The University of Manitoba

Studies on the Isocitrate Metabolism of *Chlamydomonas segnis* during Photoautotrophic Growth in Mass and Synchronous Culture

by

Samuel Siang-Kiang Foo

A Thesis
Submitted to
The Faculty of Graduate Studies
In Partial Fulfilment
of the Requirements for the Degree of
Doctor of Philosophy

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ABSTRACT

When the Chlorophyte Chlamydomonas segnis was grown photoautotrophically at 11,000 lux and aerated with 5% CO_2 in air (v/v), it metabolised isocitrate via NADP-isocitrate dehydrogenase (NADP-IDH) and isocitrate lyase (ISL). Isocitrate metabolism was 5- 15 times faster via the dehydrogenase rather than the lyase. NADP-IDH had greater affinity for Ds-threo isocitrate with a $Km_{app.}$ = 0.008 mM as compared to ISL which showed a $Km_{app.}$ of 0.1 mM.

Under carbon and nitrogen starvation (manganese and nitrogen deficiencies), ISL was undetectable while NADP-IDH showed similar activity as in normally grown cells. In photoorganotrophic (light + acetate) and mixotrophic (5% CO_2 in air + acetate) cultures, both ISL and IDH were present together with phosphoenolpyruvate carboxylase. The presence of the latter and the absence of malate synthase have indicated that the replenishment of 4C-compounds was achieved in this alga during photosynthesis by β-carboxylation of phosphoenolpyruvate rather than by the glyoxylate cycle.

Whereas substantial activity of succinyl CoA synthetase (succinyl thiokinase) was demonstrated in the algal cell free extracts, δ-aminolevulinic acid (δ-ALA) synthetase was absent. This finding and other observation have led to the

conclusion that succinyl CoA was not metabolised for the synthesis of pyrroles. Therefore, the formation of succinyl CoA may represent an intermediary step in the oxidation of α-ketoglutarate to succinate in the TCA cycle.

The presence of ISL in addition to NADP-IDH provided evidence to support the view that an ancillary pathway for isocitrate metabolism and succinate production via ISL was available in C. segnis when grown under conditions favourable for active protein synthesis and carbohydrate accumulation.

IDH was inhibited by α-ketoglutarate (Ki = 0.76 mM) and oxaloacetate (Ki = 0.34 mM) as well as high energy metabolites viz. NADPH (Ki = 0.06 mM) and ATP (0.65 mM). Glycolate, oxalate, oxaloacetate and α-ketoglutarate are potent inhibitors of ISL. One of the products of ISL, glyoxylate in combination with oxaloacetate exerted a concerted inhibition (Ki = 0.01 mM) on IDH.

Therefore, isocitrate metabolism in C. segnis is subject to regulation by the intermediates of the TCA cycle and products of ISL.

The patterns of biosynthetic processes (protein, δ-ALA, RNA, DNA and chlorophyll synthesis, photosynthetic CO_2 fixation and glycolate formation) and the levels of enzyme activities (NADP-IDH, ISL and NAD(P) glutamate

dehydrogenase) as well as the respiration rate have been studied in synchronized cells. The results have provided evidence that NADP-IDH and ISL in C. segnis were probably involved in the synthesis of metabolites and macromolecules which require a high NADPH/ATP ratio in the cell.

Inhibition of isocitrate metabolism by addition of monofluoroacetate (inhibitor of aconitase) during the S-phase, stimulated DNA synthesis. This has indicated that isocitrate metabolism might be implicated in the regulation of DNA synthesis.

This thesis is affectionately dedicated to my wife, my mother and father, brothers and sisters, who believe that the betterment of oneself through the acquirement of knowledge is the most valuable asset one can have in this world.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate
POPOP	1, 4-Bis (2-5) phenyloxazoylbenzene
PPO	2, 5-Diphenyloxazole
IDH	Isocitrate dehydrogenase
ISL	Isocitrate lyase
GDH	Glutamate dehydrogenase
δ-ALA	δ-amino levulinic acid
CoA	Coenzyme A
CMU	3-(p-chlorophenyl)-1, 1-dimethyl urea
PHMS	2-pyridylhydroxymethanosulfonate
DEAE	Diethylaminoethyl cellulose
MFA	Monofluoroacetic acid
EDTA	Ethylenediaminetetraacetic acid
Tris-HCl	Tris (hydroxymethyl) HCl
MES	2-(N-morpholino) ethanesulfonic acid
PEM	Phosphate (0.1 M, pH 7.0) containing 1 mM EDTA and 1 mM mercaptoethanol
G	Mean doubling time of the cell in hours

INTRODUCTION

Isocitrate lyase (EC 4.1.3.1) has been shown to occur in various unicellular algae when forced to grow on acetate as sole carbon source (Syrett, 1963, 1966; Haigh & Beevers, 1964; Wiessner and Kuhl, 1962; Wiessner, 1963, 1968) and these authors concluded that the enzyme was not necessary for autotrophic growth. However, the occurrence of isocitrate lyase has been reported in autotrophically grown green algae; Chlamydomonas (Badour & Waygood, 1971b; Foo et al, 1971; Badour et al, 1972), Chlamydobotrys (Wiessner, 1968), Chlorella (Harrop & Kornberg, 1966, Baechtel et al, 1970).

Whereas the role of isocitrate lyase has been elucidated in algae grown on acetate or ethanol, as well as in some obligate photoautotrophic blue greens (Smith, 1973) its function in unicellular autotrophic Chlorophytes is unknown.

Although various comparative kinetic studies have been conducted on isocitrate lyase and NADP-isocitrate dehydrogenase (EC 1.1.1.42) in bacteria, (Ozaki & Skiio, 1968 and Shiio and Ozaki, 1968) and the protozoan, Tetrahymena (Levy, 1972), no such comparative studies to our knowledge have yet been conducted on unicellular green algae. Therefore it was felt necessary to conduct similar studies on C. segnis grown photoautotrophically in an attempt to provide some evidence for the role of both enzymes in algal

metabolism. This is of interest since isocitrate lyase and NADP-isocitrate dehydrogenase occupy a pivotal position at the branch point of the TCA cycle and the glyoxylate bypass.

The physiological and biochemical studies embodied in the present thesis, were undertaken to provide evidence that isocitrate lyase might function as an ancillary pathway for isocitrate metabolism when the alga was grown with plentiful carbon supply (i.e. 5% CO_2 in air or 15 mM acetate). Under these conditions, isocitrate lyase might serve as a key enzyme in an alternative pathway for isocitrate metabolism if NADP-isocitrate dehydrogenase is inhibited.

The effect of monofluoroacetate (inhibitor of isocitrate synthesis) on DNA synthesis has been studied during the life cycle in synchronous cultures.

LITERATURE REVIEW

Advances made during the last few decades in the field of plant physiology were most remarkable and extensive especially in the area of metabolism. Not only was the construction of detailed metabolic maps and their substantiation in plant tissues achieved, but attention has naturally become increasingly focused on questions of how regulation is imposed; how the sequences are compartmented among the various organelles; how individual pathways are shut off or brought into play at appropriate times; how traffic at important branch points is controlled; and how the pace of central sequences is regulated and co-ordinated with other events in the cell (Beevers, 1974). For comprehensive reviews on regulatory metabolisms in plants, the reader is referred to the articles by Laties (1957, 1963), Preiss & Kosuge (1970), and Thomas et al (1973). The objective of this literature review is to summarise some of the works and aspects relevant to the regulation and possible role of isocitrate metabolism in microalgae. This consists mainly of results obtained from kinetic studies and physiological observations made in cells growing and developing under various environmental conditions.

General Considerations

Isocitric acid, a key tricarboxylic acid is metabolised by most microorganisms via NAD(P)-specific isocitrate dehydrogenase

and isocitrate lyase. The former enzyme primarily functions to provide α-ketoglutarate for biosynthetic processes as well as for production of energy via the tricarboxylic acid cycle (TCA cycle). But when microalgae are forced to grow on two carbon substrates, such as acetate or ethanol, isocitrate lyase is induced and in presence of malate synthase the glyoxylate cycle becomes functional so that the organisms can grow (Haigh & Beevers, 1964). Isocitrate lyase catalyses the cleavage of isocitric acid to succinate and glyoxylate. Malate synthase converts glyoxylate and acetyl CoA to malate which can serve to replenish the withdrawal of any tricarboxylic acid cycle intermediates.

The operation of a classical tricarboxylic acid cycle in green algae was conclusively established by Marsh et al, 1965 who fed Scenedesmus obliquus with acetate-1 or -2-^{14}C and pyruvate-3-^{14}C. The determination of the rates of equilibration of ^{14}C in the carboxyl carbons of TCA cycle intermediates showed that there were no differences between those in the dark and in light saturating conditions for photosynthesis. However, Pearce et al (1969) have reported that the blue green alga Anabaena variabilis possesses an incomplete TCA cycle (see Fig. 1) with a block at the steps of α-ketoglutarate dehydrogenase and succinyl CoA synthetase. The production of succinyl CoA for tetrapyrrole biosynthesis in this case was circumvented by the catalytic action of 3-keto acid CoA transferase. This enzyme catalyses the synthesis of succinyl CoA from succinate using

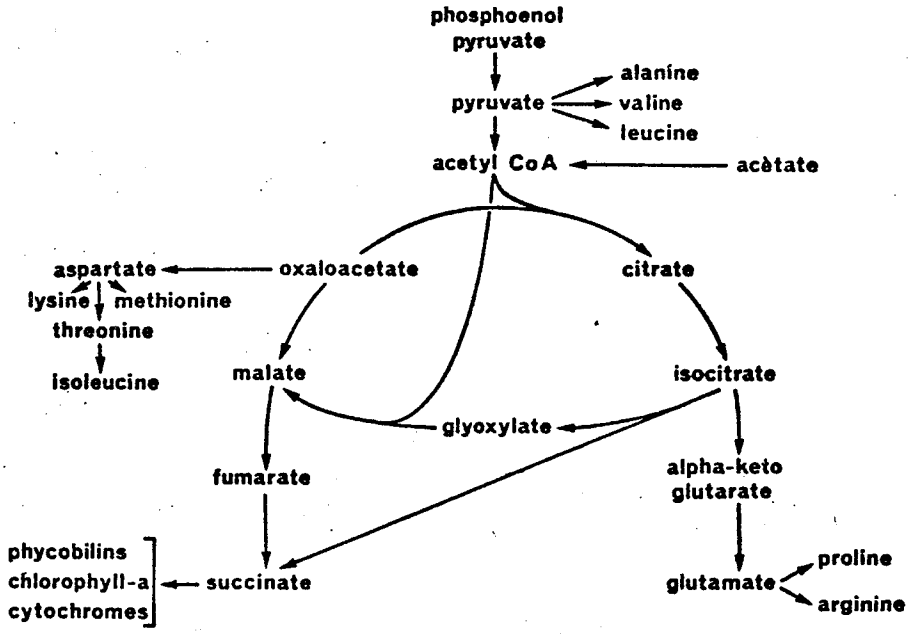
acetoacyl CoA as donor for the coenzyme A moeity. Pearce and Carr (1967) suggested that succinate could have been provided by isocitrate lyase and malate synthase.

Effect of Nutrition and Light on the Activities of Isocitrate Enzymes in Algae

Wiessner & Kuhl (1962) and Syrett et al (1963) have shown that isocitrate lyase and malate synthase activities increased in cells incubated with acetate in darkness as compared to cells placed in light with CO_2 or acetate as the C-source. On the other hand, citrate synthase, NADP-isocitrate dehydrogenase and NAD-malate dehydrogenase activities remained practically unchanged.

Haigh & Beevers (1964) surveyed a number of algal species for isocitrate lyase and concluded that acetate or some complex organic compounds were necessary to induce the synthesis of the enzyme as in bacteria. In other words isocitrate lyase was not necessary for autotrophic growth. However, the detection of substantial activities of isocitrate lyase has been reported in autotrophically grown green algae, namely Chlamydobotrys (Wiessner, 1968), Chlamydomonas segnis (Badour & Waygood, 1971b) and Chlorella vulgaris (Baechtel et al, 1970). In such autotrophic cultures, 1-5% CO_2 in air (v/v) was provided. Isocitrate lyase was undetectable in cells secured from cultures of Chlamydomonas segnis bubbled with air (0.03% CO_2) at relatively high light intensity (Badour &

Fig. 1 Routes for the synthesis of metabolites related to intermediates of the tricarboxylic acid cycle in blue-green algae (after Smith, 1973).


phosphoenol
pyruvate
pyruvate
alanine
valine
leucine
acetyl CoA
acètate
aspartate
oxaloacetate
citrate
lysine
methionine
threonine
isoleucine
malate
isocitrate
glyoxylate
fumarate
alpha-keto
glutarate
phycobilins
chlorophyll-a
cytochromes
succinate
glutamate
proline
arginine

Waygood, 1971b).

According to Matsuka and Hase (1965), isocitrate lyase was required by algae for growth only when acetate was used as sole carbon source and the enzyme was redundant when growth occurred on glucose. Interestingly, addition of glucose to acetate adapted cells of Chlorella pyrenoidosa resulted in a rapid loss of isocitrate lyase activity, and this loss became more pronounced when cells were deprived of nitrogen source (John et al, 1970; Thurston et al, 1973). These authors attributed this loss of activity to protein degradation rather than enzyme inactivation. Moreover, the rate of degradation of isocitrate lyase protein by papain was shown to be unaffected by inhibitors of isocitrate lyase such as pyruvate, oxaloacetate, succinate or phosophoenol pyruvate. But the presence of its substrate, isocitrate at a concentration of 10mM, protected the enzyme from digestion by papain.

Various authors (Wiessner & Kuhl, 1962; Syrett et al 1963; Cook & Carver, 1966; and Goulding & Merrett, 1966) have reported that light may lead to a decrease in the activity of the glyoxylate cycle enzymes whereas the activity of NADP-isocitrate dehydrogenase was not affected. Cook and Carver (1966) suggested that this might be attributed to the presence of photosynthates that would repress the synthesis of isocitrate lyase or inhibit its activity. The fact that the addition of DCMU, (Badour et al, 1972) a potent inhibitor of non-cyclic photophosphorylation can overcome the inhibitory

effects of light by inducing a 'dark type' metabolism with cells placed in the light, provides support for the conclusion of Cook & Carver (1966), and Goulding and Merrett (1966). The synthesis of isocitrate lyase in acetate grown Chlorella cells has been shown to be dependent on ATP produced by photosynthetic cyclic phosphorylation (Syrett, 1966).

Growth of Chlorella in blue instead of red light of equal quantum flux brings about a decrease in carbohydrates, an increase in protein and an enhanced production of RNA (Pirson & Kowallik, 1964). Experiments dealing with $^{14}CO_2$ fixation by Chlorella ellipsoidea cells have shown that aspartic and glutamic acids were preferentially labelled in blue light than under red light (Ogasawara and Miyachi, 1970). The activation of endogenous respiration by blue light has been reported (Kowallik and Gaffron, 1966; and Kowallik, 1967). A direct effect of blue light on carbon metabolism may be its influence upon enzymes containing chromophore groups (Kowallik, 1969; Voskresenskaya, 1972). To our knowledge, no literature is available that relates the influence of light spectrum to the levels of isocitrate lyase and NADP-isocitrate dehydrogenase. It is possible that the activities of these two enzymes are influenced by blue light.

Localization of Isocitrate Lyase and NADP-specific Isocitrate Dehydrogenase

Harrop and Kornberg (1966) found isocitrate lyase to be in the soluble fraction of Chlorella vulgaris after growth on glucose or on CO_2, but acetate-grown cells contained a major portion of their isocitrate lyase in a dense particulate fraction. These authors suggested that isocitrate lyase will participate in the glyoxylate cycle only when it is localised intracellularly in a particulate structure.

Graves et al (1972) found isocitrate lyase, malate synthase, glyoxylate reductase, citrate synthase, and malate dehydrogenase in microbodies of Euglena. On the other hand, Stabenau (1974) demonstrated that isolated microbodies from both autotrophic and heterotrophic cells of Chlorogonium did not contain isocitrate lyase. From experiments with another flagellate, Polytomella, Gerhardt (1971) also concluded that isocitrate lyase, a distinctive marker for glyoxysomes in other microorganisms was not present in microbodies.

Isocitrate dehydrogenase, one of which is NAD dependent is found mainly in the mitochondria (Ernster & Navozio, 1956) while the NADP dependent one is located primarily in the soluble fraction (Self and Weitzman, 1972) of eucaryotes. In the ciliated protozoan, Tetrahymena pyriformis, both isocitrate lyase and NADP-isocitrate dehydrogenase are localised in the peroxisomes (Muller et al, 1966).

Regulation of Isocitrate Metabolism

Control of the tricarboxylic acid cycle and the glyoxylate cycle in microalgae appears to be achieved by a combination of regulatory effectors at both the enzyme and gene level.

Isocitrate lyase, occupying a pivotal position where carbon is apportioned between TCA cycle and the glyoxylate shunt, is expected to be subjected to regulation by intermediary metabolites. John and Syrett (1968) have shown that isocitrate lyase from Chlorella pyrenoidosa was competitively inhibited by oxaloacetate and pyruvate at physiological concentration and non-competitively by phosphoenolpyruvate. These inhibitions occurred at the pH optimum of the enzyme. Sensitivity to these metabolites would regulate the rate at which intermediates of the TCA cycle are diverted into the glyoxylate cycle. By the nature of this mechanism, the overproduction of four carbon organic acid is avoided, and when these acids are drained for biosynthetic processes, the enzyme is relieved from inhibition. This fine control is generally found in all microorganisms and thus provides the cells of a feedback control of a cyclic system by end product inhibition (Umbarger, 1964; and Atkinson, 1969). In addition, in yeast cells there is another regulatory mechanism, that is the isosteric inhibition by 6-phosphogluconate. This 6-C metabolite which is endowed with some structural similarity to isocitrate, can be a suitable feedback signal for the

regulation of the glucogenetic route from acetyl CoA via the glyoxylate cycle (Hanozet & Guerritore, 1972).

On the other hand, NADP-isocitrate dehydrogenase is subjected to concerted inhibition by glyoxylate and oxaloacetate as shown in Chlamydomonas reinhardti (Ramaley & Hudock, 1973), Tetrahymena pyriformis (Levy, 1972), and in various bacteria (Shiio & Ozaki, 1968). These compounds also inhibit the NAD-specific enzyme from Thiobacillus (Hampton & Hanson, 1969), whereas glyoxylate alone stimulates the NADP specific enzyme from Pseudomonas (Hampton & Hanson, 1969). Levy reported that either glyoxylate or oxaloacetate was inhibitory to the NADP specific enzyme from Tetrahymena pyriformis. In addition, ATP has been reported to inhibit the NADP specific enzyme from Rhodospirillum rubrum (Dhillon & Silver, 1972) while NADPH was found to activate the enzyme from Rhodopseudomonas spheroides (Chung & Braginski, 1972).

A second type of control mechanism is mediated via the synthesis of the enzyme itself. Addition of 4-C intermediates such as succinate or a 3-C intermediate such as phosphoenolpyruvate or pyruvate to acetate grown cells, causes in most systems the repression of the glyoxylate cycle enzymes (Pardee, 1959; Kornberg, 1963; Bell, 1967; Wolfson & Krulwich, 1972). John et al (1970), have shown that isocitrate lyase in Chlorella pyrenoidosa was specifically degraded when acetate adapted cells were suspended in

glucose-containing medium. The mechanism, they suggested could involve an increase in the activity of intracellular proteolytic enzymes or an alteration in the susceptibility of isocitrate lyase protein to proteolytic attack.

Some TCA cycle enzymes appear to be induced or derepressed under dark heterotrophic conditions. Hellebust and Lewin (1972) have reported two to threefold increases for malate dehydrogenase, fumarase and succinic dehydrogenase in the diatom Cylindrotheca fusiformis grown in the dark on 3- and 4- carbon organic acids compared to light grown cells. Studies on Euglena gracilis, have shown that the level of NADP-isocitrate dehydrogenase in cells grown on glucose, pyruvate, malate and succinate was about the same as that of autotrophic cells (Cook & Carver, 1966).

Levels of Isocitrate Enzymes during the Cell Cycle

In the cell cycle of microorganisms of bacteria, yeast and algae, some enzymes are continuously synthesised throughout the life cycle, whereas others are synthesised only once per cell cycle, ie. periodically. During cell cycle studies of a thermophilic strain of Chlorella pyrenoidosa, Baechtel et al (1970), have shown that the low basal (uninduced) activity of isocitrate lyase in continuous light remained constant during the first eight hours and then increased in a single step coincident with that of total cellular DNA prior to cell division. Such an increase in basal

activity could not be blocked by cycloheximide (12 ug/ml) which inhibited 87% of total protein accumulation. Therefore this increase might represent activation of pre-existing enzyme or enzyme synthesis on cycloheximide insensitive ribosomes.

In contrast to an 'activation' mechanism, McCullough & John (1972) reported that isocitrate lyase was de novo synthesised during the development of Chlorella pyrenoidosa in synchronous cultures. These authors followed the increases in isocitrate lyase protein using acrylamide gel electrophoresis. The addition of 6-methyl purine (inhibitor of RNA synthesis in algae) to cells adaptively synthesising isocitrate lyase, caused a decay of the rate of enzyme synthesis. This indicated that the synthesis of isocitrate lyase was probably dependent upon the concurrent transcription of an unstable mRNA.

In synchronous cultures of Euglena gracilis, Cook (1971) measured oxygen consumption in the dark and found that it increased fairly rapidly during the light period and levelled off (after a doubling in rate) prior to the dark period. The activities of at least two of the respiratory enzymes showed no clear relationship to the overall pattern of oxygen consumption. On a protein basis, the specific activity of NADP-isocitrate dehydrogenase increased steadily during the light period, then declined to its initial value during the dark period. A similar pattern of NADP-isocitrate de-

hydrogenase was also demonstrated in synchronous cultures of Chlorella pyrenoidosa (Berger, 1966).

Role of Isocitrate Lyase and NADP-specific Isocitrate Dehydrogenase in Metabolism:-

A. Glyoxylate cycle

It is well documented that isocitrate lyase is the key enzyme of the glyoxylate cycle. It operates with malate synthase to form malate and hence the replenishment of 4-C-compounds which are drained for biosynthetic processes (Kornberg and Krebs, 1957; Syrett et al, 1963; Haigh & Beevers, 1964) is achieved. The operation of the glyoxylate cycle is evident in various microalgae when forced to grow on acetate or ethanol (Wiessner & Kuhl, 1962; Wiessner, 1963; Syrett, 1966).

During the germination of fatty seeds, the glyoxylate cycle functions to convert fatty acids to succinate which is subsequently metabolised to sugars (Kornberg and Beevers, 1957; Cooper and Beevers, 1969).

B. Glycerate Pathway

The key enzyme of the glycerate pathway is glyoxylate carboligase (sometimes referred to as glyoxylate carboxy-lyase). The enzyme catalyses the anaerobic condensation of two molecules of glyoxylate to yield one molecule of tartronic semi-aldehyde (3C-compound) with simultaneous production of one

molecule of CO_2 (Gupta & Vennesland, 1966; Gotto & Kornberg, 1961). Tartronic semialdehyde is subsequently reduced by a tartronic semialdehyde reductase (Gotto & Kornberg, 1961) to glycerate which is converted by D-glycerate kinase to 3-phosphoglycerate. Badour & Waygood (1971b) suggested that isocitrate lyase in autotrophically grown Chlamydomonas segnis might provide the glyoxylate required for the formation of glycerate via glyoxylate carboxy-lyase. Under this condition the glyoxylate cycle was probably not operating and the replenishment of 4 C-compounds could be achieved by the β-carboxylation of phosphoenol pyruvate during photosynthesis.

C. Glycolate synthesis

Both higher plants and algae form rapidly labelled glycolic acid during photosynthetic $^{14}CO_2$ fixation (Tolbert, 1963). While it has been established that the source of glycolate formation is derived from an intermediate of the photosynthetic carbon reduction (Gibbs, 1969), other carbon compounds can provide the carbon skeleton of glycolic acid. When ^{14}C-glucose was fed to Chlorella (Whittingham et al, 1963) evidence was obtained that in the light it entered directly into the photosynthetic cycle and led to formation of glycolic acid. The Brannon No. 1 strain of Chlorella growing in the dark on unlabelled glucose and $^{14}CO_2$ accumulated ^{14}C-glycolate but the increase in glycolate specific activity in the presence

of iso-nicotylhydrazide was not accompanied by any significant increase in the total quantity of glycolate excreted (Harrop & Kornberg, 1966). They suggested that $^{14}CO_2$ was incorporated into oxaloacetate first and the isocitrate produced via the action of citrate synthetase and aconitase would yield ^{14}C-glycolate via the catalytic actions of glyoxylate reductase and isocitrate lyase. Recently Zelitch (1972) reported the synthesis of glycolate from acetate-2-^{14}C and pyruvate-3-^{14}C in maize and tobacco leaves. The demonstration of substantial activity of isocitrate lyase in green leaves by Godavari et al (1973) suggests a possible role of this enzyme in the formation of glyoxylate which may then be reduced to glycolate.

D. δ-aminolevulinic acid (δ-ALA)

In animal tissues, bacteria and yeast, δ-ALA is formed by the condensation of succinyl CoA and glycine, catalysed by pyridoxal phosphate requiring enzyme: succinyl CoA-glycine succinyl transferase (δ-ALA synthetase) yielding α-amino-β-ketoadipic acid which is immediately decarboxylated to give δ-ALA and CO_2 (Bottomley & Smithee, 1968; Jordan & Shemin, 1972).

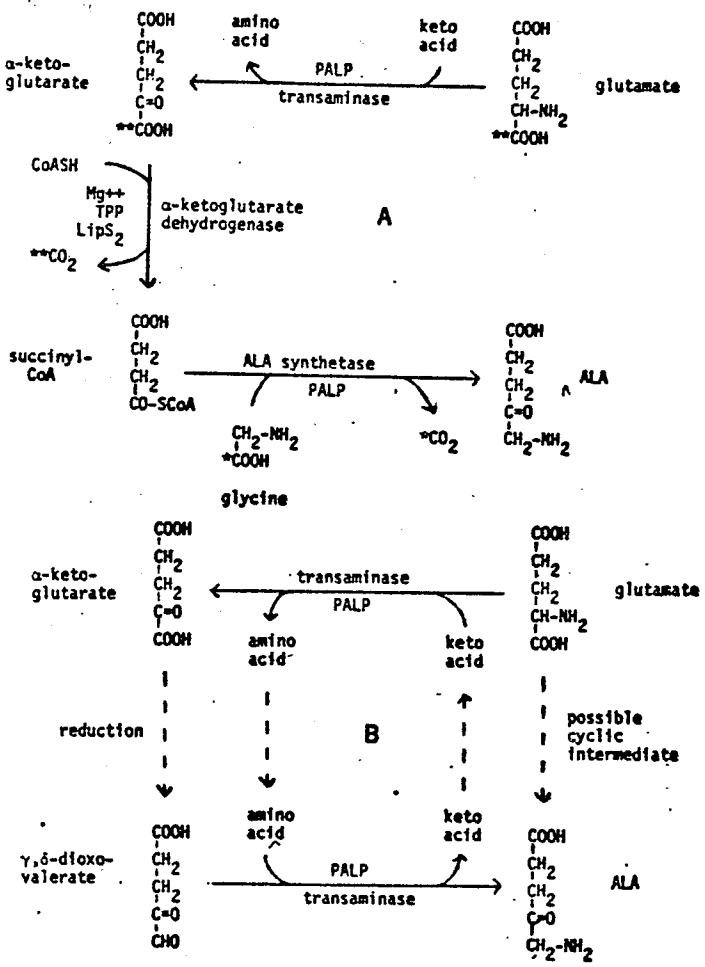
Considerably less is known about the source of δ-ALA in green algae and in higher plants. While it is tempting to suggest that the succinyl CoA and glycine are derived from the products of isocitrate lyase reaction, no unequivocal evidence exists to demonstrate the presence of δ-ALA synthetase

(Miller & Teng, 1967; Wider De Xifera, 1971; Wellburn & Wellburn, 1971; & Ramaswamy & Nair, 1973).

Recently, Beale & Castelfranco (1974), performed feeding experiments with ^{14}C-organic acids on greening cucumber cotyledons, barley and bean leaves, in the presence of levulinic acid, an inhibitor of δ-ALA dehydratase. They found that the most effective labelled precursors were the 5 C-dicarboxylic compounds glutamate, glutamine and α-ketoglutarate while ^{14}C-labelled glycine and succinate were relatively poor. According to the labelling pattern in δ-ALA, they proposed an alternate route for the formation of δ-ALA (see Fig. 2B) in which a transamination reaction with γ, δ-dioxovaleric acid (DOVA) and an amino acid participated. A similar transamination reaction has been demonstrated in Chlorella vulgaris (Gassman et al, 1968). However, some investigators have discounted the importance of this route on the basis that the transaminase levels do not follow changes in chlorophyll biosynthesis. Although the source for DOVA is unknown in plants and algae, it has been demonstrated that it could be formed via 5-hydroxy-4-keto valeric acid, the product of a α-ketoglutarate: glyoxylate carboligase reaction (Wang et al, 1970). These results suggest that α-ketoglutarate, the product of NADP-isocitrate dehydrogenase is a key metabolite in δ-ALA synthesis in green cells.

Fig. 2 A: ALA formation from succinyl CoA and glycine catalyzed by ALA synthetase, and succinyl CoA formation from glutamate or α-ketoglutarate, illustrating the loss of C_1 of glycine, α-ketoglutarate, and glutamate.

B: Hypothetical formation of ALA from glutamate or α-ketoglutarate, either via γ,δ-dioxovalerate or another, unknown route, where the C_1 of glutamate of α-ketoglutarate is incorporated into ALA. $LipS_2$: lipoic acid; PALP: pyridoxyl phosphate; TPP: thiamine pyrophosphate. (after Beale and Castelfranco, 1974).

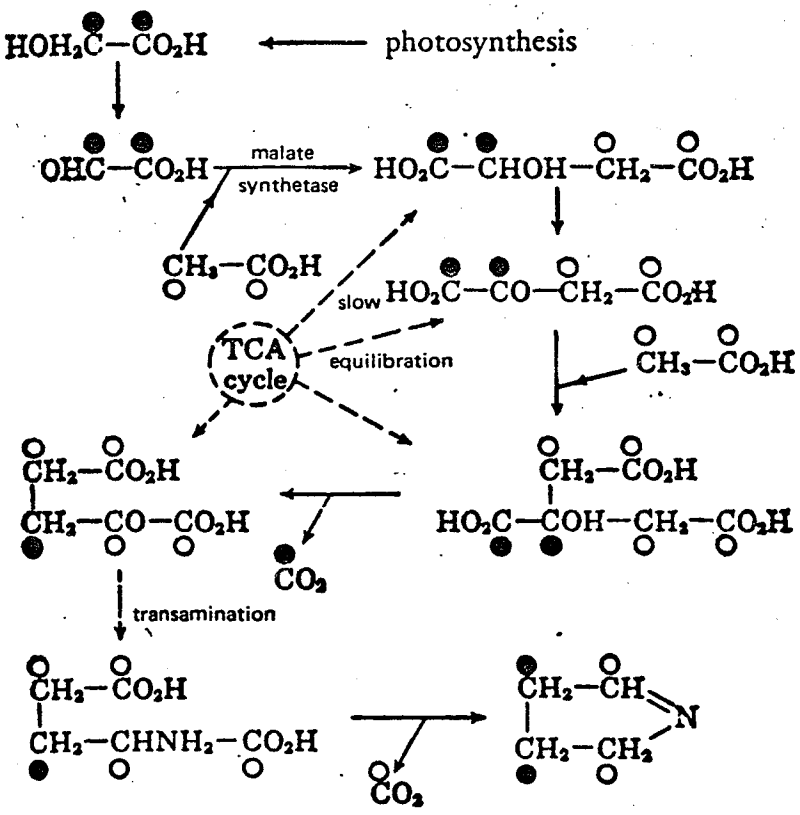

α-keto-glutarate
amino acid
keto acid
PALP
transaminase
glutamate
CoASH
Mg++
TPP
LipS2
α-ketoglutarate dehydrogenase
A
succinyl-CoA
ALA synthetase
PALP
ALA
glycine
α-keto-glutarate
transaminase
PALP
glutamate
amino acid
keto acid
reduction
B
possible cyclic intermediate
γ,δ-dioxo-valerate
amino acid
keto acid
PALP
transaminase
ALA

E. Glutamate Biosynthesis

All eucaryotic systems studied so far possess both NAD^+ and $NADP^+$ linked isocitrate dehydrogenase whereas procaryotic systems have either NAD-isocitrate dehydrogenase or NADP isocitrate dehydrogenase but never both. While most studies have concentrated in various organisms on the regulatory behaviour of the NAD-linked enzyme in eucaryotic cells (Hathaway & Atkinson, 1963; Sanwal et al, 1963, 1964; Plaut, 1970) the NADP linked enzyme has not been assigned a role in metabolic regulation. It is evident, however, that the tricarboxylic cycle intermediates are used for biosynthetic purposes during photosynthetic growth and the NADP-linked isocitrate dehydrogenase may serve to synthetize glutamate (Anderson & Fuller, 1967; Sterns & Bambers, 1966).

A hypothetical pathway for glutamic acid biosynthesis via malate synthase and isocitrate dehydrogenase and citrate synthase (Alworth et al, 1964) is suggested (see Fig. 3). These authors have employed $^{14}CO_2$ in their study of the biosynthesis of nicotine in Nicotiana glutinosa. They isolated and degraded the radioactive nicotine and determined the ^{14}C activity in the pyridine ring. Their conclusion was that the labelling pattern required was not derivable from any of the known glutamate biosynthesis and thus a pathway other than the Krebs cycle would be responsible for glutamate biosynthesis.

Fig. 3 Hypothetical glutamic acid biosynthesis pathway outside the TCA cycle (after Alworth et al, 1964).


HOH2C–CO2H
photosynthesis
OHC–CO2H
malate
synthetase
HO2C–CHOH–CH2–CO2H
CH3–CO2H
slow
HO2C–CO–CH2–CO2H
TCA
cycle
equilibration
CH3–CO2H
CH2–CO2H
CH2–CO–CO2H
CO2
CH2–CO2H
HO2C–COH–CH2–CO2H
transamination
CH2–CO2H
CH2–CHNH2–CO2H
CO2
CH2–CH
N
CH2–CH2

MATERIALS AND METHODS

A. Organism and Cultures

The Alga:

The experimental organism used in this investigation is a unicellular green alga, Chlamydomonas segnis Ettl (Ettl, 1965; and Badour et al, 1973) previously referred to as Gloeomonas sp. (Badour and Waygood, 1971a). Stock cultures of this alga were maintained on agar slants at room temperature and low light intensity.

The Nutrient Medium:

The mineral nutrient medium of Kuhl and Lorenzen (1964), enriched with phosphate and contained NH_4^+ instead of NO_3^- as the N-source (Badour & Waygood, 1971a) was used for the cultivation of C. segnis. The composition of the medium is as follows:

		moles/L
1.	NH_4Cl	1.0×10^{-2}
2.	KCl	1.0×10^{-2}
3.	$MgSO_4.7H_2O$	1.0×10^{-3}
4.	$CaCl_2$	1.0×10^{-4}
5.	$NaHPO_4.H_2O$	4.5×10^{-3}
6.	K_2HPO_4	1.0×10^{-2}
7.	$FeSO_4.7H_2O$	2.5×10^{-5}
8.	EDTA (Trisodium)	2.5×10^{-5}

9.	H_3BO_3	1.3×10^{-6}
10.	$ZnSO_4.7H_2O$	3.5×10^{-7}
11.	$MnSO_4.4H_2O$	1.2×10^{-7}
12.	$CuSO_4$	4.5×10^{-8}
13.	$CoCl_2.6H_2O$	3.4×10^{-8}
14.	$(NH_4)_6Mo_7O_{24}.4H_2O$	4.9×10^{-9}

This medium was sterilised by autoclaving at 121 C for 15 minutes and the pH was found to be consistently around 7.0.

Preparation of Mass Cultures:

Mass or the so called "batch" cultures of C. segnis were grown photoautotrophically in Fernbach culture vessels maintained at 25C in a growth chamber. The cultures were exposed to continuous light at 11,000 lux using "cool white" fluorescent lamps and were continuously bubbled with 5% CO_2 in air (v/v) or with air alone (0.03% CO_2) depending on the type of experiment.

Photoorganotrophic cultures were prepared under the same conditions of light and temperature except that the carbon source provided was acetate (15mM) and aeration was achieved by CO_2 free air. The air was passed through a concentrated solution of potassium hydroxide before being allowed into the culture vessel.

Mixotrophic cultures were also prepared under the same conditions of light and temperature except that the nutrient medium contained acetate (15mM) and the cultures were

continuously bubbled with 5% CO_2 in air (v/v).

Preparation of Synchronous Cultures:

Synchronization of algal cultures was based on the techniques developed by Pirson and Lorenzen (1966). Essentially, an inoculum maintained on agar slants was aseptically inoculated into 100 - 150 ml of sterilized nutrient medium in 250 ml Erlenmyer flasks and placed in a controlled environment chamber under the same conditions of light, temperature, and aeration employed for the preparation of mass cultures. After three days of continuous illumination these precultures were diluted with fresh mutrient medium to an algal suspension containing 10^6 cells per ml. One litre of this diluted algal suspension was collected in a 2500 ml low-form Fernbach culture flask and then subjected to alternating periods of 12 hours light and 12 hours darkness during growth. At the end of each dark period, the cultures were diluted to a constant cell number of a million cells per ml. After 3 - 4 cycles of 12:12 light-dark regimen, the cells showed synchrony with respect to their growth (increase in cell diameter during the light period), timing of cell division and zoospores release as described by Badour et al (1973) for C. segnis. The zoospores obtained from such synchronized cultures, were used as the starting material in cultures grown for studies dealing with cell development during the life cycle of the alga under various environmental conditions.

B. Analytical Methods

Cell number:

The cell number was determined by counting the cells using an haemocytometer.

Chlorophyll determination:

Total chlorophyll per ml of algal suspension was determined according to the method of Holden (1965). About ten milligrams of $MgCO_3$ and 8 ml of 95% methanol were added to a pellet obtained by centrifugation of 10 ml of algal suspension. After stirring, the mixture was maintained at 70C for 30 minutes in a water bath to allow complete extraction of the pigment. The supernatant obtained by centrifugation was made up to 10 ml with 95% methanol and absorbance readings were taken at 650 nm and 665 nm using methanol as a blank. Total chlorophyll per ml algal suspension was determined from the following equation:

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

Protein determinations:

The micro-Kjeldahl method was used for the determination of total cellular nitrogen and trichloro-acetic acid soluble nitrogen according to the procedure of Ogg (1960). Ten ml of algal suspension were washed twice with nitrogen-free nutrient medium and digested in a flask (containing 2 ml con. H_2SO_4, K_2SO_4(630 mg), and HgO(20 mg)) with an electrical

heater until the solution was colorless. The sample was quantitatively transferred to a distillation flask, and 5 ml of NaOH(50% w/v) was added. The distillate (15 ml) was collected in a graduated beaker containing 2 ml of boric acid (½% w/v) and methyl red as indicator. The distillate was titrated against 0.01 N potassium biiodate.

Calculation

mg Nitrogen in sample = $0.14\ (A-B)/25$

where A = ml of 0.01 N biiodate to titrate sample

B = ml of 0.01 N biiodate to titrate reagent blank.

For the determination of trichloroacetic acid (TCA) soluble nitrogen, the algae were washed after centrifugation using distilled water and the pellet was resuspended in 5 ml of distilled water. After the addition of 5 ml of TCA (10% w/v) and mixing, the mixture was then left for 30 minutes at room temperature to allow the extraction of TCA soluble nitrogen. It was found that the time of extraction and the concentration of TCA used did not practically cause any significant hydrolysis of protein (Pirie, 1955). After centrifugation, 5 ml of the clear TCA extract were taken and ashed in a digestion bulb as described before. The TCA insoluble-nitrogen (protein-N) was calculated from the difference between total cellular nitrogen and the total TCA soluble-nitrogen of the same volume of algal suspension.

The method of Lowry et al (1951) was routinely used for the determination of enzyme proteins. It was also employed for the determination of algal protein as follows:

To 2 ml of twice washed algal suspension, was added 2 ml of 2N NaOH (Badour & Waygood, 1971a). The mixture was incubated for 30 min. in boiling water. Half a ml of the cooled hydrolysate was used for colorimetric determination of protein according to Lowry's method. The total protein in micrograms per ml of algal suspension was calculated from a graph prepared for Bovine serum albumin.

Total and soluble carbohydrates:

Total carbohydrate was determined by the Anthrone method (Roe, 1955). To 1 ml of algal suspension, twice washed with distilled water, was added 4 ml of anthrone reagent (100 mg anthrone and 1 g thiourea dissolved in 100 ml 75% H_2SO_4). One ml of algal suspension and 4 ml of 75% H_2SO_4 served as a blank. The mixture was placed in a water bath at 100 C for 15 minutes. The absorbancy of the blue-green colour produced was measured against the blank at 620 nm using a Zeiss spectrophotometer. The total carbohydrates were expressed as micrograms glucose per ml algal suspension. A standard calibration curve was made with glucose.

The total soluble carbohydrates were determined according to the procedure described by Laudenbach & Pirson (1969). 50 ml of algal suspension were centrifuged and washed

twice with distilled water before suspending it in 0.1 M sodium acetate buffer, pH 4.9. The algal suspension was then sonicated, centrifuged and one ml of the clear supernatant was used to determine the amount of soluble carbohydrate using the anthrone method described before.

Nucleic Acids Determination

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

$$\text{Total Nucleic Acids (ug/ml)} = \frac{(OD_{268.5} - OD_{320})}{0.6} \times 25$$

For the determination of DNA, 2.0 mls of Diphenylamine Acetaldehyde reagent was added to 1.0 ml of the final extract

and incubated for 18 hours at 30 C. The absorbancy of the blue colour obtained was read at 595 nm and 650 nm.

$$\text{Total DNA (ug/ml)} = \frac{(OD_{595} - OD_{650})}{0.4} \times 25$$

RNA was obtained by subtracting total DNA from total nucleic acids. The data were expressed as micrograms per ml algal suspension according to the method of Senger (1965).

Determination of δ-aminolevulinic acid (δ-ALA)

The method for the determination of δ-ALA was essentially that of Mauzerall and Granick (1956). 0.2 ml of ethyl acetoacetate was added to the supernatant obtained from levulinic acid treated algal cells and brought to a volume of 10 ml with 0.5 M phosphate buffer, pH 6.8. The mixture was then placed in a water bath at 100 C for 15 min. to allow the condensation of δ-ALA to form ALA pyrrole. Thereafter the solution was cooled to room temperature. 2 ml of this solution was added to 2 ml of modified Erlich's reagent (1 gm of diaminobenzaldehyde was dissolved in 42 ml of glacial acetic acid and 8 ml of perchloric acid (70% w/v).). In the presence of δ-ALA pyrrole, a red colour, absorbing at 555 nm would develop.

Photosynthetic $^{14}CO_2$ fixation and glycolate determination

Ten ml of algal suspension after being washed twice with distilled water, was resuspended in a clear transparent

scintillation vial and brought to a final volume of 5 ml with 0.05 M phosphate buffer, pH 7.0. The algal suspension was allowed to equilibrate for 10 min. at 25 C and 12,000 lux. $^{14}CO_2$ fixation was initiated by the addition of 0.2 ml of $NaHCO_3$ solution containing 25 µmoles of $NaHCO_3$ and 10 uCi $NaH^{14}CO_3$ (s.a. 59 mCi/mM) with gentle constant stirring. After two minutes, CO_2 fixation was terminated by injecting 0.5 ml of glacial acetic acid to get rid of excess $^{14}CO_2$. Thereafter the suspension was air dried overnight.

For the determination of glycolate produced during $^{14}CO_2$ fixation, 2-pyridylhydroxymethanesulfonate (PHMS, 10 mM) was added to the buffer and the same procedure described above was followed except that the incubation time was extended to 15 min. After the reaction was stopped, 2 ml of the sample was withdrawn to measure the total ^{14}C fixed and the rest was used to estimate ^{14}C-labelled glycolate. The latter was separated by ion-exchange chromatography and thin layer chromatography (Gimmler et al, 1969).

Incorporation of L-leucine-^{14}C(U) into protein

The method of Morris (1966) was used. Samples taken at suitable time intervals were washed and resuspended in N-free medium (the complete nutrient medium minus NH_4Cl). The cell density of the algal suspension was adjusted to 3 - 5 mg dry weight in a total volume of 1.8 ml. The samples

were then aerated by 5% CO_2 in air (w/v) for 15 min. to deplete the nitrogenous pools in the cells. L-leucine-^{14}C(U) (final concentration 5 mM and specific activity 0.2 μCi per mM) was added to initiate the reaction. After 10 min. incubation, the reaction was terminated by addition of 3 ml of 10% TCA (w/v). The resulting precipitate was centrifuged and washed four times with cold 10% TCA containing an excess of cold L-leucine. This was followed by hot ethanol-ether (3:1) extractions to remove lipids and an ether extraction for drying. The pellets were dissolved in 1.0 ml of concentrated formic acid and the radioactivity measured by scintillation counter.

Ion Exchange chromatography of organic acids.

The methodology was as described by Canvin and Beevers (1961) and Cossins and Beevers (1963). Two types of resin (Bio-Rad Labs; Richmond, California) were used:-

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

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

Both types of resins were prepared in bulk according to Atkins and Canvin (1971). Each resin was then added to a large column plugged at the base with glass wool. The cation exchange resin, Dowex 50W-8 was treated with 10 ml of 2 N HCl per 5 ml resin and then washed with distilled water

until the effluent was nearly neutral. The anion exchange resin, Ag 1- x10 was converted to the formate form with 1M sodium formate until a negative test for chloride ion was obtained. The resin was next washed with 0.1 N formic acid (50 ml/5 ml resin), followed by distilled water until the effluent was close to neutral.

Ion exchange columns, 6x1 cm by dimension, were prepared with 13 x 1 cm glass columns plugged at the base with glass wool. The columns were rinsed with 2 bed volumes of distilled water before use.

In order to remove amino acids from water soluble extracts, the latter were loaded on to a Dowex-50-X8-H^+ column and washed with 80 ml of distilled water. The effluent containing mainly organic acids and sugar phosphates was concentrated and quantitatively transferred to the Agl-xl0-$HCOO^-$ column and rinsed with 100 ml of distilled water. For elution of organic acids, 20 ml of 1 N formic acid was added to the column and 1 ml fractions were collected. Glycolic acid was collected in the 5th - 10th tubes and its presence was confirmed by thin layer chromatography. The eluate was air dried and redissolved in 1.0 ml distilled water for radioactivity measurements.

Measurement of Radioactivity

Determination of radioactivity for samples was measured by the Picker Nuclear Liquimat Scintillation Counter.

Samples were corrected for quenching by using a quench curve prepared with picric acid and ^{14}C-toluene.

Aliquots of the aqueous samples obtained were placed in scintillation vials and made up to 0.5 ml with 20% ethanol. Ten ml of scintillation cocktail (0.3 g POPOP, 7.0 g PPO and 100 g Naphthalene dissolved in a total volume of 1 litre of p-dioxane) were added to each sample and measured for radioactivity.

Radioautograms were also made by exposing the dried plates to "Kodak Medical X-ray Film, No-screen" from Eastman Co., Rochester, New York. After an appropriate period (1 to 2 weeks) of exposure, the films were developed to locate the radioactive spots. The spots were scraped off as a rule and quantitatively transferred to scintillation vials for radioactivity determination.

Thin-Layer Chromatography (TLC)

The cellulose powder MN 300 (Machery, Nagel & Co.) was prepared as described by Cook and Bieleski (1969). Fifteen grams of washed, dried cellulose was mixed with 90 ml of water and homogenised at 20,000 rpm for 1 minute, paused 30 seconds and homogenised again for 1 minute. The slurries were then allowed to sit for 1½ minutes before they were used. With a Desaga spreader (Desaga, Heidelberg, West Germany), five plates of 250 microns thickness of cellulose were prepared. These plates were then allowed to age for one week

after spreading to ensure stability. For the separation of glycolate and malate, two dimension chromatography was employed using 100 ml of n-propanol:ammonium hydroxide: water (6:3:1 v/v) and 140 ml of n-propyl acetate:formic acid:water (11:5:3 v/v) for the first and second run respectively. For detection of δ-aminolevulinic acid, one dimension thin layer chromatogram was run in a solvent mixture which consisted of methyl acetate:isopropyl alcohol:25% NH_4OH (45:35:20 v/v), whereas the dinitro-phenylhydrazone of δ-hydroxylevulinic acid was separated with a tertiary amyl alcohol:ethanol:H_2O (5:1:4 v/v) solvent.

C. Preparation of Enzymes and Enzyme Assays

Preparation of partially purified NADP isocitrate-dehydrogenase:

Chlamydomonas segnis was grown in mass cultures and the algal suspension was collected when the cell density reached 6-8 x 10^6 cells/ml. Usually five litres of cell suspension were harvested using a Sorvall KSB continuous flow system. The algal concentrate was washed and resuspended in 50 ml of PEM buffer, pH 7.2, 0.05 M. The cells were then stored frozen at -20 C until required. For the preparation of cell-free extracts, the cells were thawed and sonically disrupted with a Biosonik sonicator, for five half minute intervals spread 1 min. apart. It was then centrifuged at 18,000 rpm for half an hour in the SS-34 rotor of the Sorvall

centrifuge, model RC2B. The clear yellowish supernatant solution (50 ml) was retained and the pellet was discarded. The following steps were carried out for partial purification of the enzyme.

a) Ammonium sulfate fractionation:

The clear supernatant was stirred magnetically and treated slowly with 12.2 g of solid ammonium sulfate (40%) The pH of the mixture was maintained above pH 7.2 by the addition of ammonia. After standing for one hour in cold (4 C), the suspension was sedimented at 15,000 rpm for 20 min. and the residue was discarded. The fractionation was repeated again with 9.1 g of solid ammonium sulfate (60%) The sediment obtained was dissolved in 10 ml of PEM buffer, pH 7.2, 0.05 M and was used for Sephadex column chromatography.

b) Sephadex G-100 Filtration:

Enzyme solution from the previous operation (10 ml) was applied to a column (3 x 80 cm) of sephadex G-100 and eluted with standard buffer. Fractions containing enzymes were combined (15 ml) and this was used for the next step of purification.

c) DEAE-Sephadex G-50:

The enzyme protein from the previous operation was passed through a column (3 x 30 cm) of DEAE-Sephadex G-50 packed in standard buffer and the enzyme was eluted with a linear gradient using 400 ml of standard buffer and 400 ml

of 0.05 M phosphate buffer, pH 7.2 containing 0.5 M KCl. The most active fractions were pooled to a volume of 12 ml and were used for the next step.

d) Hydroxyl-apatite chromatography:

The enzyme solution from previous operation was passed through a column (3 x 30 cm) of hydroxyl-apatite that was washed with standard buffer. The enzyme was eluted with a linear gradient of 400 ml of standard buffer and 400 ml of 0.05 M phosphate containing 0.5 M KCl (pH 7.2). The most active fractions were pooled to a volume of 9 ml and stored at -16 C. Before use, samples were thawed at room temperature and dialysed for two hours.

Preparation of partially purified isocitrate lyase:

The procedure described for the purification of isocitrate lyase by Foo et al, (1971) was employed in the present work.

Enzyme Assays:

All assays were carried out at 25 C unless otherwise stated. The spectrophotometric assays were done on a Gilford recording spectrophotometer. In all cases the enzymatic assays reported in this work were tested for relationship between enzyme activity and enzyme protein concentration and linear portions of enzyme activity. In studies dealing with the effect of metabolites on enzyme reaction, test

compounds were preincubated with enzyme and cofactors before addition of substrate.

1. NAD(P) - Isocitrate Dehydrogenase (EC 1.1.1.42) was assayed according to the method of Ochoa (1948). The incubation mixture contained in μmoles the following in a total volume of 3.0 ml:- DL-isocitric acid (trisodium salt), 3; NAD or NADP, 0.6; $MnCl_2$, 0.3; phosphate buffer, pH 7.3, 100; and enzyme. The reaction was initiated by addition of isocitrate and the extinction at 340 nm was read.

2. Isocitrate Lyase (EC 4.1.3.1)

a - Method I (referred to as continuous method)

Using the method of Dixon and Kornberg (1959) the assay system in 3.0 ml contained in μmoles: MES buffer, pH 6.8, 50; $MgCl_2$, 7.5; glutathione (reduced), 2; phenyl-hydrazine adjusted by NaOH to pH 6-8, 10; DL-isocitric acid (trisodium salt), 10; and enzyme protein. The reaction was conducted at 30 C and was initiated with isocitrate and the extinction at 324 nm was read.

b - Method II (referred to as the incubation method)

Using the modified method of Rao & McFadden (1965), the reaction mixtures contained in μmoles the following in a total volume of 1.0 ml: Tris-HCl, pH 7.3, 50; $MgCl_2$, 7.5; glutathione reduced, 2; and phenyl hydrazine adjusted by NaOH

to pH 7.3, 10; and enzyme. After preincubation of this mixture for 10 min. at 30 C the reaction was initiated by addition of 10 µmole of DL-isocitric acid (trisodium salt), and terminated by addition of 0.4 ml of M oxalic acid. The mixture was then immediately placed in ice for 10 min. Thereafter 2 ml of cold 4N HCl and 0.1 ml 25% (w/v) $K_3Fe(CN)_6$ were added. If glyoxylate were present, a reddish violet colour (absorbance at 520 nm) would develop. Spectrophotometric measurements were carried out after 13 min. at room temperature.

The detection of the following enzymes was carried out using crude cell extracts obtained by sonication of algal cell suspension in phosphate buffer, 0.1 M, pH 7.0 and centrifugation at 4 C for half an hour at 18,000 rpm.

3. NAD(P) glutamate dehydrogenase (EC 1.4.1.12)

This enzyme was assayed by following the oxidation of NAD(P)H. The reaction mixture in a total volume of 3.0 ml contained in µmole the following:- α-ketoglutaric acid (disodium salt), 10; NAD(P)H, 5; NH_4Cl, 150; Tris HCl, pH 9.0, 100; and crude enzyme.

4. Malate synthase (EC.4.1.3.2)

The assay is based on the principle that ^{14}C-malate formed from 1-^{14}C-glyoxylate can be rapidly isolated by thin layer chromatography and quantified by scintillation spectroscopy

(Furmanski et al, 1968). The reaction mixture contained in umoles the following in a total volume of 1.0 ml: phosphate buffer, pH 8.0, 40; $MgCl_2$, 0.5; acetyl CoA, 10; unlabelled glyoxylate (0.5 umole) with 0.16 umole 1-^{14}C glyoxylate and enzyme. Two other reaction mixtures lacking either enzyme or acetyl CoA are included as controls. The reaction rate was initiated by addition of glyoxylate, and was stopped after one hour with 0.2 ml of TCA (10% w/v). The deproteinised supernatant obtained was streaked onto MN-cellulose plates and cochromatographed with authentic malate. The radioactive spot corresponding to authentic malate was scraped off and counted.

5. Glyoxylate carboxy-lase

The enzyme was assayed by measuring the $^{14}CO_2$ evolved from 1-^{14}C-glyoxylate in the manner described by Badour & Waygood (1971b). The main compartment of a double-sided arm Warburg flask with a central well, contained in umoles:- phosphate buffer, pH 7.5, 100; $MgCl_2$, 3; thiamine pyrophosphate, 0.65, and enzyme in a volume of 1.8 ml. The enzyme assay was carried out under O_2-free N_2 at 30 C, and reaction was initiated by tipping in 0.2 ml of unlabelled glyoxylate (0.5 umole) with 0.16 umole 1-^{14}C-glyoxylate from the side arm. The reaction was terminated after 30 minutes by tipping in 2 M H_2SO_4 from the other side arm.

After shaking for a further 30 minutes, the $^{14}CO_2$ trapped in the central well by 20% KOH was transferred to a scintillation vial and the radioactivity was measured.

6. α-ketoglutarate: glyoxylate carboligase

The carboligase was assayed essentially by the method of Koch and Stokstad (1966). Incubations were carried out for 1 h at 30 C under nitrogen atmosphere in Warburg vessels (see glyoxylate carboxy-lyase) in a final volume of 2 ml. The main compartment contained the following in umoles:- potassium phosphate buffer, pH 7.0, 100; $MgCl_2$, 5; TPP, 0.5; α-ketoglutarate, 2; and the enzyme. The reaction was started after 10 minutes equilibration by tipping 2 umoles of U-^{14}C-glyoxylate or unlabelled glyoxylate when 1-^{14}C-α-ketoglutarate was used.

The reaction was stopped by adding 0.2 ml of 2 M H_2SO_4. After shaking for a further 30 min. the $^{14}CO_2$ trapped in the centre well by 20% KOH was measured with a scintillation counter.

7. α-ketoglutarate dehydrogenase.

a - Demonstration of the decarboxylase reaction:

The enzyme was assayed essentially according to the method of Schlossberg et al (1968) in Warburg flasks by incubating for 1 h at 30 C in an air atmosphere and in a total volume of 2 ml. The main compartment contained the following

in μmoles:- potassium phosphate buffer, pH 7.0, 100; $MgCl_2$, 5; TPP, 0.5; 1-^{14}C -α-ketoglutarate, 2; albumin, 20 mg; and enzyme. The reaction was terminated by the addition of 0.2 ml of 2 M H_2SO_4; $^{14}CO_2$ formed was absorbed into 0.2 ml of 20% KOH in the centre well and the trapped radioactivity was measured as described before.

b - Assay of over-all reaction:

This enzyme was assayed by measuring the NADH formation in the reaction system (final volume 3.0 ml) which contained in μmoles the following:- phosphate buffer, pH 6.8, 100; α-ketoglutarate, 10; $MgCl_2$, 5; TPP, 0.5; CoA, 0.5; NAD, 0.3; and enzyme. The reaction mixture was incubated at 25 C and the increase in optical density at 340 nm was measured.

8. Alanine-dioxovalerate transaminase

This enzyme was assayed according to the method of Neuberger and Turner, (1963). The standard assay system contained in μmoles the following in a final volume of 1.5 ml:- dioxovalerate, 1; L-alanine, 10; phosphate buffer, pH 7.0, 100; and the enzyme. The incubation was carried out at 25 C for 60 min. and δ-aminolevulinate formed was determined by the method of Mauzerall and Granick (1956) as described before.

9. Glutamate: Glyoxylate Aminotransferase (EC 2.6.1.4)

This enzyme was assayed at 25 C by following the formation of glycine-^{14}C in the manner described by Kisaki and Tolbert (1969). In a final assay volume of 1.25 ml were the following in μmoles: U-^{14}C-glyoxylate (0.2 uCi) and unlabelled glyoxylate, 2; L-glutamate, 15; pyridoxal-5-phosphate, 0.1; phosphate buffer, pH 7.5, 100; and enzyme. The reaction was initiated by addition of glyoxylate and terminated after 15 min. by boiling. Glycine-^{14}C was separated from unreacted glyoxylate by passage of the boiled reaction mixture over a Dowex-1x10-(formate) column which was then washed with 5 ml of water. The combined effluents were dried down and resuspended in 0.2 ml of distilled water and 0.1 ml aliquots were counted for ^{14}C-glycine.

10. δ-aminolevulinic acid synthetase

Two methods are used for assaying the activity of the enzyme.

Method I (Colorimetric assay)

The complete reaction mixture consisted of the following in μmoles:- glycine, 100, sodium succinate, 100; pyridoxal phosphate, 0.25; coenzyme A, 0.25; ATP, 5; EDTA, 0.05; 0.2 mg succinyl CoA synthethase protein isolated from spinach by the method described by Kaufman et al, (1955); phosphate buffer, pH 7.0, 100; and enzyme in a final volume of 2.0 ml. The reaction mixture was incubated at 25 C for

1 h and the reaction was stopped by the addition of 0.5 ml of TCA (10% w/v). δ-ALA was estimated by the method described previously.

Method II (Radiochemical assay)

For a more sensitive assay, the incorporation of 2, 3-^{14}C succinate and glycine into δ-ALA was determined as described by Irving & Elliot (1969). The reaction mixtures are the same as described in method I except that ^{14}C-succinate (0.5 uCi) with cold succinate (5 μmoles) were used.

After incubations were carried out for 1 hour, the reaction was stopped with 2 ml of 0.3 M TCA. Authentic unlabelled δ ALA (μmole) was added to the supernatant obtained and these were applied to a Dowex 50 (H^+) column, 3x1 cm, and washed with 30 ml of water and the ALA was eluted with 10 ml of 2 M pyridine acetate buffer, pH 6.0. The eluates were dried down and dissolved in 1.0 ml of distilled water and the ALA was converted to the pyrrole by the method of Urata and Granick (1963) and the solution was extracted with 25 ml of ethyl acetate. The ethyl acetate layer was washed with 3 ml of water and evaporated to dryness. The ALA pyrrole was dissolved in 1.0 ml of methanol and samples of known volume were streaked onto MN-cellulose plates and chromotographed. Pyrroles were detected by spraying the dried plates with modified Erlich reagent.

11. Glycerate Kinase (EC 2.7.1.31)

This was assayed by measuring the change in OD at 340 nm of NAD. The assay mixture contained in umoles the following in a total volume of 3.0 ml:- NAD, 15; glutathione reduced, 150; $MgCl_2$, 30; Tris HCl, pH 9.0, 300; ATP, 1: and D-3-phosphoglycerate dehydrogenase from chicken liver (1 enzyme unit catalyses the conversion of 1 umole of 3 phosphoglycerate to phosphohydroxypyruvate per minute at pH 9.0); and 0.5 ml of the following mixture:

5 volume of 1.0 M Tris-HCl pH 9.0

4 volume of 1.0 M Hydrazine Sulfate

1 volume of 0.25 M EDTA

The reaction was initiated with the addition of 10 umole of D-glycerate. The net change in absorbance of NAD in the following reaction sequence is summarised as follows:-

$$\text{D-glycerate} + \text{ATP} \xrightarrow[\text{KINASE}]{\text{GLYCERATE}} \text{ADP} + \text{2-phospho-D-glycerate}$$

$$\text{2-phospho-D-glycerate} \xrightarrow[\text{MUTASE}]{\text{P-GLYCERO}} \text{3-phospho-D-glycerate}$$

$$\text{NAD} + \text{3-phospho-D-glycerate} \xrightarrow[\text{DEHYDROGENASE}]{\text{3-P-GLYCERATE}} \text{NADH} + \text{phosphohydroxypyruvate}$$

12. Glyoxylate reductase (EC.1.1.1.26)

This enzyme was assayed by the procedure of Zelitch (1955). The assay mixture contained in umoles in a total

volume of 3.0 ml the following:- phosphate buffer, 100, pH 7.0; sodium glyoxylate, 10; and NAD(P)H, 0.3 and enzyme. The reaction was initiated by addition of glyoxylate and extinction at 340 nm was read.

13. Phosphoenolpyruvate carboxylase (EC 4.1.1.31)

The enzyme was assayed according to the method of Paulsen and Lane (1966). The incubation mixture contained in umoles: PEP (trisodium salt), 2.0; $NaH^{14}CO_3$, 25 (1 uCi); NADH, 0.8; EDTA, 0.03, $MgCl_2$, 5; Tris HCl pH 7.8, 100; and malate dehydrogenase (EC 1.1.1.37), 1 i.u. and crude enzyme. The reaction mixture was preincubated at 30 C for 10 min. The reaction was initiated by the addition of PEP. Control samples were without PEP. The reaction was terminated after 15 min. by the addition of 1 ml of 2 N HCl. The sample was then centrifuged and 0.5 ml of the supernatant was taken into a scintillation vial, dried down and redissolved in 0.5 ml distilled water for determination of radioactivity as previously described.

RESULTS AND DISCUSSION

I. Isocitrate Metabolism in Cell-Free Extracts of Chlamydomonas segnis

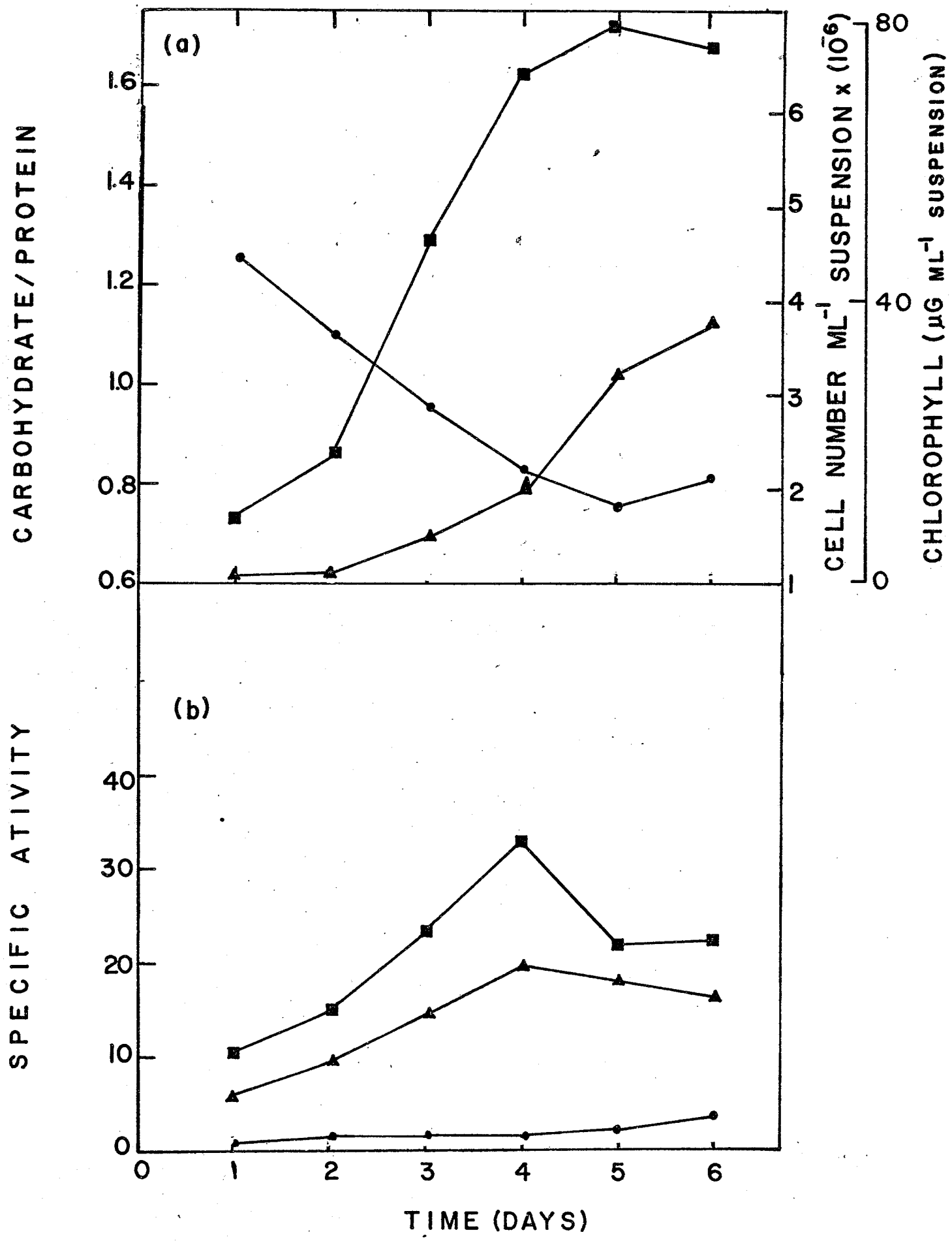
In the following experiments, cell-free extracts of C. segnis were prepared from algal cells grown under various culture conditions. The specific activities of IDH and ISL were determined in the supernatant of the sonicates after centrifugation. The levels of enzyme activities in such extracts would indicate the rates of isocitrate metabolism via IDH and ISL. Some of the enzymes which might metabolise the products of isocitrate enzymes were tested in extracts prepared from photoautotrophically grown cells. The presence or absence of such enzymes may provide some ground to elicit the possible pathways of α-keto glutarate, succinate and glyoxylate.

A. Alteration in Enzyme Level during Photoautotrophic Growth in "batch" cultures:

As shown in Fig. 4a, C. segnis exhibited the exponential phase of growth after 2 days from the inoculation time and entered the phase of declining relative growth rate at the fourth day. In such batch cultures, the cells indicated a relative growth constant (K', in $\log_{10}$ day units) of 0.165 and mean doubling time (G) of 48 hours. These values represent a relatively low rate of growth, presumably as a result of the large size of the algal cells and the dense inoculum used to

Fig. 4 (a) Changes in cell number (■——■), chlorophyll content (▲——▲) and carbohydrate/protein ratio (●——●)during the growth of C. segnis under continuous illumination (11,000 lux) with 5% CO_2 in air (v/v).

(b) Specific activities (nmoles/min/mg) of NADP-isocitrate dehydrogenase (▲——▲), isocitrate lyase (●——●) and NAD-glutamate dehydrogenase (■——■) during the photoautotrophic growth in "batch" cultures.


(a)
(b)
CARBOHYDRATE/PROTEIN
SPECIFIC ATIVITY
CELL NUMBER ML⁻¹ SUSPENSION x (10⁻⁶)
CHLOROPHYLL (µG ML⁻¹ SUSPENSION)
TIME (DAYS)
0.6
0.8
1.0
1.2
1.4
1.6
1
2
3
4
5
6
0
40
80
10
20
30
0
1
2
3
4
5
6

initiate the culture (Fogg, 1966). As indicated by the declining carbohydrate/protein ratio, the exponential phase of growth was the phase of active protein synthesis. The rate of chlorophyll synthesis was not necessarily in synchrony with that of protein synthesis.

When the activities of NADP-IDH and ISL (Fig. 4b) were compared at the phase of active protein synthesis, it was found that the activity of the former was 6 - 7 times higher than that of the latter. During the phase of declining relative growth rate and inactive protein synthesis, the activity of ISL was almost doubled while the activity of IDH remained practically unchanged. In the meantime, the activity of NAD-GDH dropped about 30% which was concomitant with the declined rate of protein synthesis.

These results have shown that regardless of the culture age, the rate of isocitrate metabolism via the dehydrogenase was 5 - 7 times faster than via isocitrate lyase. The declining rate of protein synthesis was associated with significant increase in ISL activity.

B. Fate of Organic Acids Produced by Isocitrate Enzymes:

It is obvious that the relatively high activity of NAD-GDH (Fig. 4b) would account for the conversion of most if not all of the α-ketoglutarate to glutamate. When NADPH (coenzyme for glutamate biosynthesis) was used instead of NADH (coenzyme for glutamate degradation in vivo), the

activity of the enzyme was reduced by about 80% (Talley et al, 1972, and Kinghorn & Pateman, 1973). However, the activity of NADP-GDH would be still adequate for the utilisation of about 20% of the α-ketoglutarate produced by IDH.

Other pathways of α-ketoglutarate metabolism may exist in C. segnis if the activities of some enzymes are detectable in the algal extracts. The results obtained from these enzyme tests are presented in Tables I and II. The extracts used were prepared from cells at the stationary phase of growth (see Fig. 4 at the 5th day), during which protein synthesis was almost terminated.

In such extracts, α-ketoglutarate dehydrogenase was undetectable whether fluorometric (reduction of NAD or NADP in presence of α-ketoglutarate) or radiotracer method (decarboxylation of 1-^{14}C-α-ketoglutarate in presence of CoA) was used. On the other hand, α-ketoglutarate-glyoxylate carboligase was detectable (Table II). This enzyme produces hydroxy-levulinic acid which may be metabolised to dioxovaleric acid, (Shigesada, 1972, Schlossberg et al, 1968). The latter compound can be transaminated with L-alanine via δ-ALA transaminase to form δ-ALA. However, the latter enzyme was undetectable in the algal cell free extracts regardless of using L-alanine, L-phenylalanine or L-glutamate as amino group donor.

As shown in Table III, malate synthase activity was virtually absent in the algal extract although ISL was pre-

Table I

Detection of α-ketoglutarate dehydrogenase in cell-free extracts of C. segnis

Assay system	$^{14}CO_2$ evolved (disint./min x 10^{-3})		Specific activity (mmoles/min/mg)
Complete	5.43	6.42	0.02
Minus CoA	7.84	5.80	0.03
Boiled enzyme	3.2	-	0.01

Table II

Detection of α-ketoglutarate-glyoxylate carboligase reaction in cell-free extracts of C. segnis

Assay system	$^{14}CO_2$ evolved (disint./min x 10^{-3})		Specific activity (mmoles/min/mg)
Complete	76.1	76.5	0.27
Minus $MgCl_2$	86.4	-	0.30
Minus TPP	10.4	-	0.04
Minus glyoxylate	8.5	8.3	0.03
Boiled enzyme	3.2	-	0.01

Table III

Absence of malate synthase activity in cell-free extracts of C. segnis (See Methods for enzyme assay)

Assay system	^{14}C-malate (disint./min x 10^{-3})
Complete	0.295
-$MgCl_2$	0.275
-Acetyl CoA	0.264
Boiled enzyme	0.186

Table IV

Specific activities (nmoles/min/mg) of glyoxylate carboligase, glyoxylate: L-glutamate transaminase, D-glycerate kinase, and NADH glyoxylate reductase in cell-free extracts of C. segnis

Enzyme	Specific activity
glyoxylate carboligase	0.09
glyoxylate: L-glutamate transaminase	5.60
glyoxylate reductase	42.00
D-glycerate kinase	5.40

sent. However, some of the glyoxylate produced via ISL could be metabolised via glyoxylate carboligase reaction (Table IV) in the glycerate pathway involving tartronic acid semialdehyde reductase (Badour and Waygood, 1972) and further to 3-phosphoglycerate by D-glycerate kinase. Furthermore, the algal extract catalysed the conversion of glyoxylate to glycine and glycolate.

The other reaction product of ISL, succinate was metabolised via succinyl thiokinase (Table V). However, the resulting succinyl CoA was not used for the synthesis of δ-aminolevulinic acid (Table VIa). The absence of δ-ALA synthetase in the algal extracts was not attributable to the presence of intracellular inhibitors. It is obvious from the results in Table VIb that δ-ALA synthetase from chicken liver was not inhibited by the algal extracts nor by algal cells treated with levulinic acid (an inhibitor of δ-ALA dehydratase) that will cause δ-ALA to accumulate.

From the above reported enzyme tests, it may be concluded that about 20% of the α-ketoglutarate produced via IDH was converted to glutamate. If glyoxylate was formed by ISL, its metabolism would be most unlikely mediated via malate synthase in the glyoxylate cycle. The presence of succinyl thiokinase in the algal extracts may represent a major step in the metabolism of succinate but not towards the formation of δ-ALA.

Table V

Detection of succinyl thiokinase in cell-free extracts of *C. segnis*

Assay system ≠	Specific activity (nmoles/min/mg)
Complete	3.6
-CoA	0
-ATP	0
Boiled enzyme	0

≠The method of Kaufman & Alivisatos (1955) was used.

Table VIa

Absence of δ-ALA synthetase in cell-free extracts of C. segnis

Assay is as described in Materials & Methods

Assay system	$^{14}CO_2$ evolved (dpm x 10^{-3})
Complete	1.317
-$MgCl_2$	.828
-Succinyl CoA	1.361
Pyridoxal phosphate	.852
Boiled enzyme	.574

Table VIb

Absence of inhibitors of δ-ALA synthetase in cell-free extracts of C. segnis

Treatment	Specific activity (nmoles/min/mg)
ALA synthetase (from chicken liver)#	6.6
algal extracts from exponential phase plus chicken liver	6.5
algal extracts from stationary phase plus chicken liver	6.5
algal extracts from levulinic acid treated cells plus chicken liver	6.7

#Enzyme assay was carried out according to Cowtan et al, 1973.

C. Activities of isocitrate enzymes in nitrogen - and manganese - deficient cells:

It is well known that protein synthesis, rate of growth and cell division are impaired when unicellular green algae are grown under conditions of mineral deficiencies (Pirson, 1955; and Pirson & Badour, 1961). As shown in Table VII, the protein content of C. segnis was very low in cultures supplemented with 0.4 mM NH_4Cl as compared to that of cells produced in normal complete nutrient media (10 mM NH_4Cl). In such N-deficient cultures, the rates of growth and cell division were slow and the ratio of carbohydrate to protein increased as a result of the restricted utilisation of carbon skeleton for protein synthesis. Algal extracts prepared from N-deficient cells appeared to metabolise isocitrate exclusively via IDH since ISL activity was undetectable.

When C. segnis was grown in nutrient media deprived of manganese, the cells exhibited normal rates of division although their protein content was 65% lower than that of normally grown cells. In other words, the high protein content characteristic of cells grown in complete nutrient media is not a prerequisite for cell division (Aach, 1952; and Tan, 1974). The comparatively low protein content of Mn-deficient cells is attributable to inhibition of photosynthesis which eventually provides the carbon skeletons required for protein synthesis.

Extracts from Mn-deficient cells with comparatively

Table VII

Cell number ($x10^6$/ml), mean doubling time (G, in hours), chlorophyll (ug/ml), carbohydrate (ug/ml) and protein content (ug/ml), specific activities (nmoles/min/mg) of NADP-isocitrate dehydrogenase (IDH), isocitrate lyase (ISL) & NAD-glutamate dehydrogenase (GDH) of C. segnis grown for 4 days in complete nutrient medium and in nitrogen or manganese deficient media. The initial cell number of cultures was 10^6 cells per ml.

Growth Conditions	Complete	N-deficient	Mn-deficient
Cell number	4.2	2.0	4.0
G	46.0	96.0	48.0
Chlorophyll	11.0	2.0	20.0
Carbohydrate	130.0	70.0	20.0
Protein	140.0	20.0	50.0
IDH	48.2	44.6	39.0
GDH	76	25	15.6
ISL≠	4.5	undetectable	0.01

≠continuous Method I was used

low carbohydrate content metabolised isocitrate via IDH. Isocitrate lyase was virtually absent as indicated in Table VII.

These observations suggest that in photoautotrophically grown C. segnis, which exhibits active protein synthesis, isocitrate would be partially metabolised via ISL. When protein synthesis is limited by nitrogen or carbon shortage, virtually all isocitrate would be converted to α-ketoglutarate. As seen from the difference between IDH and GDH activities, only a small portion of the α-ketoglutarate could be metabolised to glutamate in N- and Mn^{++}-deficient cells.

D. The effect of Growth in Acetate on the Activities of Isocitrate Enzymes:

When cultures of C. segnis were provided with acetate (15mM) as the sole carbon source and aerated with CO_2 free air in light, the cells divided faster than in cultures bubbled with 5% CO_2 in air (v/v) alone (Table VIII). The observed increase in cell number in the acetate cultures was not attributable to enhanced protein or chlorophyll synthesis. It was most likely the result of provision of reduced carbon. Probably, the latter required less energy for assimilation than CO_2. In this case, energy would be more available for the process of cell division. As shown in Table VIII, cell division was inhibited when CO_2 was provided to cultures already supplemented with acetate. The

Table VIII

Cell number ($x10^6$/ml), mean doubling time (G in hours), chlorophyll and protein content (ug/ml), rate of $^{14}CO_2$ fixation (nmoles/ug chlorophyll/min), and specific activities (nmoles/min/mg) of phosphoenolpyruvate carboxylase (PEP), NADP-isocitrate dehydrogenase (IDH) and isocitrate lyase (ISL) of C. segnis grown in acetate in light with or without CO_2. The initial cell number of culture was 10^6 per ml and cells were harvested for various analysis after 7 days from inoculation time.

Growth Conditions	5% CO_2	Acetate (15mM)	5% CO_2 + acetate (15mM)
Cell number	7.5	14.5	4
G	58.0	43.0	84.0
Chlorophyll	18.0	19.0	26.4
Protein	320.0	300.0	610.0
$^{14}CO_2$ fixation	40.0	48.0	33.0
PEP carboxylase	1.43	1.15	0.94
IDH	45.0	20.0	22.5
ISL*	5.0	3.3	8.3

*Continuous Method I was used

cells in such cultures with mixed carbon source continued to synthesise protein and chlorophyll instead of producing the metabolites necessary for cell division.

Growth in acetate did not practically influence the ability of the cell to fix CO_2 in light. The detection of PEP carboxylase in all extracts indicated that the replenishment of 4C-compounds in the tricarboxylic acid cycle could be achieved in C. segnis via β-carboxylation of phosphoenolpyruvate.

Whereas the activity of ISL was slightly altered in cells grown either in presence of CO_2 or acetate, it increased significantly when CO_2 and acetate were provided together (Goulding and Merrett, 1966). The IDH activity decreases 50% as a result of algal growth in acetate. Such a decrease was not associated with a decrease in the protein content, even when it is expressed on the basis of per ml of algal suspension. Alternatively, the higher activity of IDH in CO_2 grown cells was not accompanied by the production of more protein or chlorophyll as compared to that observed in acetate cultures. This may imply that a substantial portion of α-ketoglutarate produced via IDH in CO_2 grown cells was catabolised.

II. Kinetic Studies

A. Purification of NADP-isocitrate Dehydrogenase:

The steps carried out for the purification of IDH from C. segnis are summarised in Table IX. Briefly the procedure involves ammonium sulphate fractionation and column chromatography. The enzyme is labile in Tris-HCl buffer during purification but is stabilized in phosphate buffer in the presence of mercaptoethanol, EDTA and its substrate isocitrate. A highly active NAD-malate dehydrogenase is present in this alga and was eluted at the same position of IDH during Sephadex chromatography (Fig. 5). Before any kinetic studies can be undertaken, it is imperative to separate malate dehydrogenase from isocitrate dehydrogenase as the former enzyme interferes with the assay system of IDH if oxaloacetate is present in the reaction mixture. However, separation of the two enzymes was achieved by hydroxylapatite column chromatography (Fig. 6). Using the procedure as outlined above, a preparation of 50-fold purification was achieved. This enzyme preparation was stored frozen at -4 C in 3 ml aliquots. Prior to any kinetic studies, the partially purified enzyme extracts were dialysed against PEM buffer, 0.005 M, pH 7.2 for 2 hours. The enzyme kept at -4C was stable for more than a month.

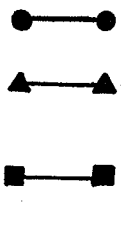
TABLE IX

Summary of Purification Steps for NADP-isocitrate dehydrogenase

Fraction		Total protein (mg)	Total *units	Specific activity	Recovery (%)	Purification (-fold)
I	Crude extracts	814	15,954	19.6	100.0	-
II	$(NH_4)_2SO_4$ precipitation	304	15,291	50.3	95.8	2.6
III	Sephadex G-100	93	14,935	160.6	93.6	8.2
IV	DEAE-Sephadex G-50	14	11,833	880.0	77.2	45.0
V	HAP-effluent (pooled)	6	5,850	975.0	36.7	49.7

*One unit of enzyme activity is the amount of enzyme catalyzing the reduction of 1 μmole of $NADP^+$ per min. Specific activity is expressed as units/mg protein.

Fig. 5 Sephadex G-100 column chromatography of fraction II (Table IX); protein extracts prepared from cells grown in 5% CO_2 (v/v) in continuous light.



Protein concentration

NADP-isocitrate dehydrogenase activity

NAD-malate dehydrogenase activity

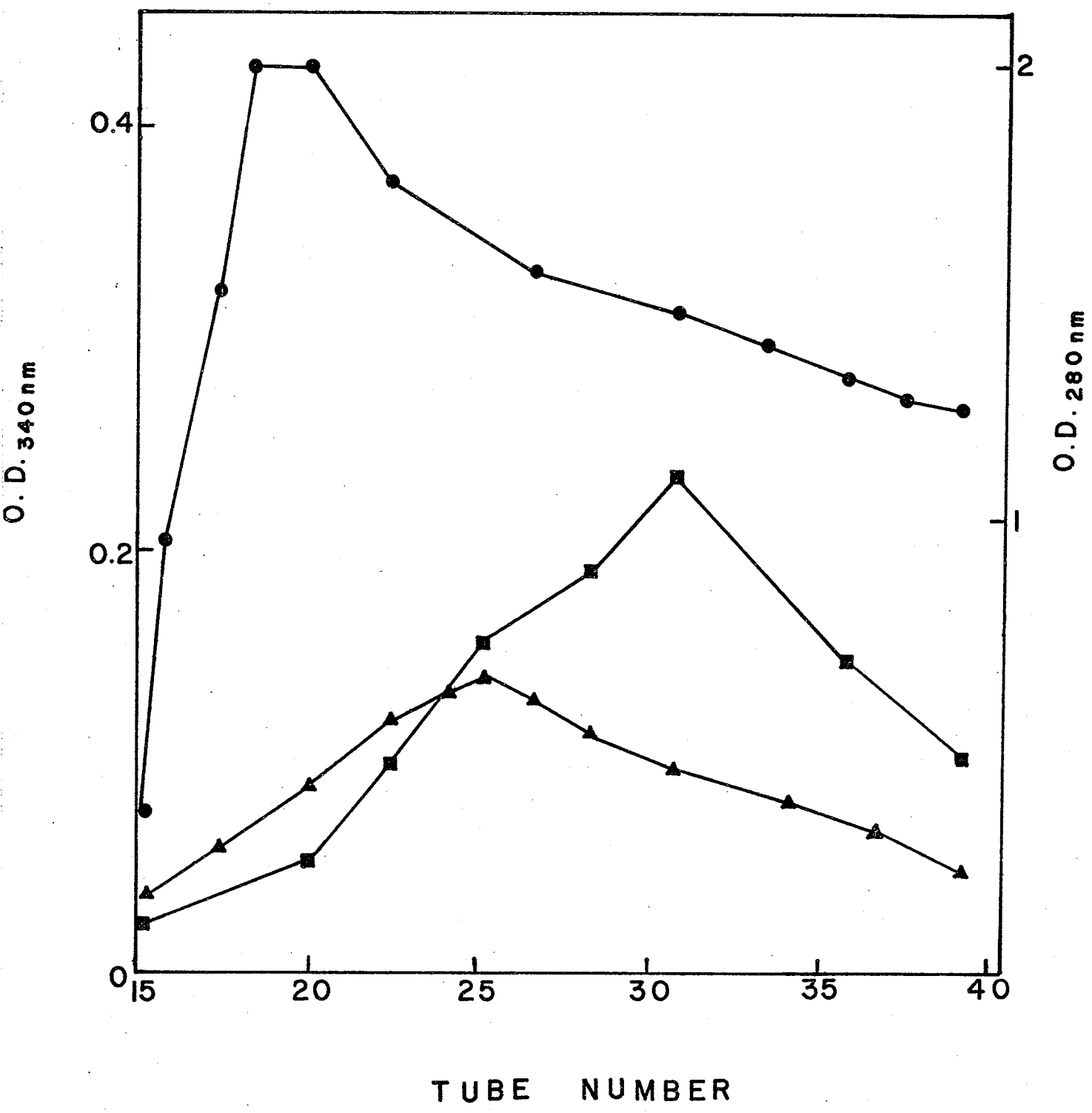
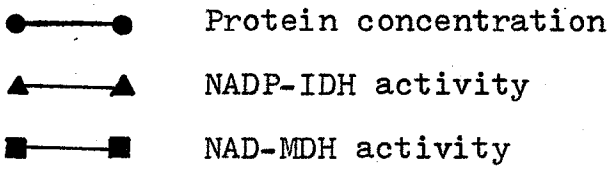
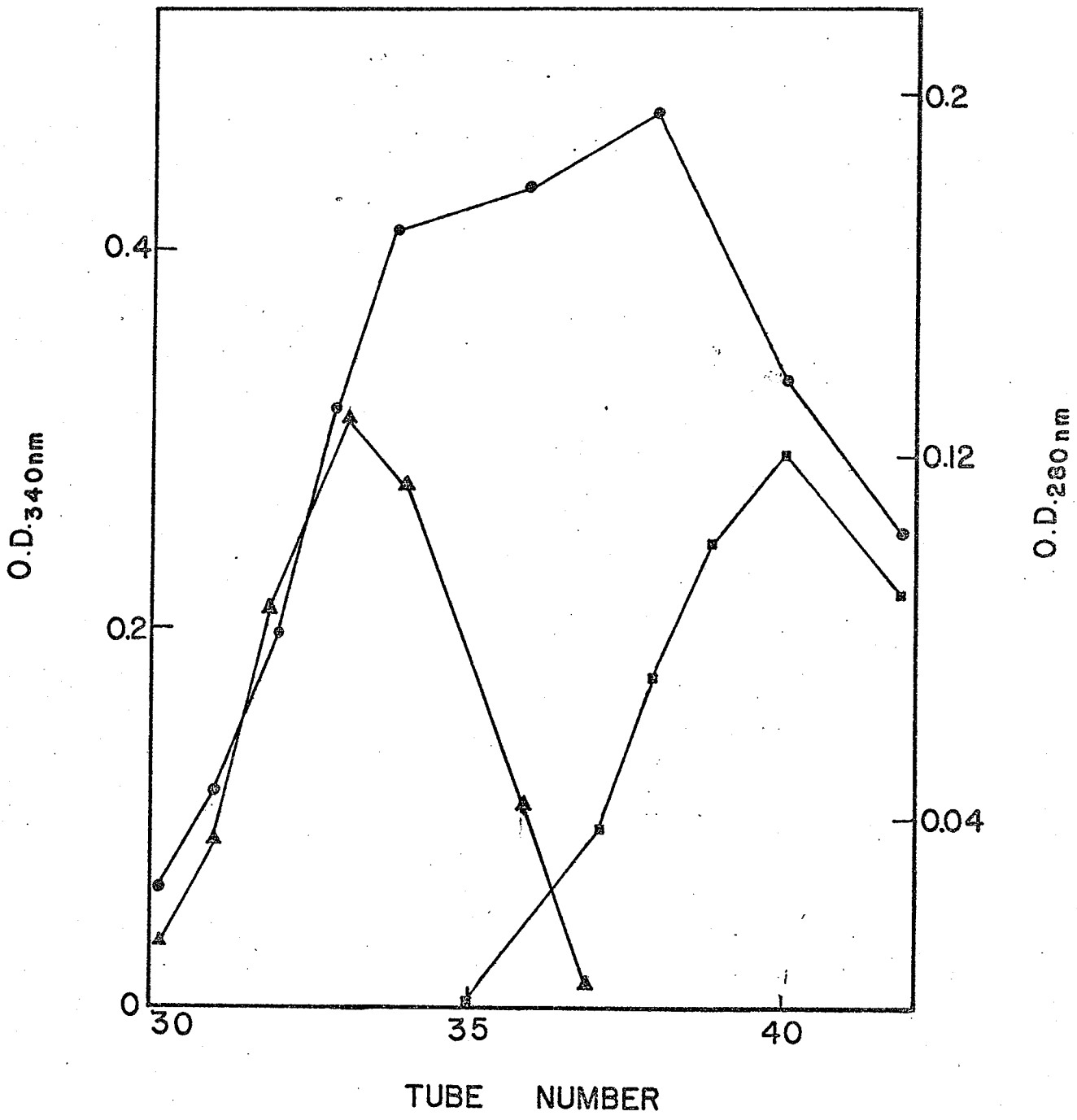

O. D. 340 nm
0.4
0.2
0
O.D. 280 nm
2
1
15
20
25
30
35
40
TUBE NUMBER

Fig. 6 Hydroxyl-apatite column chromatography of the eluate from DEAE-Sephadex G 50.




0.4
0.2
0
0.2
0.12
0.04
O.D.340nm
O.D.280nm
30
35
40
TUBE NUMBER

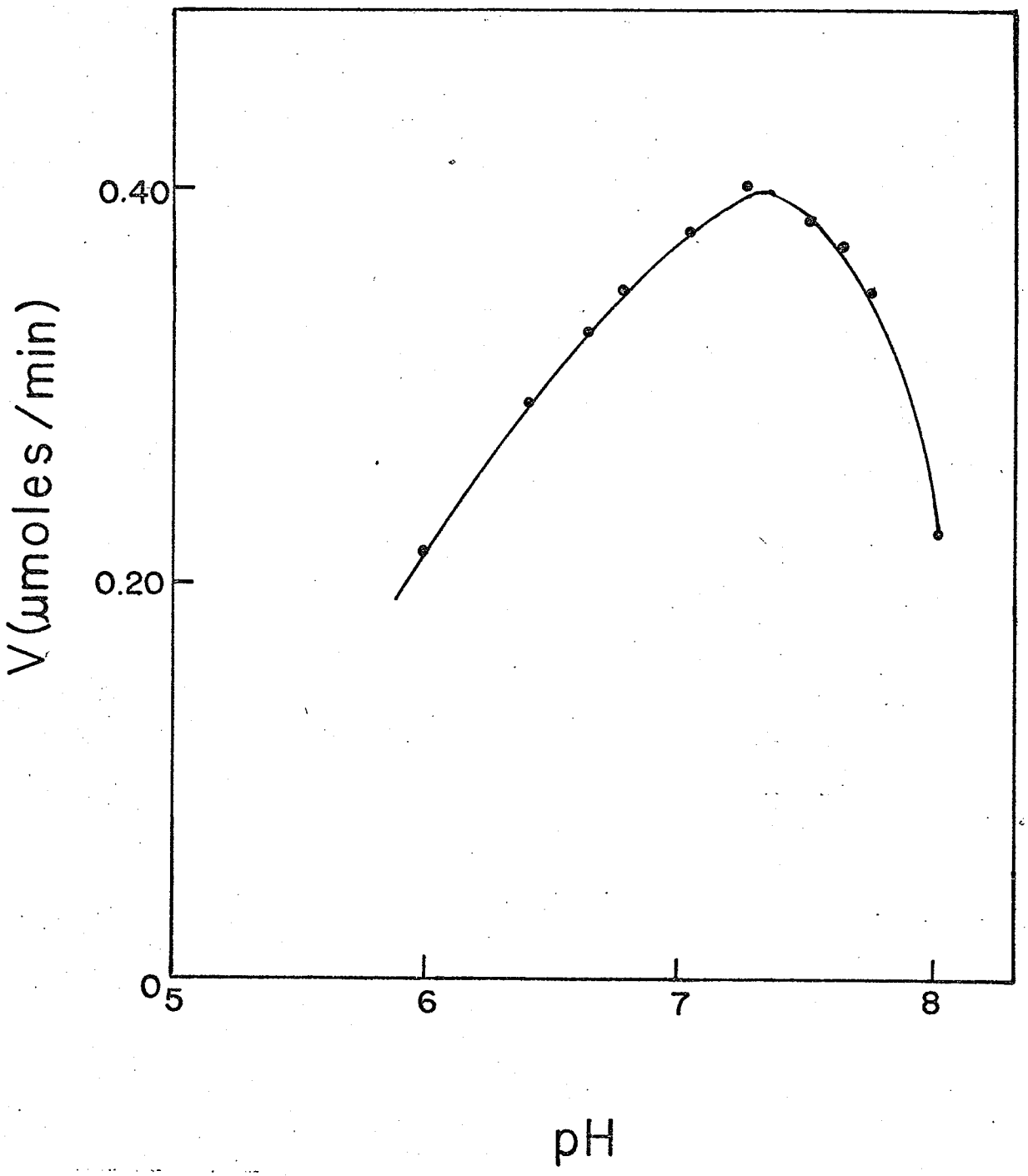
B. Effect of pH:

Isocitrate dehydrogenase exhibits a pH optimum of 7.2 in 0.1 M phosphate buffer (Fig. 7). At pH 6.0 and 8.0 the activity is about 50% lower than the activity at pH 7.2. The enzyme is, however, inactivated readily by 0.1 M Tris-HCl buffer at pH 7.0. Chung and Braginski (1972) attributed the loss of IDH from Rhodopseudomonas spheroides in Tris-acetate buffer to a change in enzyme structure.

C. Km for isocitrate, Mn, NADP of IDH:

From Figs. 8, 9, 10, 11, the apparent Km for isocitrate, Mn^{++}, NADP for IDH and ISL were determined and given in Table X. The Km was obtained for various substrates by plots of varied substrate concentration and initial velocity. Both IDH & ISL show normal Michaelis-Menten hyperbolic curves. The Km value of isocitrate for the dehydrogenase was calculated to be 7.7×10^{-6} M. This agrees with the value reported from C. reinhardtii which was 12.5×10^{-6} M (Ramaley & Hudock, 1973). The apparent affinity of the dehydrogenase for isocitrate is 10-fold higher than that of isocitrate lyase which has a Km of 1×10^{-4} M. The dehydrogenase is specific for NADP for which the Km is 11.0×10^{-6}M and does not show any activity with NAD. Requirement of metal ions such as Mn^{++} and Mg^{++} is necessary for optimum activity.

Fig. 7 The effect of pH on NADP-isocitrate dehydrogenase activity using 0.1 M phosphate buffer.


0.40
0.20
0
V (μmoles / min)
5
6
7
8
pH

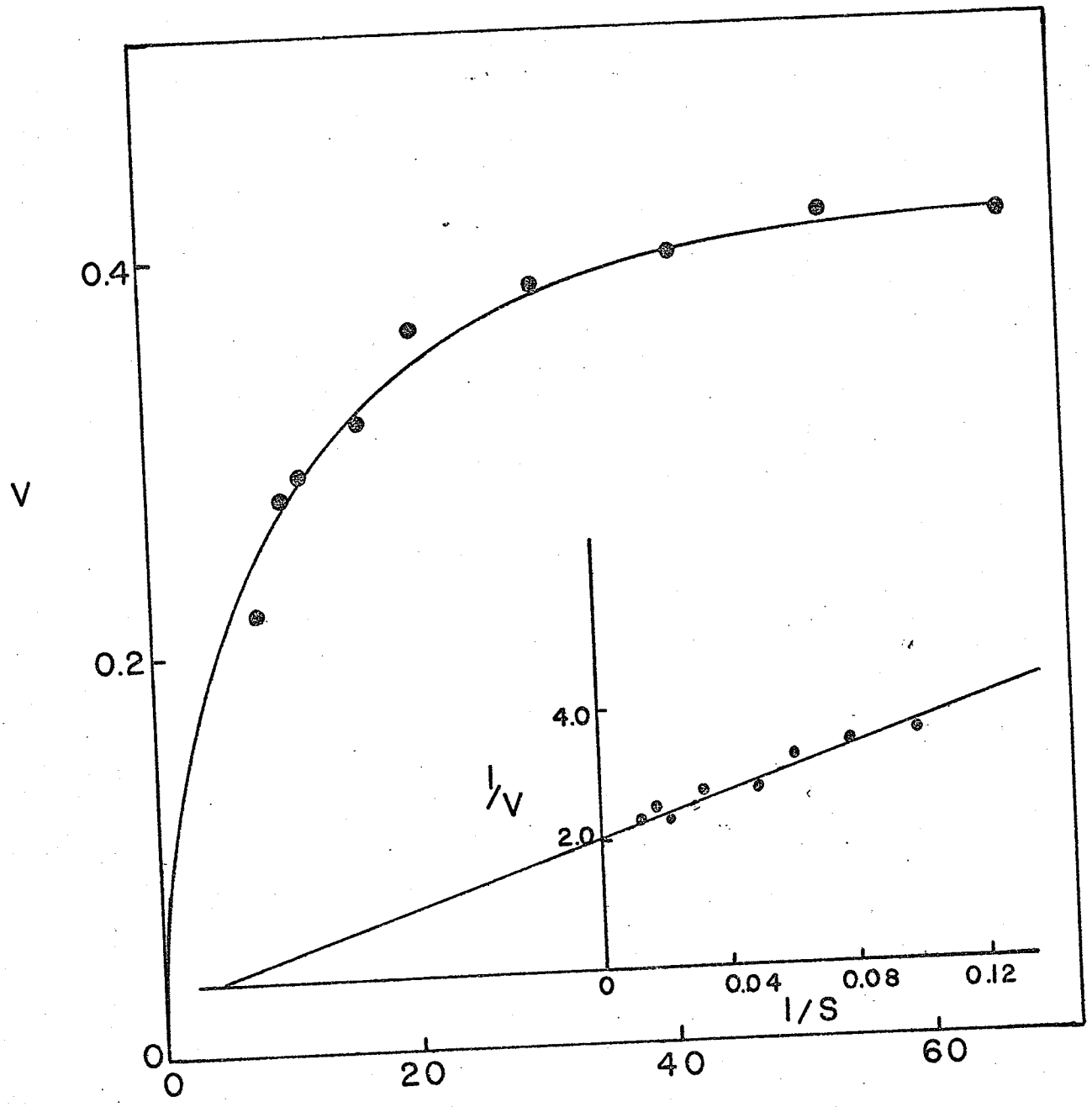

V
0.4
0.2
0
0
20
40
60
ISOCITRATE x 10⁶ M
1/V
4.0
2.0
0
0.04
0.08
0.12
1/S

Fig. 9 Initial velocity vs manganese concentration for IDH of C. segnis. Reaction mixture: 3 ml final volume, 100 µmoles phosphate buffer, pH 7.2, 0.5 µmoles of NADP, 10 µmoles of DL isocitrate, 30 ug protein, and varying amounts of manganese as indicated. The v values are expressed as µmoles NADPH formed per minute.

$Km = 6.0 \times 10^{-6} M.$

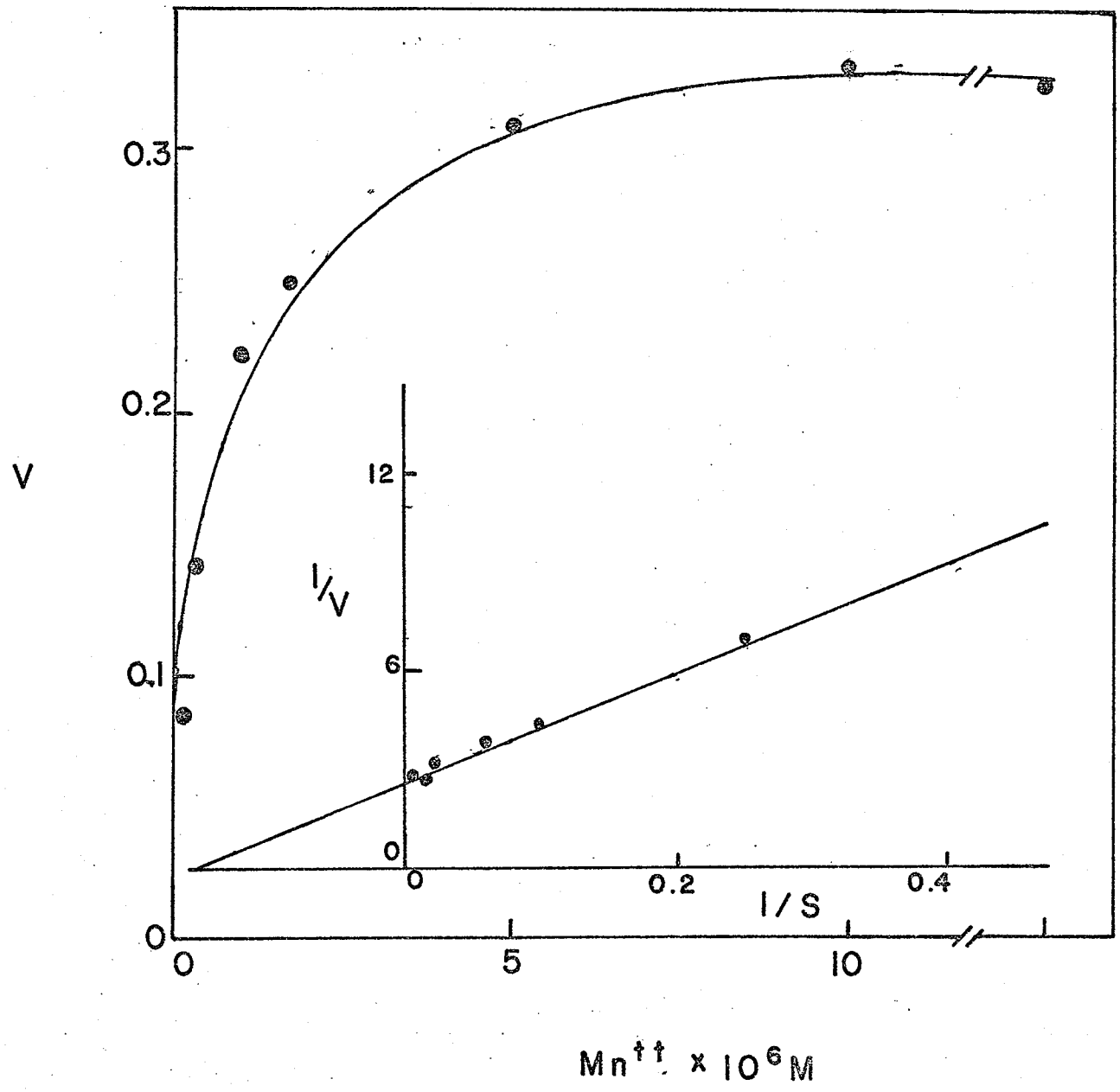

V
0.3
0.2
0.1
0
0
5
10
$Mn^{++} \times 10^6 M$
1/V
12
6
0
0
0.2
0.4
1/S

Fig. 10 Effect of NADP concentration on the initial velocity of IDH. Reaction systems (3.0 ml) contained 100 umoles of phosphate buffer, pH 7.2, 10 umoles of DL isocitrate, 1 umole $MnCl_2$, 30 ug enzyme protein and varying amounts of NADP as indicated.

Km = 11.0×10^{-6}M.

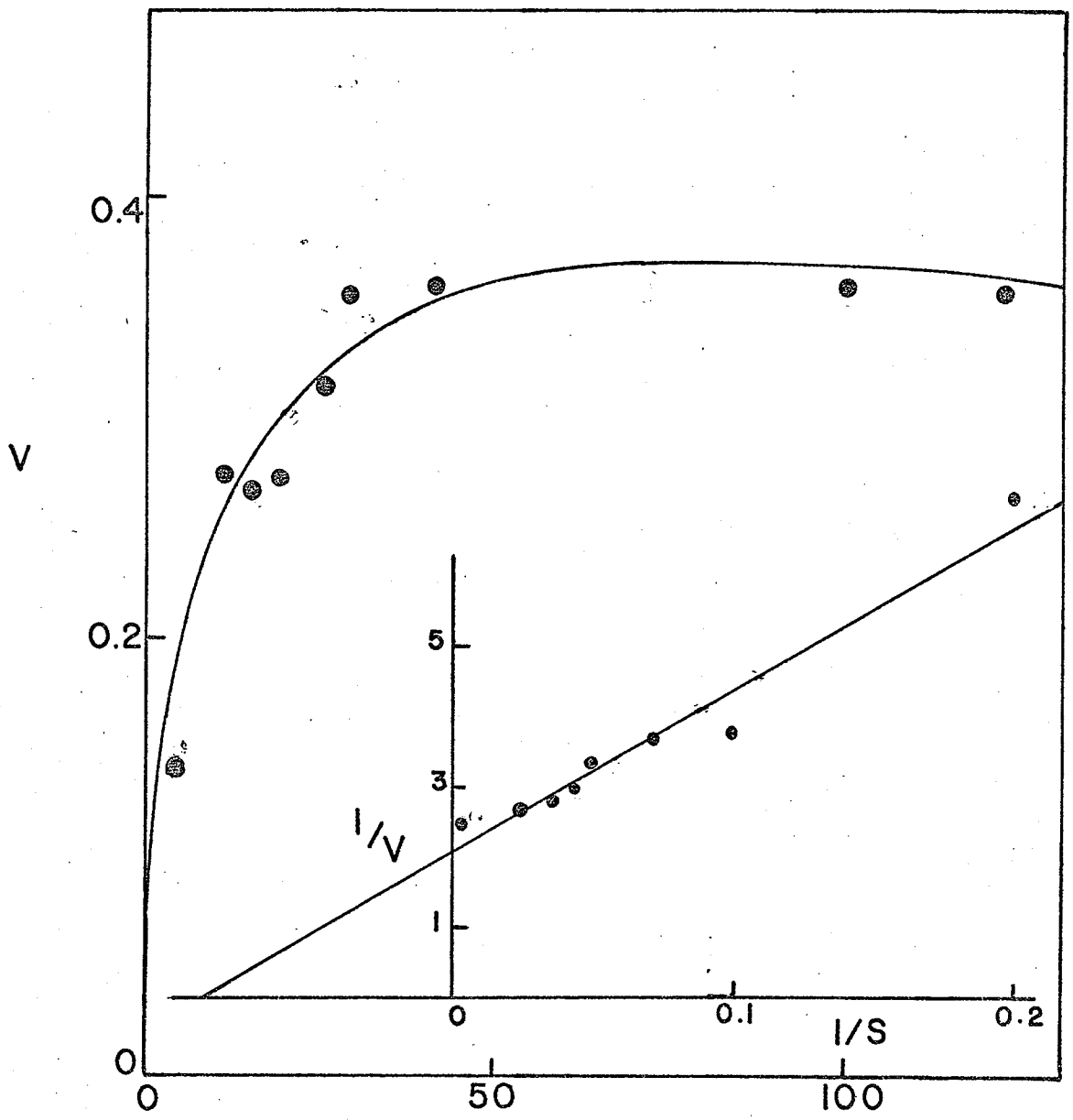

0.4
0.2
0
V
0
50
100
NADP x 10⁶ M
5
3
1
1/V
0
0.1
0.2
1/S

Fig. 11 Effect of isocitrate concentration on the initial velocity of isocitrate lyase. Assays were done according to the continuous method of Dixon and Kornberg (1958). Velocity is expressed as umoles of glyoxylate phenylhydrazone formed per minute.

$Km = 0.1 \times 10^{-3} M.$

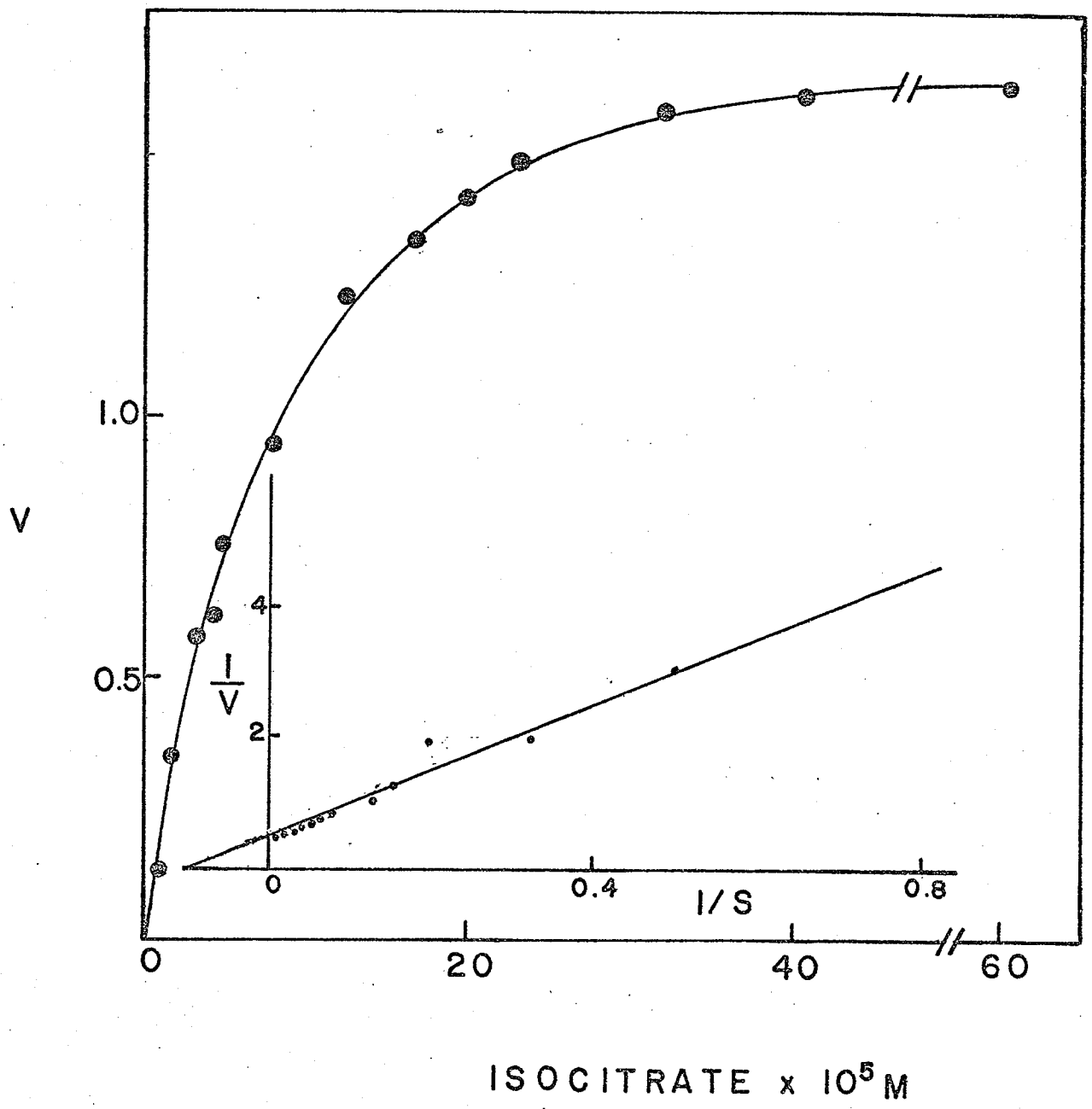

V
1.0
0.5
0
20
40
60
ISOCITRATE x 10^5 M
1/V
4
2
0
0.4
0.8
1/S

Table X

Michaelis constants (Km) for Ds-threo isocitrate, NADP, & Mn^{++}

Substrates	IDH (μM)	ISL (μM)
Ds-threo isocitrate	7.7	100
NADP	11.0	-
Mn^{++}	6.0	-

D. Inhibition of IDH and ISL by added metabolites:

In the absence of either some type of regulatory mechanisms or compartmentalization, we expect keen competition between the two enzymes for their common substrate, isocitrate. Thus, it is desirable to study the effects of certain metabolites on both enzymes in order to elicit the type of regulation which might exist at this metabolic branch point.

These studies were carried out with partially purified fractions of the enzymes and normally three concentrations (1, 5, and 10 mM) of the metabolites were tested. Among the organic acids that were tested, succinate, malate, tartarate and cis-aconitate at a concentration of 1 mM, mildly inhibited the activity of isocitrate lyase (Table XI). Oxaloacetate and α-ketoglutarate were more effective inhibitors of this enzyme. However, the inhibition of IDH by each of the latter two keto acids was about 100% greater than that obtained with ISL. The latter enzyme was also strongly inhibited by oxalate while the dehydrogenase remained unaffected. In Chlorella pyrenoidosa, the inhibition of isocitrate lyase activity by oxaloacetate, pyruvate, and phosphoenolpyruvate was reported by John & Syrett (1968).

Among the metabolites arising from photosynthesis at a concentration of 1 mM, only glycollate exerted a substantial inhibitory effect on isocitrate lyase activity while the dehydrogenase was susceptible only to inhibition by 3-phosphoglycerate (Table XI).

None of the amino acids tested showed any appreciable effect on the activities of the two enzymes. While none of

TABLE XI

Inhibition of NADP-isocitrate dehydrogenase and isocitrate lyase activities by organic acids related to the tricarboxylic acid cycle and the glyoxylate cycle. Incubation mixture was as described in Materials and Methods except that the indicated concentration of organic acids was used. The incubation Method II was employed for the determination of isocitrate lyase activity.

	NADP-Isocitrate Dehydrogenase					
	10 mM		5 mM		1 mM	
Additives	O.D./min	% of inhibition	O.D./min	% of inhibition	O.D./min	% of inhibition
Nil	0.061	0	0.061	0	0.061	0
Acetyl coA	0.061	0	0.061	0	0.061	0
Citrate	0.061	0	0.061	0	0.061	0
cis-Aconitate	0.045	26	0.051	16	0.057	6
α-ketoglutarate	0	100	0	100	0.019	68
succinate	0.061	0	0.061	0	0.061	0
fumarate	0.061	0	0.061	0	0.061	0
malate	0.061	0	0.061	0	0.061	0
oxaloacetate	0.006	90	0.008	87	0.013	79
oxalate	0.053	14	0.061	0	0.061	0
glycerate	0.061	0	0.061	0	0.061	0
tartrate	0.053	13	0.061	0	0.061	0
glyoxylate (1 mM)	-	-	0.061	0	0.061	0

Table XI . . . continued

Additives	Isocitrate Lyase					
	10 mM		5 mM		1 mM	
	O.D./min	% inhibition	O.D./min	% inhibition	O.D./min	% inhibition
Nil	0.79	0	0.79	0	0.79	0
Acetyl coA	-	-	-	-	0.78	0
Citrate	0.72	9	0.79	0	0.78	0
cis-Aconitate	0.48	39	0.51	35	0.60	24
α-ketoglutarate	0.43	45	0.47	40	0.50	37
succinate	0.36	53	0.47	40	0.62	21
fumarate	0.44	44	0.55	30	0.79	0
malate	0.43	45	0.55	33	0.52	24
oxaloacetate	-	-	-	-	0.50	36
oxalate	0.35	66	0.28	65	0.31	60
glycerate	0.64	19	0.78	0	0.77	0
tartrate	0.58	27	0.58	26	0.61	22
glyoxylate (1 mM)	-	-	-	-	0.69	13

Table XI (continued)

Inhibition of NADP-isocitrate dehydrogenase and isocitrate lyase activities by some intermediates of Pentose Reductive Cycle and glycolysis.

	NADP-Isocitrate Dehydrogenase					
	10 mM		5 mM		1 mM	
Additives	O.D./min	% of inhibition	O.D./min	% of inhibition	O.D./min	% of inhibition
Nil	0.061	0	0.061	0	0.061	0
3-phosphoglycerate	0.030	50	0.034	45	0.043	30
Dihydroxyacetone-P	0.061	0	0.061	0	0.061	0
Fructose-1, 6-DiP	0.059	3	0.061	0	0.061	0
Ribose-5-P	0.046	25	0.061	0	0.061	0
Ribulose-1, 5-DiP	-	-	-	-	0.061	0
Glycollate	0.061	0	0.061	0	0.061	0
Glucose-1-P	0.061	0	0.061	0	0.061	0
Glucose-6-P	0.061	0	0.061	0	0.061	0
Phosphoenolpyruvate	0.050	18	0.061	0	0.061	0
Pyruvate	0.061	0	0.061	0	0.061	0
Acetyl Phosphate	0.061	0	0.061	0	0.061	0
Glycoaldehyde	0.061	0	0.061	0	0.061	0

Table XI . . . continued

	Isocitrate Lyase					
	10 mM		5 mM		1 mM	
Additives	O.D./min	% inhibition	O.D./min	% inhibition	O.D./min	% inhibition
nil	0.78	0	0.78	0	0.79	0
3-phosphoglycerate	0.25	69	0.54	31	0.78	0
Dihydroxyacetone-P	0.65	15	0.79	0	0.79	0
Fructose-1, 6-DiP	0.70	11	0.78	0	0.79	0
Fructose-6-P	0.68	13	0.79	0	0.78	0
Ribose-5-P	0.61	22	0.77	0	0.79	0
Ribulose-1, 5 DiP	0.45	44	0.70	12	0.78	0
Glycollate	0.30	71	0.30	61	0.51	36
Glucose-1-P	0.62	22	0.78	0	0.79	0
Glucose-6-P	0.78	0	0.79	0	0.77	0
Phosphoenolpyruvate	0.61	22	0.78	0	0.79	0
Pyruvate	0.39	50	0.44	44	0.66	15
Acetyl Phosphate	0.63	20	0.78	0	0.77	0
Glycoaldehyde	-	-	-	-	0.78	0

Table XI (continued)

Inhibition of NADP-isocitrate dehydrogenase and isocitrate lyase activities by some amino acids, nucleotides and related compounds.

	NADP-Isocitrate Dehydrogenase					
	10 mM		5 mM		1 mM	
Additives	O.D./min	% of inhibition	O.D./min	% of inhibition	O.D./min	% of inhibition
Nil	0.061	0	0.061	0	0.061	0
NAD	0.061	0	0.061	0	0.061	0
NADP	-	-	-	-	-	-
NADH	-	-	-	-	0.061	0
NADPH (0.1 mM)	-	-	-	-	0.026	57
ATP	0	100	0	100	0.018	71
ADP	0	100	0	100	0.048	21
AMP (5')	0.061	0	0.061	0	0.061	0
AMP (3')	0.048	21	0.061	0	0.061	0
AMP (3, 5)	0.060	0	-	-	-	-
Serine	0.061	0	0.061	0	0.061	0
Glycine	0.061	0	0.061	0	0.061	0
B-alanine	0.061	0	0.061	0	0.061	0
Aspartate	0.061	0	0.061	0	0.061	0
Glutamate	0.061	0	0.061	0	0.061	0

Table XI . . . continued

Additives	Isocitrate Lyase					
	10 mM		5 mM		1 mM	
	O.D./ 10 min	% inhibition	O.D./ 10 min	% inhibition	O.D./ 10 min	% inhibition
Nil	0.78	0	0.78	0	0.78	0
NAD	0.79	16	0.79	0	0.77	0
NADP	-	-	-	-	-	-
NADH	-	-	-	-	0.79	0
NADPH (0.1 mM)	0.78	0	0.79	0	0.78	0
ATP	0.79	0	0.78	0	0.79	0
ADP	0.46	42	0.55	30	0.78	0
AMP (5')	0.79	0	0.78	0	0.77	0
AMP (3')	0.38	51	0.47	40	0.58	26
AMP (3, 5)	-	0	-	0	-	0
Serine	0.62	21	0.78	0	0.79	0
Glycine	0.77	0	0.79	0	0.78	0
B-alanine	0.79	14	0.78	0	0.79	0
Asparate	0.59	24	0.79	0	0.78	0
Glutamate	0.69	11	0.78	0	0.79	0

the nucleotides tested showed any inhibition on isocitrate lyase activity, the dehydrogenase was markedly inhibited by NADPH at a very low concentration of 0.1 mM. ADP and ATP at a concentration of 1.0 mM was an equally potent inhibitor of the dehydrogenase while ISL was inhibited only by AMP(3').

E. Kinetics of Inhibition of Isocitrate Dehydrogenase

In order to evaluate the natural role that the inhibitors might have in regulating cellular metabolism of C. segnis, it is pertinent to know the modes of inhibition of the inhibitors and their apparent affinity for their specific enzyme active site.

The kinetic patterns of inhibitions were observed in the usual double reciprocal plots of initial velocity. The methods of Dixon (1953) were used in which isocitrate was varied against changing levels of inhibitors. The results of some of the inhibitor constants and their modes of inhibition are shown in Figures 12, 13, 14, and 15 and summarised in Table XII.

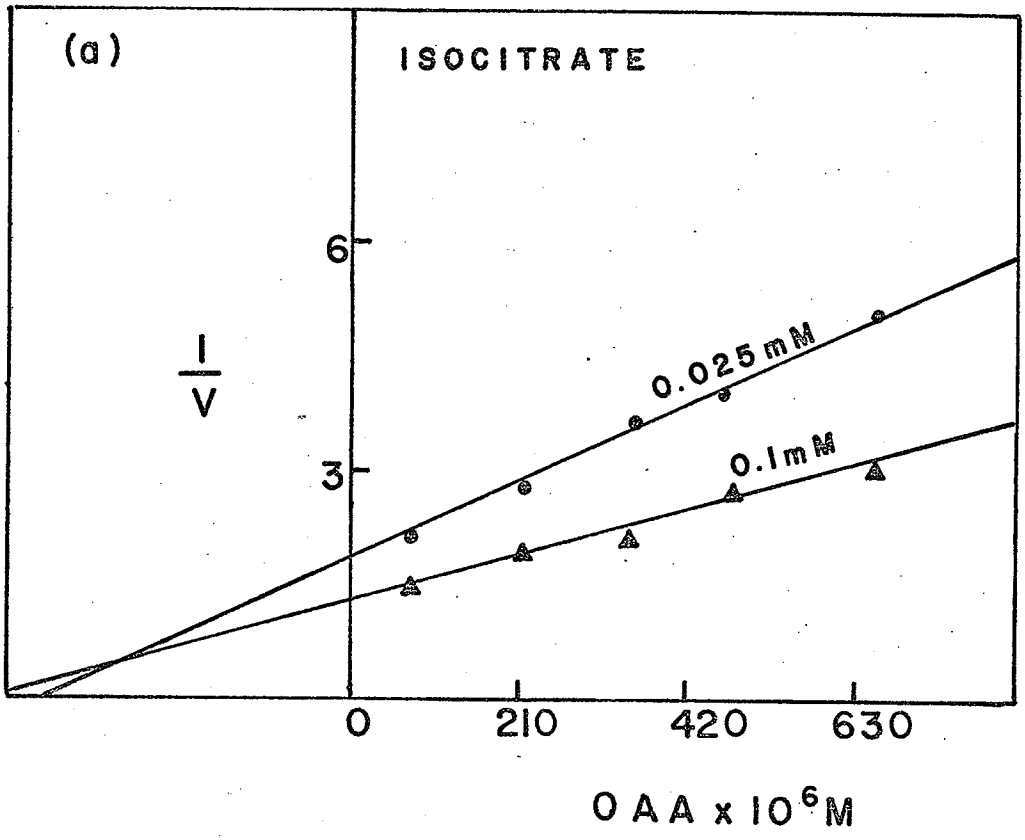
Oxaloacetate and α-ketoglutarate acting alone inhibited IDH activity in a competitive manner and the respective Ki's obtained for the inhibitors are 0.34 and 0.76 mM respectively (Fig. 12 and 13). The dehydrogenase was also sensitive to high energy metabolites. Both ATP and NADPH are non-competitive inhibitors and their respective Ki's are 0.65 mM and 0.064 mM (Fig. 14 and 15).

Fig. 12 Competitive inhibition of NADP-isocitrate dehydrogenase from *C. segnis* by oxaloacetate. The reactions were carried out in the incubation mixture as described in Materials & Methods except that the indicated concentrations of isocitrate and inhibitors were used. The v values are expressed as μmoles NADPH formed per minute.

$K_i = 0.34 \times 10^{-3}$ M.

(a) Determination of inhibition constant (Ki) for oxaloacetate (Dixon, 1953).

(b) Double reciprocal plot of the inhibition of oxaloacetate on IDH. (Lineweaver and Burke, 1934.)


(a)
ISOCITRATE
6
3
1/V
0.025 mM
0.1 mM
0
210
420
630
OAA x 10⁶M

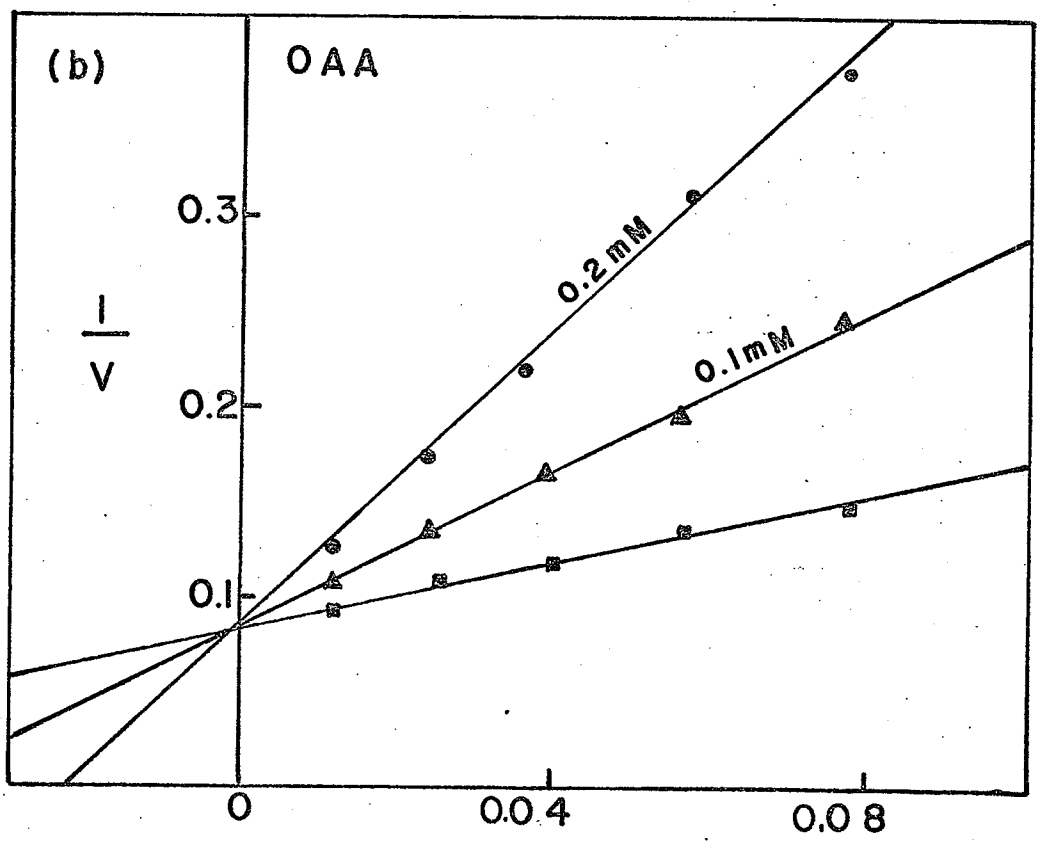
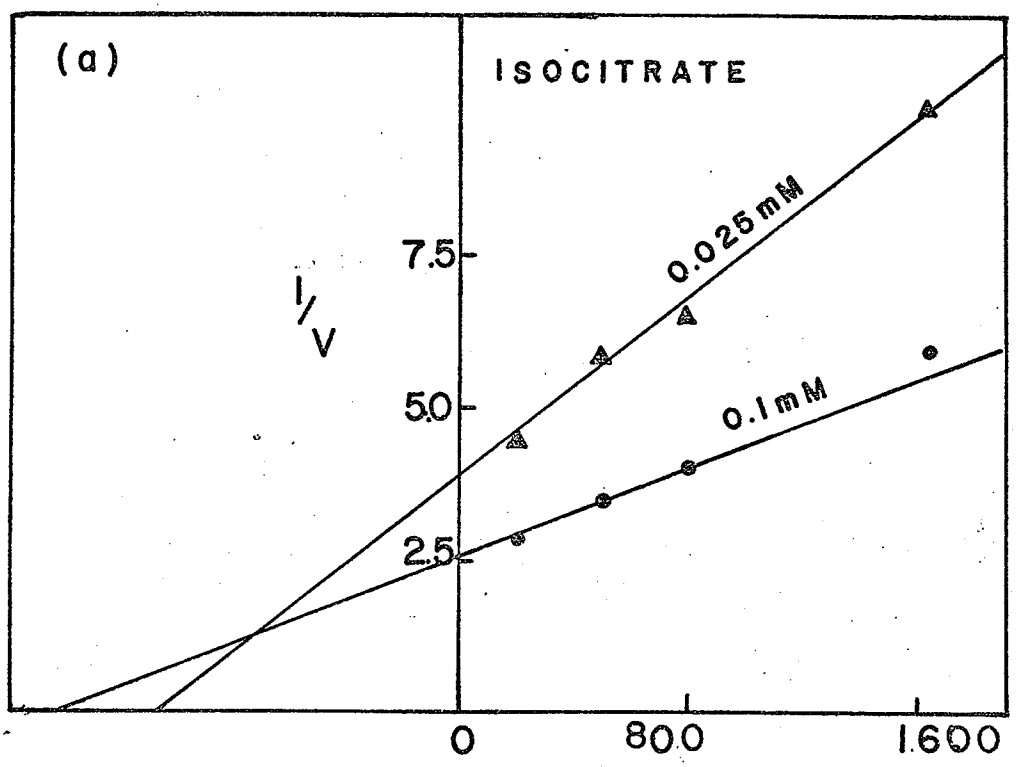

(b)
OAA
0.3
0.2
0.1
1/V
0.2 mM
0.1 mM
0
0.04
0.08
1/ISOCITRATE x 10⁶M

Fig. 13 Competitive inhibition of NADP-isocitrate dehydrogenase by α-ketoglutarate. The reactions were carried out in the incubation mixture as described in Materials and Methods except that the indicated concentrations of isocitrate and inhibitors were used. The v values are expressed as μmoles of NADPH formed per minute.

$K_i = 0.76 \times 10^{-3}$ M.

(a) Determination of inhibition constant for α-ketoglutarate.

(b) Double reciprocal plot of the inhibition of α-ketoglutarate on IDH.


(a)
ISOCITRATE
0.025 mM
0.1 mM
1/V
7.5
5.0
2.5
0
800
1.600
α- KETOGLUTARATE x 10⁶ M

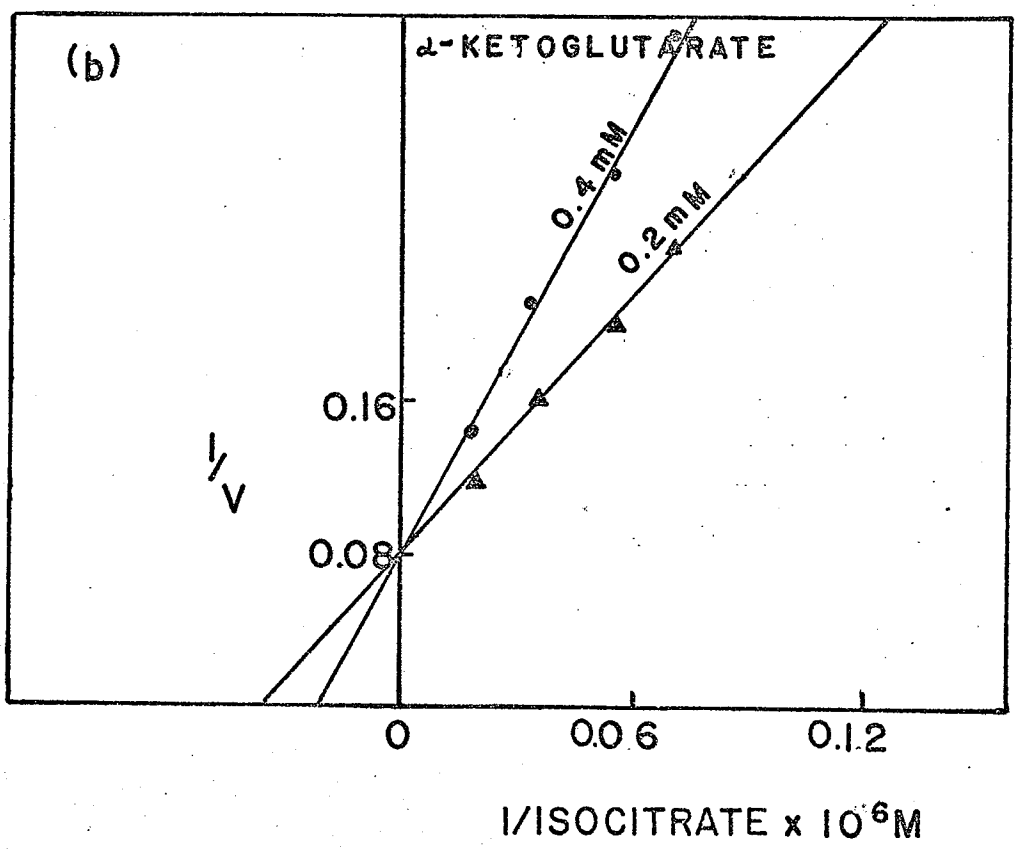
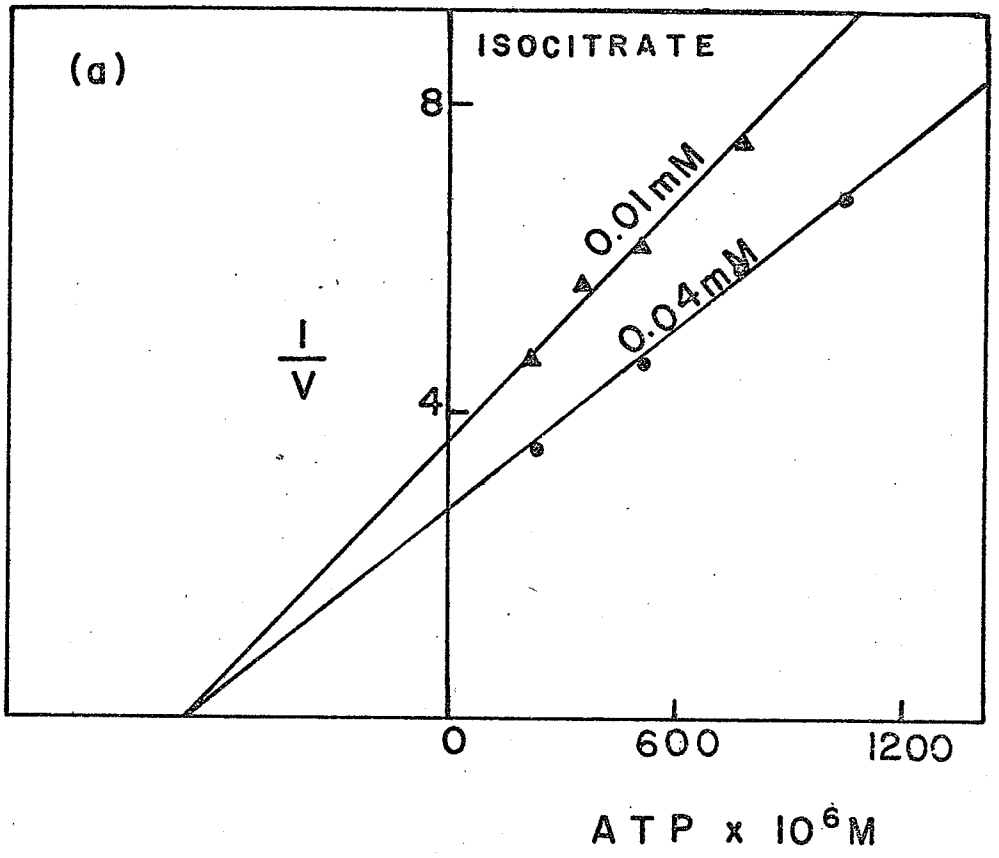

(b)
α-KETOGLUTARATE
0.4 mM
0.2 mM
1/V
0.16
0.08
0
0.06
0.12
1/ISOCITRATE x 10⁶ M

Fig. 14 Non-competitive inhibition of NADP-isocitrate dehydrogenase by ATP. The reactions were carried out in the incubation mixture as described in Materials and Methods except that the indicated concentrations of isocitrate and inhibitors were used. The v values are expressed as umoles NADPH formed per minute.

$K_i = 0.65 \times 10^{-3}$ M.

(a) Determination of inhibition constant for ATP.

(b) Double reciprocal plot of the inhibition of ATP.


(a)
ISOCITRATE
8
4
0.01mM
0.04mM
1/V
0
600
1200
ATP x 10⁶M

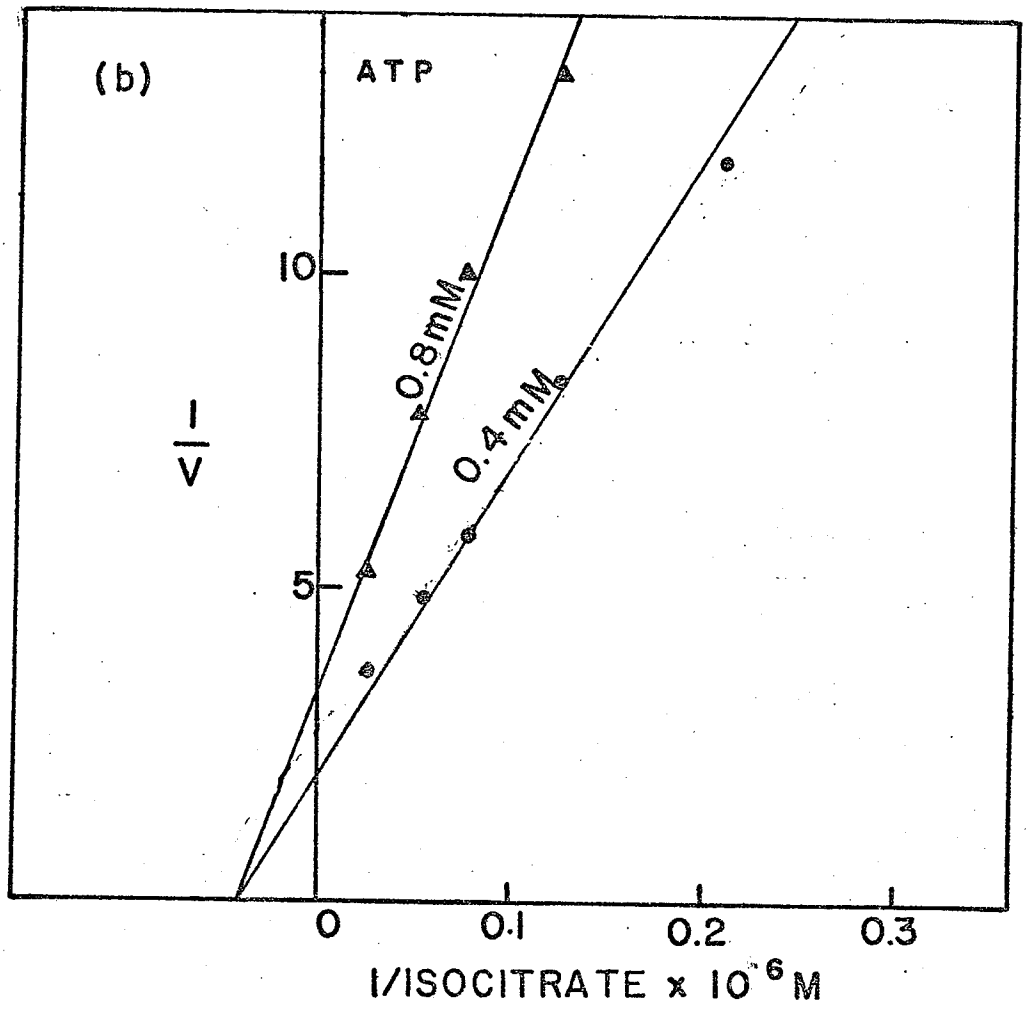
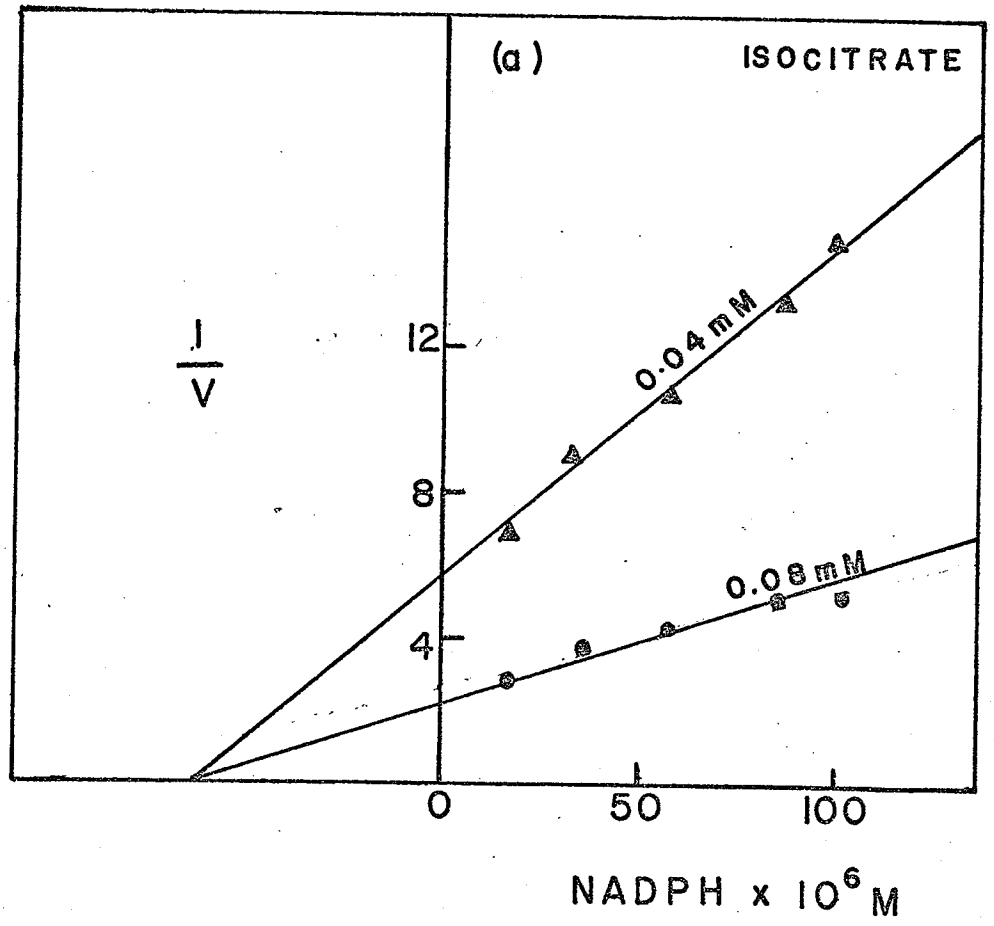

(b)
ATP
10
5
0.8mM
0.4mM
1/V
0
0.1
0.2
0.3
1/ISOCITRATE x 10⁶M

Fig. 15 Non-competitive inhibition of NADP-isocitrate dehydrogenase by NADPH, The reactions were carried out in the incubation mixture as described in Materials and Methods except that the indicated concentrations of isocitrate and inhibitors were used. The v values are expressed as umoles NADPH formed per minute.

Ki = 0.064×10^{-3} M.

(a) Determination of inhibition constant for NADPH.

(b) Double reciprocal plot of the inhibition of NADPH.


(a)
ISOCITRATE
1/V
12
8
4
0.04 mM
0.08 mM
0
50
100
NADPH x 10⁶ M

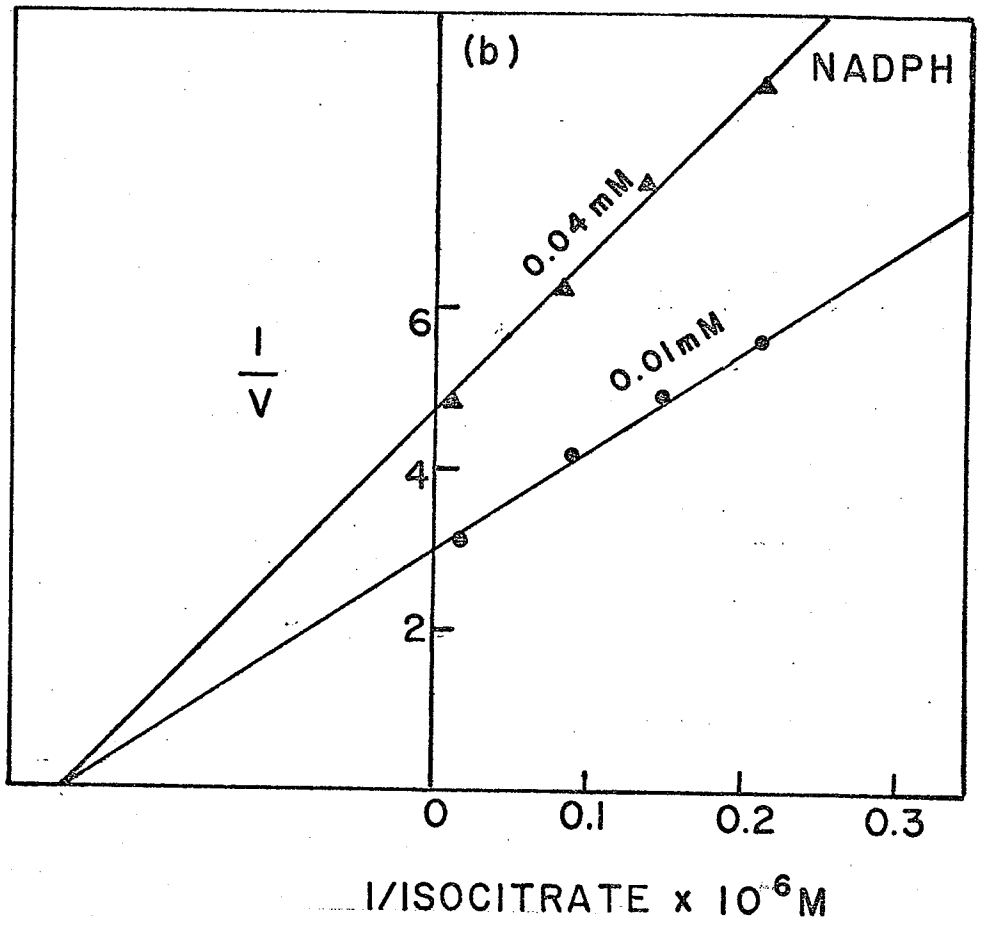

(b)
NADPH
1/V
6
4
2
0.04 mM
0.01 mM
0
0.1
0.2
0.3
1/ISOCITRATE x 10⁶ M

TABLE XII

Inhibition constants and mode of inhibition of NADP-isocitrate dehydrogenase

Metabolites	Type of inhibition	Ki x 10^{-3}M
ATP	Non-competitive	0.65
α-ketoglutarate	Competitive	0.76
3-PGA	Non-competitive	0.85
NADPH	Non-competitive	0.064
OAA	Competitive	0.34

F. Concerted Inhibition of isocitrate dehydrogenase

Many investigators have reported on a concerted inhibitory mechanism by oxaloacetate and glyoxylate (in combination) on the activity of the dehydrogenase (Shiio & Ozaki, 1968; Marr & Weber, 1969; Levy, 1972). This unique mechanism was also observed in Chlamydomonas segnis dehydrogenase (Table XIII). This mode of inhibition is specific and very sensitive. Only 0.1 mM of each of oxaloacetate and glyoxylate can produce virtually complete cessation of the dehydrogenase activity. While oxaloacetate alone at a concentration of 0.2 mM can inhibit the activity of the dehydrogenase by 40%, glyoxylate alone has no effect. Glyoxylate alone has been reported to inhibit the activity of the dehydrogenase from Tetrahymena (Levy, 1972) and activate one of the isozymes in Acinetobacter lwoffi (Self & Weitzman, 1972).

The degree of concerted inhibition by oxaloacetate and glyoxylate is greatly dependent upon the sequence in which reactants are added to the reaction mixture. A marked inhibition (90%) of the dehydrogenase was observed when the reaction was initiated by the addition of its substrate, isocitrate. On the other hand the inhibition was only 60% when the reaction was initiated by addition of NADP (Fig. 16). This may suggest that the substrate could provide protection of the enzyme from inhibitors.

The nature of the inhibition due to glyoxylate and

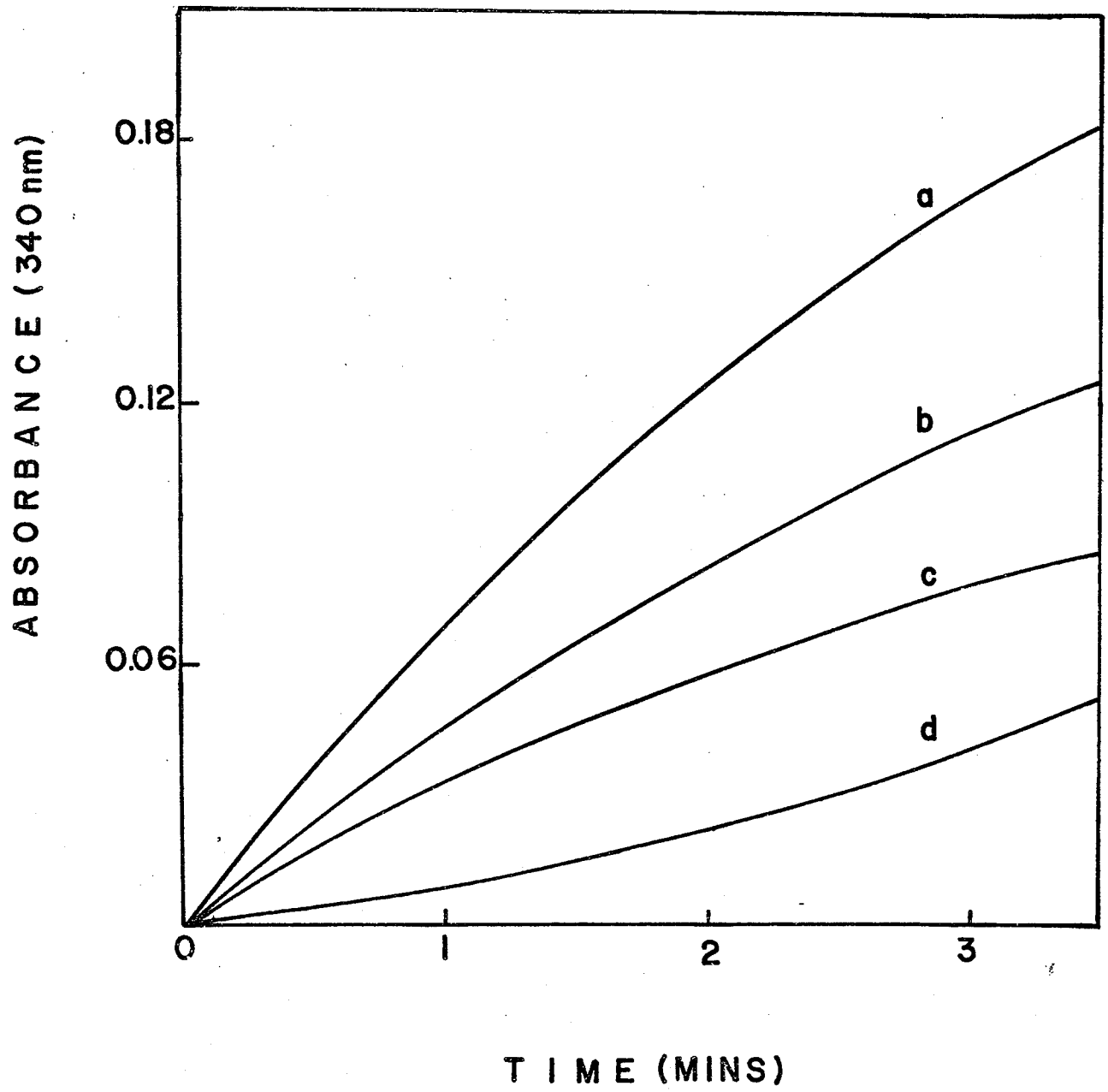
Table XIII

Specificity of concerted inhibition by oxaloacetate and glyoxylate. Incubation mixture was described in Materials and Methods except that the indicated concentration of organic acids was used. The reaction was initiated by the addition of DL-isocitrate at 30 C.

Addition (0.1 mM)	GLYOXYLATE (0.1 mM)		OXALOACETATE (0.1 mM)	
	O.D./min (340 nm)	% of inhibition	O.D./min.	% of inhibition
None	0.061	0	0.041	33
Citrate	0.061	0	0.061	0
Cis-Aconitate	0.054	12	0.049	20
α-ketoglutarate	0.040	37	0.039	37
Succinate	0.061	0	0.041	33
Fumarate	0.053	14	0.039	37
L-Malate	0.053	14	0.041	33
Oxaloacetate	0.004	93	0.036	40
Glyoxylate	0.058	6	0.004	93
Glycollate	0.058	6	0.041	33
Pyruvate	0.061	0	0.032	47

Fig. 16 Protection of IDH from concerted inhibition by oxaloacetate and glyoxylate with its substrate, Ds-threo-isocitrate.

Curve a = no inhibitor, reaction started with isocitrate. Curves b, c and d: 100 μM each of glyoxylate and oxaloacetate, b - started with enzyme (20 ug protein); c - started with NADP; and "d" started with 100 μM isocitrate.


ABSORBANCE (340 nm)
0.18
0.12
0.06
0
1
2
3
a
b
c
d
TIME (MINS)

oxaloacetate, with respect to substrate was studied. These inhibitors were competitive and displayed a Ki of 1.0×10^{-5} M (Fig. 17).

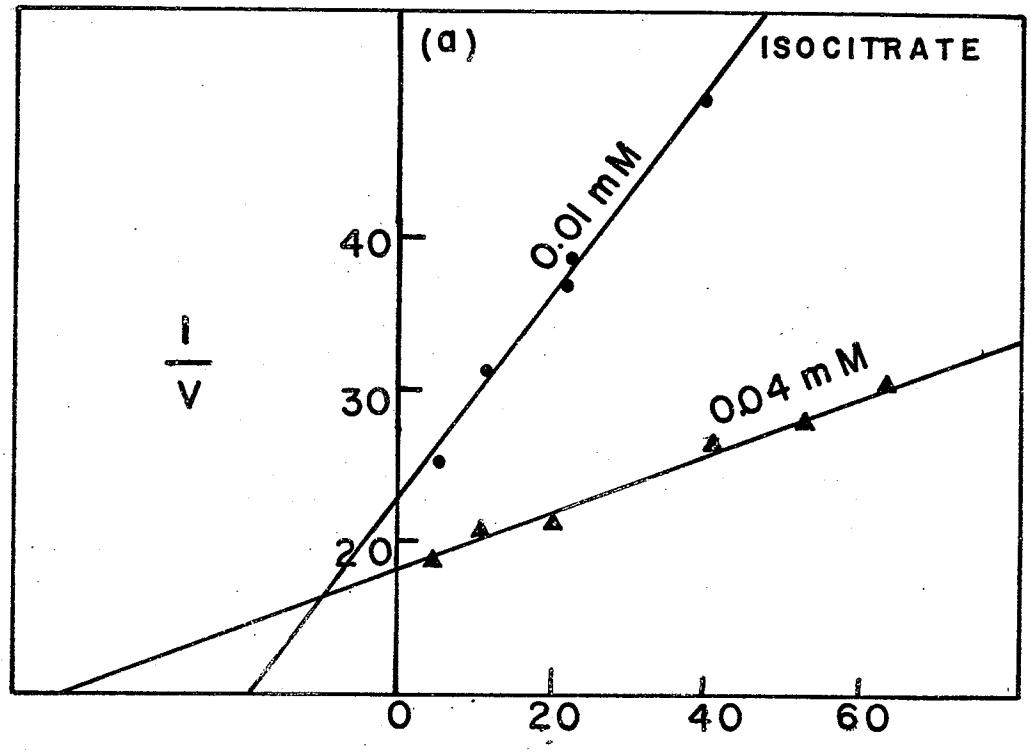
These kinetic results, therefore, suggest that both isocitrate lyase and isocitrate dehydrogenase are subject to different regulatory mechanisms and we expect these regulatory mechanisms to operate in vivo to ensure an efficient way to meet the cells demand.

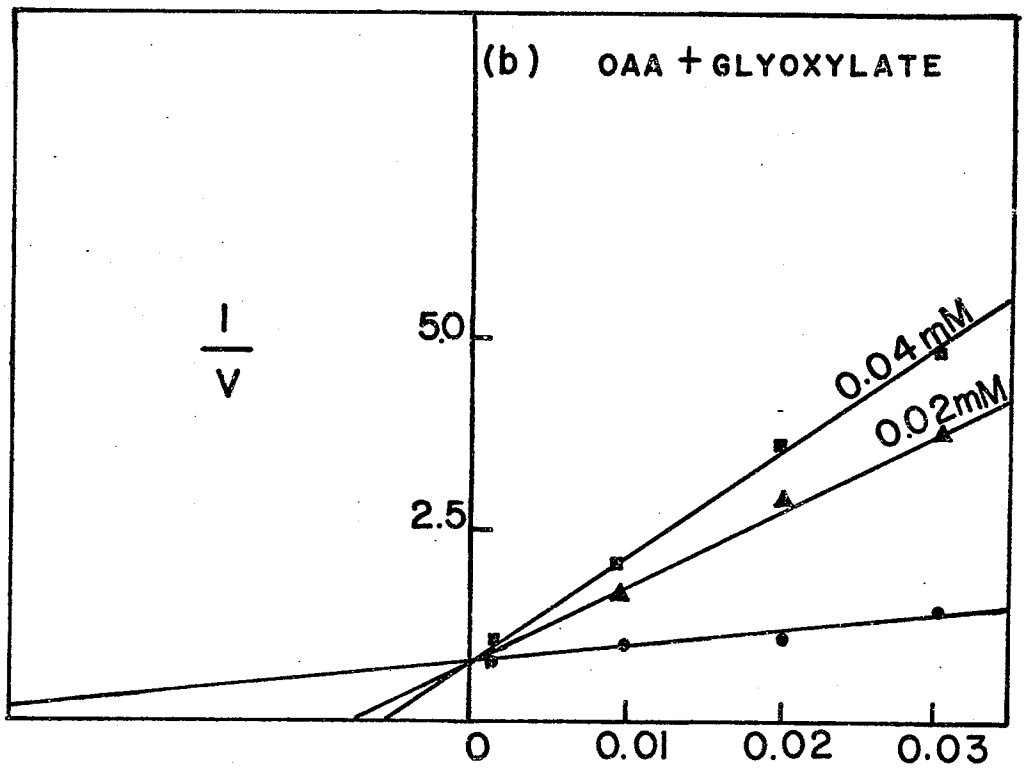
Fig. 17 Competitive inhibition of NADP-isocitrate dehydrogenase from C. segnis by oxaloacetate and glyoxylate (both were added in equal amounts). The reactions were carried out in the incubation mixture as described in Methods except that the indicated concentrations of isocitrate and inhibitors were used. 30 ug protein of IDH preparation was included in the reaction mixture. The v values are expressed as μmoles NADPH formed per minute.

$K_i = 1.0 \times 10^{-5}$ M.

(a) Determination of inhibition constant for oxaloacetate and glyoxylate.

(b) Double reciprocal plot of the inhibition of oxaloacetate and glyoxylate.

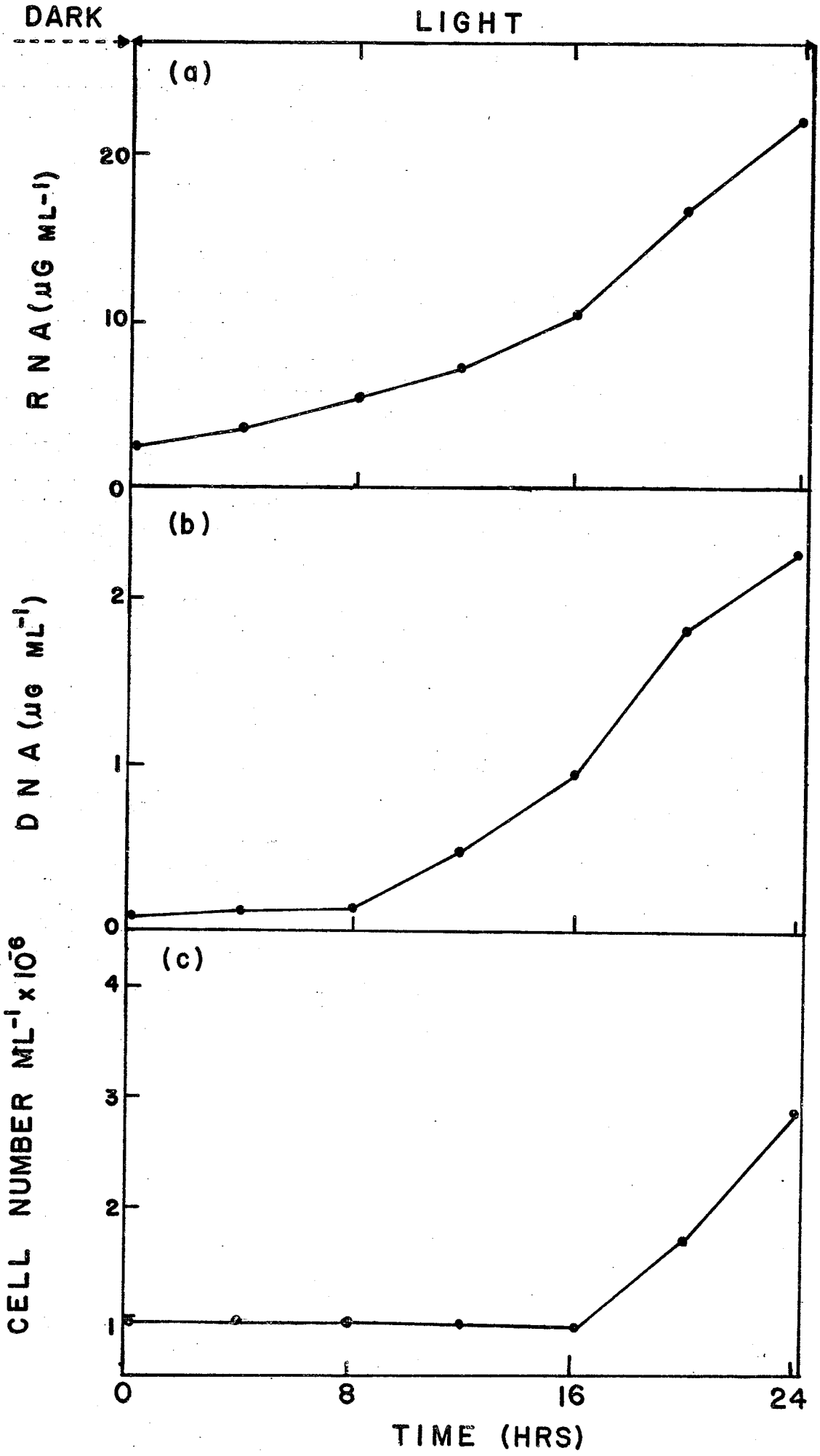

(a)
ISOCITRATE
0.01 mM
0.04 mM
1/V
40
30
20
0
20
40
60
OAA+ GLYOXYLATE x 10⁶M


(b) OAA + GLYOXYLATE
1/V
5.0
2.5
0.04 mM
0.02mM
0
0.01
0.02
0.03
1/ISOCITRATE x 10⁶ M

III. Isocitrate Metabolism during the Development of Chlamydomonas segnis in Synchronous Cultures

In this and the following experiments, the zoospores of C. segnis produced in the dark were collected from well synchronised cultures and resuspended in a fresh nutrient medium. The cultures were then exposed to continuous light (11,000 lux) and bubbled with 5% CO_2 in air (v/v) for 24 hours. Under these conditions, the zoospores would grow and complete their life cycle, without being influenced by the long dark period (12 hr) normally used during the synchronisation method (alternating light dark regime). Physiological and biochemical studies during the algal development have been conducted in continuous illuminated cultures to avoid any effects that may occur by the imposed dark period (Mitchison, 1971; John et al, 1973; and Tan, 1974). Under the described growth conditions, the length of the life cycle of C. segnis, as calculated from the commencement of illumination till the time of attainment of 50% of the final percent increase in cell number (cf. Schmidt, 1969; and Tan, 1974), was about 22 hours (Fig. 18c). Whereas RNA was continuously synthesised during the life cycle as indicated by the increases in the RNA contents of the cell (Fig. 18a), DNA synthesis commenced after an elapse of 8 hours from the beginning of illumination (Fig. 18b). These results indicated that the G_1 and S-phases occupied roughly 0.4 and 0.6 of the life cycle respectively. A shorter growth phase associated with a relatively larger S-phase

Fig. 18 Periodic increases in RNA(a), DNA(b) and cell number (c) of C. segnis during the development in synchronous cultures exposed to continuous light (11,000 lux) and bubbled with 5% CO_2 in air (v/v).


DARK
LIGHT
(a)
RNA (µG ML-1)
20
10
0
(b)
DNA (µG ML-1)
2
1
0
(c)
CELL NUMBER ML-1 x 10-6
4
3
2
1
0
8
16
24
TIME (HRS)

is characteristic of C. segnis when grown in 5% CO_2 (Tan, 1974).

A. Isocitrate Metabolism and Macromolecules Biosynthesis during the G_1-phase:

As shown in Fig. 19, cell free extracts prepared from algal cells during the G_1 phase (0 - 8 hr light) metabolised isocitrate 30 - 44 times faster via the dehydrogenase than the lyase. As indicated from the specific activities of NAD-GDH and NADP-GDH, it was evident that only 11 - 20% of the product (α-ketoglutarate) of IDH would be used for the formation of glutamate via NADP-GDH. The lower activity of the latter during the G_1 phase is in agreement with the observations of Berger (1966), Berger & Pirson (1967), using autotrophically grown Chlorella and Kates and Jones (1967) using Chlamydomonas reinhardtii.

As shown in Fig. 20, active incorporation of U-^{14}C-leucine into the algal cells and the increases in the TCA-insoluble cellular nitrogen occurred during the G_1-phase. Determination of the total cellular protein by the Folin method as described in Materials and Methods, resulted in a linear increase in contradiction to the curve obtained by the Kjeldahl method. Obviously, this is attributable to the fact that the colorimetric Folin Method determines tyrosine which might be present as free amino acid rather than in protein. Protein accumulation during the G_1-phase was associated with chlorophyll synthesis as indicated by the rate of δ-ALA (precursor of pyrrole synthesis) excretion and the increases in chlorophyll content of the cells (Fig. 21). In addition

Fig. 19 NADP-isocitrate dehydrogenase (o——o), NAD-glutamate dehydrogenase (■——■), NADP-glutamate dehydrogenase (▲——▲) and isocitrate lyase (●——●) activities during the development of C. segnis in synchronous cultures exposed to continuous light (11,000 lux) and bubbled with 5% CO_2 in air (v/v). Enzyme activities are expressed as nmoles min^{-1} per mg protein. Arrow indicates the addition of 20 uM of cycloheximide and the dotted lines represent the specific activities after this treatment. ISL activity was determined by the incubation Method II.

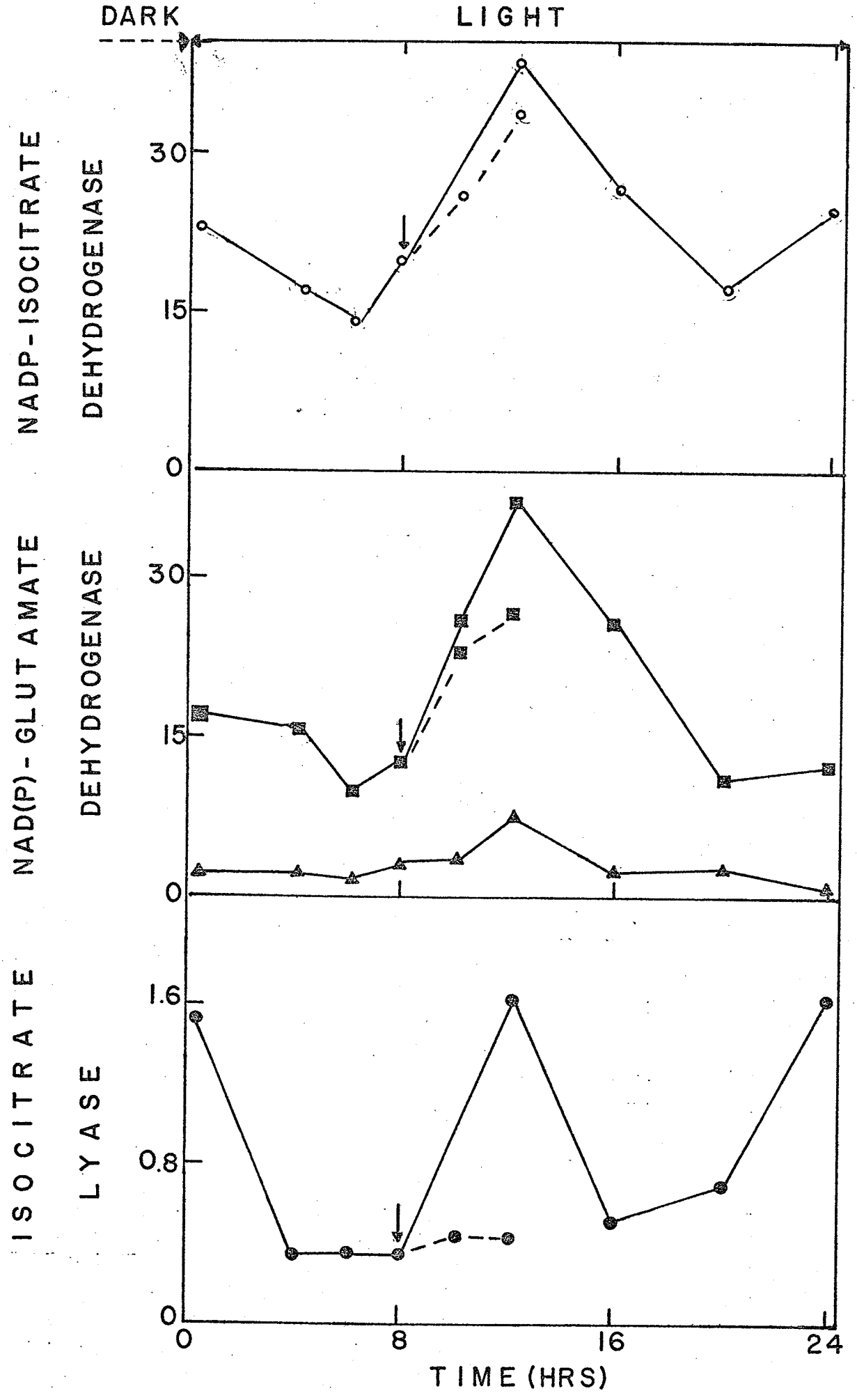

DARK
LIGHT
NADP-ISOCITRATE DEHYDROGENASE
NAD(P)-GLUTAMATE DEHYDROGENASE
ISOCITRATE LYASE
30
15
0
30
15
0
1.6
0.8
0
0
8
16
24
TIME (HRS)

Fig. 20 Changes in the rate of U-^{14}C-Leucine incorporation (a) and total protein (b) determined either colorimetrically by the Folin method (●——●) or by the Kjeldahl method (▲——▲) in terms of trichloroacetic acid insoluble-nitrogen.

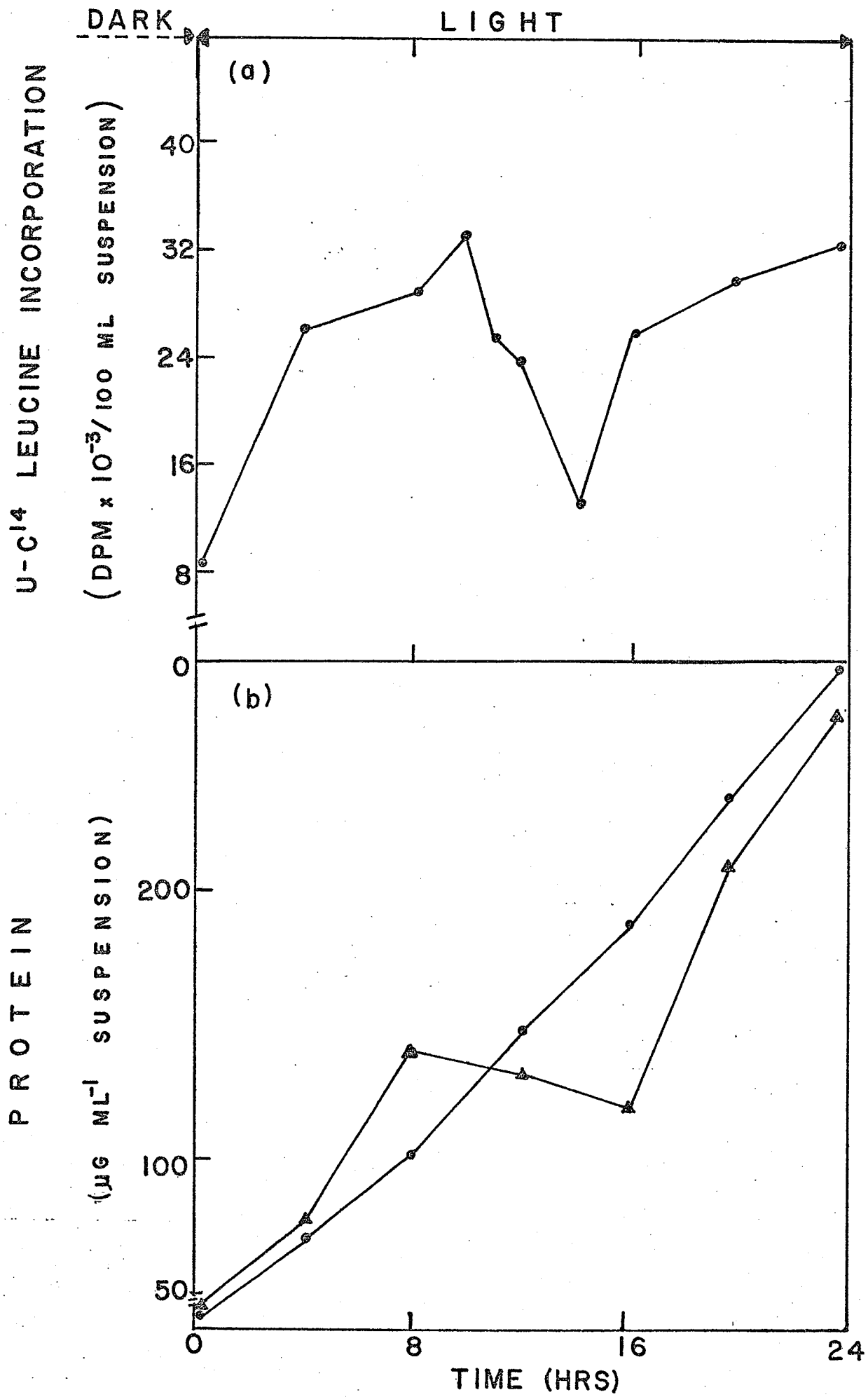
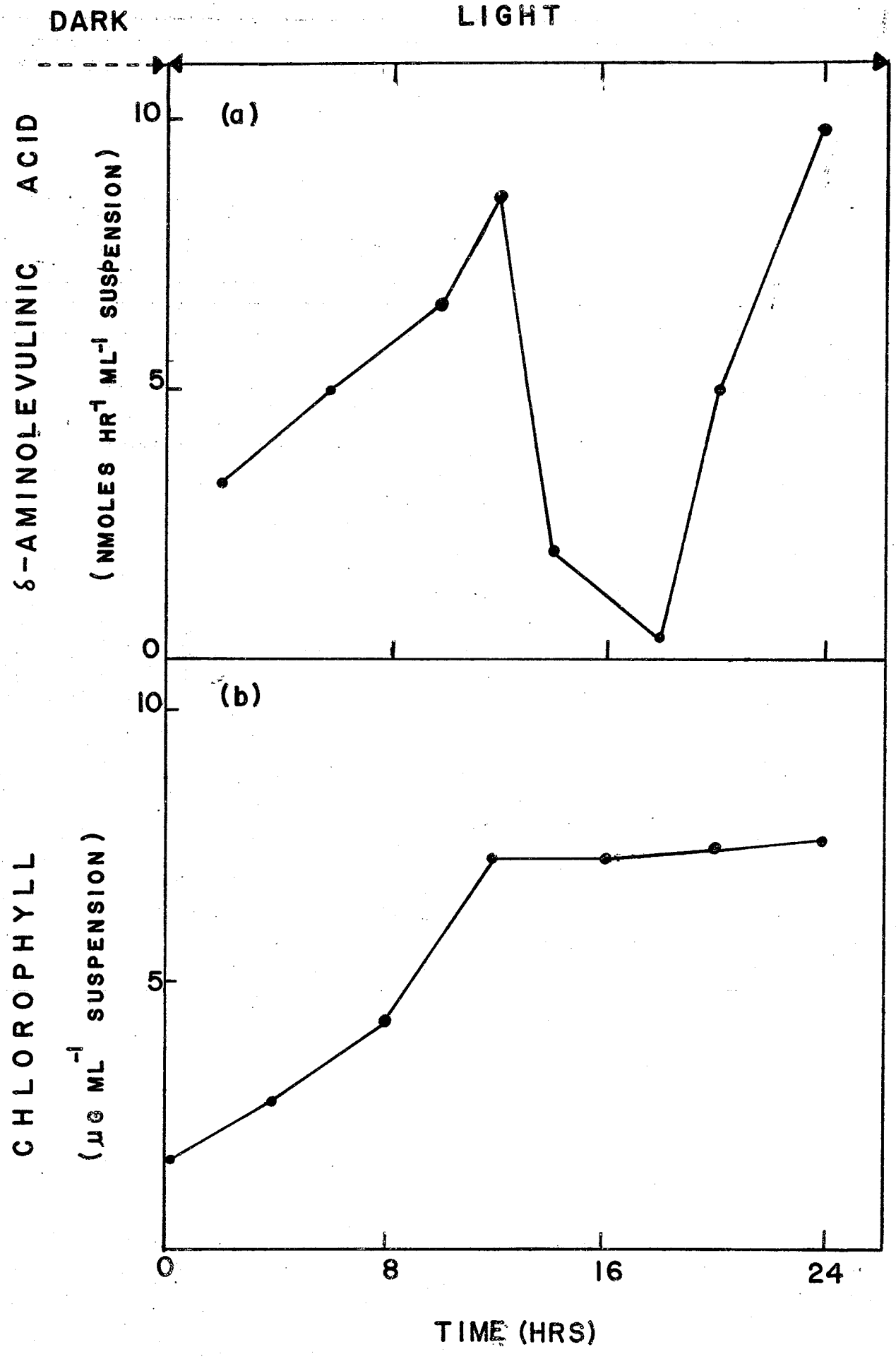

DARK
LIGHT
(a)
U-C14 LEUCINE INCORPORATION
(DPM x 10-3/100 ML SUSPENSION)
40
32
24
16
8
0
(b)
PROTEIN
(µG ML-1 SUSPENSION)
200
100
50
0
8
16
24
TIME (HRS)

Fig. 21 Changes in the rate of δ-aminolevulinic acid (δ-ALA) excretion (a) and total chlorophyll (b) of C. segnis during the development in synchronous cultures. Culture conditions were the same as in Fig. 18.

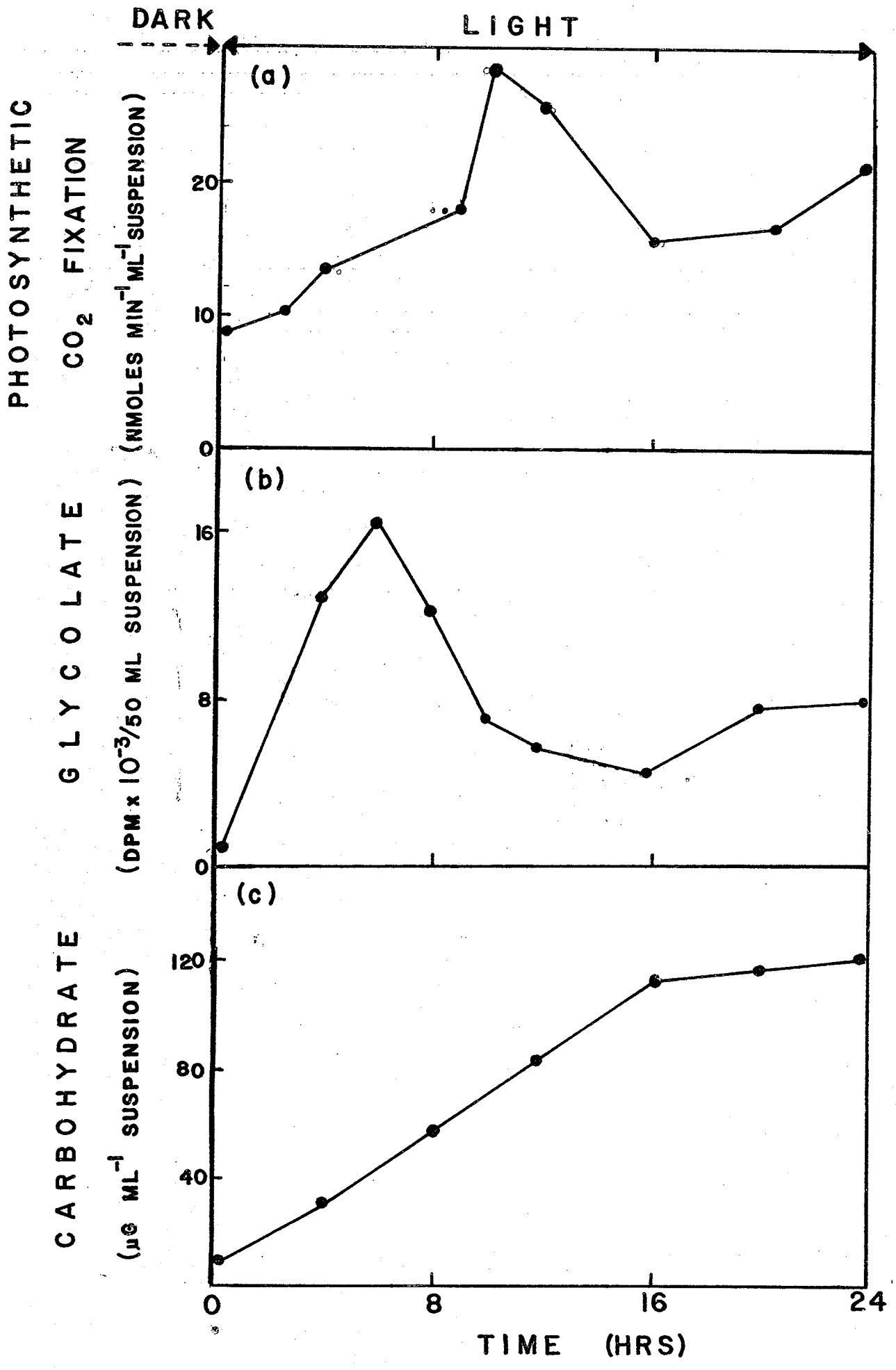

DARK
LIGHT
δ-AMINOLEVULINIC ACID
(NMOLES HR⁻¹ ML⁻¹ SUSPENSION)
(a)
10
5
0
CHLOROPHYLL
(μg ML⁻¹ SUSPENSION)
(b)
10
5
0
8
16
24
TIME (HRS)

to protein and chlorophyll synthesis, accumulation of carbohydrate occurred. It was obvious that most if not all of the C-skeleton required for the synthesis of these macromolecules during the G_1-phase was derived from the products of photosynthetic CO_2 fixation and active glycolate formation (Fig. 22). Although NADP-IDH showed substantial activity (15 - 22 nmoles min^{-1} mg^{-1}), it would not account for the entry of all the ammonium ions necessary for active protein synthesis via its product, α-ketoglutarate. Only about 20% or less of the latter would be converted to glutamate as indicated by the low activity (2 - 3 nmoles min^{-1} mg^{-1}) of NADP-GDH (Fig. 19). In other words, the greater portion of α-ketoglutarate appeared to be decarboxylated rather than being incorporated into protein. Furthermore, the low activity of ISL would not account for the active production of δ-ALA for chlorophyll synthesis during the G_1 phase.

B. Isocitrate Metabolism and DNA-synthesis:

At the beginning of the S-phase (i.e. 8 - 12 hr light) IDH doubled its activity whereas the activities of ISL and NAD-GDH were almost tripled (Fig. 19). The observed increase in ISL activity could be virtually abolished in presence of cycloheximide, whereas the activities of the other two enzymes were slightly influenced by this antibiotic. This observation may suggest that IDH and NAD(P) GDH were synthesised on cycloheximide insensitive ribosomes or the existing enzymes were activated at this particular stage of development. It is worthwhile to note that the addition of cycloheximide has led to complete inhibition of cell division

Fig. 22 Changes in rates of photosynthetic $^{14}CO_2$ fixation (a), photosynthetic glycolate formation (b) and total carbohydrates of C. segnis (c) during development in synchronous cultures. Culture conditions were the same as in Fig. 18.


DARK
LIGHT
(a)
PHOTOSYNTHETIC CO_2 FIXATION (NMOLES $MIN^{-1}ML^{-1}$ SUSPENSION)
0
10
20
(b)
GLYCOLATE (DPM x 10^{-3}/50 ML SUSPENSION)
0
8
16
(c)
CARBOHYDRATE (µG ML^{-1} SUSPENSION)
40
80
120
0
8
16
24
TIME (HRS)

(cf. Mihara and Hase, 1971).

Again, isocitrate metabolism was 8-fold greater by the dehydrogenase than the lyase and NAD(P) GDH could possibly utilise around 15 - 20% of the α-ketoglutarate produced by IDH.

At the early stages of DNA synthesis (8 - 12 hr), the rate of U-^{14}C-leucine incorporation and protein synthesis was slowed down and then declined (Fig. 18, 19). At this developmental stage, formation of chlorophyll (Fig. 21) would occur probably at the expense of pre-existing δ-ALA. The accumulation of carbohydrate during the S-phase did not coincide with the rate of photosynthetic $^{14}CO_2$ fixation nor with glycolate formation. The latter decreased rapidly as DNA-synthesis commenced (Ahmed & Ries, 1969, & Gimmler et al., 1969). It appeared, therefore, that carbohydrate accumulation during the S-phase (8 - 16 hr) was attributable to declining rate of protein synthesis and δ-ALA formation.

The higher activity of NADP-IDH, NAD(P)-GDH and ISL was characteristic of early stages of DNA synthesis (8 - 12 hr) which were conducive to the first cell cleavage at 12 hour. This, however, was not the case at later stages which led to the second cleavage at 16 hr. This observation might provide evidence that DNA synthesis could occur independent of isocitrate metabolism.

This conclusion was further substantiated by the results obtained from treatments with monofluoroacetate

(MFA). The latter inhibits isocitrate metabolism since metabolically formed fluorocitrate has been known to block the formation of isocitrate by the aconitase reaction (Vagelos et al, 1963, Cooksey, 1974). The addition of 10 mM MFA at the beginning of the S-phase (8 hr) resulted in three fold increase of DNA synthesis without influencing cell division (Table XIV). A higher concentration of MFA enhanced DNA synthesis but inhibited cell division.

C. Effect of Acetate on DNA synthesis:

When acetate was added instead of mono-fluoroacetic acid, DNA synthesis was stimulated too (table XV). This stimulatory effect was not attributable to the presence of glycolate contamination in acetate (cf. Merrett & Lord, 1973, Tolbert, 1974), since addition of 15 mM glycolate failed to enhance DNA synthesis.

Addition of glycolate, however, stimulated carbohydrate synthesis by approximately 50% as compared to the control. It is well established that photosynthetic tissues and algae can metabolise glycolate to hexoses via the glycine-serine metabolism in the glycolate pathway (Wang & Waygood, 1962; Shah & Cossins, 1970; Bruin et al, 1970 & Tolbert, 1974). Furthermore, the uptake of glycolate at this particular developmental stage of the life cycle was shown by Nelson et al, (1969) in Ankistrodesmus braunii.

The slight decrease observed in the DNA content of

Table XIV

Effect of monofluoroacetate (MFA) on DNA synthesis in light

Time*	Treatments	Cell number (x 10^6/ml)	DNA (ug/ml)
8 hr	Nil	1.1	0.25
24 hr	Nil	3.1	1.26
	+5 mM MFA	3.8	1.68
	+10 mM MFA	3.3	3.77
	+20 mM MFA	1.5	2.51

*The cells were collected before the addition of MFA at the 8 hr of the life cycle and the various concentrations of MFA were then added to cultures which were placed in light at 11,000 lux under continuous bubbling with 5% CO_2 in air (v/v) for 16 hours. The cells were harvested for analysis at the 24 hr of the life cycle.

Table XV

Effect of Acetate on DNA synthesis in light

Time*	Treatment	DNA	RNA	Total carbohydrate
8 hr	Nil	0.20	5.6	15.5
16 hr	Nil	0.96	9.2	23.7
	+15 mM acetate	1.60	8.6	24.0
	+15 mM glycolate	0.72	6.7	34.9
	+10 mM MFA	1.74	8.6	28.2
	+10 mM MFA + 15 mM acetate	1.32	8.3	24.0

*Same conditions of growth (light and CO_2) as indicated for Table XIV, except other additions were made and the cells were harvested for analysis after 8 hours incubation, ie. at the 16 hr of the life cycle. Data are given in ug/ml algal suspension.

the cell due to addition of MFA and acetate might be due to the competition of both analogues for the enzyme (eg. Acetyl CoA synthetase) which metabolises them.

Figure 23 illustrates the level of 2 - ^{14}C acetate uptake in light by C. segnis during its life cycle. Two peaks of incorporation were evident, one at the beginning of the G_1 phase and another at the end of the S-phase prior to cell division into four zoospores. If acetate were involved in DNA synthesis, then it would follow that the two peaks might represent the S-periods for two different species of DNA (i.e. chloroplastic and nuclear DNA). The level of incorporation of tritiated adenine into cytoplasmic DNA of synchronised Euglena (Cook, 1966), showed two peaks similar to those shown in Fig. 23 with 2 - ^{14}C acetate. On the other hand, the first peak might indicate the synthesis of nucleotides in preparation for the first nuclear division at 12 hr whereas the second peak would account for the second nuclear division prior to the onset of liberation of four zoospores in C. segnis at the 16 hr.

D. Activation of NADP-Isocitrate Dehydrogenase during the S-phase by Light:

From Table XVI it was evident that the observed increase in NADP-IDH activity at the beginning of the S-phase (8 - 12 hr) was dependent on light. Such an increase could be abolished by placing the cells in darkness. However, the

Fig. 23 Periodic 2-^{14}C-acetate uptake in light by C. segnis during the development in synchronous cultures. Culture conditions were the same as in Fig. 18.

●------● Cell number

●——● 2-^{14}C-acetate uptake

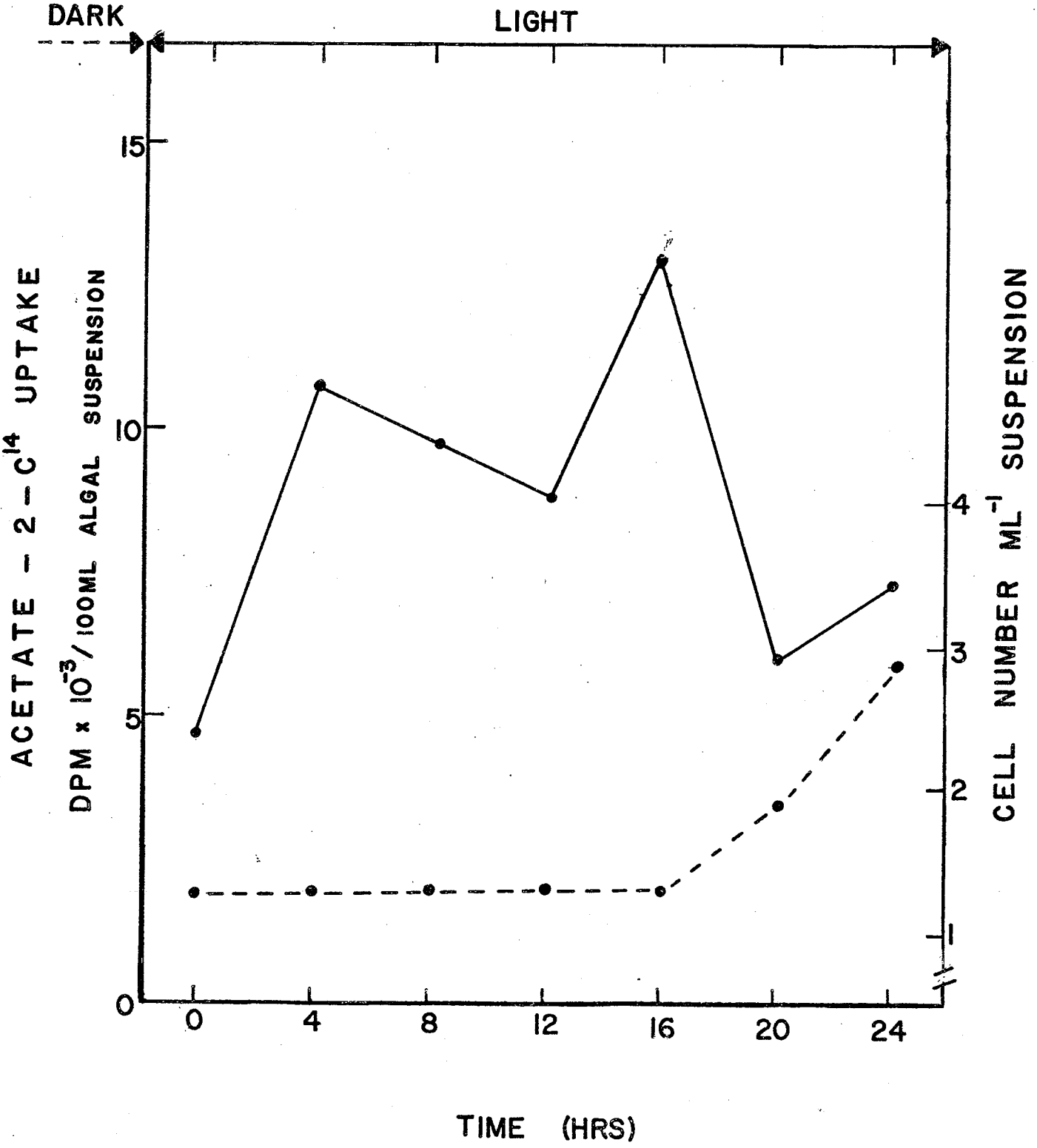

DARK
LIGHT
ACETATE - 2 - C14 UPTAKE
DPM x 10-3/100ML ALGAL SUSPENSION
CELL NUMBER ML-1 SUSPENSION
0
5
10
15
1
2
3
4
0
4
8
12
16
20
24
TIME (HRS)

Table XVI

Effect of light, anaerobiosis, and CMU on NADP-isocitrate dehydrogenase, isocitrate lyase, and NAD-glutamate dehydrogenase activities

Time*	Treatment	IDH	ISL	GDH
8 hr	light	21.5	0.10	21.7
12 hr	light	32.3	0.43	32.3
	dark	18.9	0.50	37.7
	light + N_2	49.1	0.42	24.6
	dark + N_2	32.8	0.50	36.1
	light + CMU (10 uM)	39.1	0.70	49.1

*The cells were collected before treatments at the 8 hr of the life cycle. All cultures were bubbled with 5% CO_2 in air (v/v) except in N_2 treatment where oxygen was absorbed by passing air through pyrogallol. After 4 hours incubation (ie. at the 12 hr of the life cycle), cells were harvested for analysis. Specific activities of enzymes are given in nmoles min^{-1} mg^{-1}.

inhibitory effect of dark incubation could be overcome by bubbling the cultures with N_2 in presence of 5% CO_2.

Incubation in light under N_2 - 5% CO_2 atmosphere resulted in two fold activation of the enzyme as compared to darkness. This activation appeared to be partially due to inhibited oxidative phosphorylation. Inhibition of non-cyclic photophosphorylation by CMU did not result in the magnitude of activation observed in cultures bubbled with N_2 in light. This could imply that the higher activity of NADP-IDH during the S-phase may not necessarily be associated with oxidative phosphorylation nor with non-cyclic photophosphorylation. Furthermore, the comparatively low activity of NAD(P) glutamate dehydrogenase in N_2-cultures would account for the utilisation of only 10% of the α-ketoglutarate produced by NADP-IDH.

Imposing anaerobic conditions on cells entering the S-phase (at 8 hr) in light, would not influence the subsequent stages of development since the cells divided as usual and produced four zoospores. On the other hand, if the cells were darkened or treated with CMU during that period, no cell division ever occurred. Inhibition of cell division in darkness was not due to lack of DNA or RNA synthesis but rather to other unknown light dependent factors as shown in C. reinhardtii (Kates et al, 1968).

E. Isocitrate metabolism and endogenous respirations:

Figure 24 shows clearly that the endogenous respiration was relatively low and remained almost unchanged during the G_1-phase as well as the early stages of the S-phase (0 - 12 hr). Thereafter the rate of respiration increased enormously. This pattern of O_2 uptake is in agreement with the earlier work of Tamiya et al (1953) and Tamiya (1963), who showed that L3 and L4 cells (cells which have grown in light prior to the second cell cleavage resulting in the production of more than two spores) are characterised by the highest respiratory activities. Such activities did not coincide with maximal specific activities of the isocitrate enzyme whether expressed on protein basis (Fig. 19) or volume basis (nmoles min^{-1} ml^{-1} algal suspension) as shown in Fig. 25. This result may indicate that the products of NADP-IDH and probably ISL were not utilised for the production of ATP in respiration during the G_1-phase and early stages of DNA synthesis. Conversely, such products might be used in respiration prior to the second cell cleavage albeit the level of NADP-IDH activities was declining. Such low rate of enzyme activities were found at this stage to be due to inhibition rather than proteolysis of enzyme protein as shown in Chlorella by Berger & Pirson (1967).

Fig. 24 Endogenous respiration of C. segnis during development in synchronous cultures. Culture conditions were as in Fig. 18. Cells were suspended in 0.05 M phosphate buffer pH 6.5 and oxygen uptake (nmoles min^{-1} ml^{-1} suspension) was measured by the oxygen electrode.

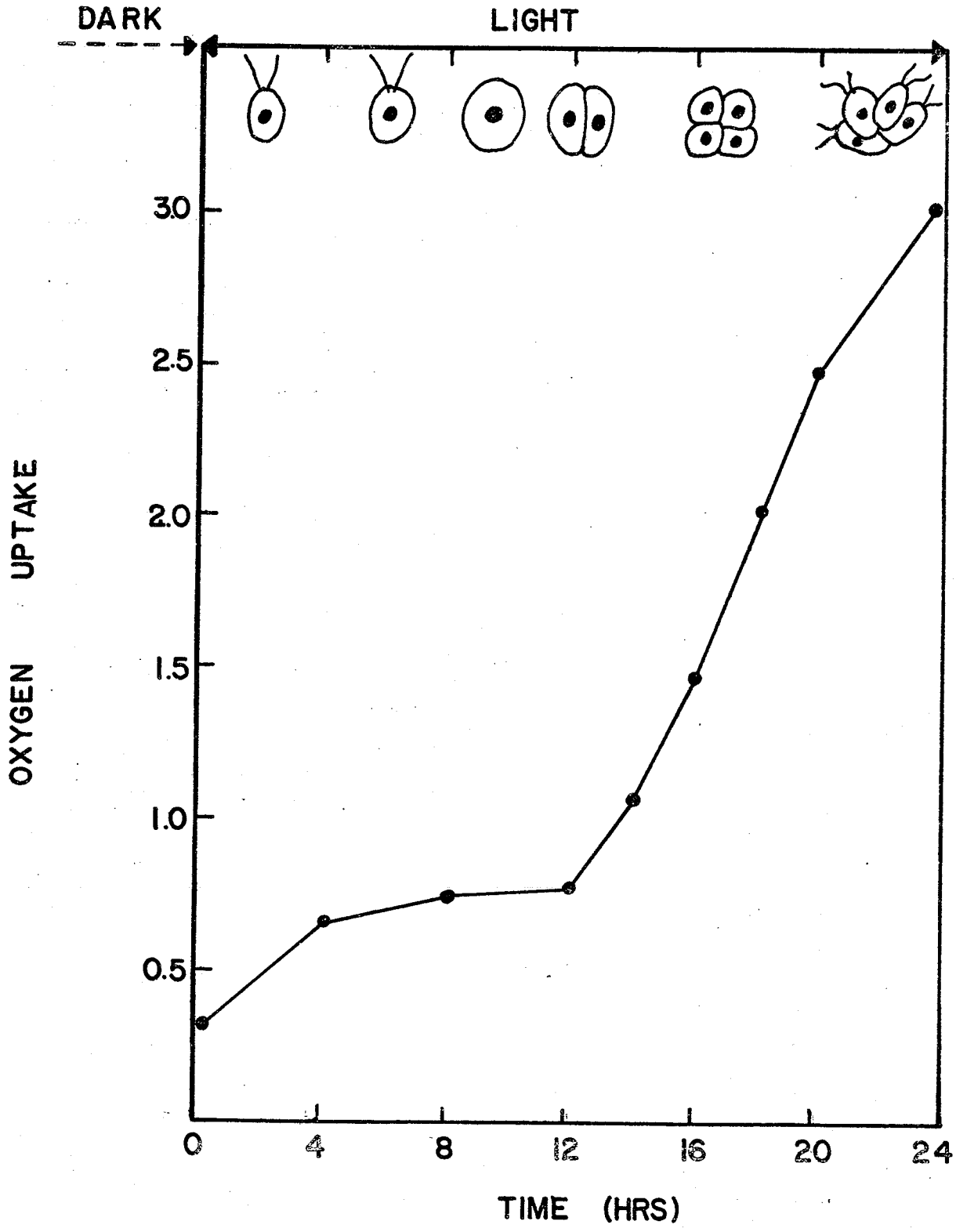
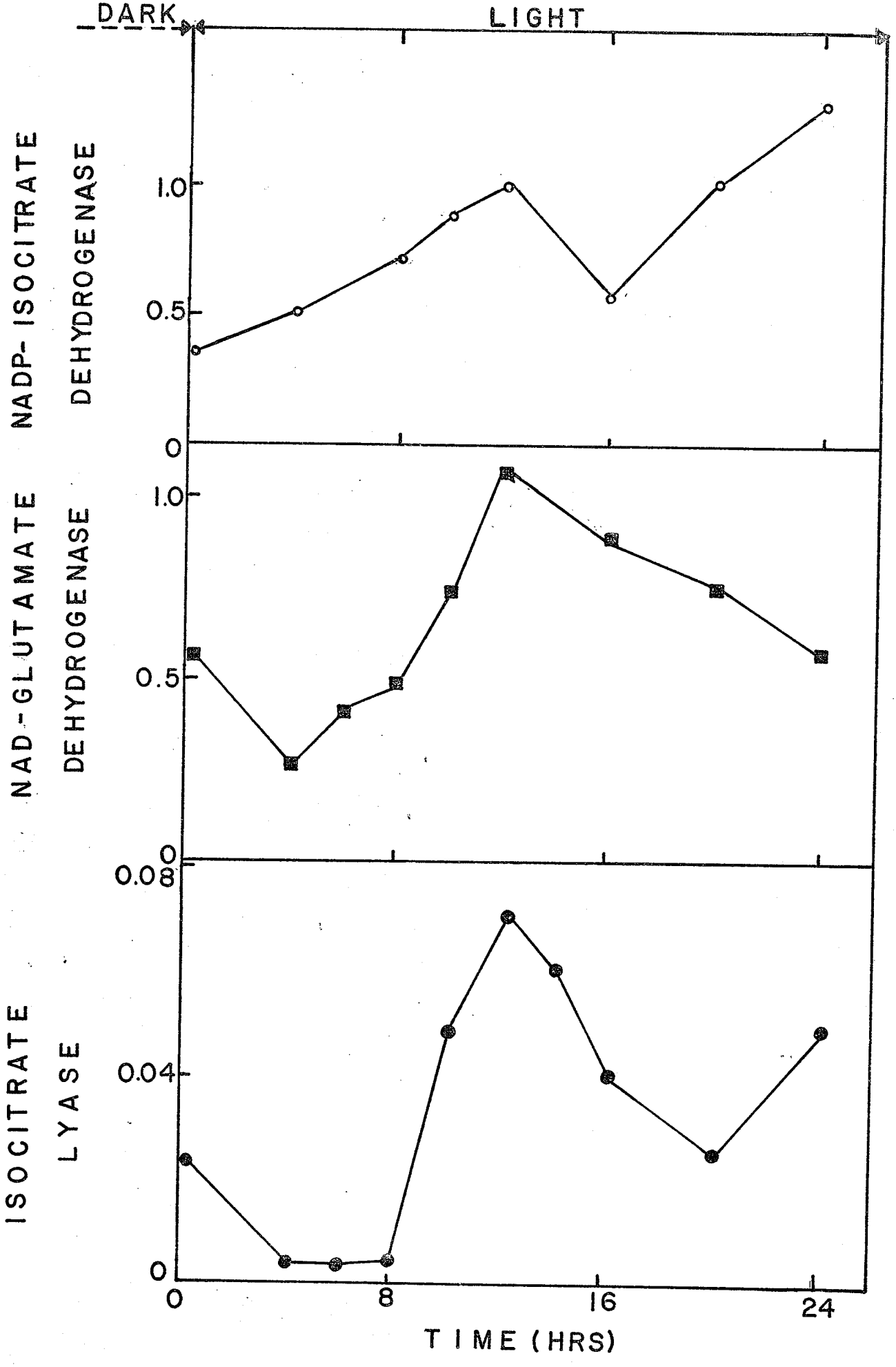

DARK
LIGHT
OXYGEN UPTAKE
3.0
2.5
2.0
1.5
1.0
0.5
0
4
8
12
16
20
24
TIME (HRS)

Fig. 25 NADP-isocitrate dehydrogenase (o——o), NAD-glutamate dehydrogenase (■——■), and isocitrate lyase (●——●) activities during the development of C. segnis in synchronous cultures exposed to continuous light (11,000 lux) and bubbled with 5% CO_2 in air (v/v). Enzyme activities are expressed as nmoles min^{-1} per ml algal suspension.


DARK
LIGHT
NADP- ISOCITRATE DEHYDROGENASE
1.0
0.5
0
NAD-GLUTAMATE DEHYDROGENASE
1.0
0.5
0
ISOCITRATE LYASE
0.08
0.04
0
0
8
16
24
TIME (HRS)

DISCUSSION

It is well known that in photoautotrophic organisms, the tricarboxylic acid cycle is necessary for the biosynthesis of α-ketoglutarate and oxaloacetate which are precursors of amino acids and also for the production of succinyl CoA which is required for tetrapyrrole biosynthesis. However, its role in the generation of assimilatory power (ie. ATP and NADPH) is dubious. In some species of blue-green algae (Anabaena variabilis & Anacystis nidulans), the biosynthetic function of the cycle can in principle be fulfilled without α-ketoglutarate dehydrogenase and succinyl CoA synthetase, provided that the glyoxylate cycle is operating (Smith, 1973). In these organisms which lack α-ketoglutarate dehydrogenase; α-ketoglutarate is formed via NADP-isocitrate dehydrogenase; oxaloacetate is provided from oxidation of malate produced by malate synthase; and succinyl CoA by the succinate derived from isocitrate lyase catalysis with the subsequent reaction mediated by α-keto acid CoA transferase. Although the levels of isocitrate lyase and malate synthase, in this case, were very low (Pearce and Carer, 1967), they should be adequate to account for the metabolic sequences as described above and this was verified by the pattern of incorporation of labelled organic acids substrates.

In C. segnis, however, we have relied solely on measuring the levels of enzyme activities as well as detecting

certain key enzymes of some related pathways. Admittedly, the failure to demonstrate the activity of a specific enzyme in cell free extracts, does not by itself constitute sufficient evidence for the absence of an enzyme. The use of unsatisfactory procedures for the preparation of extracts or from the presence of inhibitory metabolites in the extracts would contribute to this pitfall. Therefore, our results should be complemented by the determination of patterns of incorporation of various labelled substrates into the cell. However, the results obtained from Fig. 4b have clearly shown that isocitrate can be metabolised 5 - 7 fold faster via IDH rather than by ISL (Fig. 4b, Tables VII, VIII). The presence of the latter even at low activity would undoubtedly indicate the production of glyoxylate and succinate from isocitrate (cf. Pearce and Carr, 1967). In addition, it was shown that ISL of C. segnis was undetectable in cells previously grown under carbon or nitrogen starvation (Tables VII), whereas substantial activity of IDH was evident. Under normal conditions of growth (Table VII, VIII) and regardless of the nature of carbon source, ISL was present besides IDH.

In other words the enzymes which would account for the formation of oxaloacetate (PEP carboxylase, Table VIII), α-ketoglutarate (NADP-IDH, Tables VII & VIII), succinate (ISL, Table VII & VIII) were present in C. segnis. Furthermore, succinyl CoA synthetase activity was demonstrated in Table V and its product may be utilised for pyrrole ring formation.

The detection of these enzymes and the failure to detect α-ketoglutarate dehydrogenase (Table I) would imply that isocitrate metabolism in C. segnis might operate in an interrupted TCA cycle as in blue-green algae. However, the presence of α-ketoglutarate dehydrogenase in the diatom Cocconeis dimunata has been reported by Cooksey (1972). This author added NaCN to the reaction mixture presumably to inhibit the utilisation of NADH which probably in our case would be oxidised in air as NaCN was not included in the reaction mixture. However, the use of radiotracer method (1-^{14}C-α-ketoglutarate) did not result in any significant activity (Table I) (cf. Pearce et al, 1969).

The absence of δ-ALA synthethase (Table VIa & VIb) and the presence of succinyl CoA thiokinase (Table V) may suggest that the formation of succinyl CoA represents a step in the TCA cycle although α-ketoglutarate dehydrogenase was undetectable. If this assumption holds true, then two sources of succinate would be available in the eucaryotic unicellular green alga C. segnis as succinate can be formed via ISL as well as from the TCA cycle. The activation of ISL by succinyl CoA (Foo et al, 1971) represents a mechanism by which succinate production via the lyase could be regulated. Therefore, if succinate production via α-ketoglutarate dehydrogenase is blocked at the step of succinyl CoA synthetase which converts succinly CoA to succinate, the accumulation of succinyl CoA would activate ISL to produce succinate.

From the kinetic studies on IDH in C. segnis, it was found that the enzyme is inhibited by its products, α-ketoglutarate and NADPH (Table XI, Fig. 13 and Fig. 15) and also by OAA (Table XI, Fig. 12). As a result of keto acids accumulation due to declining rate of amino acids biosynthesis, isocitrate would be metabolised via ISL. The latter has been shown to be far less sensitive to such metabolic inhibitors (Table XI) as can be seen from the magnitude of their inhibitor constants (Foo et al, 1971, and Table XII). It is also evident that very low concentration of OAA and glyoxylate together at a concentration of 100 uM has exerted a profound inhibition (90%) on IDH (Table XIII and Fig. 16) whereas ISL is insensitive to such inhibitors. Furthermore, a declining rate of macromolecules biosynthesis would lead to an increase in the cellular levels of ATP which in turn would inhibit IDH activity (Table XI and Fig. 14). It is obvious that IDH is more susceptible to inhibition by various intermediary metabolites (α-ketoglutarate, ATP, NADPH, and oxaloacetate alone or with glyoxylate at 1mM) rather than ISL which apparently displayed less sensitivity to organic and α-keto acids such as glycolate, α-ketoglutarate, and oxaloacetate. Only oxalate was found to be a strong inhibitor of ISL.

These results would also provide evidence supporting the view that an ancillary pathway for isocitrate metabolism via ISL is available in C. segnis when grown under conditions

favorable for active protein synthesis and carbohydrate accumulation. In this way, the flow of carbon toward the formation of α-ketoglutarate via IDH could be regulated by diverting isocitric acid via ISL.

Besides its function as a key enzyme for an alternative pathway for succinate production, the proposed function of ISL would provide glyoxylate which could be converted to 3-phosphoglycerate via the glycerate pathway or metabolised in the glycine-serine pathway (Tolbert, 1974). Recent studies of the glycolate pathway in Euglena and Chlorella pyrenoidosa suggest that one carbon units required in serine synthesis are derived from glyoxylate during serine synthesis (Murray et al, 1971; Lord & Merrett, 1970). Furthermore, glyoxylate is required for hydroxylevulinic acid synthesis via α-ketoglutarate: glyoxylate carboligase, and the latter compound was shown to be further metabolised to dioxovaleric acid (Wang et al, 1970). Dioxovaleric acid has been shown by Gassman et al (1968) to undergo a transamination reaction with L-glutamate or L-alanine catalysed by δ-ALA transaminase to give δ-ALA. Hence, the above mentioned pathways would be in operation whenever the depletion of photosynthetically formed glycolate reaches a critical level.

The results obtained from the experiments conducted on isocitrate metabolism during synchronous cultures have revealed that the reductive amination of α-ketoglutarate would not represent the major entry of ammonia. This conclusion was

made on the ground that NADP-GDH consistently showed less activity (Fig. 19) as compared to NAD-GDH during the active phase of protein synthesis (cf. Talley et al, 1972). Tan (1974), using C. segnis found that alanine, glycine, serine and aspartate were the major amino acids formed from $^{14}CO_2$ fixation in photosynthesis during the G_1-phase and early S-phase. ^{14}C-alanine appeared prior to ^{14}C-glutamate during photosynthetic $^{14}CO_2$ fixation in presence of NH_4^+ in Chlorella (Smith et al, 1961). On the other hand, ^{14}C-glutamate formation was enhanced when Cocconeis diminuta was fed with ^{14}C-acetate or lactate (Cooksey, 1972). Furthermore, Kates and Jones (1967) reported higher activity of alanine dehydrogenase and comparatively low NADP-GDH activity during the phase of active protein synthesis in Chlamydomonas reinhardtii. Berger (1966) has also shown that higher activities of NAD(P)-GDH in Chlorella could be shown under heterotrophic cultural conditions, whereas low enzyme activities were consistently demonstrated in autotrophic cultures. This may lend support to the conclusion that a great portion of the products of IDH, ie. α-ketoglutarate is most likely metabolised by decarboxylation rather than via a reductive amination process. On the other hand a relatively low rate of respiration (Fig. 24) during the G_1-phase, may suggest that the decarboxylation of α-ketoglutarate was probably associated with biosynthetic processes that require a high ratio of NADPH/ATP (eg. fatty acids and lipid synthesis). Furthermore the activation of IDH

in light, under anaerobic conditions (Table XVI) and during the early stages of S-phase (8 - 12 hour, Fig. 19 and 25) may support this view. It is tempting to speculate that such reducing power may be used for lipid synthesis, and membrane formation before the onset of the first cytoplasmic cleavage (cf. Fig. 24). The biosynthesis of other macromolecules which occurs during the G_1 phase would directly utilise NADPH arising from photosystem II. During the S-phase (8 - 16 hr), the drop in the activity of photosystem II during the S-phase (Senger & Bishop, 1969, Tan, 1974), would result in NADPH shortage but this can be compensated by the IDH activity.

It appears that IDH was protected by its substrate during the S-phase as suggested by Tan, 1974, who reported the accumulation of isocitrate lactone at this particular stage of the life cycle. The activation of IDH by light was associated with de novo synthesis of isocitrate lyase (Table XVI, Fig. 19), and the increases in activities of both enzymes were concomitant with the onset of DNA synthesis (Fig. 18 and 19). If the one carbon fragment is required at this phase for the biosynthesis of purines and pyrimidines as suggested by Lor and Cossins (1973, 1974) and Shah & Cossins (1970), it should originate from glyoxylate via ISL rather than by photosynthetically formed glycolate. The rate of synthesis of the latter was shown in Fig. 22 to decrease dramatically during the S-phase as demonstrated by Gimmler (1969) and Ahmed and Ries (1969). Alternatively, the possibility of glycolate forma-

tion from acetate as a by-product during the synthesis of fatty acids would account for the active one carbon metabolism during DNA synthesis. However, the addition of glycolate at the commencement of the S-phase did not enhance DNA synthesis (Table XVI). Rather it resulted in carbohydrate synthesis. Only added acetate has stimulated synthesis of DNA (Table XV).

The results from Table XIV, have provided evidence that DNA synthesis occurred independently of isocitrate metabolism. Inhibition of isocitrate formation by MFA (10 mM) at the step of aconitase will arrest the formation of isocitrate from citrate and thus respiration will eventually be impeded. On the other hand, the stimulation of DNA synthesis by MFA as a result of inhibiting isocitrate metabolism, may suggest that the latter could be implicated in the control of DNA synthesis. If acetate which was shown to stimulate DNA synthesis (Table XV) would not be consumed partially via isocitrate metabolism, then the processes of DNA synthesis would unnecessarily continue. Although the role of acetate in stimulating DNA synthesis at present is unknown, it appears that such stimulation is linked in some way to fatty acid metabolism and lipid synthesis.

Assimilation of labelled acetate in light by autotrophic organisms (Hoare et al, 1967; Smith et al, 1967, Cooksey, 1972), has shown that the incorporation of this metabolite is primarily directed to lipids and only some

incorporation into proteins and amino acids.

It is conceivable that aerobic respiration is dependent on isocitrate metabolism. In eucaryotes, isocitrate is decarboxylated via NAD(P)-IDH and further metabolised by the enzymatic sequences in the TCA cycle. In the prokaryotes, such as the blue-green algae, isocitrate lyase provides succinate which is oxidised via succinic dehydrogenase and NAD(P) oxidases. It is not known whether or not the control of the latter enzyme is the step at which respiration in C. segnis is regulated. Comparison of the pattern of isocitrate metabolism via NADP-IDH and ISL activities (Fig. 19, 24) and the rate of oxygen uptake (Fig. 25) has revealed that both processes were inconcomitant. NAD-IDH was barely detectable in cell-free extract of C. segnis, presumably because of using inefficient extraction methods. The periodicity of this enzyme during the life cycle might have accounted for the observed changes in respiration shown in Fig. 24 (c.f. Tager, 1961).

Furthermore, it is desirable to know about the mechanism by which α-ketoglutarate metabolism is regulated in order to understand why a great portion of this metabolite is diverted away from glutamate formation.

The present studies do not provide enough evidence to elucidate completely the role of ISL in autotrophically grown C. segnis. The results obtained have led to some logical conclusions concerning the role of this enzyme as indicated by

the fate of its product; succinate as discussed before. Further studies are required to elicit the fate of the other product, glyoxylate, particularly in conjunction with its reduction to glycolate (Harrop & Kornberg, 1966; Wiessner, 1968; Zelitch, 1972, & Godavari, 1973) and its metabolism via serine-glycine pathway or via carboligases (Badour & Waygood, 1971b; and Codd & Stewart, 1973).

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