**MONTANA STATE UNIVERSITY**

Triglyceride accumulation and the cell cycle in chlorella
by Robert Michael Thomas

A thesis submitted in partial fulfillment pf the degree requirements for the degree of Master of Science
in Microbiology
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Abstract:
Triglyceride (TG) storage lipid accumulates in microalgae during exposure to stresses which inhibit algal cell cycles. Such inhibition is typically induced by nutrient limitations such as depletion of nitrogen for green algae and silicate for diatoms. No direct mechanism has been described for low nutrient-induced lipid accumulation. It is proposed that TG accumulation in microalgae could be based on a synthesis/utilization pattern which is constitutive within the cell division cycle, as opposed to a specifically-induced synthesis of TG. Triglyceride accumulation occurs when a lag is induced in the cell cycle such that TG synthesis exceeds utilization. Historically, total lipids have been quantified and the cycling of TG independent of other lipid fractions has often been overlooked.

Recognizing the limitations of earlier researchers, trends in TG levels were examined using the neutral lipid (NL) specific fluorochrome, Nile Red, throughout complete cell cycles of synchronized cultures of Chlorella CHLORl under conditions of alkaline stress, nitrogen deprivation, and with monofluoroacetate (MFA) an inhibitor of the tricarboxylic acid (TCA) cycle. Alkaline pH stress, nitrate deprivation and TCA inhibition affects lipid accumulation by inhibiting the cell division cycle prior to TG utilization. Low levels of calcium were not found to have any noticeable effects on TG synthesis.

An accumulation of 2.5 to 5.5 times the initial NL level per cell resulted after 25 hours of culturing under alkaline conditions. Nitrogen deprivation yielded increases of 1.4 and 2.5 times the initial NL level after 36 hours and 14 days, respectively. In less than 30 hours, inhibition of the tricarboxylic acid (TCA) with MFA resulted in NL levels of 1.4 to 3.8 times the initial NL level per cell.

Radiolabeled acetate is assimilated at a constant rate into the neutral lipid fraction. Cultures with growth inhibited by MFA assimilated more radiolabel into neutral lipids and less radiolabel into the glyco- and polar lipid fractions than in control cultures.

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Date _September 4, 1990_

## ACKNOWLEDGEMENTS

# TABLE OF CONTENTS

# TABLE OF CONTENTS--Continued

# TABLE OF CONTENTS--<u>Continued</u>

## LIST OF TABLES

## LIST OF FIGURES

LIST OF FIGURES--<u>Continued</u>

## ABSTRACT

Triglyceride (TG) storage lipid accumulates in microalgae during exposure to stresses which inhibit algal cell cycles. Such inhibition is typically induced by nutrient limitations such as depletion of nitrogen for green algae and silicate for diatoms. No direct mechanism has been described for low nutrient-induced lipid accumulation. It is proposed that TG accumulation in microalgae could be based on a synthesis/utilization pattern which is constitutive within the cell division cycle, as opposed to a specifically-induced synthesis of TG. Triglyceride accumulation occurs when a lag is induced in the cell cycle such that TG synthesis exceeds utilization. Historically, total lipids have been quantified and the cycling of TG independent of other lipid fractions has often been overlooked.

Recognizing the limitations of earlier researchers, trends in TG levels were examined using the neutral lipid (NL) specific fluorochrome, Nile Red, throughout complete cell cycles of synchronized cultures of Chlorella CHLOR1 under conditions of alkaline stress, nitrogen deprivation, and with monofluoroacetate (MFA) an inhibitor of the tricarboxylic acid (TCA) cycle. Alkaline pH stress, nitrate deprivation and TCA inhibition affects lipid accumulation by inhibiting the cell division cycle prior to TG utilization. Low levels of calcium were not found to have any noticeable effects on TG synthesis.

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## INTRODUCTION

Microorganisms growing under optimal conditions synthesize fatty acids primarily for esterification into membrane lipids -- phospholipids for plasma and organelle membranes; and in the case of photosynthetic organisms, glycolipids for chloroplast membranes. Under stressed conditions of nutrient limitation, lipid biosynthesis patterns change. Lipid accumulation has been reported for microalgae under a variety of nutrient limiting conditions including nitrogen (Suen, et al., 1987), silicate (Taguchi et al., 1987), selenium, (Doucette et al., 1987), and potassium (MacCarthy and Patterson, 1974). Triglyceride droplets have been observed in the cytoplasm of microeukaryotic algae under both optimal, nutrient-limited and alkaline stress conditions (Cooksey et al., 1987; Guckert & Cooksey, 1990; Thomas et al., 1990).

In microeukaryotic algae triglyceride (TG) droplets enriched in the precursor fatty acids 16:0 and 18:1w9c (Shaw, 1966) form in the cytoplasm (Cooksey et al., 1987; Guckert & Cooksey, 1990).

The ability of TG to accumulate in microalgae and its molecular similarity to mineral-oil derived hydrocarbons make these storage lipids an attractive renewable alternative fuel (Neenan et al., 1986).

Although lipid accumulation during nutrient limitation

is well documented, no direct biochemical mechanism for the change in metabolism resulting in TG accumulation has been determined.

Aside from these few observations of TG accumulation, most researchers have missed this TG cycling phenomena because they have typically quantified total lipids. As a result, the cycling of TG independently of the other lipid fractions was often overlooked (e.g. Shifrin & Chisholm, 1981). A likely reason such cycling was missed was that quantification of each lipid fraction at frequent points across the cell cycle is logistically very difficult. However, with the Nile Red (NR) technique developed earlier in our lab, observations of trends in TG levels are performed more easily (Cooksey et al., 1987).

Our investigations into cell cycle effects on triglyceride accumulation were inspired by earlier observations that alkaline pH stress resulted in TG accumulation in Chlorella independent of medium nitrogen or carbon levels (Guckert & Cooksey, 1990). Furthermore, we believe that some of the energy to fuel cell division may come from beta-oxidation and tricarboxylic acid (TCA) Cycle oxidation of the storage lipids. On this note, one would expect an inhibition of the TCA cycle to result in an elevated pool of acetyl-CoA available for fatty acid synthesis. Monofluoroacetate was chosen as an inhibitor of the TCA cycle (Cooksey, 1972).

## Project Goal

The goal of my thesis was to develop a better understanding of the effects of extracellular factors on the control of triglyceride accumulation. In order to do so, it is necessary to consider all aspects of lipid synthesis and turnover and the role of the cell cycle in TG dynamics. To accomplish this goal, the following objectives were established:

1. Confirm the existence of a constitutive triglyceride synthesis-utilization pattern across the cell cycle of synchronized cultures of <u>Chlorella</u> grown under optimal conditions of nutrient sufficiency and neutral pH.

2. Contrast the cell cycle dynamics of triglyceride levels in optimally grown cultures with TG levels in cultures subjected to artificially-induced extracellular stresses. The extracellular stresses employed include:

      a.    alkaline stress

      b.    calcium deprivation

      c.    nitrate deprivation

3. Determine the effect of inhibition of the tricarboxylic acid cycle on triglyceride levels using monofluoroacetate (MFA), a recognized inhibitor of the TCA cycle.

The aim is to find a reproducible method to initiate TG accumulation.

In consideration of the logistics of operating a
system for large-scale triglyceride production, it would
be preferable to not wait for a medium component to
disappear during normal cell growth (e.g. N-, Si-, Se-,
etc.) or to have to remove cells from a replete medium and
resuspend in a deficient one. The best "switch" would be
one that works when it is added to the medium. This would
not only be biochemically more convenient and decrease the
likelihood of disturbances and artifacts, but it would
also be more realistic in a large-scale production sense
for efficient TG production. The work of Fisher and
Schwartzenbach (1978) indicate that an addition which
decreases cell division rates, even temporarily, can have
an influence on TG proportions. Such reliable control
allows for analysis of the relationship between TG
synthesis and TG accumulation in microaglae and the
biochemistry involved therein.

## Literature Review and Rationale

Microalgae are currently receiving considerable
attention for their high concentrations of lipids of
potential commercial value. Among the phytoplankton
recognized as sources of useful lipids are Spirulina spp.
and Chlorella spp. with their high contents of linoleic
acid 18:3 and 20:5 respectively considered as a source of
polyunsaturaed fatty acids for use in health foods and

pharmaceuticals (Seto, 1984). Microalgae are also of interest for their Omega-3-fatty acids (w3) (Seto, 1984) and for their nonpolar lipids which may serve as an alternative source of hydrocarbon fuels (Neenan et al., 1986).

The term "lipid" is an operational term for molecules soluble in nonpolar solvents. The microalgal lipids include the triglycerides (TG) of cytoplasmic storage droplets, the glycolipid (GC) of chloroplast membranes and the polar membrane lipids (PL) (Figure 1). The term neutral lipid (NL) defines all those molecules of neutral polarity which are co-extracted in nonpolar solvents. Neutral lipids consist primarily of TG but also include some isoprenoids and hydrocarbons (Figure 1).

Triglycerides, particularly those in the more saturated straight chain form can be used as alternatives to conventional hydrocarbon fuels (Neenan et al., 1986). For this reason, TG can easily enter the fuel and other petroleum-based industries with minimal modification.

## Aquatic Species Program Overview

The Aquatic Species Program was initiated in 1979 by the U.S. Department of Energy (DOE) and the Solar Energy Research Institute (SERI) as part of the renewable biofuels effort.

# LIPID COMPOSITION of MICROALGAE

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                    LIPID COMPOSITION of MICROALGAE
                                    |
        ┌───────────────────────────────────────────────────┐
        |                                                   |
POLAR, MEMBRANE BOUND LIPIDS                    NONPOLAR, STORAGE LIPIDS
    ┌───────────┴───────────┐            ┌───────────┬───────────┐
    |                       |            |           |           |
Glycolipids           Phospholipids  Triglycerides Isoprenoids Hydrocarbons
```
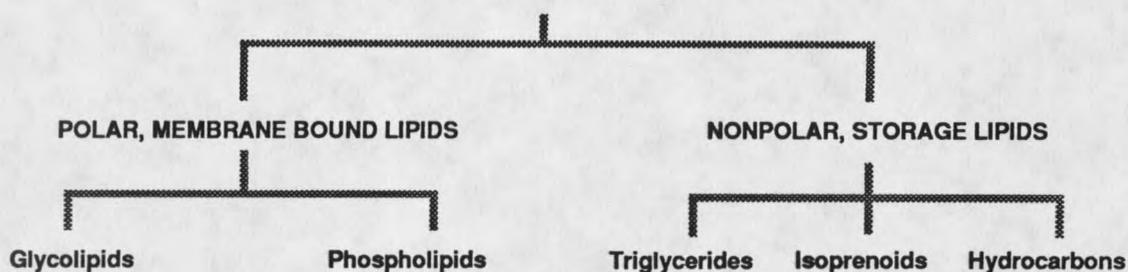
Figure 1.  Lipid Composition of Microalgae.

Currently, 42% of the energy market in the U.S. is for liquid fuels and 38% of these fuels are imported (Bollmeier, 1989).  It is estimated that up to 7% of the total current energy demand could be satisfied by the technology being developed in the Aquatic Species Program.

The DOE/SERI program emphasizes an initial commercial development of microalgal systems in the United States desert Southwest where there is an abundance of flat land and saline aquifers with few competing uses and high incident solar radiation.  As the technology is envisioned today, microalgae would be grown in large, shallow ponds of saline water, harvested, and processed for the production of liquid or gaseous fuels including ethanol, triglyceride-based diesel fuel, ester fuel, methane, and gasoline (Neenan et al., 1986).

The goal of the SERI Aquatic Species Program is to attain a lipid accumulation of 50% of the algal cell dry weight, while maintaining high cellular productivity with yields of 50g dry weight per square meter of pond surface per day (Neenan et al., 1986). With this yield, 150 to 400 barrels of oil acre$^{-1}$ yr$^{-1}$ could be produced from microalgae (Bollmeier, 1989).

Recently, the program has been recognized for its potentially important role in reducing the dangers of the "Greenhouse effect," which is primarily due to the release of carbon dioxide in the combustion of fossil fuels. The utilization of this carbon dioxide through the mass culturing of photosynthetic organisms could significantly reduce the contribution to the greenhouse effect, particularly when microalgal fuel plants are coupled to fossil fuel plants as scrubbers of carbon dioxide from flue gases (Chelf & Brown, 1989).

In order to improve the cost effectiveness of microalgal-based fuels, the productivity of all system components must be enhanced. Specific advances in the engineering design of the mass culture system in photosynthetic efficiency, nutrient supply, harvesting and processing are ongoing.

Microalgal strains have been screened to select species that are temperature and salinity tolerant with high lipid production (Brown, 1989). Genetic engineering

techniques are also being investigated to improve microalgal production and lipid content.

Before genetic engineering is selected to enhance NL yields, it is essential to identify the genes and their associated gene products. Currently, the biochemistry of microalgal lipids is for the most part only surmised from what is known of the higher plants, higher animal liver, and bacteria.

Presently, SERI is investigating the potential for genetic enhancement of triglyceride yield by focusing on characterization of the acetyl-CoA carboxylase. Efforts are currently underway in SERI labs to determine a partial amino acid sequence for the enzyme and to produce antibodies against the enzyme in order to be able to examine the properties of the acetyl-CoA carboxylase-encoding gene (Roessler, 1990). The focus of the Aquatic Species Program microalgal genetics project is to obtain algae with altered lipid metabolism by mutagenesis and selection of mutants with desired traits. A second planned approach will utilize recombinant DNA technology to obtain algae with desired lipid accumulating properties with introduction of genetic material into the cell through protoplast production, particle bombardment, electroporation and other methods. Several of these approaches must first be customized for introduction of genetic material into microalgae. Selectable markers

which can be used to assess the efficiency of
transformation must still be identified. Likewise, a
system for introducing the genetic material for
replication in microalgae must be identified. This can be
achieved by integration of the plasmid into the host
genome or with an autonomously replicating plasmid (Brown,
et al., 1989).

Recognizing that microalgal recombinant technology is
still in the early stages of evolution, the most promising
short term techniques for maximizing TG accumulation are
emerging in the recognition and understanding of the
constitutive and induced cell cycle effects on TG
accumulation. An empirical attempt at defining the
conditions for maximizing TG accumulation would at best
yield an inefficient system (Cooksey & Jackson, 1985). In
order for the program to succeed, both the physiology and
possibly the genetics of the organisms must be altered so
that the cells become enriched in the desired fuel
fraction. Additionally, aside from the product-oriented
focus of the SERI program, this work has important
implications in basic plant lipid biochemistry.

Microalgae are convenient models for higher plant
biochemistry. The chlorophyte group of algae (Chlorella,
Dunaliella) are considered to be evolutionary precursors
of higher plants (Christensen, 1964; Peerasso et al.,
1989) and are currently being utilized as models for

higher plant lipid biochemistry research (e.g. Lynch &
Thompson, 1982).

## Lipids as Storage Molecules

In eukaryotic microorganisms and in animal cells,
lipids may serve as energy storage molecules. Although
the oxidation of extracellularly supplied fatty acids has
been demonstrated in bacteria, there is little evidence
that this process can be used for the breakdown of
cellular lipids (Lennarz, 1966).

In animals the triglycerides are stored in specialized
tissue, the adipose tissue; in oil-bearing plants they are
stored in the seeds; in algae TG molecules are stored in
droplets dispersed throughout the cytoplasm (Cooksey et
al., 1987). Although some turnover of phospholipids has
been demonstrated (Lennarz, 1966), the neutral lipids,
particularly the glycerides, are the primary lipid storage
molecules in eukaryotes.

Apparently, there is no evidence of such lipid storage
capacity in bacteria (Lennarz, 1966). For lipids to
serve as storage molecules in bacteria, a mechanism for
the hydrolysis or oxidation of fatty acids is necessary.

Triglyceride is an efficient molecule for energy
storage. During the controlled oxidation of fatty acids,
ATP is generated yielding an energy supply equivalent of
38 kJoules of fatty acid catabolized, compared with 17

kJoules of carbohydrate or protein. Carbohydrate has to be stored in a bulky hydrated form, whereas three fatty acids can be stored per TG molecule in anhydrous form in animal adipose tissue or plant and microalgal TG droplets. The importance of TG is that they enable the animal, plant, or alga to store a much larger reservoir of energy than would be possible by storing carbohydrate or protein.

## Stress Effects and the Cell Cycle

The accumulation of neutral lipids under stressed conditions of nutrient limitation (Suen et al., 1987; Taguchi et al., 1987; Doucette et al., 1987; MacCarthy & Patterson, 1974) and alkaline stress (Guckert & Cooksey, 1990) is well documented. Otsuka and Morimura (1966) and Fisher and Schwartzenbach (1978) have suggested associations of TG levels with the cell cycle. It appears that NL accumulation is a result of the cell cycle being inhibited such that NL synthesis continues (during photosynthesis), but NL utilization decreases.

The term accumulation describes an important concept in this project. In fact, the word "accumulation" should be emphasized rather than synthesis in discussing changes in TG patterns across the cell cycle. Triglyceride accumulation is defined as:
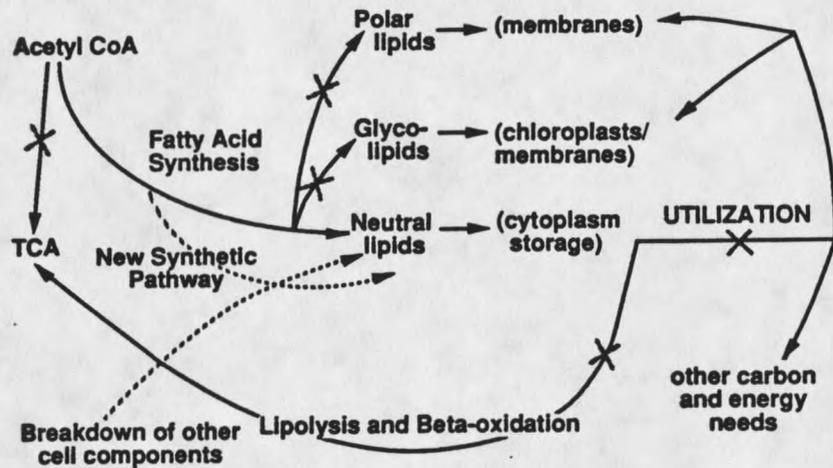
$$\text{Accumulation} = \text{Synthesis} - \text{Utilization}$$

12

An accumulation of triglycerides occurs when TG synthesis exceeds the utilization phase of the cell cycle. This is an important distinction in comparison to earlier researchers who sought to identify a trigger for a separate TG synthesis pathway. Recognition of synthesis-utilization patterns is important when trying to define parameters for maximum TG production on an industrial scale.

Earlier work has suggested the existence of sequential synthesis patterns for different lipid classes during cell cycles (Sicko-Goad et al., 1988, Suen et al., 1988). An accumulation of TG may then be a result of an uncoupling of a synthesis/utilization pattern within one cell cycle (Figure 2). Studies with the diatom Thalassiosira have suggested that storage TG is utilized during cellular energy demand, such as during the dark or during cell division and an accumulation occurs when the cell has excess energy available as with non-dividing cells in constant light (Fisher and Schwarzenbach, 1978). A different study with Chlorella in 1966 (Otsuka & Morimura) reported a synthesis of TG throughout the photosynthetic growth phase of the cell cycle and a utilization during cell division. The concept of TG synthesis and utilization stages in the cell cycle appeared to be a very good hypothesis for the lipid accumulation story; but there was weak evidence to support this idea.
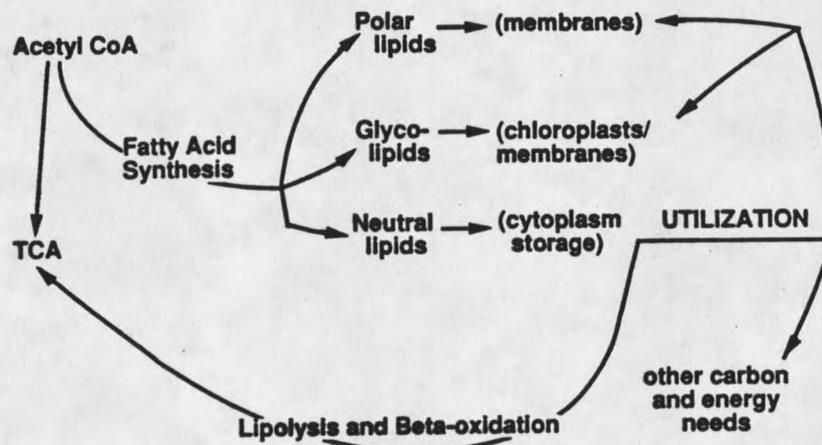
13



Figure 2. Proposed Neutral Lipid Accumulating and Non-Accumulating Conditions.

Tamiya (1963) published a map of the cell cycle-dependent stages of uptake of various nutrient elements in Chlorella. The data revealed that carbon or nitrogen depletion would result in inhibition during the early growth stages of the cell cycle before NL has accumulated.

Additionally, depletion of sulfur, which is assimilated during the ripening/post ripening stage (Tamiya, 1963) might increase cell yields of NL as cells would be inhibited during the cell cycle phase which results in the most NL accumulation just before cytokinesis.

The progression of the Chlorella cell cycle cannot be represented in the usual G1-S-M format since the S-stage of DNA synthesis is overlapped by the first mitotic event (M). The stages described by Otsuka and Morimura (1966) are compared with the more familiar G-S-M nomenclature. Using the Otsuka and Morimura nomenclature, neutral lipid is utilized in the post-ripening or L3-L4 phase.

Although lipid accumulation during nutrient limitation is well documented, no direct biochemical mechanism for the change in metabolism resulting in TG accumulation has been determined. A review of the known pathways for lipid biosynthesis will help to illustrate the hypothesized effects of nutrient deprivation and cell cycle inhibitors on lipid accumulation.