EFFECT OF ETHANOL ON TRIACYLGLYCEROL SYNTHESIS IN *CHLORELLA SPECIE*S UTILIZING A NOVEL FLUORESCENCE-BASED ASSAY

A Thesis
by
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Submitted to the Graduate School
Appalachian State University
In partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2010
Department of Biology

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FOREWORD

The organization and formatting of this thesis strictly follows the instructions to the author for article submission to *Lipids*, a journal of the American Oil Chemists' Society.

ABSTRACT

EFFECT OF ETHANOL ON TRIACYLGLYCEROL SYNTHESIS IN *CHLORELLA SPECIE*S UTILIZING A NOVEL FLUORESCENCE-BASED ASSAY
(August 2010)

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In this study, I set out to develop a novel method of assaying acyl-CoA: diacylglycerol acyltransferase (DGAT) activity employing a fluorescently labeled substrate, and then using that protocol to determine the effects of ethanol-induced stress on DGAT activity in *Chlorella species*. Microsomes from *Chlorella sp.* were used to construct protein and time curves to characterize the method in microalgae. Microsomes from *Rattus* liver were used to construct a substrate concentration curve and a Lineweaver-Burk plot to characterize the method in mammalian tissue. Optimal reaction conditions were determined to be 10 μg microsomal protein with 20 min incubation. The mammalian microsomes provided a K_m of 6.26 μM substrate concentration and a V_{max} of 0.211 pmol product x min^{-1} x μg $protein^{-1}$. This method represents the first fluorescence based DGAT assay. *Chlorella sp.* cultures were exposed to 0.33%, 0.66%, and 1.00% ethanol concentrations for 3 hr, and the DGAT activity assayed under optimal conditions. Increases in DGAT activity were observed in all cultures exposed to ethanol with a maximum seen at 0.66% concentration. These results have implications towards improving biodiesel production in mass algal cultures.

ACKNOWLEDGEMENTS

I would first like to thank my thesis committee members: Mark Venable, Howard Neufeld, and Shea Tuberty. All were very helpful and integral to the completion of my thesis. I would especially like to thank Mark Venable, my advisor and chairperson of the thesis committee. I feel very lucky to have had the opportunity to work under Mark, as he was a great mentor. I would also like to thank Richard Pagano (Mayo Clinic, Rochester, MN) for his generosity in providing fluorescent lipid standards free of charge. This study was funded by an Appalachian State University (ASU) Graduate Student Association Senate Award, ASU Office of Student Research Awards, and the ASU – University Research Council.

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INTRODUCTION

Reliable methods for the mass production of renewable fuel are at the forefront of numerous research efforts. Currently the energy sources of choice are petroleum based, which are non-renewable and exacerbate several environmental concerns. Biodiesel is seen as a possible substitute for petroleum products. It is biodegradable, has relatively lower emissions than petroleum based fuel, and is completely renewable, as vegetable oils and animal fats serve as the raw materials. There are several methods for the production of biodiesel: (1) blending raw vegetable oil with diesel fuel, (2) the use of microemulsions, (3) pyrolysis, and (4) transesterification [1-3].

Transesterification, also known as alcoholysis, represents the most logical mean of producing biodiesel. It describes the reaction between an alcohol and oil or fat that yields fatty methyl esters (fuel component) and glycerol. The method usually employs a base catalyst and displays high levels of conversion to biofuel. This is the method of choice for industrial level biodiesel production because it is simple, relatively inexpensive, and the resultant biodiesel is very similar to petroleum-based diesel in respect to its chemical and physical properties. Biodiesel produced in this manner also has improved environmental characteristics, as its combustion has reduced amounts of particulates, CO, and SO_x when compared to petroleum based diesel [3,4].

There have been numerous research efforts to find the best candidates for the production of the oils and fats required for biodiesel. Seed oils from terrestrial plants have

dominated this field of research, because their oils are almost entirely in the form of triacylglycerol (TAG), which is the oil of choice for transesterification/biodiesel production. However, the oil yield from these crops is not sufficiently high enough with respect to the amount of land required, so alternatives need to be explored [5].

The use of microalgae for oil production could provide a solution. Microalgae are very simple to raise and maintain, they grow rapidly (biomass can double in 24 hrs), and they require only a small fraction of the land needed to produce an equivalent amount of oil from terrestrial plants (based upon bench-scale yields). For example, to replace 50% of the transport fuels used in the United States with biodiesel from oil palm, which has the highest oil yield per unit of land for a terrestrial plant, it would require 24% of the existing farmland. Only 1-3% of existing farmland would be required (based upon 80% dry biomass in oil), to produce an equivalent amount using microalgae. Because of the large reduction in the land requirement, the use of microalgae for oil production would have a minimal effect on other products obtained from crops [5-8].

Chemically, oils are lipids. A lipid is classically defined as any chemical compound produced by a living organism that is not soluble in water, but readily soluble in an organic solvent. Lipids include a large variety of compounds. The focus of this paper is with the glycerolipid class, which all contain a glycerol backbone. The glycerolipid category encompasses the majority of lipids found in plants, the majority of which are membrane and storage lipids. In general, membrane glycerophospholipids are composed of the glycerol backbone, two fatty acids (FA)s esterified to the sn-1 and sn-2 positions of the glycerol backbone, and a polar headgroup, such as phosphocholine, bound to the sn-3 position.

Storage lipids (TAGs) have the same general structure as glycerophospholipids, except a third FA occupies the glycerol sn-3 position [9-15].

A major site of glycerolipid synthesis in plants, and the only site involved in TAG formation, occurs in the endoplasmic reticulum (ER) via the Kennedy pathway [10,11]. FAs are synthesized *de novo* in the plastid and activated as acyl-CoAs on the outside of the organelle. They are shuttled into the ER along with glycerol 3-phosphate (G3P) produced in the cytosol [11]. In the ER, G3P is acylated twice to form phosphatidic acid (PA). PA is then dephosphorylated via the activity of phosphatidic acid phosphatase to form diacylglycerol (DAG). DAG plays a key role in lipid metabolism, because it serves as the immediate precursor for phosphatidylcholine (PC), a prominent membrane lipid, and TAG, the primary storage lipid. Thus, DAG represents a central branch point between cellular growth and storage [16-18]. Scheme 1 displays the aforementioned fates of DAG and the enzymes involved.

The ultimate fate of DAG is determined by the activities of the governing enzymes. The terminal step in PC production from DAG is accomplished through the action of DAG phosphocholine transferase (PCT) [19]. Conversely, a family of diacylglycerol acyltransferases (DGAT)s can achieve the final step in TAG biosynthesis. There is type-1 acyl-CoA:diacylglycerol acyltransferase (DGAT)-1, type-2 DGAT (DGAT-2), and phospholipid:diacylglycerol acyltransferase (PDAT). As evidenced through the names, DGAT-1 and DGAT-2 are dependent upon the presence of acyl-CoA for the addition of the third FA, while PDAT is independent of acyl-CoA, as it transfers a FA from a phospholipid to DAG [20,21]. A type-3 acyl-CoA:diacylglycerol acyltransferase (DGAT-3) has been discovered in peanuts, but homologs have not been found in other species [22]. Interestingly,

while DGAT-1 and DGAT-2 both catalyze the same reaction, they share no sequence homology [20]. In plants, it appears that DGAT-1 is responsible for the majority of oil accumulation, while DGAT-2 and PDAT have been implicated in the production of TAGs with unusual fatty acids, such as eleostearic acid in tung oil [20]. In animals, it has been suggested that DGAT-1 is responsible for the esterification of exogenous fatty acids to DAG, while DGAT-2 is responsible for the incorporation of endogenous fatty acids to DAG [15].

The activities of the DGAT enzymes appear to be influenced by several factors. In mammals, both tissue location and nutritional status play central roles. The former is evidenced through the fact that DGAT activity is maximal in those organs associated with increased levels of TAG synthesis, such as the liver and adipose tissue [14]. The latter is exhibited through hormonal control of DGAT, as administration of insulin and glucagon, which are directly related to glucose status, resulted in increased and decreased DGAT activity, respectively [23-25]. In oilseed plants, DGAT activity is heavily reliant upon the developmental stage of the organism. It has been reported that flaxseed enter a stage of oil accumulation commencing about 8 days after flowering and ending about day 16. DGAT activity is at maximum during this period [26,27]. Similar findings were observed in *Brassica napus* (species of rapeseed) [28,29]. Otsuka and Morimura (1966) reported that *Chlorella ellipsoidea* accumulate oils during their growth phase in light and that those oils are utilized during cell division [30]. Furthermore, it has been suggested that accumulated oils in *Chlorella* are specifically consumed during the cell division stage of autospore release [31].

Oil production differs according to the type of organism and its physiological requirements, and the environmental conditions under which it finds itself at any given time.

While there are some major differences in the regulation of oil accumulation across species, there is one general commonality, which is the influence of stress on TAG production. When subjected to ideal conditions, the majority of lipids produced by eukaryotes tend to be structural or membrane lipids, such as PC [15,17]. However, when experiencing stress, eukaryotes display a proclivity for storage lipid accumulation, as reduced carbon is shunted towards the TAG pathway, while structural lipid synthesis is attenuated [31-41]. In the case of microalgae, they will then enter a semi-dormant state, in which cell division and cellular growth are no longer primary functions, and survival through the stress-inducing event becomes paramount [17,31,42].

Stress-induced oil (storage lipid) accumulation at the expense of membrane lipids in green microalgae is well documented. Notable examples involve the response of green microalgae to nutrient availability. Suen et al. [33] and Tornabene et al. [34] have demonstrated that nitrogen (N)-deprivation in *Nannochloropsis sp*. and *Neochloris oleoabundans*, respectively, elicited increased TAG accumulation. Furthermore, Suen et al. [33] suggested that the increase seen in storage carbon was at the expense of phospholipids (PL). This was evidenced not only through an increase in the TAG/PL ratio, but also through a decrease in cell density observed during N-deprivation (restricted growth) [33]. Another example involves the response of *Chlorella vulgaris* to ethanol-induced stress. Goedhart et al. [36] have demonstrated large increases in the TAG content of *C. vulgaris* when exposed to ethanol. They reported a 30-fold increase in TAG concentration upon exposure to 0.33% ethanol with decreases in PA and PC content. These results were not presented with respect to enzymatic activity, but rather to overall changes in the lipid composition [36].

In light of the information presented, one could infer that stress affects microalgae through inhibition of the cell cycle and accumulation of TAG at the expense of PL. However, it is not known what impact stress has on the specific activity of DGAT, which could provide integral information towards elucidation of this stress-mediated pathway. Schema 2 demonstrates the hypothesized effect of ethanol stress on the TAG production pathway. This could also provide an important step towards making algae-based biodiesel production a reality, as current methods of production do not provide sufficient amounts of TAG per microalgal cell to make the project cost efficient. The purpose of the research presented here was to investigate the effects of ethanol stress on acyl-CoA dependent DGAT activity in *Chlorella sp.* utilizing a novel fluorescence-based DGAT assay. We report significant increases in the specific activity of acyl-CoA dependent DGAT as a result of ethanol exposure. These results are discussed in the context of stress-mediated cell signaling and the overall goal of increasing TAG synthesis in microalgal mass cultures towards the production of biodiesel that can financially compete with petroleum derived fuel.

EXPERIMENTAL PROCEDURES

Algal Cultures, Hepatic Tissue, and Reagents

All algal cultures were purchased from Carolina Biological Supply (Burlington, NC), and the hepatic tissue was from the genus *Rattus*. Dithiothreitol (DTT) was purchased from Bio-Rad (Hercules, CA), and 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-phosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL) and enzymatically converted to NBD-DAG through phospholipase C (Sigma-Aldrich, St. Louis, MO). The NBD-TAG standard was graciously provided by Dr. Richard Pagano. Sucrose and Tris base were purchased from Fisher Scientific (Hampton, NH), and thin layer chromatography (TLC) silica gel G plates were purchased from Analtech (Newark, DE). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

NBD-DAG Preparation

The synthesis of NBD-DAG from NBD-PC was performed, with modification, according to the procedure by Kates [43]. In a 13 x 100 mm glass reaction tube equipped with Teflon cap and stir bar, NBD-PC was suspended in diethyl ether:ethanol (98:2, v:v) and 0.02M $CaCl_2$. The reaction was then initiated through the addition of phospholipase C from *Bacillus cereus* (Sigma-Aldrich, St. Louis, MO). The reaction was allowed to proceed for 2

hrs at room temperature (approximately 23 °C) with stirring. The solution was brought to dryness with a stream of N_2 and resuspended in 3 ml of methanol. Lipids were extracted according to the method of Bligh and Dyer [44] and brought to dryness in a stream of N_2. Lipids were resuspended in 2 ml chloroform:methanol (1:1, v:v), spotted in their entirety on a silica gel G TLC plate then developed in acetic acid:methanol:diethyl ether:hexane (1:5:30:70, v:v). The plate was analyzed for NBD-DAG product with a UV light. NBD-DAG product was scraped off of the TLC plate into a new 13 x 100 mm glass reaction tube and extracted according to the method of Bligh and Dyer [44]. NBD-DAG solution was brought to dryness in a stream of N_2 and resuspended in chloroform.

Preparation and Separation of Microsomal Fraction

Microsomes from *Chlorella species*, *Chlamydomonas reinhardtii*, *Cyclotella cryptica*, and *Rattus norvegicus* hepatic tissue were prepared, with modification, by the procedure of Sewada and Shiraiwa [45]. Approximately 0.8 g (wet cell weight) were isolated through centrifugation at 2500*g* for 10 min at 4 °C. Samples were kept on ice or at 4 °C for the remainder of the microsomal preparation. Cell pellets were suspended in 1 ml of grinding buffer (125 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 50 mM Tris-MES, pH 7.48), transferred to a 1 ml Potter-Elvehjem homogenizer (Reacti-Ware, Pierce, IL), and homogenized with a Barnant Mixer, Series 10 (speed of 7 for three 30 sec periods; Barrington, IL). Algal homogenates were centrifuged at 1230*g* for 10 min at 4 °C and the supernatant decanted. The pellets were resuspended in 1 ml of grinding buffer and homogenization repeated. The rat liver sample did not require a second homogenization. All samples were then centrifuged at 270*g* for 13 min at 4 °C and the supernatants isolated. The supernatants were placed in a Beckman TLA 100.4 (Fullerton, CA) rotor and centrifuged at

89,000*g* for 30 min at 4 ^{o}C (Beckman Optima TL Ultracentrifuge, Fullerton, CA). Microsomal pellets were resuspended in 500 μl of grinding buffer through brief sonication (1-3 s) with a W-380 Sonicator (Heat Systems-Ultrasonics, Inc, Plainview, NY; Settings: continuous cycle, 40% duty cycle, and 4 output control) and microsomal protein quantified through the Bradford method using γ-globulin as the standard [46]. The microsomal samples were used fresh or stored at -80 ^{o}C. The enzymatic activity for algal microsomes appeared to be somewhat degraded by freezing and thawing. The enzymatic activity for rat liver microsomes was not affected by the freezing and thawing process.

DGAT Assays

Enzyme assays were conducted, with modification, by the procedure from Little et al. [47]. The assays were performed in 8 ml polypropylene snap cap tubes with shaking at 35 ^{o}C in a C25KC Incubator Shaker (New Brunswick Scientific, Edison, NJ). Each reaction tube received 50 μl of standard reaction mixture (0.2 M HEPES/NaOH buffer, pH 7.41, with 3 mM $MgCl_2$, 1 mM ATP, 330 mM oleoyl-CoA, 118 mM NBD-DAG, 0.02% Tween-20, and 0.5% (w/v) BSA, 10 μg microsomal protein (unless otherwise indicated), and was brought to a final volume of 100 μl with deionized water. The reaction was initiated by addition of the microsomes, allowed to proceed for 20 min (unless otherwise indicated), then terminated through the addition of 1 ml of methanol. Lipids were extracted according to the method of Bligh and Dyer [44]. The $CHCl_3$ phase was brought to dryness in a stream of N_2 and placed in solution by 50 μl of $CHCl_3$. The samples were spotted in their entirety onto channeled TLC plates, from Analtech (Newark, DE), coated with 250 mm Silica Gel G and a

preadsorbent zone. The plates were developed in acetic acid:methanol:hexane:diethyl ether (1:5:40:60, v:v) then allowed evaporate. The plates were then analyzed for NBD-TAG using a Typhoon Trio Variable Mode Imager (Amersham Biosciences, Piscataway, NJ). Assays were conducted in duplicate or triplicate as indicated.

Determination of Picomoles of NBD-TAG

A standard curve was constructed using known amounts of standard NBD-DAG, which was utilized to determine the concentration of an NBD-TAG standard solution. A standard curve using the NBD-TAG standard was then constructed. The "volumes" of fluorescence, represent the level of fluorescence, obtained from experimentally synthesized NBD-TAG were compared to the NBD-TAG standard curve, in order to determine moles of product produced. It should be noted that the "volumes" refer to the area under the curve determined by the fluorescence imager.

Exposure of *Chlorella species* Cultures to Ethanol

Chlorella sp., in log phase growth, at a volume of 1000 ml was centrifuged at 2500g for 10 min at room temperature. The supernatant was decanted, and the algal pellet was resuspended in spring water to an approximate optical density of 1.00 at 680 nm using a Beckman DU640B Spectrophotometer (Fullerton, CA). In 250 ml glass media bottles (dimensions: 11 cm height and 5 cm diameter), 0% ethanol, 0.33% ethanol, 0.66% ethanol, and 1.00% ethanol solutions were made consisting of *Chlorella sp.* and ethanol to a final

volume of 200 ml. The glass media bottles were capped with a lid that contained a hole in the center for insertion of a stone bubbler. The bubbler had an approximate airflow of 20 ml/min with approximately 10% CO_2 in air. The cultures were placed under constant fluorescent illumination (approximately 78 μmol photons m^{-2} s^{-1}; average illumination per algal cell was approximately 34.6 μmol photons m^{-2} s^{-1}) for 3 h. Illumination was determined with a LI-190 quantum sensor connected to a LI-250 light meter (Li-Cor, Inc., Lincoln, NE). Microsomes were prepared and DGAT activity assayed as described above.

RESULTS

DGAT Assay Characteristics

I used the existing radiolabel based assay parameters as a starting point of development. The buffer conditions were optimized for that assay. In order to perform a fluorescence assay I first needed the fluorescent substrate. I decided that it would be most appropriate to use labeled DAG instead of the acyl-CoA due to greater stability and fewer side reactions. DAG with an omega-labeled fatty acid is not commercially available. The simplest route to this substrate is to purchase ω-NBD-fatty acyl-phosphatidylcholine, treat with phospholipase C then purify the diacylglycerol product via TLC. The published protocols work well and generated product in quantitative amounts.

A concern I had was that the stearic hindrance of the NBD label would interfere with the enzyme. However, I had confidence that it would work since whole cell experiments show incorporation of NBD-labeled fatty acids into lipid droplets such as in *Schistoma mansoni* [48].

Once I had this substrate I proceeded to determine whether the assay would be sensitive enough to measure endogenous enzyme activity in algae. In preliminary experiments (not shown) I optimized the amount of substrate such that enough product would be formed to measure and that the substrate mass was such that only a small percentage of the substrate was used so that the enzyme did not become substrate limited.

I wanted to characterize the assay for linearity with respect to protein concentration and time using the common *Chlorella sp.* algae. An NBD-TAG standard curve was constructed for determination of product formation (Fig. 1). I found that the DGAT activity was linearly proportional to microsomal protein concentration up to 10 μg (Fig. 2). The activity continued to climb up to about 25 μg and then declined slightly. In these experiments I did not cut the NBD-DAG with unlabeled DAG substrate so the sensitivity could not be increased much by increasing the ratio of fluorescence to mass. The use of a more sensitive imager or increasing the scan time may allow accurate measurements at protein concentrations below 1 μg.

A time course was then performed using the 10 μg optimal protein concentration. I found that the activity increases linearly up to 20 min after which activity continues to climb through approximately 30 min and begins to level off at approximately 40 min (Fig. 3). The progress was not limited by the amount of substrate or by conversion to polar lipid products.

Next I wanted to determine whether the assay was generally applicable relative to species. I conducted the assay using microsomes from another green alga (*Chlamydomonas sp.*), the saltwater diatom (*Cyclotella cryptica*) and mammalian liver (*Rattus*). The assay worked well in all three algal taxa and in measuring liver enzyme activity (Fig. 4). The activity is presented in terms of specific activity (pmole product x min^{-1} x μg $protein^{-1}$) with the assays being performed at the standard 10 μg protein and 20 min time.

Additional study was performed with the *Rattus* liver microsomes to further characterize the kinetics of the assay in mammalian tissue. A substrate concentration curve was constructed using increasing concentrations of NBD-DAG (Fig. 5). Standard conditions were used (10 μg protein and 20 min incubation time). The curve is linear with respect to the

rate of product formation through approximately 50 μM NBD-DAG. The curve then begins to approach a horizontal asymptote at a product rate of approximately 0.2 pmoles x min^{-1} x μg $protein^{-1}$. A Lineweaver-Burk (double reciprocal) plot was constructed from the substrate concentration curve (Fig. 6), and the K_m and V_{max} were determined to be 6.26 μM NBD-DAG and 0.211 pmoles NBD-TAG x min^{-1} x μg $protein^{-1}$, respectively.

With respect to the *Rattus* liver microsomes, it should be noted that some of the volumes (amount of fluorescence) of NBD-TAG obtained were outside of the NBD-TAG standard curve. However, the extrapolation of data points was not of concern, because volume (fluorescence) plotted as a function of substance amount (pmoles NBD-TAG) will always be linear as long as the volumes obtained are less than the saturation limit of the detector. All data obtained were below the saturation limit of the detector

Effects of Ethanol on Acyl-CoA Dependent DGAT Activity in *Chlorella species*

In preliminary experiments, a variety of stressors that increase oil accumulation in algae were found. One of those was ethanol and since its delivery is straightforward I decided to assay for changes in acyl-CoA:DGAT in response to ethanol. I exposed cultures to 0.33%, 0.66%, and 1.00% ethanol concentrations. It was important that the optical density of the culture, directly related to culture density, be at an approximate absorbance of 1.00 ± 0.05 at 680 nm before ethanol is administered. Optical densities significantly less than 1.00 have demonstrated tendencies towards ethanol toxicity and cell death, while densities significantly greater than 1.00 have shown little to no effect from ethanol exposure (data not shown).

I wanted to allow ample time for all ethanol-induced cellular responses to take place (post-translational modification and changes in gene expression). Sanchez-Amate et al. [49] described persistent effects on enzyme activities in chick-liver membranes after 15 min exposure to ethanol. Experimentation with nutrient depletion in *Chlamydomonas reinhardtii* demonstrated stress-related gene expression within 15 min to approximately 2 h [50-52]. Thus, a 3 h ethanol exposure period was utilized to allow for a full response.

The activity of PDAT was of concern, because it could produce fluorescently labeled TAG independent of acyl-CoA [21]. However, this was accounted for through assays performed in the absence of exogenous acyl-CoA (data not shown). Those assays showed no TAG production, or at least the TAG produced was below the limit of detection. Thus, it was determined that PDAT of *Chlorella sp.* did not significantly contribute to TAG production under ethanol stress. Similar results were reported in *Arabidopsis thaliana* seeds [53].

To assay acyl-CoA-dependent DGAT activity in *Chlorella sp.* in response to varying ethanol concentrations, the previously determined optimal conditions of 10 μg protein and 20 min reaction time were used. Trials were performed in duplicate, and the results are presented in terms of the specific activity (Fig. 7). Increases in the specific activity, relative to the control, were observed in all cultures exposed to ethanol with a maximum exhibited at an ethanol concentration of 0.66% (approximate 3-fold increase).

DISCUSSION

The study of TAG biosynthesis and its regulation is of utmost importance. The overproduction and storage of TAG in humans has implications towards cardiovascular disease and diabetes, while synthesis of TAG in plants has relevance towards biodiesel production. Due to the importance of ongoing research in TAG metabolism, better methods of assaying the controlling enzymes are necessitated. The DGAT assays currently in use employ radioactively labeled substrates or some sort of heterologous expression of DGAT through transfection [26,47,54-57]. Recently, Siloto et al. [57] have published a novel method that determines TAG content, which can be used to indirectly measure TAG synthesizing enzymes, through the use of Nile red fluorescence in yeast, so that radioactive isotopes could be avoided. They also demonstrated that the assay was useful for the study of various DGAT mutants [57]. However, all results were obtained from a single species of yeast, inferring that in order for their technique to be ubiquitous, the DGAT of interest must first be raised through transfection. This raises the probability of aberrant results without extensive controls.

The fluorescence-based assay developed for this study represents a novel method of measuring acyl-CoA:DGAT activity. It represents the first DGAT assay to utilize a fluorescently labeled substrate (NBD-DAG) alternative to radiolabeled substrate. The assay can be directly used on plant and animal tissue preparations, thus demonstrating the broad applicability of the technique.

The assay is sensitive enough to measure endogenous DGAT and so does not require transfection. It appears to give comparable results to the radiolabeled assays in plants. Byers et al. [56] reported an acyl-CoA:DGAT specific activity of approximately 33 pmol TAG x min^{-1} x mg $protein^{-1}$ in canola at the same $MgCl_2$ and ATP concentrations as our assay. Sorensen et al. [26] reported an acyl-CoA:DGAT specific activity of approximately 1.5 pmol TAG x min^{-1} x mg $protein^{-1}$ in developing flaxseed using oleoyl-CoA. These values compare well with our range of specific activities of 8.3 – 15.2 pmol TAG x min^{-1} x mg $protein^{-1}$ reported for the ethanol portion of the study (Fig. 7).

Using the fluorescent assay I found somewhat lower specific activity than was reported using the radiolabeled assays in mammalian tissue. Ganji et al. [58] reported an approximate K_m, V_{max}, and combined DGAT-1 and DGAT-2 specific activities of 100 μM DAG, 2 pmol TAG x min^{-1} x μg $protein^{-1}$, and 1.2 pmol TAG x min^{-1} x μg $protein^{-1}$, respectively, in HepG2 cells. Young and Lynen [59] and Andersson et al. [60] reported approximate DGAT activities of 3.3 pmol TAG x min^{-1} x μg $protein^{-1}$ and 5.9 pmol TAG x min^{-1} x μg $protein^{-1}$, respectively, in *Rattus* liver microsomes using palmitoyl-CoA. When compared to my results from the *Rattus* liver microsomes (K_m = 6.26 μM NBD-DAG, V_{max} = 0.211 pmol TAG x min^{-1} x μg $protein^{-1}$, and specific activity = 0.2 pmol TAG x min^{-1} x μg $protein^{-1}$), it appears that the fluorescence-based assay is consistently lower by an approximate factor of 10. I was not able to compare the two methods side by side so the difference could be accounted for by several factors, including rat physiological status or age, particulars in how substrate or enzyme were prepared or differences in how the fluorescent and radioactive substrate fit into the enzyme active site.

Ethanol increases acyl-CoA:DGAT activity in *Chlorella sp*. The 0.66% ethanol treatment provided the highest activity, with an approximate 3-fold increase, relative to the control. Goedhart et al. [36] demonstrated that treatment of algae with 0.33% ethanol lead to an approximate 30-fold increase in cellular TAG concentration; however, their results are based upon overall TAG content and not enzymes activities. Their results are also based upon much longer exposures to ethanol (approximately 60 hr) [36]. It is tempting to speculate that this 3-fold increase in DGAT activity could lead to a 30-fold increase in algal oil content after 60 hr of treatment.

At the cellular level, it appears that ethanol induces stress by affecting membrane fluidity. It is believed to disturb the organization of the acyl chains on membrane phospholipids, thus increasing the fluidity of the membrane [61]. This is believed to occur because of the hydrogen bond breaking activity of ethanol at the membrane-water interface [62]. This effect on membrane fluidity can in turn affect the activities of enzymes throughout the cell [63]. Sanchez-Amate et al. [49] demonstrated marked inhibition of microsomal NADH cytochrome b_5, an enzyme involved in the FA desaturase complex of the ER. Inhibition of this complex suggests decreases in membrane desaturation resulting in decreased cellular fluidity. Increased rigidity would aid in ameliorating the increased fluidity brought upon by ethanol.

In order to bring about such responses, enzymatic activities must be altered. This can be accomplished through post-translational modification and/or modulation in expression levels of the enzyme. There are several publications demonstrating both cases in microalgae [50-52,64-69]. It appears that acyl-CoA:DGAT is not constitutively expressed in oilseeds. However, during TAG accumulation, DGAT-1 transcripts are observed in abundance. The

expression is then reduced as the lipid content reaches a maximum [26,28,29,70]. This suggests that algae may not constitutively express acyl-CoA:DGAT either, but rather cater the expression to the needs of the cell.

In order for external stimuli, such as ethanol administration, to upregulate acyl-CoA:DGAT expression, signal transduction must be occurring. A well-known signal transduction pathway in animals involves phosphatidylinositol 4,5-bisphosphate (PIP2). A primary function of this pathway is to relay information across the plasma membrane through the hydrolysis of PIP2, which produces inositol triphosphate (IP3) and DAG, two well-documented signaling molecules. This is accomplished via the activity of a PIP2-specific phospholipase C (PLC) [71-74]. This pathway has been observed in microalgae and found to be active in response to hypoosmotic shock (a stressor). Furthermore, PIP2 levels comparable to those found in animal cells have been demonstrated in microalgae [71,74].

Subsequently, IP3 and DAG stimulate the release of intracellular calcium and activate protein kinase C (PKC), respectively [72]. Interestingly, a putative protein kinase-targeting motif is conserved in acyl-CoA:DGAT and other acyltransferases, so some regulation could be exhibited at the protein level [54,75]. In addition, Augert et al. [72] suggested that intracellular calcium release and activated PKC stimulate a PC-specific PLC, which hydrolyzes PC to DAG and cytidine 5'-phosphocholine. This could have a profound effect on intracellular DAG content, as PC is an abundant membrane lipid [17]. Therefore, this signaling pathway could play an integral role in the conversion of membrane lipids to storage lipids in algae.

The exact mechanism through which ethanol elicits increased acyl-CoA:DGAT activity is unknown. However, based upon evidence presented in this paper, a partial

mechanism can be proposed: (1) ethanol exposure results in increased membrane fluidity, (2) this activates a PIP2-specific PLC, (3) PIP2 is hydrolyzed into signaling molecules IP3 and DAG, (4) IP3 stimulates the release of intracellular calcium stores, which with DAG activates PKC, (5) a PC-specific PLC is activated, which produces DAG, (6) increased DAG pool allows for greater chance of interaction with acyl-CoA:DGAT to produce TAG, and (7) activated PKC phosphorylates putative protein kinase-targeting motif of acyl-CoA:DGAT. Obviously, there are holes in the proposed mechanism, such as the state of the acyl-CoA pool and whether acyl-CoA:DGAT expression is up-regulated through this pathway or if the activity of pre-existing acyl-CoA:DGAT is stimulated by activated PKC. It could be both. Furthermore, the effect of ethanol on PCT activity is also unknown, so additional experimentation is required to fully elucidate the mechanism.

Three technical problems currently under assault are how to grow such large amounts of a waterborne crop, how to economically harvest a microscopic crop from water, and how to dewater the algae once harvested. Biologically, the primary limitations revolve around inadequate molecular knowledge. In order for microalgal biofuel to become competitive, the microalgae must produce large amounts of biomass (near theoretical limits), which will require a much-expanded foundation of knowledge with respect to the factors regulating factors of carbon flux in microalgae [76].

Several other biological limitations in microalgae have also been observed. These include, but are not limited to, organism survival, seasonality, and light penetration. With respect to organism survival, open-system mass culture methods, most notably raceway ponds, are very susceptible to contamination by exogenous algal species, which can take over the culture. It is suggested that herbicide resistance conferred through transgenics may solve

this problem [77]. The seasonality of biomass yields is of concern, because the highest yields only occur during certain seasons. This must be addressed so that near-maximal yields can be obtained year-round. Lastly, the insufficient penetration of light into dense mass cultures must be dealt with. This is because microalgae located near the surface absorb the majority of the light energy. This can result in photoinhibition, which drastically reduces photosynthetic capacity. Conversely, the microalgae that are not located in direct sunlight receive very little irradiance, which decreases their ability to assimilate carbon [77].

The pathway I believe that researchers will most likely follow to make this overall project cost-efficient begins with the utilization of native microalgae. Mass cultures should use species of microalgae native to that area. Through the use of native species, raceway ponds become a viable option, which could greatly limit costs [76]. Insufficient light penetration into raceway ponds would still be a major problem. However, extensive work has been performed on truncating chlorophyll antenna size in *C. reinhardtii*, which shows promise towards reducing photoinhibition with a concomitant increase in light penetration depth [78]. Mixing with paddlewheels also increases yields [76].

C. reinhardtii also shows promise for elucidation of molecular controls. The genome has been sequenced and substantial amounts of molecular data already exist for this species, so it could serve as an *in vitro* model [6]. Furthermore, it has been shown that starchless *C. reinhardtii* mutants demonstrate excessive TAG accumulation (10-fold increase) [37,79]. Therefore, I believe the next step is to determine the effects of different types of stress on this particular *C. reinhardtii* mutant to see if TAG is further accumulated. In summary, my results show that with better understanding of the underlying signaling and metabolic

processes, yields can be improved, and the nation will be closer to carbon neutrality and energy independence.

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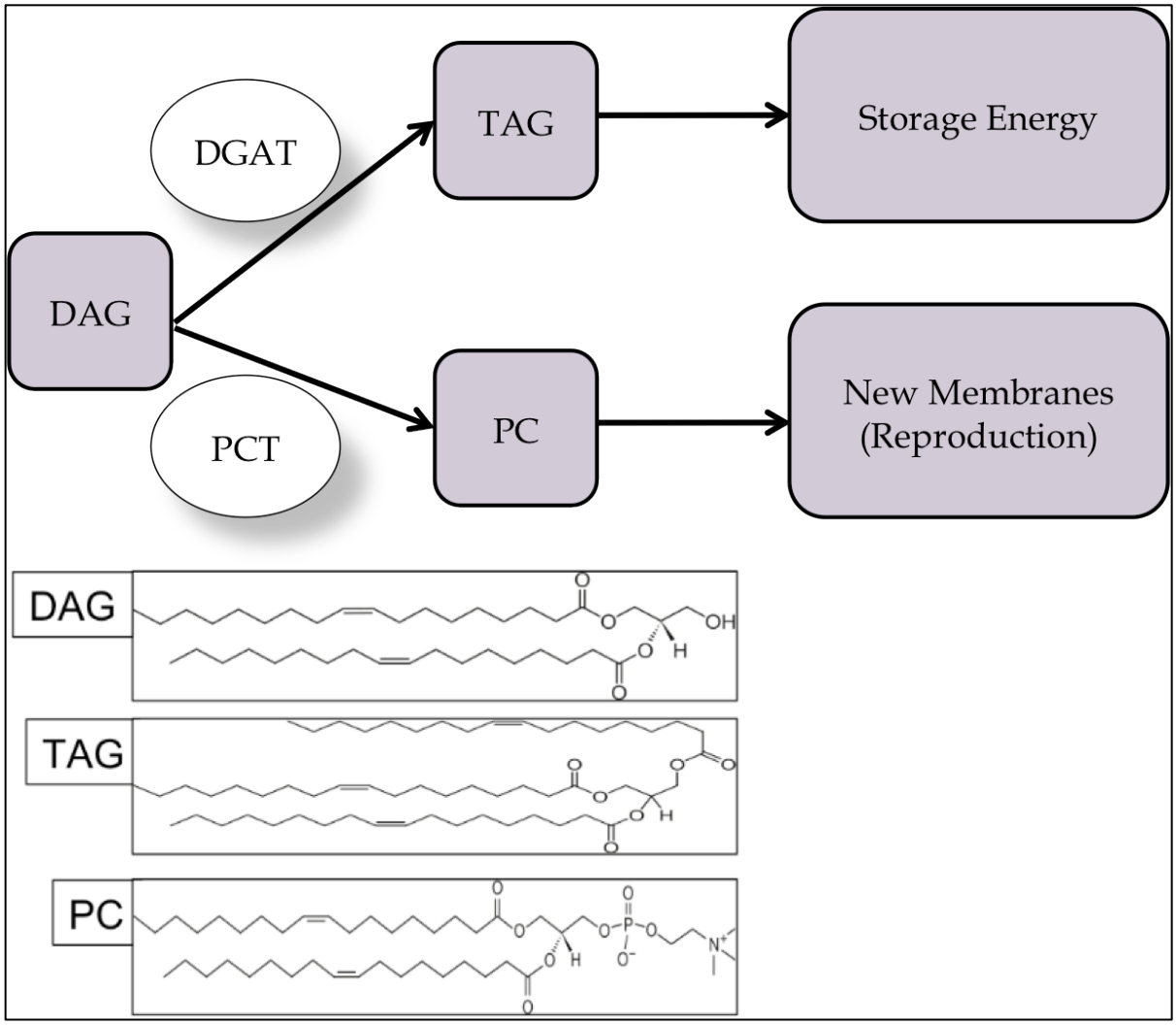
SCHEMA LEGENDS

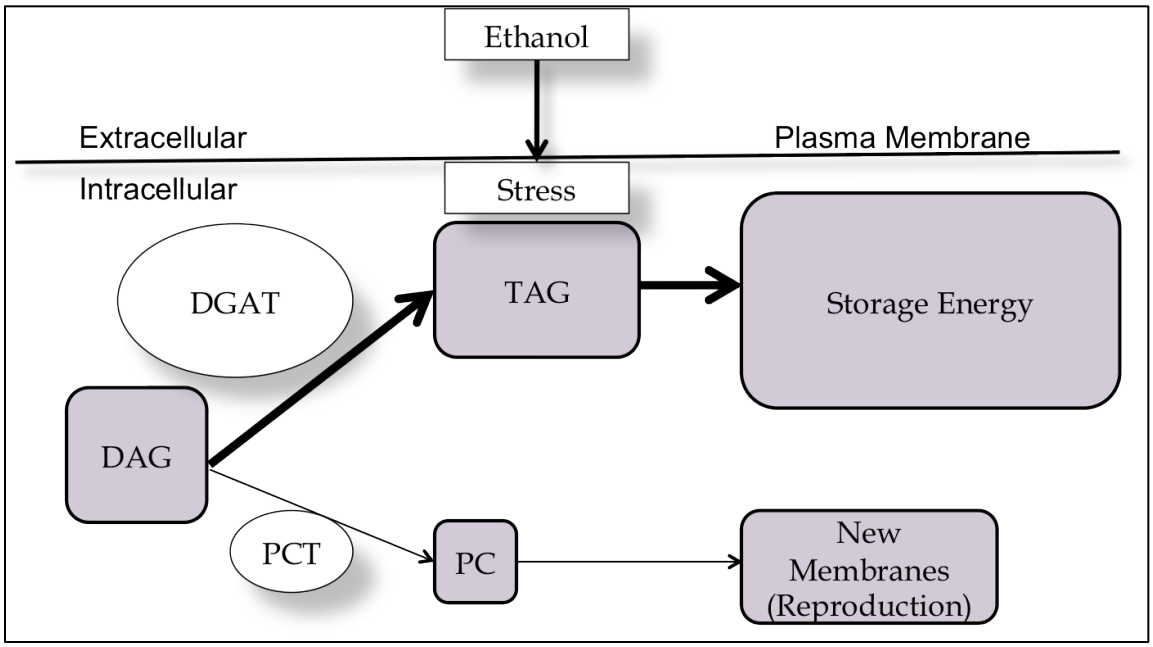
Schema 1. The schema represents two possible fates of DAG, the enzymes involved, and the molecular structures of DAG, TAG, and PC. DGAT and PCT denote the enzymes diacylglycerol acyltransferase and phosphocholine transferase, respectively. Representations of DAG, PC, and TAG were kindly supplied by Avanti Polar Lipids, Inc.

Schema 2. The schema depicts the hypothesized effect of ethanol stress on the TAG production pathway. Exposure to ethanol will elicit a stress response that results in increased DGAT activity, thus increasing TAG production and decreasing PC production.

SCHEMAS



Schema 1


Ethanol
Extracellular
Plasma Membrane
Intracellular
Stress
DGAT
TAG
Storage Energy
DAG
PCT
PC
New
Membranes
(Reproduction)

Schema 2

FIGURE LEGENDS

Fig. 1 The NBD-TAG standard curve was utilized to determine product formation. A range of NBD-TAG amounts were spotted on a TLC plate, developed in chloroform/methanol (99:1), and an image obtained using a Typhoon Trio Variable Mode Imager. The following settings were used on the imager: Emission Filter – 520 BP 40 CY2, ECL+, Blue FAM; PMT – 420; Laser – Blue (488); Sensitivity – Normal. These settings were used for all other fluorescent images. The imager provides quantification in terms of volume, which is directly related to the amount of fluorescence. Data represent results from 3 separate experiments. P-values for the y-intercept and x-variable are 0.0032 and <0.0001, respectively.

Fig. 2 DGAT activity is linear with protein mass up to 10 μg microsomal protein. DGAT activity was assessed using microsomes from *Chlorella sp*. and NBD-DAG as substrate. Total lipids were extracted, separated via TLC, and visualized and quantitated using a fluorescence imager (**a**). Picomoles of NBD-TAG produced were calculated from a standard curve and plotted (**b**). Data represent results from 3 separate experiments. P-values for the y-intercept and x-variable are 0.0299 and 0.0159, respectively.

Fig. 3 DGAT activity is linear with time up to 20 min. DGAT activity was assayed using *Chlorella sp*. (10 μg microsomal protein) and NBD-DAG substrate. Total lipids were extracted, separated via TLC and visualized and quantitated using a fluorescence imager. Data represent mean ± standard error results from 2 separate experiments.

Fig. 4 Fluorescence based DGAT assay is useful in various algae and mammalian hepatic tissue. Reaction contained 10 μg of microsomal protein and proceeded for 20 min. Total lipids were extracted, separated via TLC and visualized and quantitated on a fluorescence imager. Data represent mean ± standard error results from 2 (liver and *Chlorella*) or 3 (*Chlamydomonas* and *Cyclotella*) separate experiments.

Fig. 5 A substrate concentration curve was constructed using 10 μg *Rattus* liver microsomes and 20 min incubation time. Increasing concentrations of NBD-DAG were utilized in the reaction mixture to assess acyl-CoA:DGAT enzyme kinetics in mammalian tissue. Total lipids were extracted, separated via TLC and visualized and quantitated on a fluorescence Imager. Data represent results from 2 separate experiments.

Fig. 6 A Lineweaver-Burk (double reciprocal) plot was constructed from the *Rattus* substrate concentration curve. Fig. 5 was used to construct this plot. The y-intercept represents $1/V_{max}$ and the x-intercept represents $-1/K_m$. The V_{max} and K_m were determined to be 0.211 pmoles NBD-TAG/min/μg protein and 6.26 μM NBD-DAG, respectively. Data represent results from 2 separate experiments. P-values for the y-intercept and x-variable are both <0.0001.

Fig. 7 Ethanol treatments increase acyl-CoA:DGAT specific activity in *Chlorella*. *Chlorella* were exposed to various ethanol concentrations and the acyl-CoA:DGAT activity assessed. Reaction mixtures received 10 μg microsomal protein and incubated for 20 min. Total lipids were extracted, separated via TLC and visualized and quantitated on a fluorescence Imager. Data represent mean ± standard error results from 2 separate experiments.

FIGURES

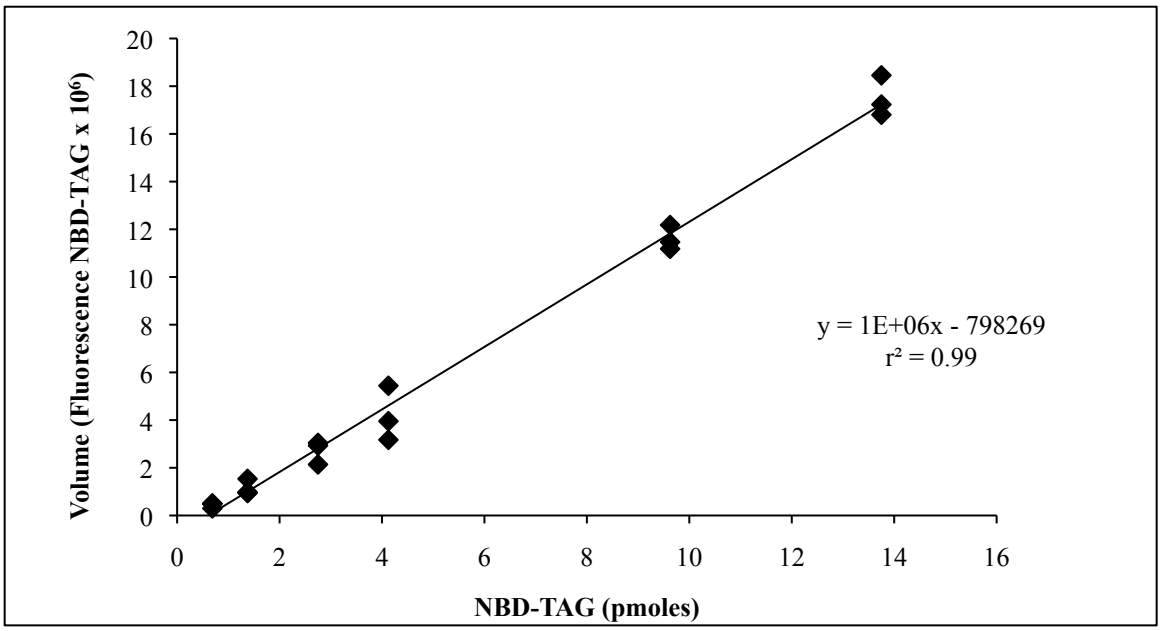


Figure 1

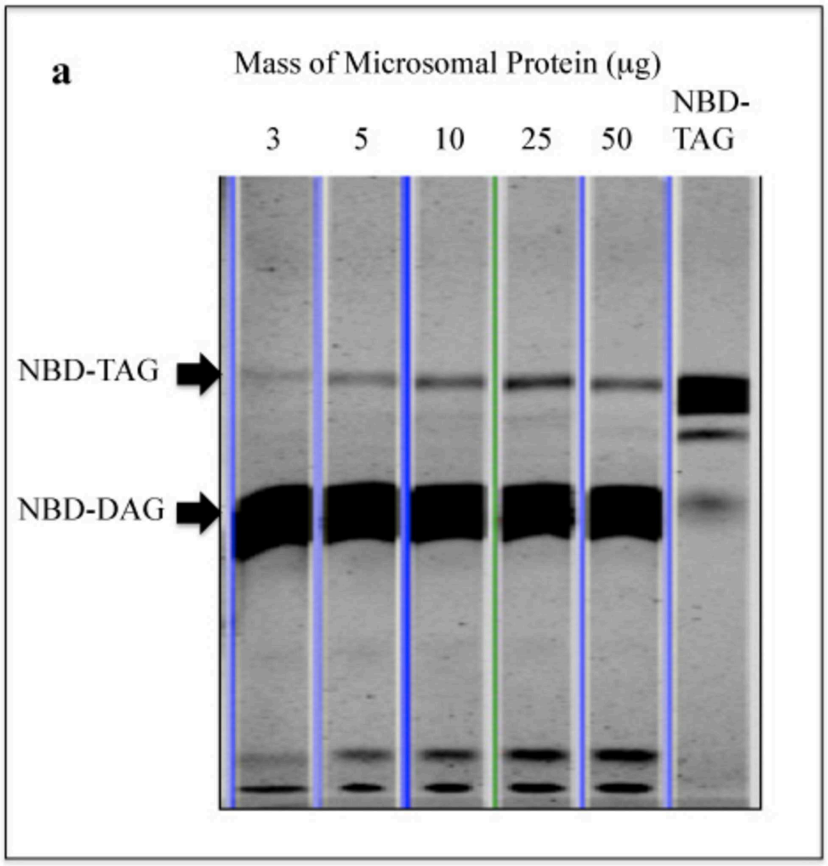


Figure 2a

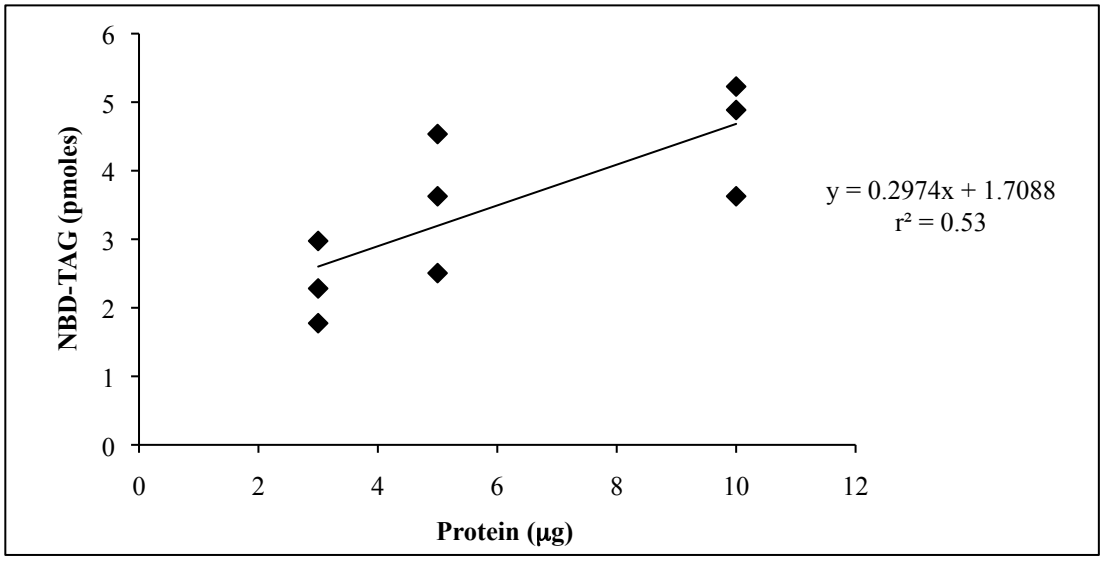


Figure 2b

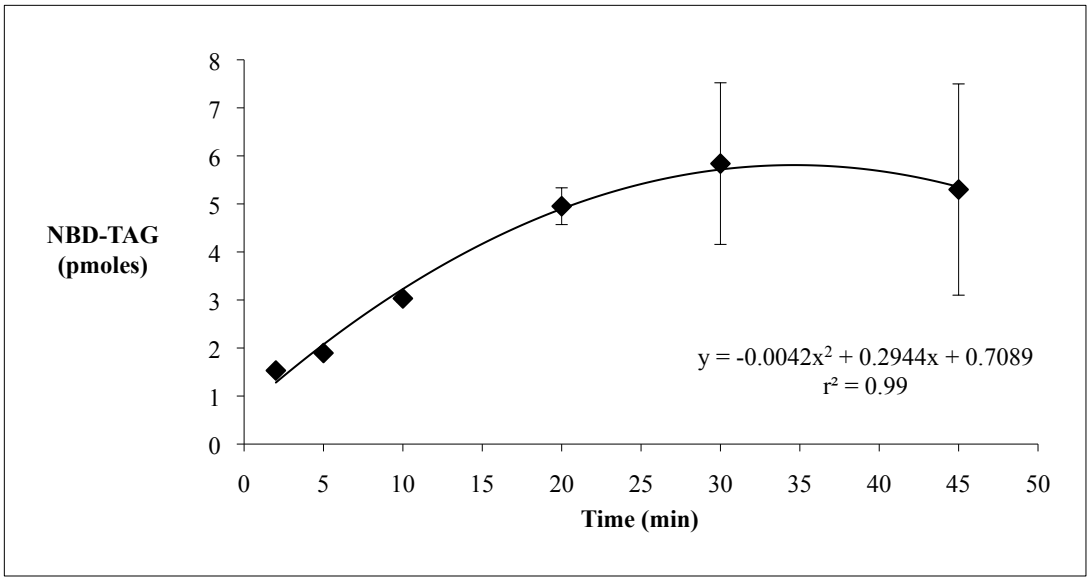

NBD-TAG
(pmoles)
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
40
45
50
Time (min)
y = -0.0042x² + 0.2944x + 0.7089
r² = 0.99

Figure 3

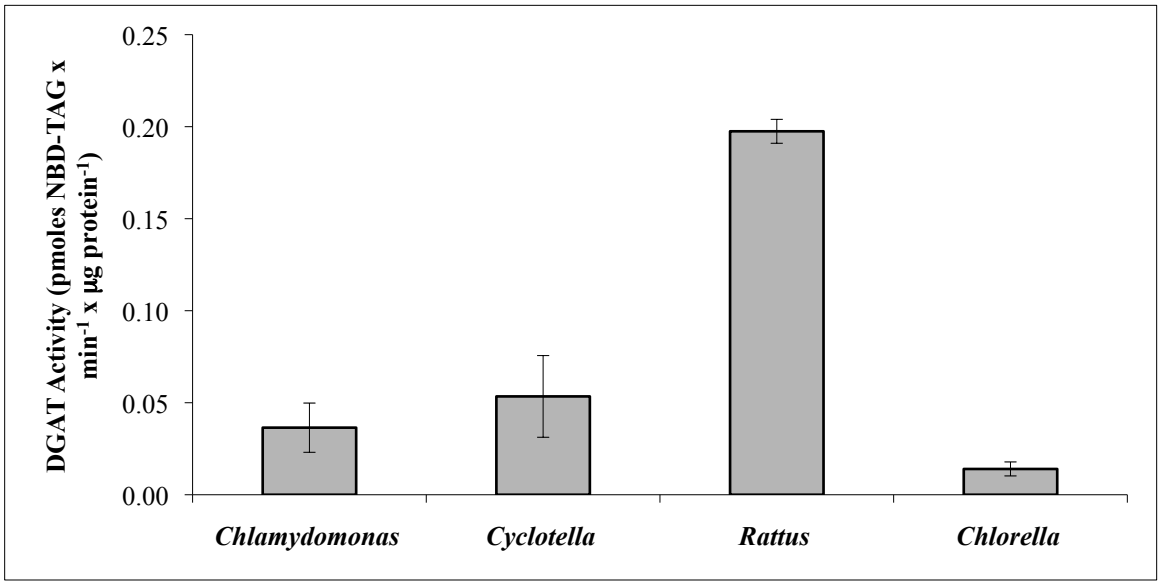

DGAT Activity (pmoles NBD-TAG x min-1 x µg protein-1)
0.25
0.20
0.15
0.10
0.05
0.00
Chlamydomonas
Cyclotella
Rattus
Chlorella

Figure 4

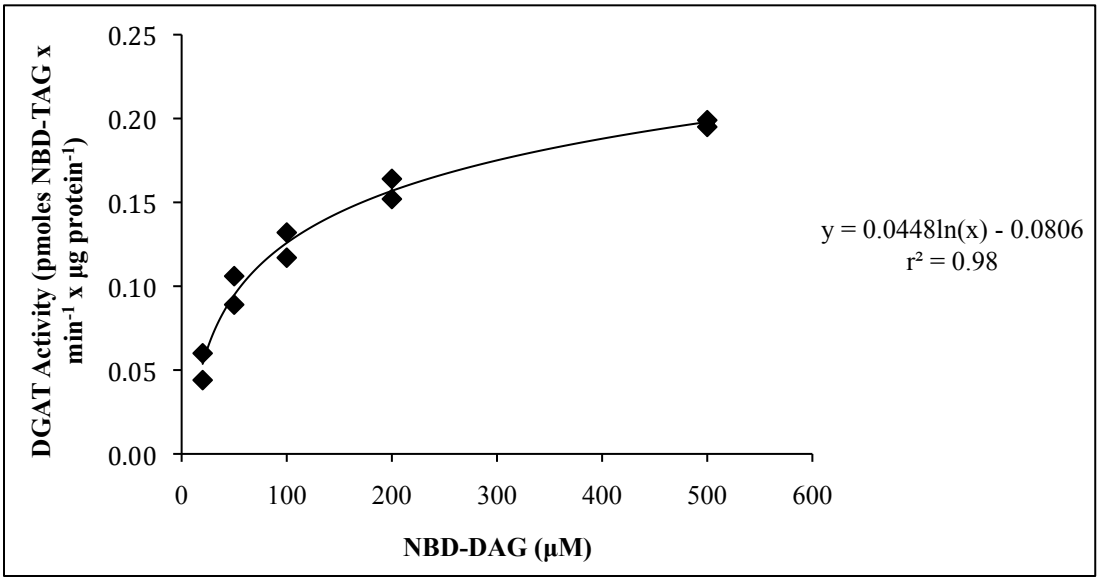

DGAT Activity (pmoles NBD-TAG x min-1 x µg protein-1)
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
NBD-DAG (µM)
y = 0.0448ln(x) - 0.0806
r² = 0.98

Figure 5

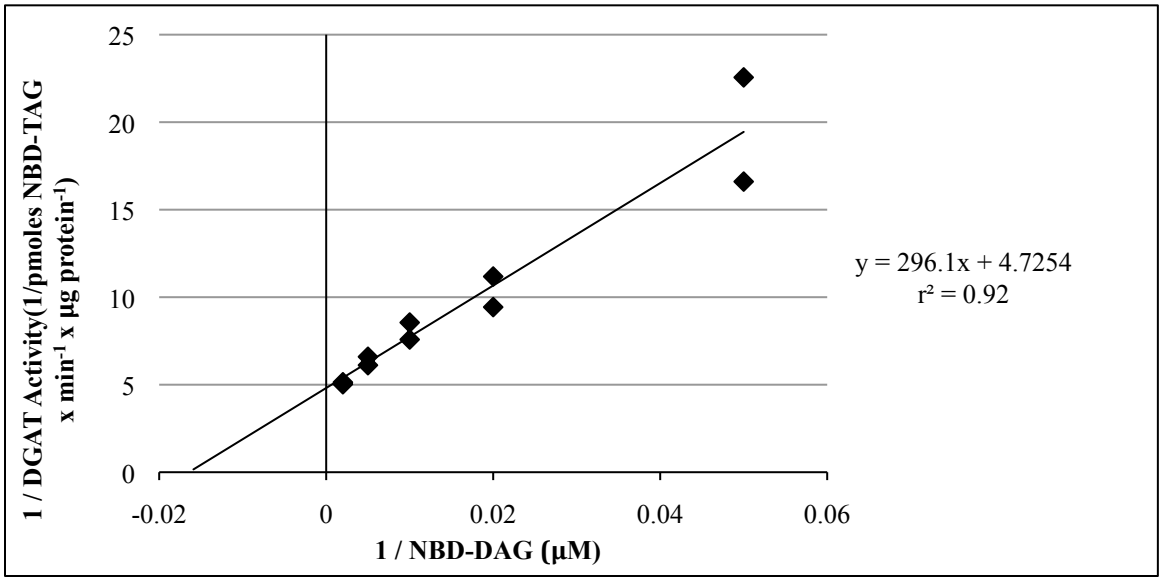

25
20
15
10
5
0
-0.02
0
0.02
0.04
0.06
1 / DGAT Activity(1/pmoles NBD-TAG x min-1 x µg protein-1)
1 / NBD-DAG (µM)
y = 296.1x + 4.7254
r² = 0.92

Figure 6

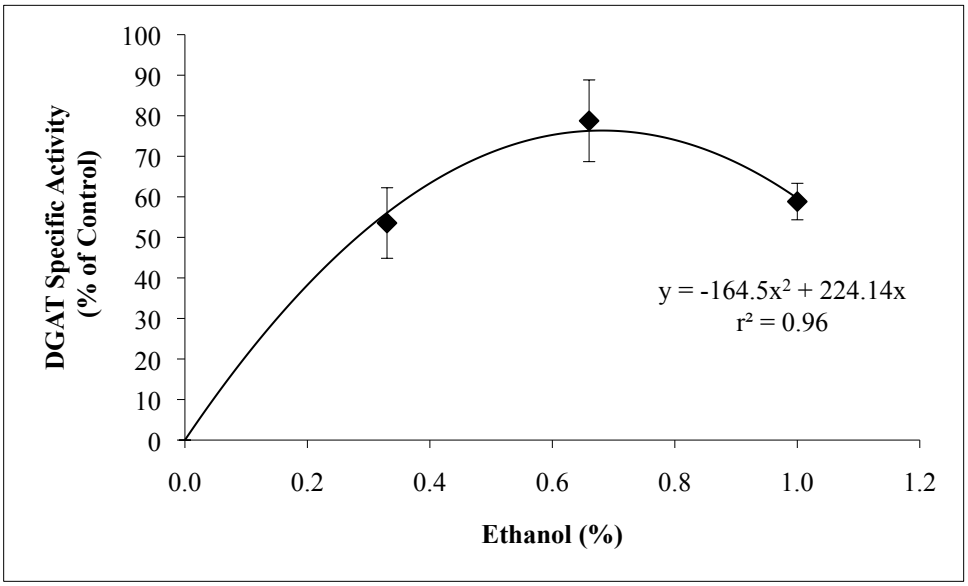

DGAT Specific Activity
(% of Control)
100
90
80
70
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Ethanol (%)
y = -164.5x² + 224.14x
r² = 0.96

Figure 7

BIOGRAPHICAL SKETCH

Matthew Conway Sanderson was born in Asheville, NC, on May 12, 1984. He attended high school at Tuscola High School in Waynesville, NC and graduated in June of 2002. The following autumn, he entered Appalachian State University to study Chemistry, and in May 2007, he was awarded the Bachelor of Science degree. The ensuing autumn, he entered graduate school in the Appalachian State University Biology Department and began study toward a Master of Science degree with a concentration in cellular and molecular biology. In August of 2010, the M.S. was awarded. Mr. Sanderson has aspirations of attending medical school in the near future.

Mr. Sanderson is currently living in Wilmington, NC. His parents are Mr. John Clarke Sanderson of Waynesville, NC and Mrs. Kathryn Tremble Murphree of Athens, OH.