The University of Manitoba

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

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Samuel Siang-Kiang Foo

A Thesis
Submitted to
The Faculty of Graduate Studies
In Partial Fulfilment
of the Requirements for the Degree of
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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 $\beta$-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, $\delta$-aminolevulinic acid ($\delta$-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 $\alpha$-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 $\alpha$-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 $\alpha$-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, $\delta$-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.*

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

## 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

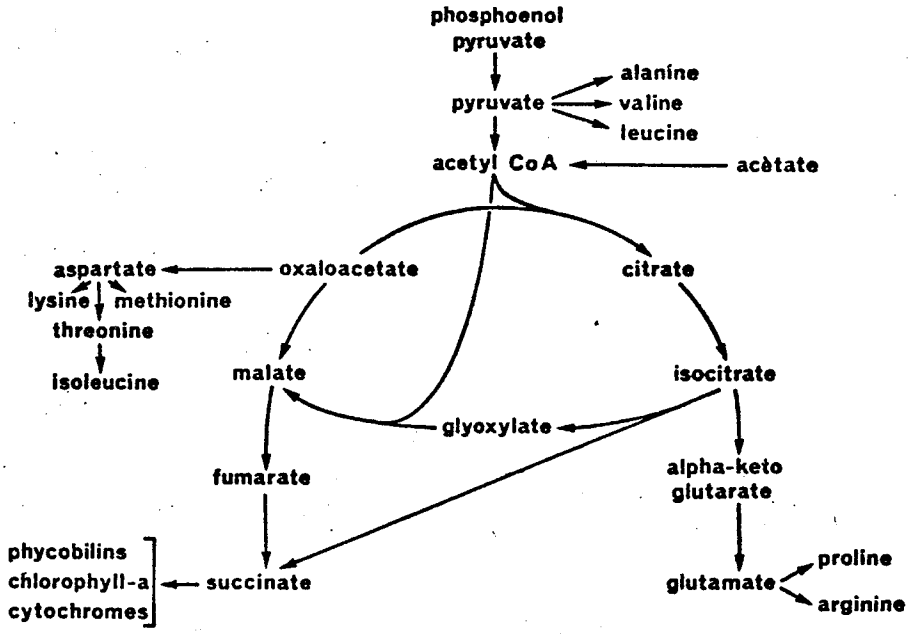
acetoacyl CoA as donor for the coenzyme A moeity. Pearce and Carr (1967) suggested that succinate could have been provided by isocitrate lyase and malate synthase.

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.