. It is also clear that the second reductive pentose phosphate cycle enzyme, phosphoribulokinase, in carbon dioxide-limited chemostat cultures was not regulated in the same way as RuBPCase activity in response to the same changes in the external environmental conditions.

The capacity to alter enzyme specific activities with respect to changing growth rate was limited since, apart from RuBPCase, all the other enzymes examined which showed variable activities, exhibited only 2 to 4 fold changes over the growth rate ranges examined. Moreover, three of the enzymes under carbon dioxide-limited conditions and six under light-limited conditions showed no changes at all substantiating the concept of little transcriptional control (Carr, 1973).

With the exception of FBAase and PRKase all the enzymes examined showed considerably higher specific activities in organisms grown under carbon dioxide-limited conditions compared with light-limited conditions. Under carbon dioxide-limited conditions the intracellular concentration of carbon compounds would be expected to be lower than under conditions of excess carbon dioxide, a situation which was likely to result in the derepression of synthesis of enzymes involved in carbon metabolism. However, the maximum

differences were only 4 fold, again suggesting a limited capacity to alter enzyme levels in response to different growth environments. It is interesting to note that the observed changes occurred in the specific activities of those enzymes at the beginning of different pathways and which may be involved in the regulation of carbon flow in that pathway.

PART 6. GROWTH OF ANACYSTIS NIDULANS DURING WASHOUT FROM CHEMOSTAT

6.1. LIGHT-LIMITED CHEMOSTAT CULTURE

The growth of Anacystis nidulans during washout from light-limited chemostat cultures at three different initial steady state cultures was measured in terms of culture absorbance and cell number. None of these cultures, assessed by either absorbance or cell number, showed the theoretical pattern of immediate and continuous exponential growth at a rate equal to μ_{max} after changing to a culture washout dilution rate. Furthermore differences existed between the washout curves for absorbance and cell number for the same culture washout experiment.

Cell number washout curves for light-limited cultures were biphasic showing a marked change in the organism's specific growth rate over a relatively short period of time during washout growth (Figures 6.1 and 6.3) with an exception for the culture at D = 0.08 h^{-1} which showed a continuous exponential growth during up to 6.5 hours of washout time (Figure 6.2); this washout curve was not continued for a long enough period. In all cases the specific growth rate of the first phase, μ_1, was less than the specific growth rate of the second phase, μ_2 (Table 6.1), where the rates were determined from the coefficients of regression line analysis. The values of μ_1 were variable, increasing with increases in the initial growth rate of the culture prior to washout and indeed, within the limits of experimental

Figure 6.1.

The washout curves in terms of cell number (O) and absorbance (●) for a light-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.37 h^{-1}.

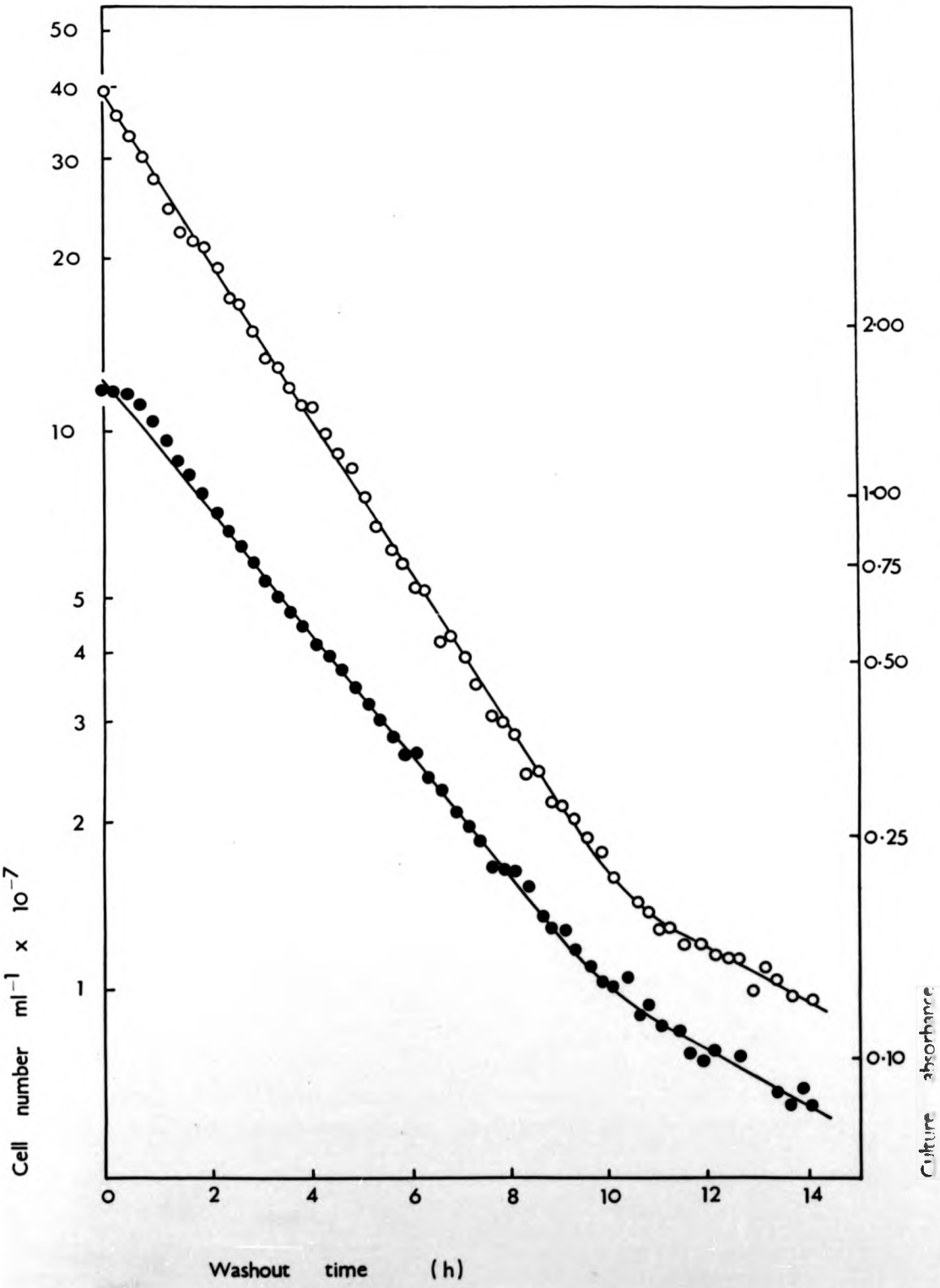

50
40
30
20
10
5
4
3
2
1
2·00
1·00
0·75
0·50
0·25
0·10
0
2
4
6
8
10
12
14
Cell number ml^{-1} x 10^{-7}
Culture absorbance
Washout time (h)

Figure 6.2.

The washout curves in terms of cell number (○) and absorbance (●) for a light-limited culture grown at an initial dilution rate of 0.08 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.32 h^{-1}.

(O) and
grown
At t = 0
reased to

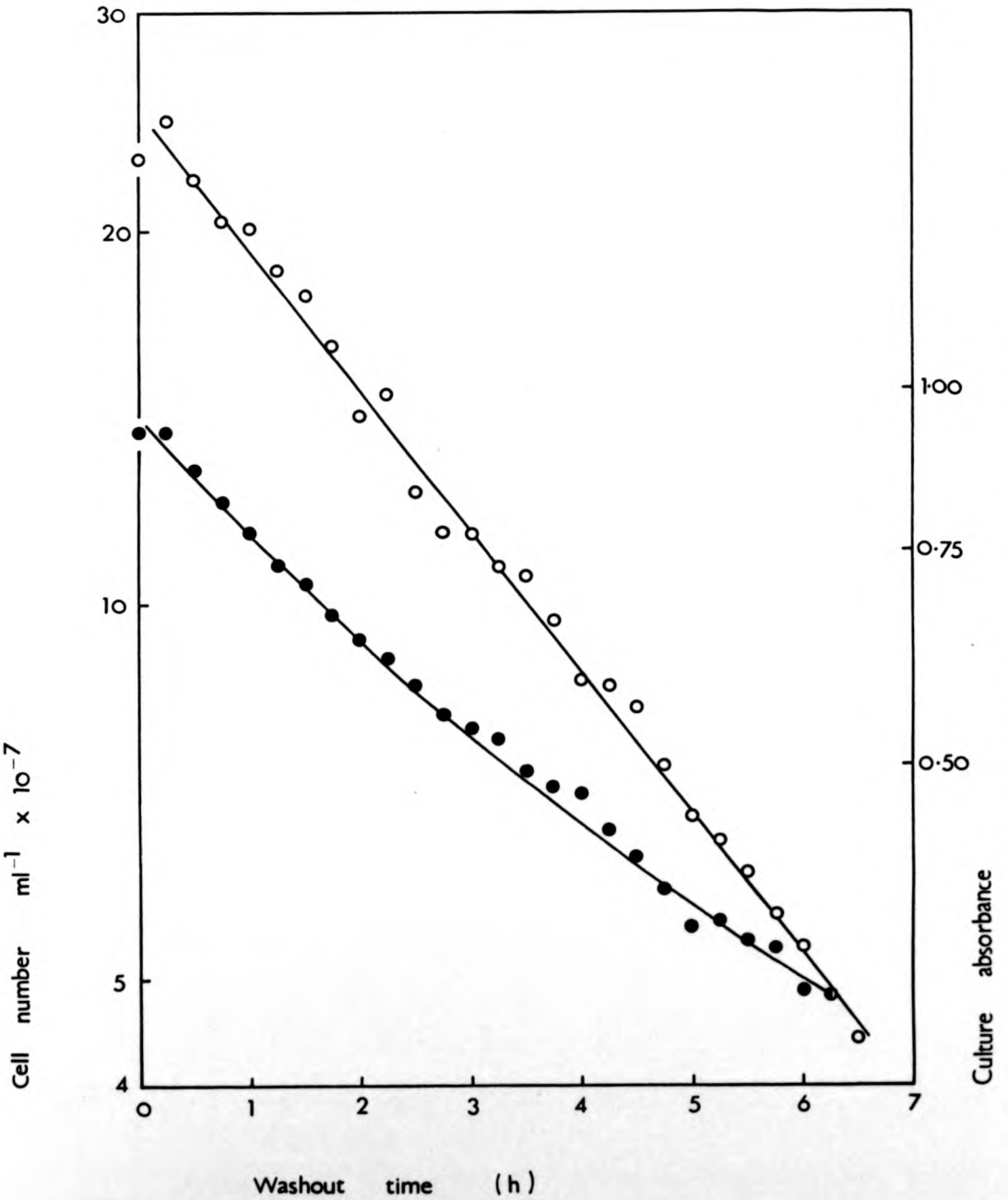


Figure 6.3.

The washout curves in terms of cell number (O) and absorbance (●) for a light-limited culture grown at an initial dilution rate of 0.15 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.31 h^{-1}.

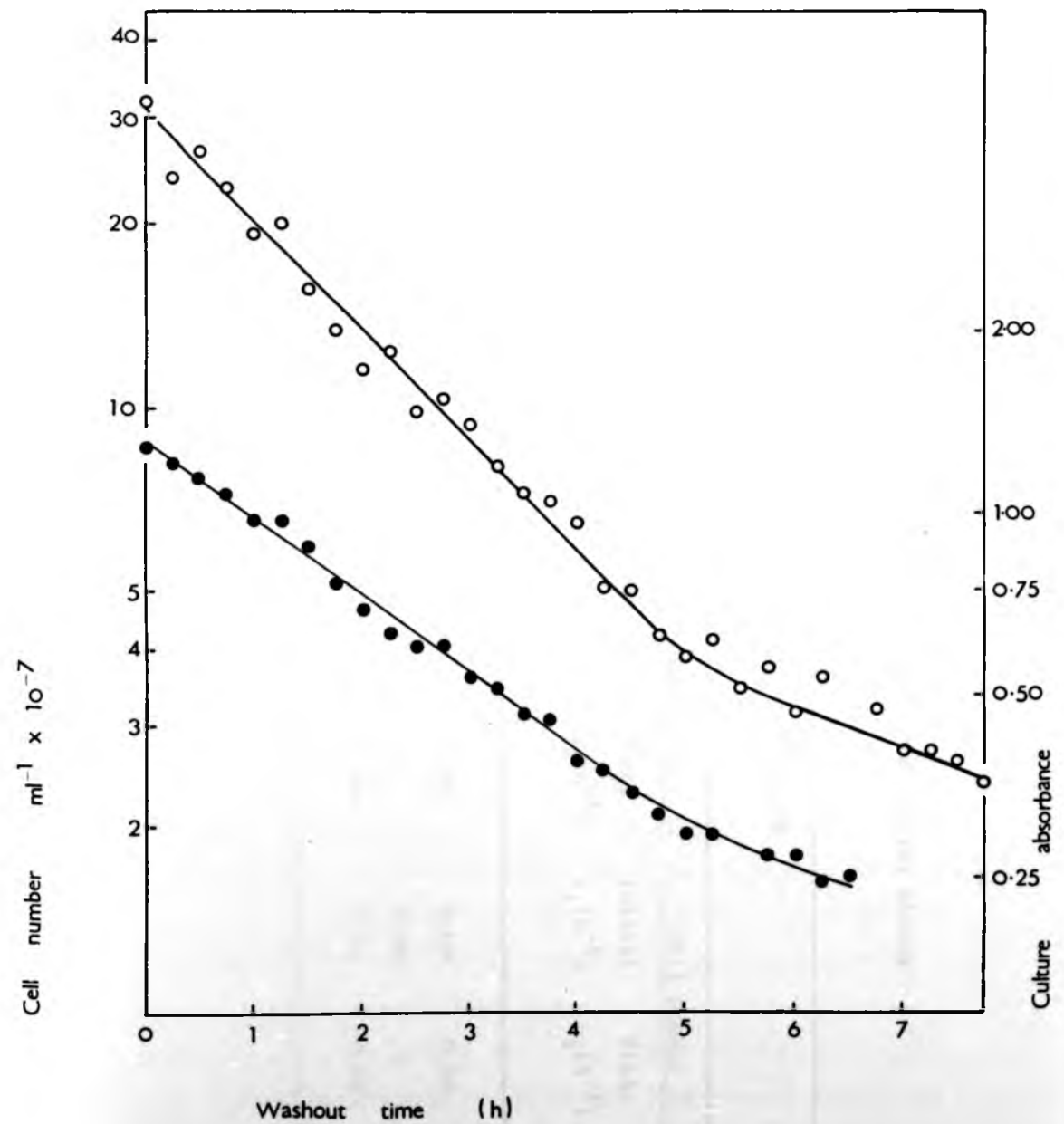

40
30
20
10
5
4
3
2
0
1
2
3
4
5
6
7
2·00
1·00
0·75
0·50
0·25
Cell number ml⁻¹ x 10⁻⁷
Culture absorbance
Washout time (h)

Table 6.1. The values of transient washout parameters from various initial steady-state light-limited cultures.

Initial steady-state culture conditions			Transient washout parameters					
				Absorbance		Cell number		
Dilution rate (h^{-1})	Absorbance (600 nm)	Cell number ($ml^{-1}10^{-7}$)	Washout dilution rate (h^{-1})	Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1})$	Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1})$	Length μ_1 phase (min)
0.03	1.54	39.6	0.37	0.10	0.26	0.05	0.26	615
0.08	0.92	23.0	0.32	0.14	-	0.06	-	> 390
0.15	0.54	9.3	0.31	0.16	0.24	0.11	0.23	285

error, the values determined from the cell number curves were probably the same as the dilution rates of the preceding steady-state cultures (Table 6.1). The specific growth rates of the second phase of washout were independent of the initial growth rate and hence the μ_1 phase and were similar to μ_{max} values for the given growth conditions as determined in closed culture experiments. The length of the μ_1 phase, that is the time taken to reach growth at a μ_{max} rate, increased with declining initial culture dilution rate such that the length of the μ_1 phase for the culture with the slowest initial growth rate examined, $D = 0.03\ h^{-1}$, was approximately 2.5 times longer than that for the fastest growing culture where $D = 0.15\ h^{-1}$.

It is obvious from Figures 6.2 and 6.3 that the washout curves measured by culture absorbance were different to the pattern of the cell number washout curves. During the course of washout the specific growth rate increased gradually with no indication of a biphasic curve, although washout from slow-growing cultures, at $D = 0.03\ h^{-1}$, resulted in a pattern similar to the biphasic cell number washout curve (Figure 6.1). The initial specific growth rates, μ_i, measured over the first 1 to 2 h were always less than μ_{max} greater than the cell number μ_i phase values (Table 6.1). There was an immediate 4 fold increase in growth rate for the slower growing culture compared with the steady state growth rate. By the time the cell number curves had reached μ_{max} values, the absorbance curves had also attained final μ_2 growth rates equal to the expected μ_{max} value for those conditions (Table 6.1). The difference

between the absorbance washout curves and those of cell number was probably due to the additional influence, on the absorbance measurements caused by changes in cell volume.

Mean cell volumes determined from Coulter Counter measurements showed that the cell volumes began to increase almost immediately after the shift to washout dilution rates. For the slower growing cultures the cell volumes began to increase after 4 hours of washout growth, showing a 3 fold increase in cell volume after 15 hours of washout growth (Figure 6.4). For cultures with medium and high initial growth rate, the cell volumes began to increase within the first 1 to 2 hours of washout (Figures 6.5 and 6.6). For faster growing organisms the cell volumes increased only 1.2 to 1.3 fold after 8h of washout growth.

To determine whether or not the delay in attaining μ_{max} growth rates could be circumvented if the cultures were saturated with the growth-limiting substrate at the same time as switching to the washout dilution rate, the following experiment was made. In a steady state light-limited culture at $D = 0.03\ h^{-1}$ with a low biomass concentration produced by lowering the incident light intensity (compared with the preceding light-limited cultures), the incident light intensity was simultaneously increased 5 fold when the dilution rate increased to a value greater than D_{crit} and equal to $D = 0.32\ h^{-1}$. The washout growth was followed (Figure 6.7) by measuring the culture absorbance and cell

Figure 6.4.

The change of the mean cell volumes during washout growth for light-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.37 h^{-1}.

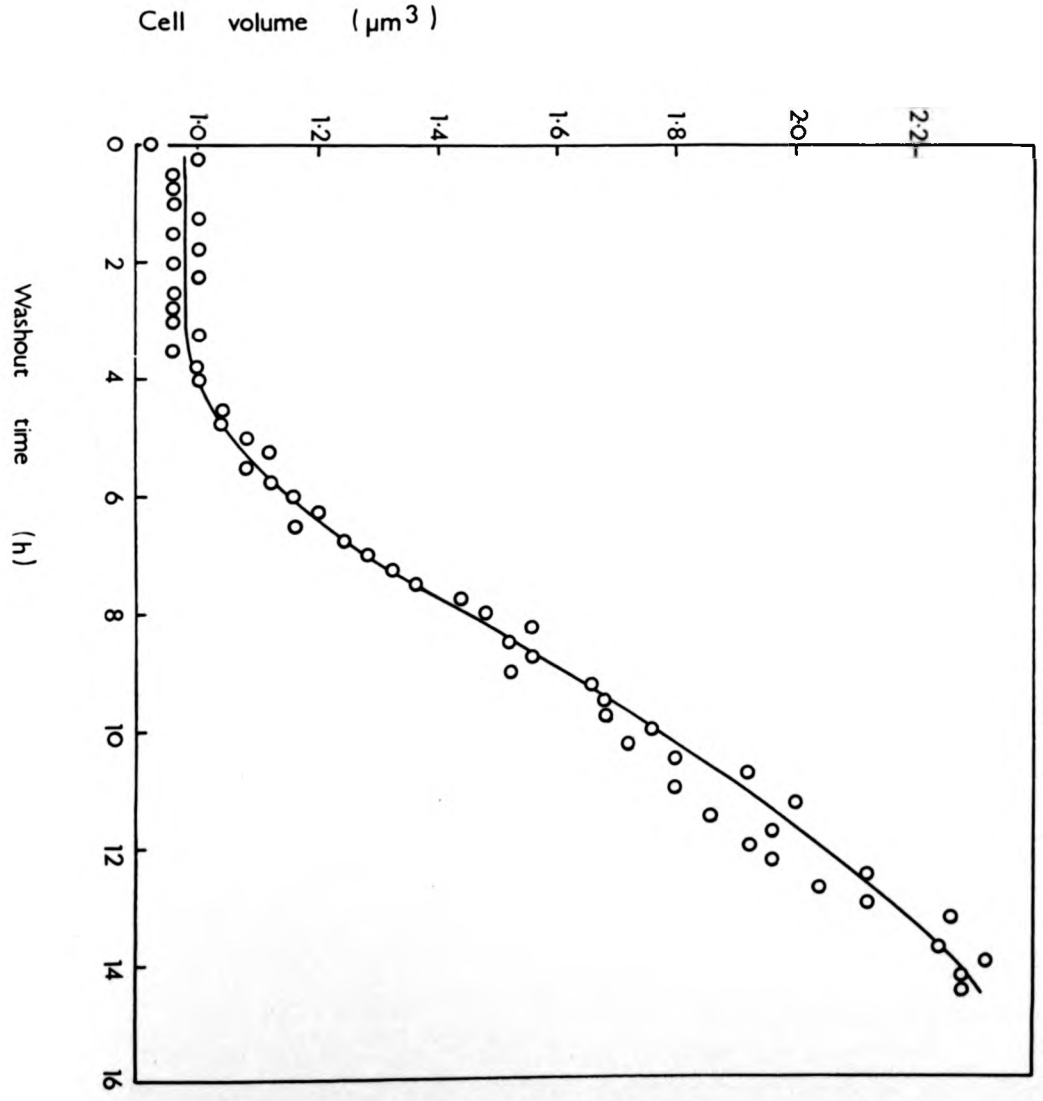
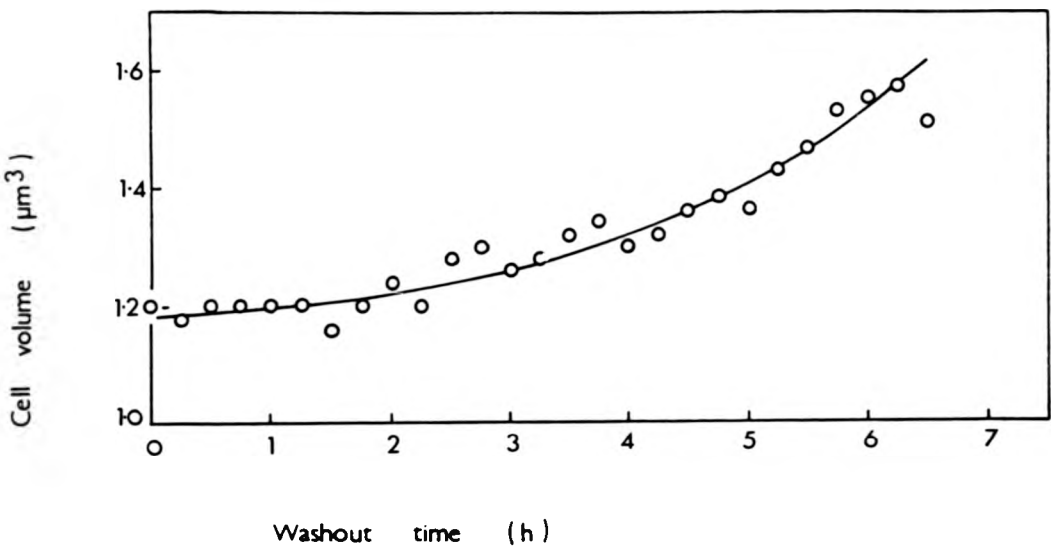

Cell volume (μm3)
1·0
1·2
1·4
1·6
1·8
2·0
2·2
Washout time (h)
0
2
4
6
8
10
12
14
16

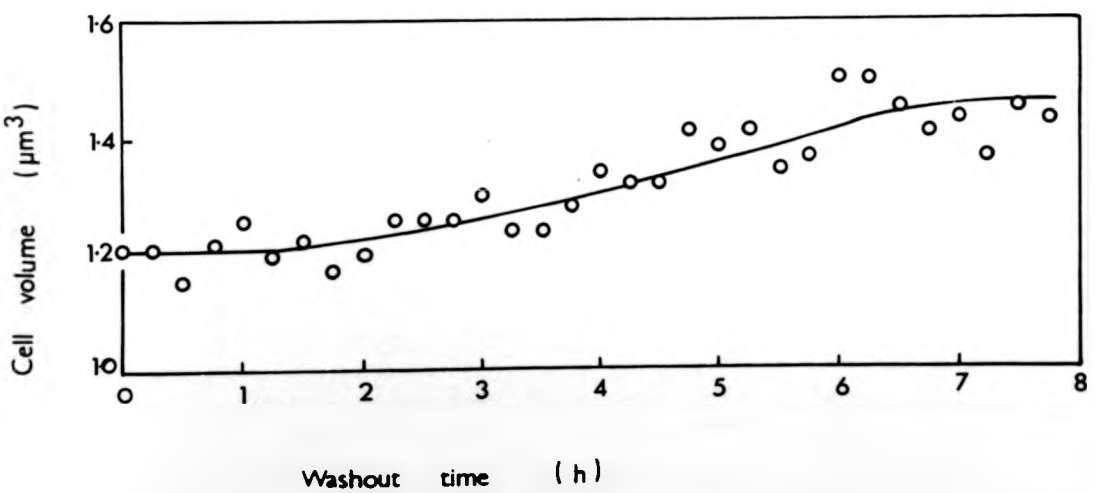
Figure 6.5.

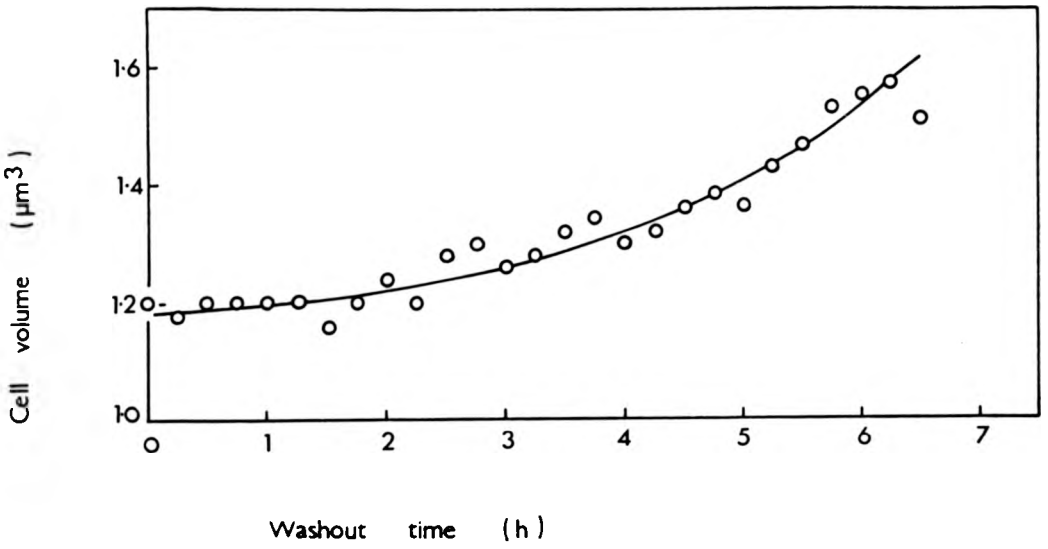
The change of the mean cell volumes during washout growth for light-limited culture grown at an initial dilution rate of 0.08 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.32 h^{-1}.

Figure 6.6.

The change of the mean cell volumes during washout growth for light-limited culture grown at an initial dilution rate of 0.15 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.31 h^{-1}.


1·6
1·4
1·2
1·0
Cell volume (μm³)
0
1
2
3
4
5
6
7
Washout time (h)


1·6
1·4
1·2
1·0
Cell volume (μm³)
0
1
2
3
4
5
6
7
8
Washout time (h)


Cell volume (μm3)
1·6
1·4
1·2
1·0
0
1
2
3
4
5
6
7
Washout time (h)

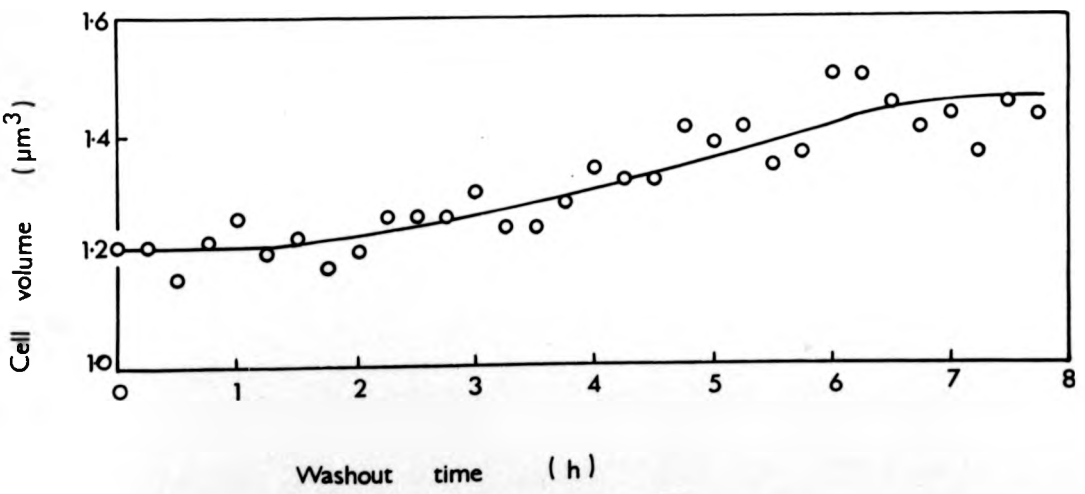
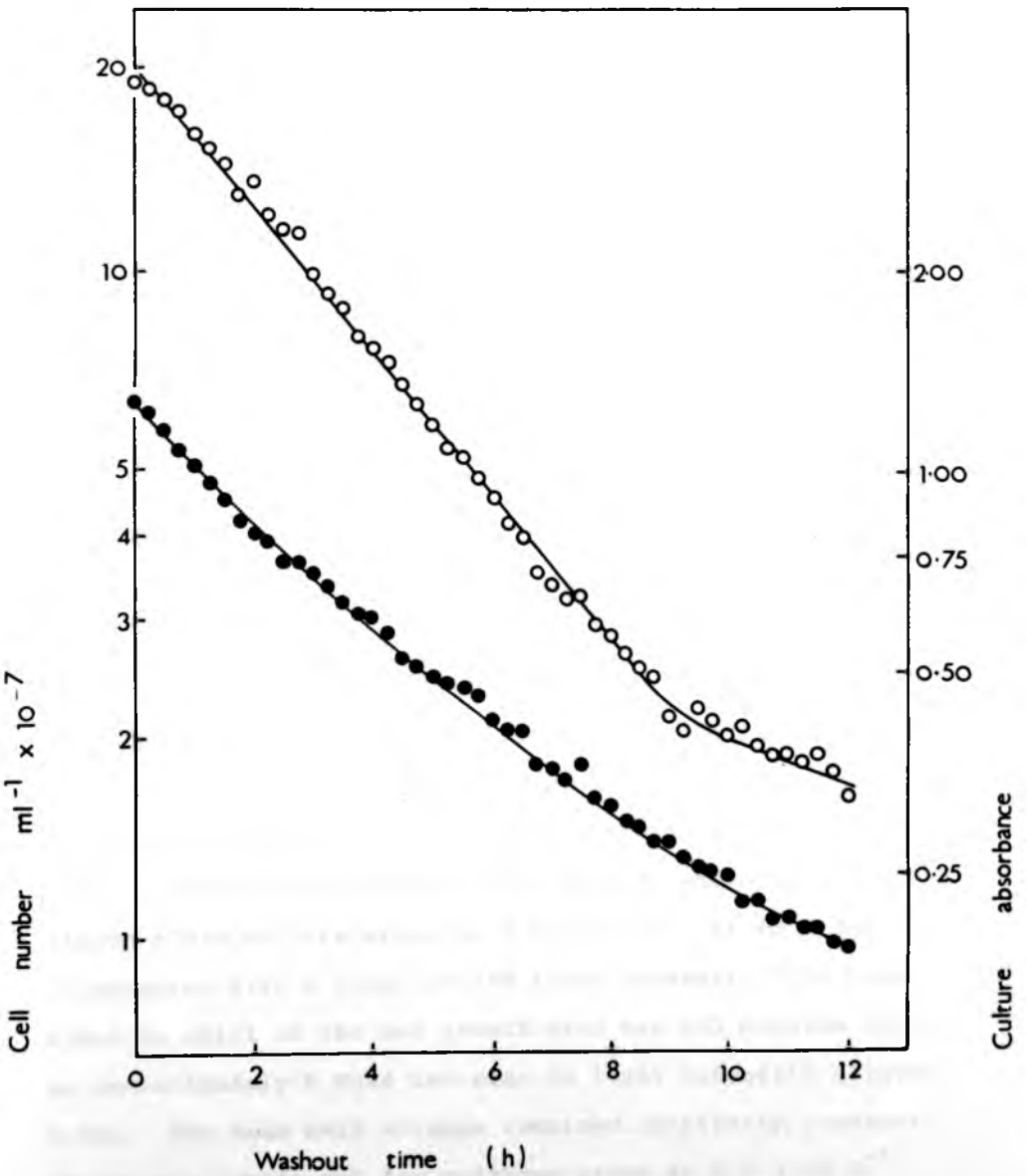

Cell volume (μm3)
1·6
1·4
1·2
1·0
0
1
2
3
4
5
6
7
8
Washout time (h)

Figure 6.7.

The washout curves in terms of cell number (○) and absorbance (●) for a light-limited culture with low biomass and grown at an initial dilution rate of 0.03 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.32 h^{-1} and simultaneously the incident light intensity increased 5 fold.

(o)
ture

d


20
10
5
4
3
2
1
0
2
4
6
8
10
12
200
1·00
0·75
0·50
0·25
Cell number ml⁻¹ x 10⁻⁷
Culture absorbance
Washout time (h)

number. The μ_1 phase lasted approximately 550 minutes. For the cell number washout curve the specific growth rate μ_1 was 0.07 h^{-1} and the specific growth rate of the second phase μ_2 was 0.26 h^{-1}. The mean cell volume increased approximately two fold during 8h of washout growth (Figure 6.8).

Two experiments were undertaken to determine the time taken for a steady state light-limited chemostat culture of Anacystis nidulans grown at D = 0.03 h^{-1} and D = 0.10 h^{-1}, to achieve a new growth rate after an increase in the incident light intensity and to compare this period of time with the length of the μ_1 phase. For a steady state light-limited culture grown at a dilution rate of 0.03 h^{-1} at 40°C and illuminated with a total of 48W light intensity, the incident light intensity was increased 12 fold at t = 0 (Figure 6.9). The culture maintained the old growth rate for a characteristic length of time before the new growth rate was established. This time taken to shift to the expected growth rate at higher light intensity was approximately 180 min whilst for a steady state culture grown at D = 0.10 h^{-1}, at 40°C and illuminated with a total of 48W light intensity, the time taken to shift to the new growth rate was 240 minutes with an approximately 8 fold increase in light intensity (Figure 6.10). The mean cell volumes remained relatively constant during the "shift-up" for cultures grown at D = 0.03 h^{-1} (Figure 6.11) whilst there was an approximately 2 fold

Figure 6.8.

The change of the mean cell volumes during washout growth for a light-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.32 h^{-1} and simultaneously the incident light intensity increased 5 fold.

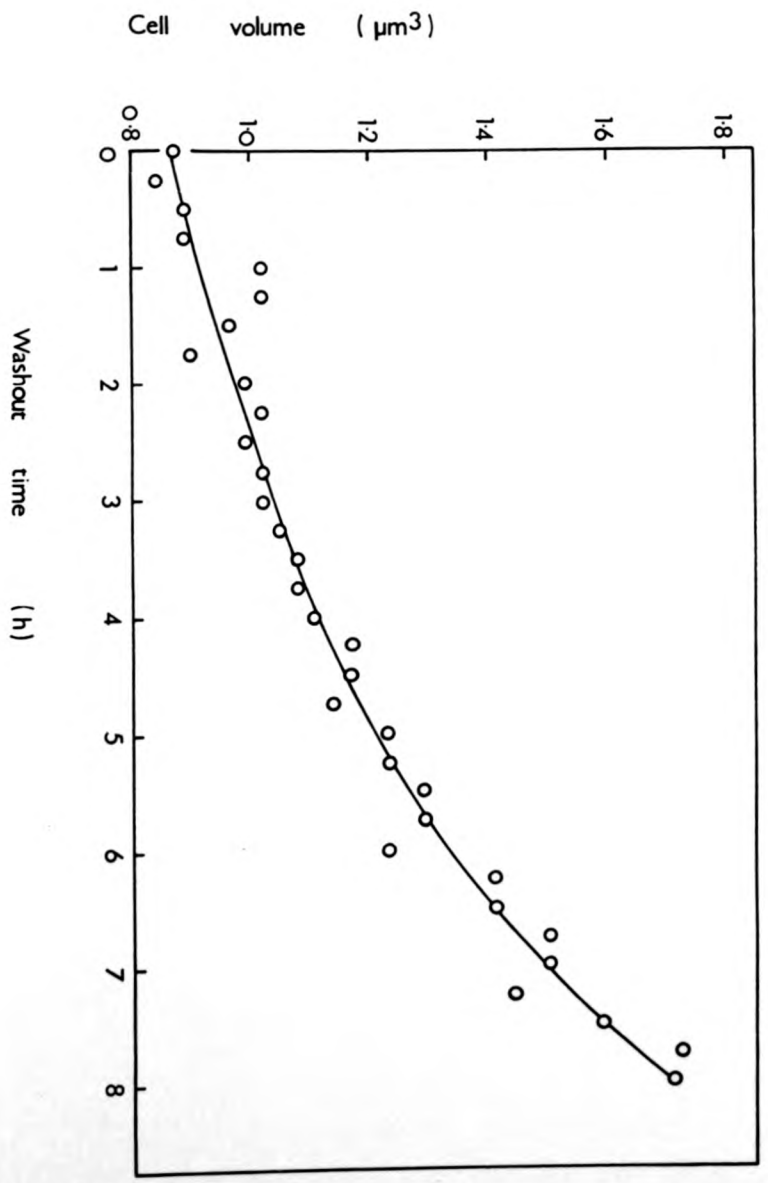

Cell volume (μm3)
0·8
1·0
1·2
1·4
1·6
1·8
0
1
2
3
4
5
6
7
8
Washout time (h)

Figure 6.9.

The increase in culture absorbance (●) and cell number (O) of steady state light-limited culture of Anacystis nidulans grown at a growth rate of 0.03 h^{-1}, during a "shift-up" induced by increasing the incident light-intensity, at t = O.

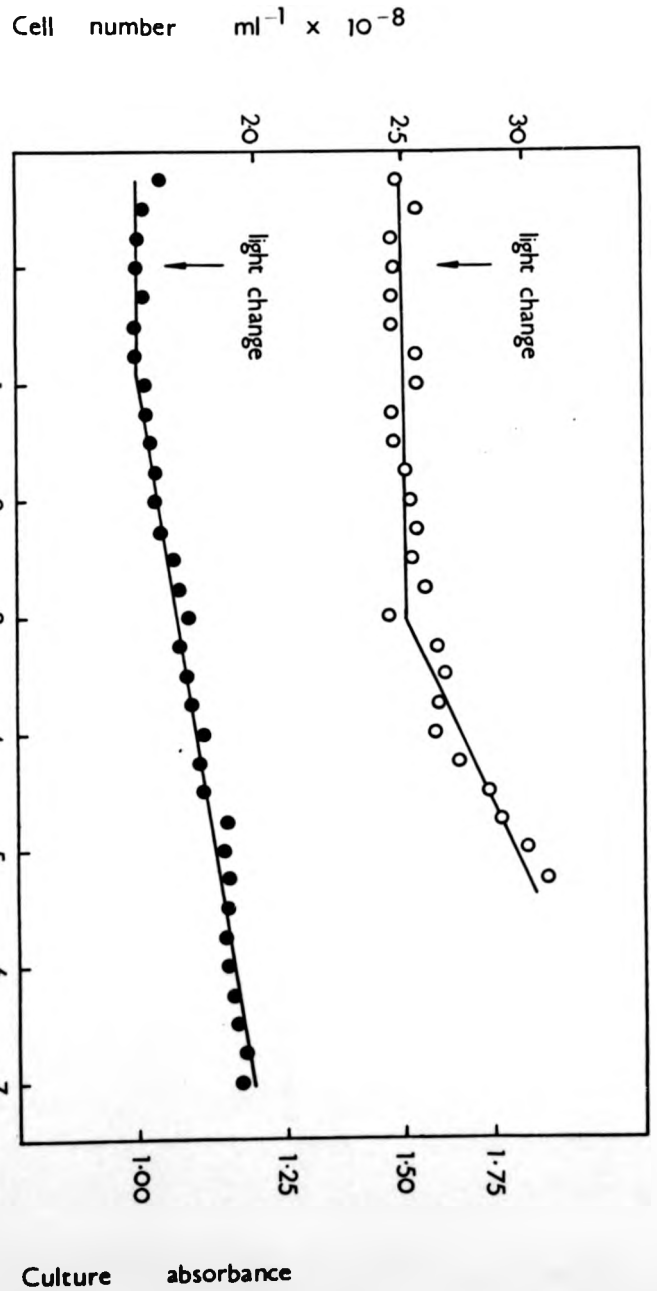

Cell number ml⁻¹ × 10⁻⁸
30
25
20
light change
light change
0
1
2
3
4
5
6
7
Time (h)
1·75
1·50
1·25
1·00
Culture absorbance

Figure 6.10.

The increase in culture absorbance (●) and cell number (○) of steady state light-limited culture of Anacystis nidulans grown at a growth rate of 0.10 h^{-1}, during a "shift-up" induced by increasing the incident light intensity at t = 0.

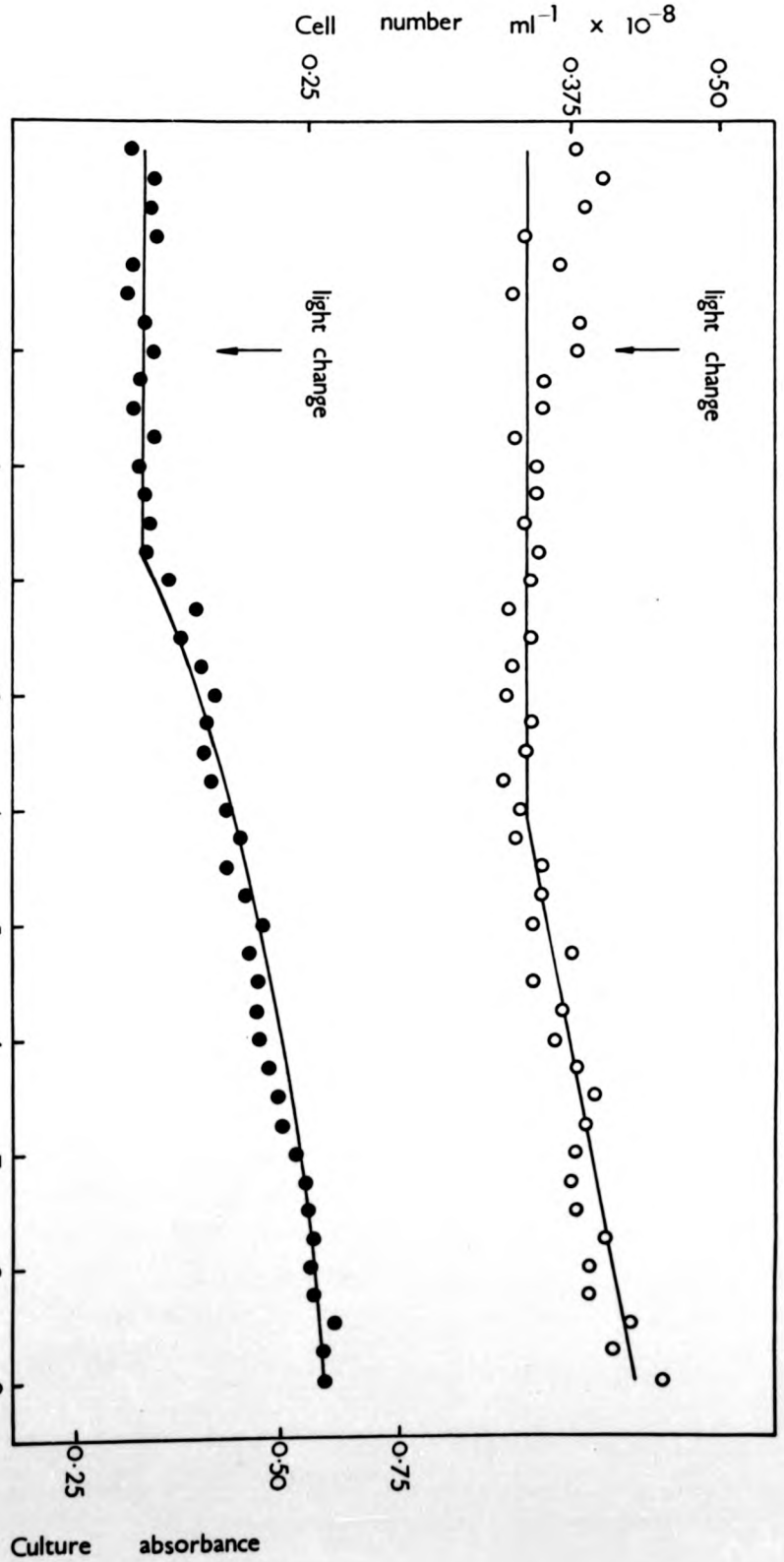
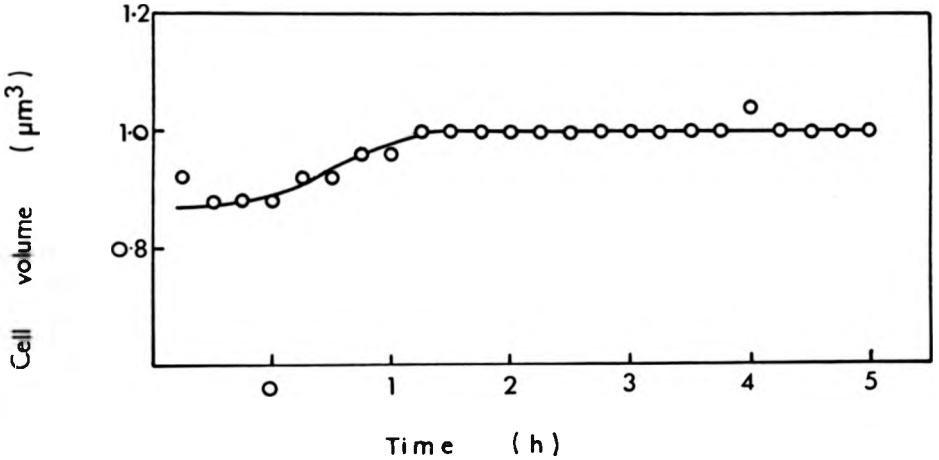

Cell number ml^{-1} x 10^{-8}
0·25
0·375
0·50
light change
light change
0
1
2
3
4
5
6
7
8
9
Time (h)
0·25
0·50
0·75
Culture absorbance

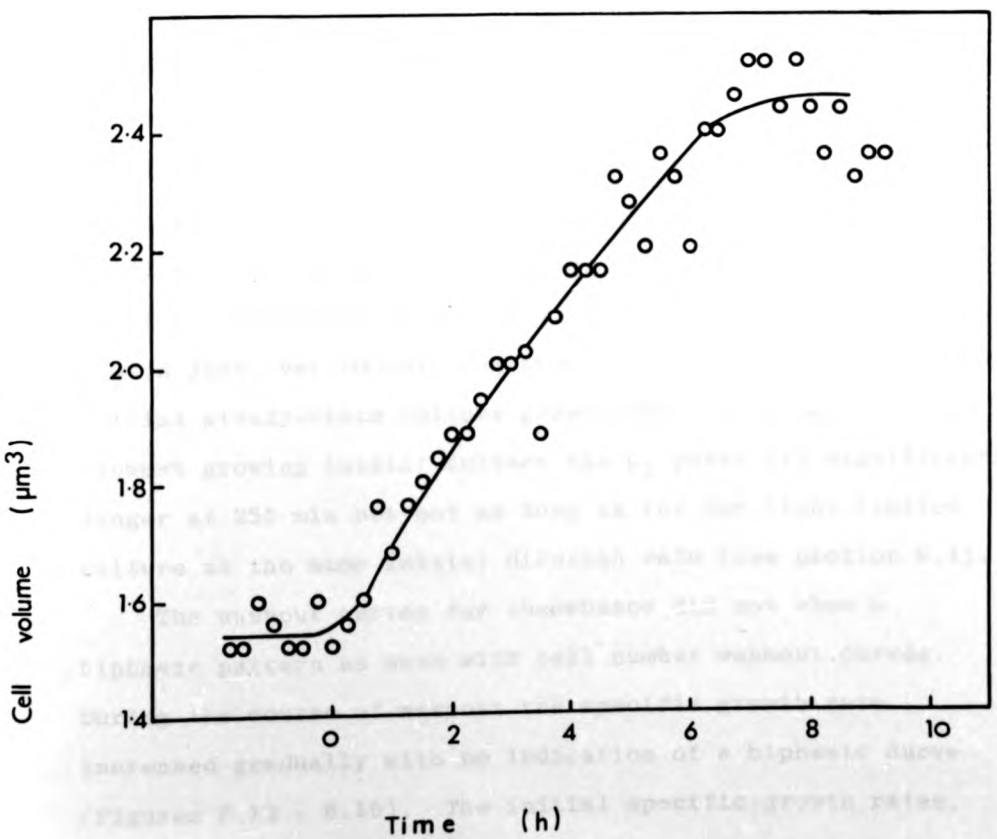
Figure 6.11.

The change of the mean cell volumes during a "shift-up" induced by increasing the incident light intensity at t = 0 for steady state light-limited culture grown at D = 0.03 h^{-1}.

Figure 6.12.

The change of the mean cell volumes during a "shift-up" induced by increasing the incident light intensity at t = 0 for steady state light-limited culture grown at D = 0.10 h^{-1}.


1·2
1·0
0·8
Cell volume (μm³)
0
1
2
3
4
5
Time (h)


2·4
2·2
2·0
1·8
1·6
1·4
Cell volume (μm³)
0
2
4
6
8
10
Time (h)

increase in cell volume in cultures grown at D = 0.10 h^{-1} (Figure 6.12).

6.2. CARBON DIOXIDE-LIMITED CHEMOSTAT CULTURE

Results similar to those for light-limited cultures were obtained for the growth of Anacystis nidulans during washout from three different initial steady state carbon dioxide-limited cultures.

The cell number washout curves were also biphasic and different from the absorbance washout curves (Figures 6.13 - 6.15). In all cases the specific growth rate of the first phase, μ_1, was equal to the initial dilution rate and less than the μ_2 phase (Table 6.2). The different μ_2 values shown in Table 6.2 were the result of the different incident light intensity used for each case. For cultures grown at D = 0.03 h^{-1} the light intensity was 296W, for cultures grown at D = 0.10 h^{-1} the illumination was 396W and for cultures grown at D = 0.17 h^{-1}, 596W. The μ_1 phase lasted just over 200 min and seemed to be independent of the initial steady-state culture growth rate, although for the slowest growing initial culture the μ_1 phase was significantly longer at 255 min but not as long as for the light-limited culture at the same initial dilution rate (see section 6.1).

The washout curves for absorbance did not show a biphasic pattern as seen with cell number washout curves. During the course of washout the specific growth rate increased gradually with no indication of a biphasic curve (Figures 6.13 - 6.15). The initial specific growth rates,

Figure 6.13.

The washout curves in terms of cell number (O) and absorbance (●) for a carbon dioxide-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.27 h^{-1}.

Figure 6.13.

The washout curves in terms of cell number (O) and absorbance (●) for a carbon dioxide-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.27 h^{-1}.

er (O) and
ted culture
3 h^{-1}. At
sly

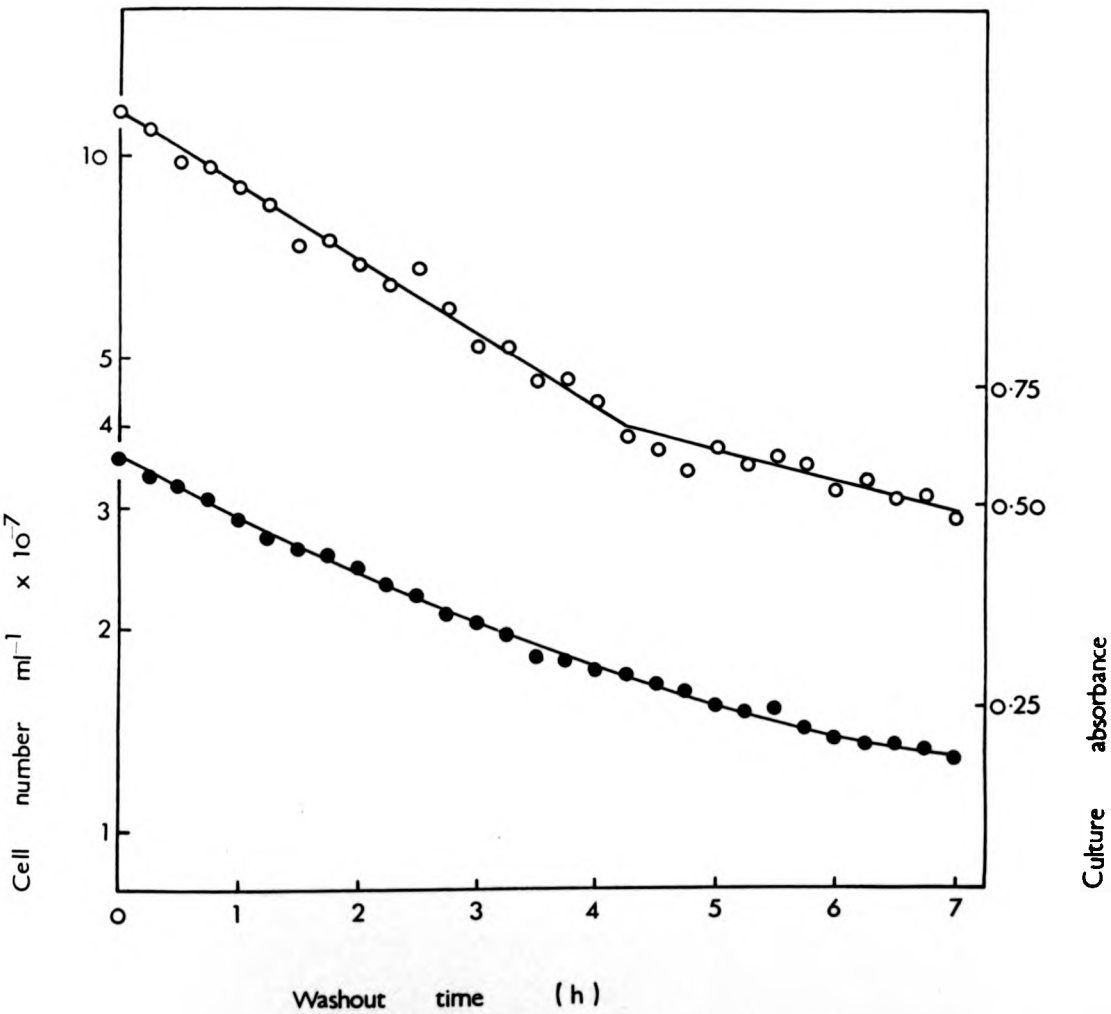


Figure 6.14.

The washout curves in terms of cell number (O) and absorbance (●) for a carbon dioxide-limited culture grown at an initial dilution rate of 0.10 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.28 h^{-1}.

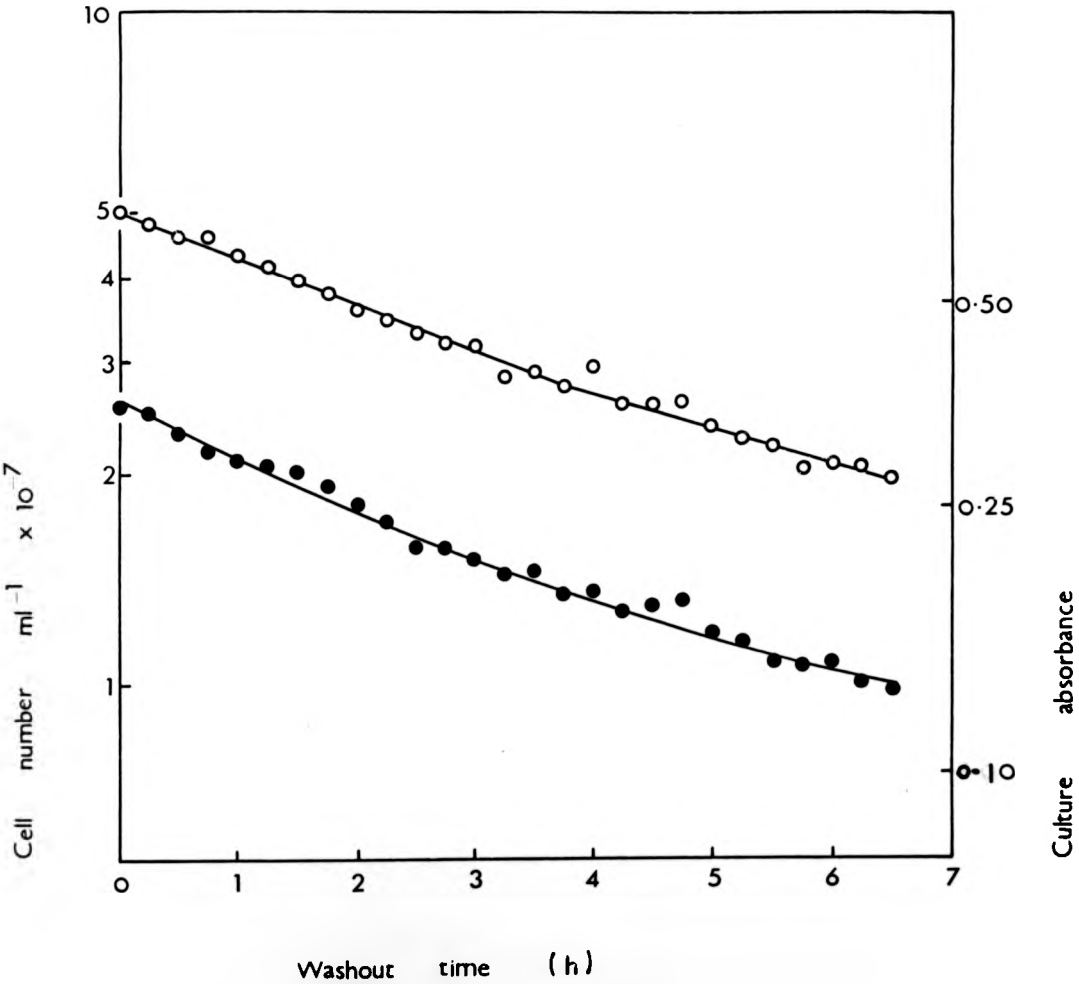

10
5
4
3
2
1
0
1
2
3
4
5
6
7
0·50
0·25
0·10
Cell number ml⁻¹ x 10⁻⁷
Culture absorbance
Washout time (h)

Figure 6.15.

The washout curves in terms of cell number (O) and absorbance (●) for a carbon dioxide-limited culture grown at an initial dilution rate of 0.17 h^{-1}. At t = O the dilution rate was instantaneously increased to D = 0.28 h^{-1}.

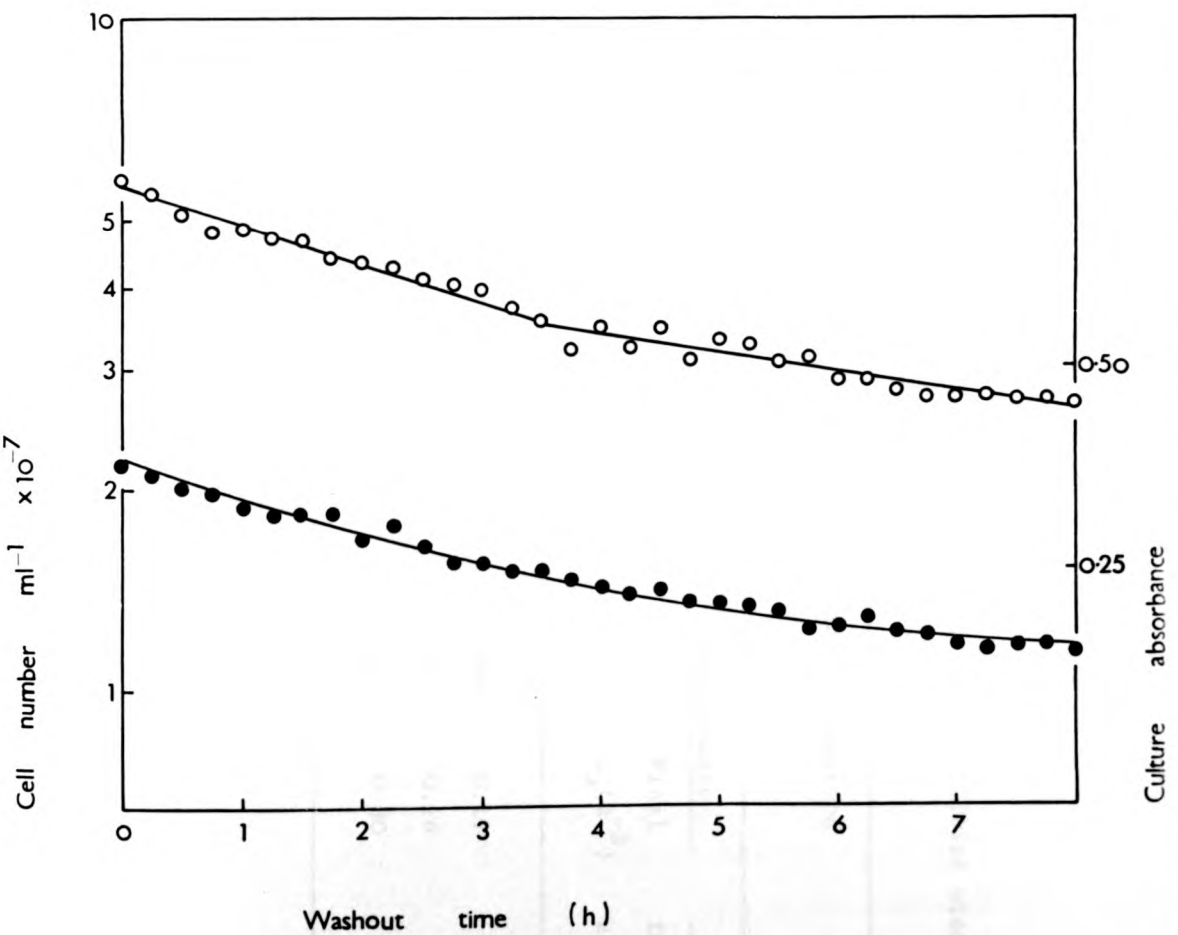

10
5
4
3
2
1
0
1
2
3
4
5
6
7
Cell number ml⁻¹ x 10⁻⁷
Washout time (h)
0·50
0·25
Culture absorbance

Table 6.2. The values of transient washout parameters from various initial steady state carbon dioxide-limited cultures

Initial steady state culture conditions			Transient washout parameters					
				Absorbance		Cell number		
Dilution rate (h^{-1})	Absorbance (600 nm)	Cell number (ml^{-1} 10^{-7})	Washout dilution rate (h^{-1})	Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-2})$	Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-2})$	Length μ_1 phase (min)
0.03	0.52	11.6	0.27	0.09	0.17	0.03	0.17	255
0.10	0.35	5.0	0.28	0.11	0.16	0.11	0.16	215
0.17	0.33	3.0	0.28	0.16	0.20	0.15	0.22	220

[α] Higher incident light intensity than preceding CO_2-limited cultures.

μ_1, measured over the first 1 to 2 h were always less than μ_{max} (Table 6.2). For organisms grown at D = 0.03 h^{-1} initial dilution rate the specific growth rate μ_1 was greater than the cell number μ_1 phase value whilst for organisms grown at D = 0.10 h^{-1} and D = 0.17 h^{-1} initial dilution rates the μ_1 phase values were equal to the cell number μ_1 phase values (Table 6.2). There was an immediate 3 fold increase in growth rate for the slower growing culture compared with the steady state growth rate. By the time the cell number curves had reached μ_{max} values, the absorbance curves had also attained final μ_2 growth rates equal to the expected μ_{max} value for these conditions (Table 6.2). The difference between the absorbance washout curves and those of cell number was probably due to the additional influence on the absorbance measurements due to changes in cell volume. The mean cell volumes determined from Coulter Counter measurements showed that cell volumes began to increase within the first one hour after the shift to washout dilution rates, an effect which was most apparent with the slow growing cultures (Figure 6.16). Therefore the mean cell volumes increased 1.4 fold for slow growing cultures, an increase which was 2 times lower compared with slow growing light-limited cultures (section 6.1). The mean cell volumes for organisms grown at initial growth rate of 0.10 h^{-1} and 0.17 h^{-1} remained relatively constant (Figures 6.17 and 6.18).

μ_1, measured over the first 1 to 2 h were always less than μ_{max} (Table 6.2). For organisms grown at D = 0.03 h^{-1} initial dilution rate the specific growth rate μ_1 was greater than the cell number μ_1 phase value whilst for organisms grown at D = 0.10 h^{-1} and D = 0.17 h^{-1} initial dilution rates the μ_1 phase values were equal to the cell number μ_1 phase values (Table 6.2). There was an immediate 3 fold increase in growth rate for the slower growing culture compared with the steady state growth rate. By the time the cell number curves had reached μ_{max} values, the absorbance curves had also attained final μ_2 growth rates equal to the expected μ_{max} value for these conditions (Table 6.2). The difference between the absorbance washout curves and those of cell number was probably due to the additional influence on the absorbance measurements due to changes in cell volume. The mean cell volumes determined from Coulter Counter measurements showed that cell volumes began to increase within the first one hour after the shift to washout dilution rates, an effect which was most apparent with the slow growing cultures (Figure 6.16). Therefore the mean cell volumes increased 1.4 fold for slow growing cultures, an increase which was 2 times lower compared with slow growing light-limited cultures (section 6.1). The mean cell volumes for organisms grown at initial growth rate of 0.10 h^{-1} and 0.17 h^{-1} remained relatively constant (Figures 6.17 and 6.18).

Figure 6.16.

The change of the mean cell volumes during washout growth for a carbon dioxide-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.27 h^{-1}.

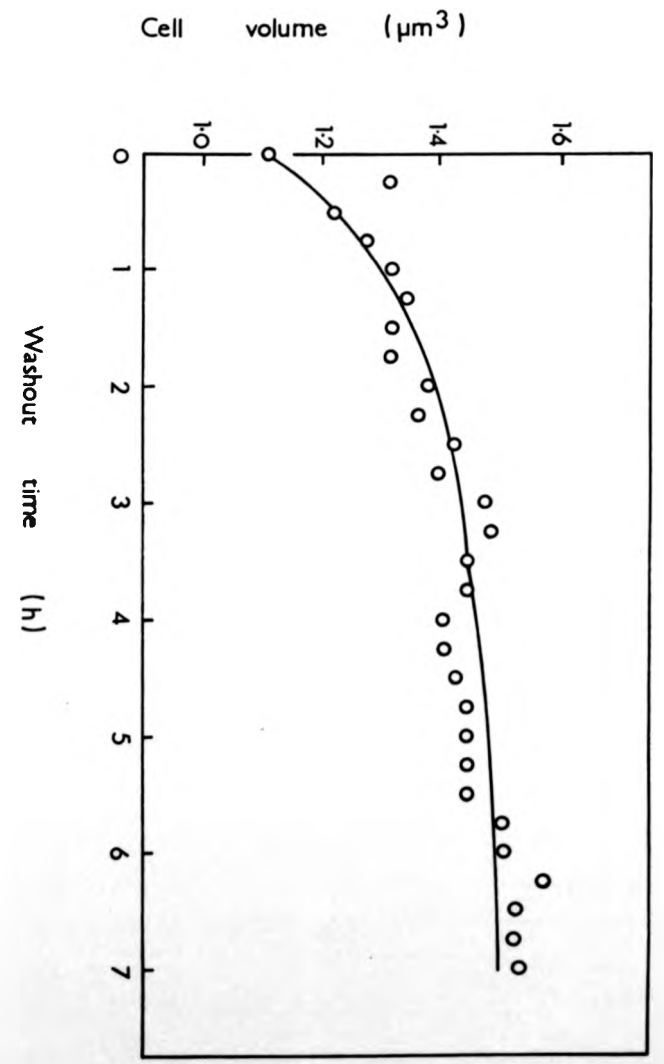
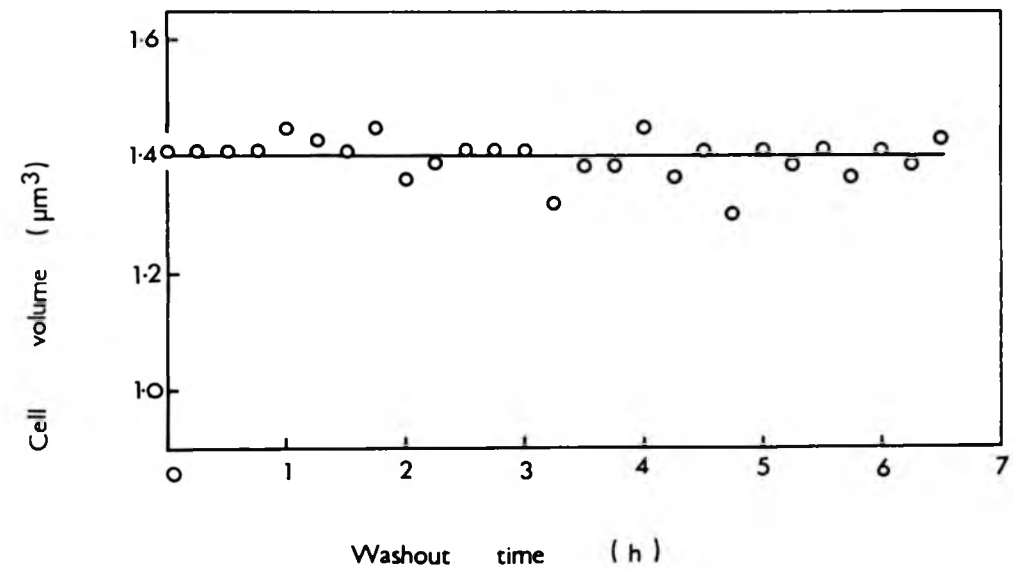

Cell volume (μm3)
1·0
1·2
1·4
1·6
0
1
2
3
4
5
6
7
Washout time (h)

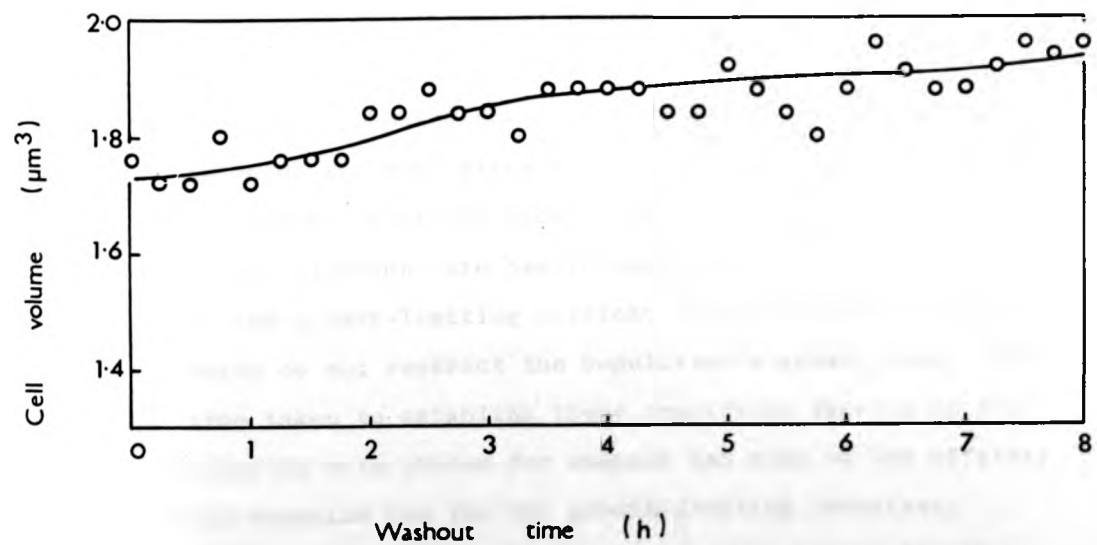
Figure 6.17.

The change of the mean cell volumes during washout growth for a carbon dioxide-limited culture grown at an initial dilution rate of 0.10 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.28 h^{-1}.

Figure 6.18.

The change of the mean cell volumes during washout growth for a carbon dioxide-limited culture grown at an initial dilution rate of 0.17 h^{-1}. At t = 0 the dilution rate was instantaneously increased to D = 0.28 h^{-1}.


1·6
1·4
1·2
1·0
Cell volume (μm3)
0
1
2
3
4
5
6
7
Washout time (h)


2·0
1·8
1·6
1·4
Cell volume (μm3)
0
1
2
3
4
5
6
7
8
Washout time (h)

DISCUSSION

The chemostat washout procedure adopted in these experiments was the conventional method used to determine μ_{max} values by continuous-flow culture washout. For Anacystis nidulans under these growth conditions, the initial stages of culture washout did not give a specific growth rate which was equal to the expected μ_{max} value and therefore were not reliable for determing μ_{max} values during the initial stages of culture washout. Two reasons could account for the inability of Anacystis nidulans to grow at a μ_{max} value from the outset of culture washout.

Firstly it is normally assumed that simply by increasing the dilution rate of the growth system, the conditions within the growth vessel immediately, or at least very quickly, are optimised for growth at μ_{max} for the prevailing physico-chemical conditions. For growth systems in which the population's growth rate is controlled by the concentration of one growth-limiting substrate, an increase in the dilution rate has to ensure that the concentration of the growth-limiting nutrient rises rapidly to levels which do not restrict the population's growth rate. The time taken to establish these conditions depends on the dilution rate chosen for washout and also on the affinity the organism has for the growth-limiting substrate. Organisms, with a high affinity for the growth-limiting substrate, or in other words those with low saturation

constants, require a relatively small increase in the growth-limiting substrate concentration, hence a short period of washout time before growth at or close to μ_{max} is possible. Conversely, for organisms with a low growth-limiting substrate affinity much greater substrate concentration increase would be needed to produce high specific growth rates; clearly a significant time period could elapse in this case and could have a marked effect on the washout kinetics.

These saturation constants for carbon dioxide (bicarbonate) or light have not reported for Anacystis nidulans. In an attempt to determine the saturation constant for bicarbonate, a value was calculated from the observed biomass concentration and a knowledge of the elemental composition. The value obtained, K_s = 2.5 mM, seemed high and may well explain why this initial growth rate during washout was not equal to μ_{max}. Further experiments need to be undertaken in which at the time of increase the dilution rate to the washout rate a simultaneously addition of sodium bicarbonate to growth saturating concentration is made. This ought to eliminate effects due to the build up of concentration of the growth-limiting substrate. If this is not done, may be a long period of time during which the concentration of the growth-limiting substrate builds up to a non-growth-limiting concentration is required and this may have a transient effect on the specific growth rate.

However, the effect of the saturation constants for bicarbonate and light on washout kinetics cannot be fully assessed at this stage. The delay in attaining μ_{max} growth rates could be circumvented if the cultures were saturated with the growth-limiting substrate at the same time as switching to the washout dilution rate. In an experiment to determine whether or not this was the principal reason for the long μ_1 phases observed during the washout of low dilution rate light-limited culture, a low biomass steady state culture at D = 0.03 h^{-1} was simultaneously switched to a washout dilution rate and the incident light intensity increased 5 fold. This culture still took about 550 min to reach growth at μ_{max} values compared with the 615 min for washout by the original method suggesting that possibly factors additional to changes in the concentration of the limiting nutrient influenced the length of the μ_1 phase.

The second reason for growth at rates lower than μ_{max} during the first phase of culture washout may be due to a more fundamental physiological reason. The classical "shift-up", closed culture experiments showed that heterotrophic microorganisms maintained a growth rate which was characteristic of the poor medium for a fixed period of time after the shift-up before changing to an increased specific growth rate characteristic of the richer growth medium (Kjeldgaard, 1961; Maaløe and Kjeldgaard, 1966). The initial post shift-up growth phase represents a transitional stage during which the organisms adapt to growth

in the richer medium, particularly by increasing the rate of chromosomal replication (and ultimately cell division) by increasing the frequency of initiating new rounds of DNA replication. The length of this transitional stage is equal to the sum of the time taken to complete one round of DNA replication and the time between the end of DNA replication and subsequent cell division - a period of time known as C + D time (Cooper and Helmstetter, 1968; Donachie, Jones and Teather, 1973). Continuous-flow culture washout experiments are analogous to closed culture shift-up experiments and so the μ_1 phase is a measure of the C + D time. For carbon dioxide-limited cultures the C + D time was approximately 210 min and appeared to be independent of the initial steady-state culture growth rate, although the length of the μ_1 phase for the slowest growing initial culture was significantly longer, and compares with the previous C + D times determined in closed cultures of about 180 min (Herdman, Faulkner and Carr, 1970; Mann and Carr, 1974). In contrast for light-limited cultures the length of the μ_1 phase was dependent on the initial growth rate of the culture, suggesting that perhaps under these growth conditions some process involved in chromosome replication and/or cell division was growth-rate dependent. This possibility is strengthened by the inability to shorten the length of the μ_1 phase significantly by increasing

the incident light intensity at the same time as switching to a washout dilution rate.

In two experiments to determine the C + D time taken to achieve a new growth rate, for a steady state light-limited culture of Anacystis nidulans grown at D = 0.03 h^{-1} and D = 0.10 h^{-1} the incident light intensity was increased 12 fold at t = 0. The C + D time was approximately 180 min for the steady state culture grown at D = 0.03 h^{-1} and this is in agreement comparing with the previous C + D time determined in closed cultures by Mann and Carr (1974) whilst for the steady state culture grown at D = 0.10 h^{-1} the C + D time was as long as 240 min.

The distribution of the cell volumes in light-limited cultures was between 0.8 and 2.52 μm^3 over the dilution rate range examined and for carbon dioxide-limited cultures was between 1.1 and 1.96 μm^3. These changes in mean cell volumes were within the distribution of cell volumes (between 0.37 and 4.57 μm^3) observed by Mann and Carr (1974) for Anacystis nidulans grown in closed cultures.

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APPENDIX

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GROWTH OF THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS* DURING WASHOUT FROM LIGHT- AND CARBON DIOXIDE-LIMITED CHEMOSTATS

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1. Introduction

For microorganisms growing in continuous-flow culture systems, the organism's specific growth rate reaches a maximum at the critical dilution rate, D_{crit}. D_{crit} is a good approximation to the maximum specific growth rate, μ_{max}, for the organism under the prevailing conditions provided that the concentration of the growth-limiting substrate in the inflowing medium is considerably greater than the organism's saturation constant for the limiting nutrient. At dilution rates above D_{crit} steady-state conditions are unobtainable and, in theory, the organism should grow at μ_{max} during the transient conditions which occur during washout from the growth vessel. Washout kinetics have been used to determine μ_{max} values [1–3] according to the relationship:

$$\mu_{max} = \frac{(\ln x_2 - \ln x_1)}{(t_2 - t_1)} + D$$

where x_1 is the initial steady state organism concentration at time t_1 and x_2 is the organism concentration at time t_2 after an instantaneous change in the dilution rate to a value $D > \mu_{max}$ at t_1.

Some attention has been paid to the factors which may influence the μ_{max} values determined during washout; for example, wall growth in the culture vessel [4] may lead to erroneously high values. Moreover the technique depends on the organism's immediate capacity to grow at its maximum rate during washout and in some cases this does not seem to be true. Jannasch [2] described some experiments in which the organisms either ceased to grow or washed out at rates in excess of the μ_{max} rate suggesting that cell lysis and death was responsible for accelerating the rate of culture washout. It has also been proposed that non-growing culture washout could be due to population density effects [5]. It has been shown for *Escherichia coli* that growth at μ_{max} may not always be physiologically attainable in the first stages of culture washout, particularly for large changes in the dilution rate [6,7].

This communication describes the pattern of growth of the blue-green alga, *Anacystis nidulans*, during washout from various steady state cultures with either light or carbon dioxide as the growth-limiting nutrient.

2. Materials and Methods

The organism used in this study was *A. nidulans* (strain number 625, Indiana University Culture Collection and obtained from N.G. Carr) and stock cultures were maintained as previously described [8]. The details of the light-limited chemostat, growth media, inoculum growth and growth conditions were as previously described [9] except that the cultures were illuminated from two sides by a total of 18 8W Warm White fluorescent tubes placed 10 cm away from the culture vessel.

A similar system was used for the carbon dioxide-limited chemostat with the addition of pH monitoring and controlling facilities (EIL Model 9150 pH controller) which maintained the culture at pH 7.5. In most experiments the culture was illuminated by 12 8W Warm White fluorescent tubes but in some experiments additional illumination was used. The

growth medium was a modified Kratz and Myers' Medium C [9,10] supplemented with sodium bicarbonate to give a final concentration of 0.42 g l^{-1}. Under these conditions carbon dioxide (bicarbonate) was the limiting nutrient.

Culture washout was established by a stepwise increase in the dilution rate to a value greater than the expected maximum specific growth rate. There was no concomitant saturation of the growth-limiting nutrient either by the addition of excess bicarbonate or by an increase of the incident light intensity.

Culture absorbance was measured in a Unicam SP600 spectrophotometer in 1 cm light path cuvettes at 600 nm. Samples for cell number determination were fixed in an equal volume of 2.5% (v/v) gluteraldehyde solution and the cell number was determined in a Model ZBI Coulter Counter.

3. Results

The growth of *Anacystis nidulans* during washout from three different initial steady state cultures for both light limited and carbon dioxide-limited chemostats was measured in terms of culture absorbance and cell number. None of the cultures, assessed by either absorbance or cell number, showed the theoretical pattern of immediate and continuous exponential growth at a rate equal to μ_{max} after changing to a culture washout dilution rate. Furthermore difference existed between the washout curves for absorbance and cell number for the same culture.

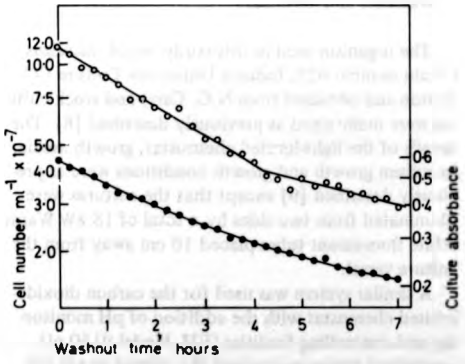

Fig. 1. The washout curves in terms of cell number (○) and absorbance (●) for a carbon dioxide-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At $t = 0$ the dilution rate was instantaneously increased to 0.27 h^{-1}.

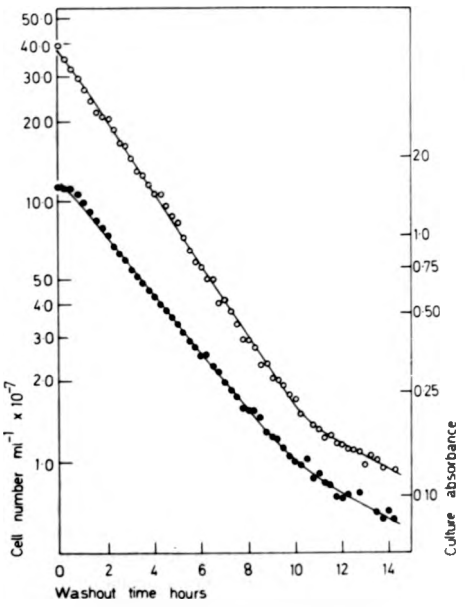

Fig. 2. The washout curves in terms of cell number (○) and absorbance (●) for a light-limited culture grown at an initial dilution rate of 0.03 h^{-1}. At $t = 0$ the dilution rate was instantaneously increased to 0.37 h^{-1}.

Cell number washout curves for both limitations were biphasic showing a marked change in the organism's specific growth rate over a relatively short period of time during washout growth (Figs. 1 and 2). In all cases the specific growth rate of the first phase, μ_1, was less than the specific growth rate of the second phase, μ_2 (Table 1), where the rates were determined from the coefficients of regression line analysis. For both growth-limiting conditions the values of μ_1 were variable, declining with decreases in the initial growth rate of the culture prior to washout. Indeed, within the limits of experimental error,

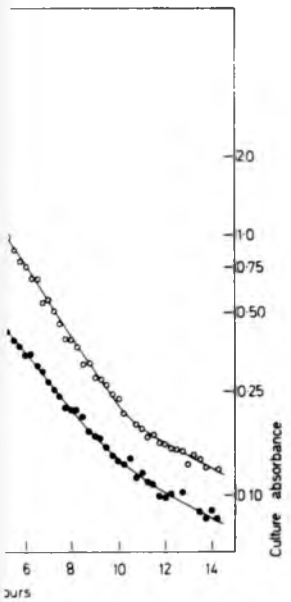

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TABLE 1

The values of transient washout parameters from various initial steady-state light-limited and carbon dioxide-limited cultures

Culture limitation	Initial steady-state culture conditions			Transient washout parameters					
				Washout dilution rate (h^{-1})	Absorbance		Cell number		
	Dilution rate (h^{-1})	Absorbance A_{600nm}	Cell number $ml^{-1} \cdot 10^{-7}$		Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1}$ –	Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1})$	Length μ_1 phase (min)
Light	0.03	1.54	39.6	0.37	0.10	0.26	0.05	0.26	615
Light	0.08	0.92	23.0	0.32	0.14	–	0.06	–	>390
Light	0.15	0.54	9.3	0.31	0.16	0.24	0.11	0.23	285
CO_2	0.03	0.59	11.6	0.27	0.09	0.17	0.03	0.17	255
CO_2	0.10	0.35	5.0	0.28	0.11	0.16	0.11	0.16	215
CO_2 [a]	0.17	0.33	3.0	0.28	0.16	0.20	0.15	0.22	220

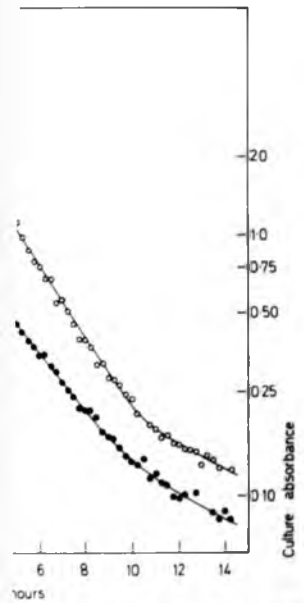
[a] Higher incident light intensity than preceding CO_2-limited cultures.

the μ_1, values were the same as the dilution rates of the preceding steady-state cultures (Table 1). The specific growth rates of the second phase of washout were independent of the initial growth rate and hence the μ_1 phase and were equal to μ_{max} values for the given growth conditions as determined in closed culture experiments. The different μ_2 values shown in Table 1 were the result of the different growth conditions used, particularly the incident light intensity. The length of the μ_1 phase, that is the time taken to reach growth at a μ_{max} rate, depended on the type of growth limitation. For carbon dioxide-limited cultures the μ_1 phase lasted just over 200 min and seemed to be independent of the initial steady-state culture growth rate, although for the slowest growing initial culture the μ_1 phase was significantly longer at 255 min. For the light-limited culture the length of the μ_1 phase increased with declining initial culture dilution rate such that the length of the μ_1 phase for the culture with the slowest initial growth rate examined, $D = 0.03\ h^{-1}$, was approx. 2.5 times longer than that for the fastest growing culture where $D = 0.15\ h^{-1}$.

The washout curves measured by culture absorbance were different to the pattern of the cell number washout curves. During the course of washout the specific growth rate increased gradually usually with no indication of a biphasic curve (Fig. 2) although washout from slow-growing light-limited cultures resulted in a pattern similar to the biphasic cell number washout curves (Fig. 1). The initial specific growth rates, μ_1, measured over the first 1 to 2 h were always less than μ_{max} and equal to or greater than the cell number μ_1 phase values (Table 1). For the slower growing cultures there was an immediate 3- to 4-fold increase compared with the steady state growth rate. By the time the cell number curves had reached μ_{max} values, the absorbance curves had also attained final μ_2 growth rates equal to the expected μ_{max} values for those conditions (Table 1). The adsorbance washout curves were different to the cell number curves probably due to the additional influence on the measurement of the population size due to changes in cell volume. Mean cell volumes determined from Coulter Counter measurements showed that cell volumes began to increase almost immediately after the shift to washout dilution rates, an effect which was most apparent with the slow growing cultures.

4. Discussion

The chemostat washout procedure adopted in these experiments was the conventional method used to determine μ_{max} by continuous-flow culture washout. Clearly, for *Anacystis nidulans* under these growth conditions, the initial stages of culture washout were not reliable for determining μ_{max} values. Two reasons



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	Dilution rate (h^{-1})	Absorbance A_{600nm}	Cell number $ml^{-1} \cdot 10^{-7}$		Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1}$ –	Initial $\mu_1(h^{-1})$	Final $\mu_2(h^{-1})$	Length μ_1 phase (min)
Light	0.03	1.54	39.6	0.37	0.10	0.26	0.05	0.26	615
Light	0.08	0.92	23.0	0.32	0.14	–	0.06	–	>390
Light	0.15	0.54	9.3	0.31	0.16	0.24	0.11	0.23	285
CO_2	0.03	0.59	11.6	0.27	0.09	0.17	0.03	0.17	255
CO_2	0.10	0.35	5.0	0.28	0.11	0.16	0.11	0.16	215
CO_2 [a]	0.17	0.33	3.0	0.28	0.16	0.20	0.15	0.22	220

[a] Higher incident light intensity than preceding CO_2-limited cultures.

the μ_1, values were the same as the dilution rates of the preceding steady-state cultures (Table 1). The specific growth rates of the second phase of washout were independent of the initial growth rate and hence the μ_1 phase and were equal to μ_{max} values for the given growth conditions as determined in closed culture experiments. The different μ_2 values shown in Table 1 were the result of the different growth conditions used, particularly the incident light intensity. The length of the μ_1 phase, that is the time taken to reach growth at a μ_{max} rate, depended on the type of growth limitation. For carbon dioxide-limited cultures the μ_1 phase lasted just over 200 min and seemed to be independent of the initial steady-state culture growth rate, although for the slowest growing initial culture the μ_1 phase was significantly longer at 255 min. For the light-limited culture the length of the μ_1 phase increased with declining initial culture dilution rate such that the length of the μ_1 phase for the culture with the slowest initial growth rate examined, $D = 0.03\ h^{-1}$, was approx. 2.5 times longer than that for the fastest growing culture where $D = 0.15\ h^{-1}$.

The washout curves measured by culture absorbance were different to the pattern of the cell number washout curves. During the course of washout the specific growth rate increased gradually usually with no indication of a biphasic curve (Fig. 2) although washout from slow-growing light-limited cultures resulted in a pattern similar to the biphasic cell number washout curves (Fig. 1). The initial specific growth rates, μ_1, measured over the first 1 to 2 h were always less than μ_{max} and equal to or greater than the cell number μ_1 phase values (Table 1). For the slower growing cultures there was an immediate 3- to 4-fold increase compared with the steady state growth rate. By the time the cell number curves had reached μ_{max} values, the absorbance curves had also attained final μ_2 growth rates equal to the expected μ_{max} values for those conditions (Table 1). The adsorbance washout curves were different to the cell number curves probably due to the additional influence on the measurement of the population size due to changes in cell volume. Mean cell volumes determined from Coulter Counter measurements showed that cell volumes began to increase almost immediately after the shift to washout dilution rates, an effect which was most apparent with the slow growing cultures.

4. Discussion

The chemostat washout procedure adopted in these experiments was the conventional method used to determine μ_{max} by continuous-flow culture washout. Clearly, for *Anacystis nidulans* under these growth conditions, the initial stages of culture washout were not reliable for determining μ_{max} values. Two reasons

could account for the inability of *Anacystis nidulans* to grow at a μ_{max} value for the outset of culture washout both of which are likely to be applicable to washout μ_{max} determinations with other organisms.

Firstly it is normally assumed that simply by increasing the dilution rate of the growth system, the conditions within the growth vessel immediately, or at least very quickly, are optimised for growth at μ_{max} for the prevailing physico-chemical conditions. For growth systems in which the population's growth rate is controlled by the concentration of one growth-limiting substrate, an increase in the dilution rate has to ensure that the concentration of the growth-limiting nutrient rises rapidly to levels which do not restrict the population's growth rate. The time taken to establish these conditions depends on the dilution rate chosen for washout and also on the affinity the organism has for the growth-limiting substrate. Organisms with low saturation constants require a relatively small increase in the growth-limiting substrate concentration, hence a short period of washout time, before growth at or close to μ_{max} is possible. These constants for carbon dioxide (bicarbonate) or light have not been determined or reported for *Anacystis nidulans*, thus their effect on washout kinetics cannot be fully assessed at this stage. However, the delay in attaining μ_{max} growth rates could be circumvented if the cultures were saturated with the growth-limiting substrate at the same time as switching to the washout dilution rate. In an experiment to determine whether or not this was the principal reason for the long μ_1 phases observed during the washout of low dilution rate light-limited culture, a low biomass steady state culture at $D = 0.03\ h^{-1}$ was simultaneously switched to a washout dilution rate and the incident light intensity increased 4-fold. This culture still took about 550 min to reach growth at μ_{max} values compared with the 615 min for washout by the original method suggesting that possibly factors additional to changes in the concentration of the limiting nutrient influenced the length of the μ_1 phase.

The second reason for growth at sub-μ_{max} rates during the first phase of culture washout may be due to a more fundamental physiological reason. The classical "shift-up" closed culture experiments from nutritionally poor to rich growth environments showed that heterotrophic microorganisms maintained a growth rate which was characteristic of the poor medium for a fixed period of time after the shift-up before changing to an increased specific growth rate characteristic of the richer growth medium [11,12]. The initial post shift-up growth phase represents a transitional stage during which the organisms adapt to growth in the richer medium, particularly by increasing the rate of chromosomal replication (and ultimately cell division) by increasing the frequency of initiating new rounds of DNA replication. The length of post shift-up submaximal growth phase is equal to the sum of the time taken to complete one round of DNA replication and the time between the end of DNA replication and cell division – the C + D time [13,14]. Continuous-flow culture washout experiments are analogous to closed culture shift-up experiments and so the μ_1 phase is a measure of the C + D time. For carbon-dioxide limited cultures the C + D time was approx. 210 min and appeared to be independent of the initial steady-state culture growth rate, although the length of the μ_1 phase for the slowest growing initial culture was significantly longer, and compares with the previous C + D times determined in closed cultures of about 180 min [15,16]. In contrast for light-limited cultures the length of the μ_1 phase was dependent on the initial growth rate of the culture, suggesting that perhaps under these growth conditions some process involved in chromosome replication and/or cell division was growth-rate dependent. This possibility is strengthened by the inability to shorten the length of the μ_1 phase significantly by increasing the incident light intensity at the same time as switching to a washout dilution rate and is currently being examined in greater detail.

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