

**STIMULATION OF MICROSOMAL DIACYLGLYCEROL ACYLTRANSFERASE
ACTIVITY FROM MICROSPORE-DERIVED CELL SUSPENSION CULTURES
OF OILSEED RAPE**

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ABSTRACT

Several factors including an unidentified endogenous substance were found to stimulate microsomal diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) from a microspore-derived cell suspension culture of oilseed rape (*Brassica napus* L. cv Jet Neuf). Mg^{2+} salts were found to stimulate microsomal DGAT 14 to 23-fold. ATP and CoA were also found to stimulate the enzyme 2.4 and 12 fold respectively, although the effects were decreased in the presence of high Mg^{2+} concentrations. While microsomal DGAT activity was only slightly increased by the concentration of exogenous diacylglycerol in the reaction mixture it was increased substantially by the addition of exogenous phosphatidate. Other phospholipids tested were not found to have this stimulatory effect. During attempts to investigate possible covalent modification of the enzyme, the soluble fraction obtained from cell suspension homogenate was found to contain a small metastable organic molecule(s) which stimulated DGAT activity. Stimulation of microsomal DGAT by this factor was concentration dependent but not dependent on preincubation time.

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LIST OF ABBREVIATIONS

ACCase	acetyl-CoA carboxylase
ACP	acyl carrier protein
ATP	adenosine triphosphate
BSA	bovine serum albumin
CaM-PKII	calcium/calmodulin dependent protein kinase II
cDNA	complementary deoxyribonucleic acid
CDP	cytidine diphosphate
CF	cytosolic fraction
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CL	cardiolipin
CMC	critical micelle concentration
CoA	coenzyme A
CPT	choline phosphotransferase
DAG	diacylglycerol
dcdAMP	dibutyryl cyclic adenosine monophosphate
DGAT	diacylglycerol acyltransferase
DGDG	digalactosyldiacylglycerol
DGTA	diacylglycerol transacylase
DTT	dithiothreitol
EDTA	ethyldiamine tetraacetic acid

ER	endoplasmic reticulum
FA	fatty acid
G-3-P	<i>sn</i> -glycerol-3-phosphate
GPAT	<i>sn</i> -glycerol-3-phosphate acyltransferase
Hepes	<i>N</i> -[2-hydroxyethyl]piperazine- <i>N'</i> -[2-ethanesulfonic acid]
HPLC	high performance liquid chromatography
LPA	lysophosphatidate
LPAAT	lysophosphatidate acyltransferase
LPC	lysophosphatidylcholine
MD	microspore-derived
MEGA-8	<i>N</i> -octanoylmethylglucamide
MGDG	monogalactosyldiacylglycerol
Mops	3-[<i>N</i> -morpholino]propanesulfonic acid
MW	molecular weight
NAD⁺	nicotinamide adenine dinucleotide
NEM	<i>N</i> -ethylmaleimide
PA	phosphatidate
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol

PI	phosphatidylinositol
PKC	protein kinase C
PS	phosphatidylserine
SDS	sodium dodecylsulphate
SQDG	sulphoquinovosyldiacylglycerol
TAG	triacylglycerol
TLC	thin layer chromatography
Tween 20	polyoxyethylenesorbitan monolaurate
UDP	uridine diphosphate
VLDL	very low density lipoprotein

INTRODUCTION

The production of seed oils is an important part of Alberta's economy. About 36% of Canada's total canola production occurs within the province of Alberta (Alberta Agriculture Food and Rural Development, 1998). In total, Canada supplies about 18% of the world's demand for oilseed rape products (Alberta Agriculture Food and Rural Development, 1998). Oil accumulated in both fruits and seeds can be used for a wide range of applications from cooking and salad oils to commercial lubricants and paint products. Rapeseed oil is used mainly for industrial applications due to its high levels of erucic acid. Canola, which is low in erucic acid and glucosinolates, was developed in Canada from rapeseed as a more nutritionally suitable oil for human consumption (Canola Council of Canada, 1998). Low levels of saturated fatty acids also contribute to the nutritional value of canola oil. Approximately 40% of the seed is comprised of oil which can be extracted quite economically (Canola Council of Canada, 1998). The meal produced during the extraction of canola oil from the seed is also used as a highly nutritional animal feed (Canola Council of Canada, 1998).

The main form of storage oil in the seed is triacylglycerol (TAG) which is comprised of a glycerol backbone to which three fatty acyl chains are attached via ester linkages. TAG is produced by a series of enzymatically catalyzed reactions known as the Kennedy pathway (Kennedy, 1961; Stymne and Stobart, 1987). These reactions involve the sequential acylation of glycerol-3-phosphate (G-3-P) to

produce TAG. The enzymes responsible for catalyzing the reactions in this pathway are found in the membrane of the endoplasmic reticulum (ER). In order to study these enzymes, researchers must first obtain tissue that is actively accumulating TAG. This can be difficult since seeds accumulate oil in a discontinuous manner during development, and seeds found on the same plant are not necessarily at the same stage of development. The use of oil-forming cell cultures ensures a relatively uniform source of biological material throughout the year (Rutter *et al.*, 1997). A cell suspension culture of *Brassica napus* L. cv Jet Neuf was developed from immature pollen grains in 1983 (Simmonds *et al.*, 1991). The cell suspension culture was found to produce TAG and has been used in biochemical and molecular genetic studies of TAG accumulation (Weselake *et al.*, 1993; Little *et al.*, 1994; Weselake *et al.*, 1997; Weselake *et al.*, 1998).

Differential centrifugation is routinely used to obtain subcellular fractions enriched in ER and associated TAG biosynthetic enzymes. Due to the membrane bound nature of these enzymes, they have proven to be difficult to solubilize and purify, which, in turn, has hampered attempts to use molecular genetic approaches to improve TAG content and composition. Obtaining a detailed understanding of the mechanisms of action and regulation of these enzymes may lead to the development of biotechnological strategies for altering seed oil content and/or fatty acid composition. For example, Calgene has developed a strategy for obtaining *B. napus* L. seed oil containing over 40 % laurate (12:0). Seeds of the California bay tree, which accumulate laurate (12:0), were used to purify a 12:0-acyl carrier protein

thioesterase. The amino acid sequence allowed the isolation of a cDNA clone which was expressed first in *Arabidopsis thaliana* and subsequently in *B. napus* L. (Voelker *et al.*, 1992). The resulting increase in laurate produced an oil which will provide an economic alternative to imported coconut and palm kernel oils for use in the soap and detergent industry (Ohlrogge, 1994).

Diacylglycerol acyltransferase (DGAT)(EC 2.3.1.20) catalyzes the final step in TAG formation and may play a rate limiting role in TAG formation in both plant (Ichiara *et al.*, 1988; Perry and Harwood, 1993; Kocsis and Weselake, 1996) and animal systems (Hillmar *et al.*, 1983; Mayorek *et al.*, 1989). The regulation of this enzyme has been studied extensively in mammalian tissues and has been found to be regulated by both allosteric modulation and covalent modification (Haagsman *et al.*, 1982; Rodriguez *et al.*, 1992). The presence of a phosphorylation / dephosphorylation mechanism for the regulation of mammalian DGAT has been reported by several research groups (Haagsman *et al.*, 1982; Soling *et al.*, 1989; Rodriguez *et al.*, 1992). In plants, the regulation of DGAT has gone largely unexamined although the effects of some compounds on its activity have been reported. The purpose of this research was to determine the effects of various factors on microsomal DGAT activity in microspore derived (MD) cell suspension cultures of *B. napus* and to investigate whether the plant enzyme was regulated by a phosphorylation/dephosphorylation mechanism. In doing so, an endogenous stimulator of the enzyme was discovered.

LITERATURE REVIEW

Triacylglycerols

There are several hundred species of oilseed plants throughout the world and oils extracted from many of these plants can be used for human consumption, animal feeds and industrial applications. Paints, lubricants and cosmetics are some examples of the industrial uses of these seed oils. The main species that are currently being used commercially are soybean, cotton, sunflower, palm, groundnut (peanut), coconut and rapeseed (Ohlrogge and Browse, 1995). These plants store oil within the seed itself while others, such as avocado, olive and palm store oil in the mesocarp surrounding the seed. TAG is the major form of lipid stored in these oilseeds and can represent as much as 80% of the dry weight of the seeds (Gurr and Harwood, 1991). This storage lipid serves as the major carbon source during germination and seedling growth (Imeson *et al.*, 1993) and is analogous to the use of starch by cereals as a source of fuel for seed germination and seedling growth (Gurr, 1980). TAG is stored within the mature seed in densely packed, more or less spherically-shaped lipid bodies. A phospholipid monolayer containing proteins called oleosins surrounds the lipid bodies (Huang, 1992; Murphy *et al.*, 1993).

Each TAG molecule is comprised of a glycerol backbone to which fatty acyl moieties are attached via ester linkages to each of the three hydroxyl groups (Gurr, 1980). The three carbon atoms of the glycerol backbone are distinguishable from

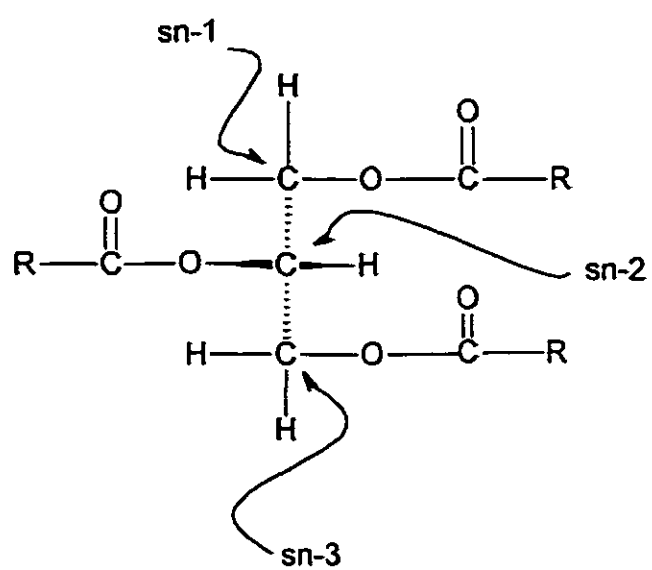
each other because the glycerol molecule lacks rotational symmetry. The carbon atoms are therefore classified as *sn*-1, *sn*-2 and *sn*-3 (stereochemical numbering) as shown in Fig. 1 (Stymne and Stobart, 1987). Hundreds of different naturally occurring fatty acids are found in plant tissues (Harwood, 1980). While many of these may constitute a major proportion of fatty acid in an individual species, there are five fatty acids that predominate in seed TAGs. These are: palmitic (16:0), stearic (18:0), oleic (*cis*-9-18:1), linoleic (*cis,cis*-9,12-18:2) and alpha-linolenic (*cis,cis,cis*-9,12,15-18:3). The *sn*-2 position is usually esterified with 18 carbon unsaturated acyl groups while the *sn*-1 and *sn*-3 positions are usually esterified with unsaturated fatty acids and/or fatty acids of other chain lengths (Gurr, 1980; Frentzen, 1993). Many species of oilseed plants accumulate TAGs that contain unusual fatty acids which do not occur in the membrane lipids of the plant. Castor bean (*Ricinus communis*), for example, accumulates oil containing ricinoleic acid. The targeting of these unusual fatty acids to TAG is thought to be the result of the spatial separation of two diacylglycerol (DAG) pools rather than being attributable to the specificity of TAG biosynthetic enzymes (Vogel and Browse, 1996).

Fatty Acid Biosynthesis

The *de novo* synthesis of fatty acids in plant cells is localized almost exclusively in the plastids (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). The acetyl-coenzyme A (acetyl-CoA) pool present in the plastids of plant cells provides all of the carbon atoms used in the production of fatty acids.

6

Figure 1. Stereochemical numbering of triacylglycerol. R = fatty acid acyl chains.



Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) catalyzes the first step of fatty acid biosynthesis in which malonyl-CoA is formed from acetyl-CoA and CO₂. In animals, yeast, *E. coli* and plants, the rate of fatty acid biosynthesis has been shown to be regulated, at least in part, by ACCase (Murphy *et al.*, 1993; Ohlrogge and Browse, 1995). In plants, there are two forms of ACCase. One form is a multifunctional enzyme found outside of the plastids and is similar to that found in the cytosol of animals and yeast. It is speculated that this form of ACCase is used in the elongation of oleic acid (C18:1) to erucic acid (C22:1) which occurs primarily in the cytosol. The second form is a multisubunit complex located in the plastids and is similar to that found in prokaryotes. This form is thought to be essential for fatty acid biosynthesis which occurs in the plastids (Ohlrogge and Browse, 1995).

Fatty acids in cells are almost never found in the form of free fatty acids (Ohlrogge and Browse, 1995). Instead, their carboxyl group is esterified or otherwise modified by binding to a protein or other ligand. Malonyl-CoA produced by ACCase is the predominant carbon donor for fatty acid synthesis and is transferred from CoA to an acyl carrier protein (ACP) prior to proceeding through the pathway. From this point on the fatty acid precursor is associated with ACP until the 16- or 18-carbon product is either transferred to a glycerolipid or exported from the plastid (Ohlrogge, 1994). Malonyl-ACP enters into a series of condensation reactions with acyl-ACP (or acetyl-CoA acceptors) resulting in the formation of a C-C bond and the release of CO₂ (Harwood, 1988). In each cycle of elongation, incorporation of a 2-carbon unit from malonyl-ACP also requires two reduction

reactions and one dehydration reaction. The initial condensation reaction with acetyl-CoA is catalyzed by beta-ketoacyl-synthetase III while beta-ketoacyl-synthetase I performs condensation reactions leading from C4 to C16 and beta-ketoacyl-synthetase II performs the last condensation reaction resulting in an 18-carbon chain (Ohlrogge and Browse, 1995). The initial product of each condensation reaction is a 3-ketoacyl-ACP which is reduced at the carbonyl group by 3-ketoacyl-ACP reductase and then dehydrated by hydroxyacyl-ACP dehydratase before being reduced by enoyl-ACP reductase to form a saturated fatty acyl-ACP product which is then further condensed in the next cycle (Browse and Somerville, 1991).

The fatty acid biosynthetic pathway produces saturated fatty acids which must then undergo desaturation because, in most plant tissues, over 75% of fatty acids are unsaturated. The first double bond is introduced by the catalytic action of the soluble enzyme stearoyl-ACP desaturase located within the plastid. All other known desaturases in plants are integral membrane proteins making this soluble desaturase unique (Ohlrogge and Browse, 1995). The elongation of fatty acids in the plastid is terminated by removal of the acyl group from ACP (Murphy *et al.*, 1993). In most cases, an acyl-ACP thioesterase catalyzes the hydrolysis of acyl-ACP releasing free fatty acid which is then able to leave the plastid either by transport or diffusion across the membrane (Ohlrogge and Browse, 1995). This thioesterase activity is presumed to be selective for 16:0 and 18:1 acyl groups (Ohlrogge *et al.*, 1978; Browse and Somerville, 1991). On the outer membrane

surface of the plastid, an acyl-CoA synthetase is thought to catalyze the assembly of acyl-CoA thioesters that diffuse to the endoplasmic reticulum (ER) (Ohlrogge and Browse, 1995) where the molecules serve as substrates for membrane and TAG bioassembly (Stymne and Stobart, 1987). Elongation of fatty acids to chain lengths of more than 18 carbons occurs subsequently in the cytosol (Somerville and Browse, 1991).

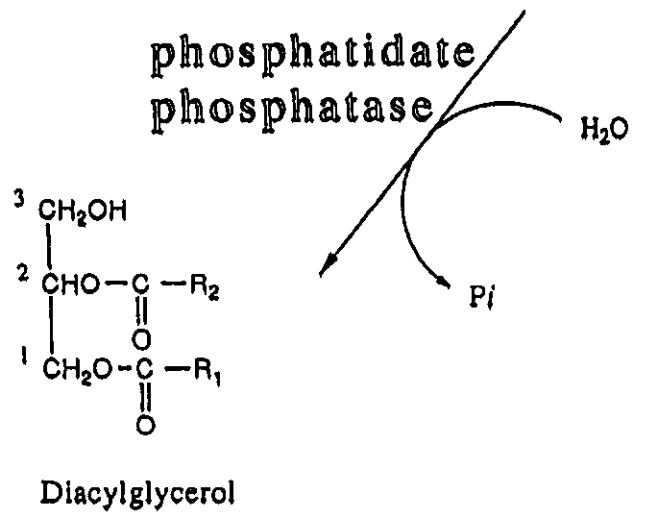
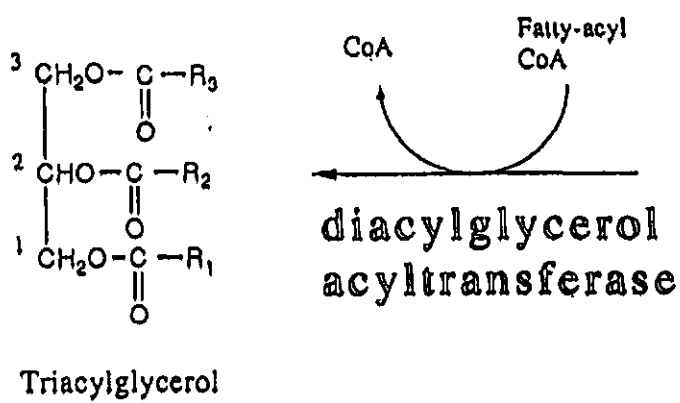
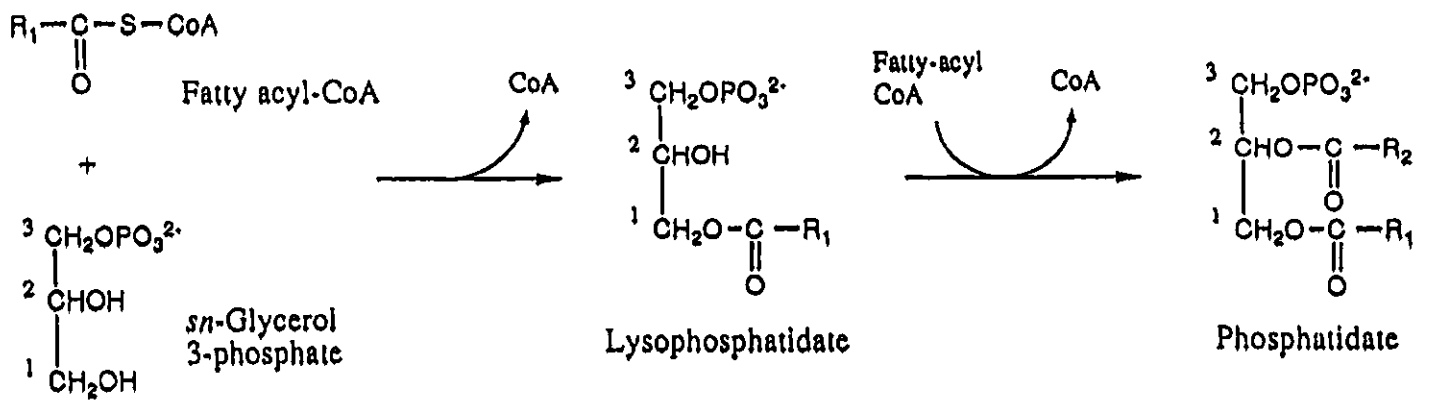
In plants, there are two mechanisms for the production of very long chain fatty acids. The first process is quite well known and involves an acyl-CoA elongase which utilizes acyl-CoA as a primer. This elongation process begins with the condensation of acyl-CoA to malonyl-CoA and, through a series of reduction and dehydration reactions, results in the formation of the elongated acyl-CoA (Lessire *et al.*, 1998). In a recent study on fatty acid elongation in leek and *Brassica napus*, however, Hlousek-Radojicic *et al.* (1998) found that acyl-CoA synthetase may not play a direct role in fatty acid elongation. Their results indicated that phosphatidylcholine (PC) or another glycerolipid was a more likely source of the elongation primers than acyl-CoA. The elongation mechanism for this second elongase still remains unknown but the process should not be overlooked (Lessire *et al.*, 1998).

Triacylglycerol Biosynthesis

In all plant tissues, the major glycerolipids are first synthesized using only these 16:0 and 18:1 acyl groups, which are subsequently desaturated by membrane

bound desaturases of the plastid and ER (Browse and Somerville, 1991). The fatty acids produced in the plastid are transported to the ER where the bulk of TAG is synthesized in the plant. The reactions of the Kennedy pathway (Fig. 2) lead to TAG formation and are carried out by the microsomal membranes (Kennedy, 1957; Kennedy, 1961; Stymne and Stobart, 1987). The reduction of dihydroxyacetone phosphate in the cytosol is considered to be the source of *sn*-glycerol-3-phosphate (G-3-P) from which the glycerol backbone of TAG is formed. In the pathway, G-3-P is first acylated at the *sn*-1 position through the action of *sn*-glycerophosphate acyltransferase (GPAT, EC 2.3.1.15) to yield lyso-phosphatidate (LPA). A second enzyme, *sn*-1-acyl-glycerol-3-phosphate acyltransferase (LPAAT, EC 2.3.1.51) then catalyzes the acylation at the *sn*-2 position to form phosphatidate (PA). A third enzyme, PA phosphatase (EC 3.1.3.4) catalyzes the cleavage of the phosphate group resulting in the production of DAG. The DAG can then follow one of two routes, either being channeled towards phosphatidylcholine (PC) or undergoing acylation at position *sn*-3 to yield TAG. The synthesis of PC from DAG by choline phosphotransferase (CPT) is reversible, so that PC is a direct precursor of the highly unsaturated forms of DAG used for TAG production in many oilseeds. PC is the major substrate for 18:1 and 18:2 desaturation by microsomal enzymes and exchange of fatty acids at position *sn*-2 of PC with those from the acyl-CoA pool provides input of these fatty acids via PA as well. The pool of DAG used for TAG synthesis is therefore fed by both PA phosphatase and CPT (Browse and Somerville, 1991).

Figure 2. Schematic of TAG biosynthetic pathway.



Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the transfer of a fatty acid from acyl-CoA to the *sn*-3 position of DAG and is dedicated to TAG biosynthesis (Stobart and Stymne, 1990). In leaf tissue, DGAT activity is associated with plastidial membranes, but in oil-storing tissue there is no indication that plastidial DGAT is involved in the accumulation of TAG (Frentzen, 1993). While it has been suggested that, in developing oilseeds, PA phosphatase is the rate limiting enzyme in TAG production (Frentzen, 1993), in seeds of certain plant species, including oilseed rape, there are indications that DGAT rather than PA phosphatase catalyzes the rate-limiting step (Kocsis and Weselake, 1996). In contrast, diacylglycerol transacylase (DGTA) also catalyzes the formation of TAG through the acyl-CoA independent transfer of an acyl moiety from one DAG molecule to another (Stobart *et al.*, 1997). This enzyme has been shown to be present in the microsomal fraction of both mammalian cells (Lehner and Kuksis, 1996) and developing oilseeds (Stobart *et al.*, 1997). In microsomes from developing safflower seeds, DGTA activity was found to be 25 % that of DGAT indicating that DGTA may contribute substantially to the total amount of TAG produced (Stobart *et al.*, 1997). The activity of this enzyme may allow for the introduction of more desaturation and the enrichment of TAG with polyunsaturated C18 acyl moieties.

The distribution of various fatty acyl moieties in TAG is non-random and reflects the selectivity of the various acyltransferase enzymes. The fatty acid composition of plant storage TAG is generally species specific and in crop species may even be cultivar specific. The control of acyl composition is genetically controlled, although temperature during seed maturation can have a modifying

influence (Roughan and Slack, 1982). When developing seeds are exposed to elevated temperatures they accumulate an increased amount of 18:1 acyl groups whereas lower temperatures cause an increase in 18:2 and 18:3 acyl groups (Raison, 1980).

The majority of TAG within the seed is stored within an organelle known as the oil body. TAG is found within the lumen of the oil body and is encapsulated by phospholipid and oleosin. Oleosins, which are ~19 kDa proteins which associate with oil bodies, are thought to play a role in the stabilization of oil bodies during seed maturation and the mobilization of oil bodies after seed germination (Murphy, 1993). It was initially thought that these oil bodies were small particles which bud off from the endoplasmic reticulum, but it has since been proposed that they are formed from a membranous matrix of lipid and protein contained within the cytoplasm (Murphy, 1993). This second theory of oil body formation proposes that TAG synthesis occurs at the surface of the ER and results in the release of oil droplets which are then surrounded by oleosins and phospholipids (Napier *et al.*, 1996). A similar type of particle, known as a lipid-protein particle, is present in carnation petals and is also thought to be formed by "blebbing" from membranes (Thompson *et al.*, 1997). These lipid-protein particles, however, differ from lipid bodies in their lipid composition, containing lower levels of TAG and higher levels of free fatty acids and steryl and wax esters. Cytosolic lipid-protein particles contain a protein similar in size to oleosins and are thought to play a role in the removal of lipids from the membrane to prevent destabilization of the bilayer (Thompson *et al.*, 1997).

Many of the TAG biosynthetic enzymes located in the ER are highly hydrophobic and tend to lose activity rapidly upon solubilization and during purification attempts (Stobart and Stymne, 1990). In order to gain more insight into the regulation of TAG biosynthesis, however, studies with purified enzymes and their encoding genes are required. GPAT has been solubilized and partially purified from avocado mesocarp (Eccleston and Harwood, 1995), but has not yet been purified to homogeneity. cDNAs for GPAT of chloroplasts have been cloned from several plants and the gene itself has been cloned from *Arabidopsis thaliana* (Murata and Tasaka, 1997). The gene has allowed the modification of tobacco to increase its ability to tolerate chilling temperatures. LPAAT has been solubilized from immature coconut endosperm (Davies *et al.*, 1995) and a cDNA encoding LPAAT has been isolated (Knutson *et al.*, 1995). Lassner *et al.* (1995) have isolated a cDNA from meadowfoam that encoded LPAAT and were able to express the enzyme in maturing oilseed embryos. DGAT has not been purified to homogeneity from any source but there has been a report on the identification of a mouse cDNA encoding DGAT (Cases *et al.*, 1998). DGTA has been purified from rat intestinal villus cells by Lehner and Kuksis (1993, 1996). Understanding the regulatory mechanism of TAG biosynthesis will provide a foundation for the development of methods to increase seed lipid content and to improve the fatty acid composition of seed oils.

The fatty acyl-CoAs used by the various acyltransferase enzymes as sources for fatty acids can be broken down into fatty acid and CoA by thioesterases. It has been suggested that there may be sufficient thioesterase activity present in

microsomal preparations to interfere with assays of acyltransferase enzymes (Taylor *et al.*, 1990). Acyl-CoA synthetase catalyzes the reverse reaction and forms acyl-CoA from free fatty acid and CoA. Within the cell, these antagonistic enzymes may provide a possible mechanism for regulation of the acyl-CoA pool.

While TAG is the storage fuel for cells, other glycerolipids play a crucial role as membrane lipids (Harwood, 1989). These glycerolipids are formed via the first two reactions of the Kennedy pathway with the acylation of G-3-P at both the *sn*-1 and *sn*-2 positions to produce PA. This phospholipid acts as an intermediate for phospholipase A which catalyzes a hydrolysis reaction to produce G-3-P and for PA phosphatase which catalyzes a dephosphorylation reaction to produce DAG (Harwood, 1989). DAG is required for the production of both storage lipid in the form of TAG and important membrane phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). PA is also a key intermediate which can be used to produce both phosphatidylglycerol (PG) and phosphatidylinositol (PI) via cytidine diphosphate (CDP)-diacylglycerol (Frentzen, 1993). By distinguishing glycerolipids based on the polar headgroup, they can be divided into two major classes, phosphoglycerols and glycosylglycerols (Harwood, 1989). Alternatively, glycerolipids can be divided into groups according to the acyl groups they contain. Glycerolipids containing either C₁₈ or C₁₆ fatty acids at the *sn*-1 position and C₁₈ fatty acids at the *sn*-2 position are termed "eukaryotic". This pattern is found in glycerolipids both inside and outside the plastids (Harwood, 1989; Frentzen, 1993). A second pattern of acyl group distribution, called "prokaryotic", is characterized by C₁₆ fatty acids at the *sn*-2 position with the *sn*-1 position being comprised of mainly

C₁₈ fatty acids (Frentzen, 1993).

The biosynthesis of glycosylglycerols within the plastids yields three major forms of lipid: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulphoquinovosyldiacylglycerol (SQDG). MGDG is formed by the transfer of galactose from uridine diphosphate (UDP)-galactose to diacylglycerol and DGDG can be synthesized either by the transfer of a second galactose from UDP-galactose to MGDG or transfer between two MGDG molecules (Harwood, 1989). The mechanism for the formation of SQDG has been subject to much debate, but the enzymes responsible for the synthesis of sulpholipid synthesis are thought to be present in the chloroplasts (Gurr and Harwood, 1991). The glycerol backbone in both MGDG and DGDG typically contains polyunsaturated fatty acids at the *sn*-1 and *sn*-2 positions (Harwood, 1980). Both eukaryotic and prokaryotic patterns of acyl composition have been shown to be present in MGDG (Harwood, 1989).

Properties of Mammalian DGAT

Studies with membrane fractions have indicated that mammalian DGAT is regulated by both allosteric modulation and covalent modification (Haagsman *et al.*, 1981; Rodriguez *et al.*, 1992). DGAT activity is located primarily in the microsomal fraction of the cell and has been shown to occur assymmetrically on the cytoplasmic surface of the ER (Coleman and Bell, 1978). Many thiol-specific reagents (eg. NEM) have been shown to inhibit mammalian DGAT during skeletal myogenesis which indicates that there may be a thiol group near the active site of the enzyme (Sauro

and Strickland, 1990).

In addition to being thoroughly characterized in membrane-bound form, mammalian DGAT has been solubilized and partially purified. Hosaka *et al.* (1977) partially purified DGAT from rat liver microsomes by gel filtration chromatography and sucrose density gradient centrifugation following solubilization with Triton X-100. Polokoff and Bell (1980) also partially purified DGAT from rat liver microsomes by Sepharose 4B chromatography and sucrose density gradient centrifugation following solubilization with cholate. This partially purified DGAT was shown to be stimulated by the addition of phospholipids and Mg^{2+} . Ozasa *et al.* (1989) reported that DGAT in rat liver microsomes was a protein with a functional size of approximately 72 kDa. This was determined by the technique of radiation inactivation in which the rate of loss of enzyme activity is dependent on both the duration of irradiation and the mass of the molecules involved. The only report of DGAT from mammalian tissues being purified to near homogeneity was by Andersson *et al.* (1994). They reported partially purifying two proteins which exhibited DGAT activity from rat liver. The proteins were reported to be 60 and 77 kDa in size and each was purified to near homogeneity. Cases *et al.* (1998) reported the identification of a mouse cDNA encoding DGAT. When the cDNA was expressed in H5 insect cells an approximately 47 kDa protein was found at high levels in the membrane fraction and the membrane fraction was found to contain more than 5-fold higher DGAT activity than wild-type cells.

DGAT has been implicated as a rate-limiting step in the formation of TAG in rat liver cells (Mayorek *et al.*, 1989). The molecule 2-bromooctanoate has been

shown to inhibit TAG synthesis in cultured rat hepatocytes with a concomitant build-up of DAG. Mayorek *et al.* (1989) found that 2-bromooctanoate was acting as a competitive inhibitor of DGAT with the inhibition being ascribed to its thioesterification by medium chain fatty acyl-CoA synthetase. By analyzing the overall synthetic flux with respect to TAG production in cultured rat hepatocytes both in the absence and presence of 2-bromooctanoate, Mayorek *et al.* (1989) demonstrated that under those conditions DGAT was rate-limiting in the formation of TAG.

Incubation with short chain FAs such as palmitic acid has been shown to stimulate DGAT in rat liver cells by up to 354%. This increase in DGAT activity occurred along with an increased secretion of very low density lipoproteins (VLDL) and persisted during cell homogenization and preparation of microsomes (Haagsman *et al.*, 1981). Hillmar *et al.* (1983) found that various long-chain fatty acids increased DGAT activity to between 114% and 191% of control activities after 72-h incubation with primary cultures of rat hepatocytes. This corresponded with cellular TAG levels suggesting that DGAT played a regulatory role in TAG biosynthesis. The addition of the various fatty acids did not influence the subcellular distribution of the enzyme which was concentrated in the microsomal fraction during differential centrifugation.

Monochain phospholipids have also been shown to activate DGAT in rat liver microsomes (Parthasarathy *et al.*, 1981). DGAT activity in rat liver microsomes was found to be stimulated by 1-acyl-*sn*-glycero-3-phosphocholine at low concentrations, but was inhibited above 0.2 mM. The acylation of DAG was found to be optimal at

75 μ M lysophosphatidylcholine (LPC), resulting in more than 2-fold activation of the enzyme, but DGAT activity disappeared above 0.5 mM LPC. The data suggested a direct effect of the lysophospholipids on the pathway (Parthasarathy *et al.*, 1981). Stals *et al.* (1994) have shown that saturating levels of DAG are present in microsomes and that the rate of TAG synthesis depends on fatty acid supply and the affinity of DGAT for acyl-CoAs (Stals *et al.*, 1994).

Haagsman *et al.* (1981) found that DGAT activity in isolated rat hepatocytes decreased by 53% after incubation with glucagon in the presence of F⁻ (50 mM) and EDTA (2.5 mM) whereas the activity of cholinephosphotransferase (CPT, EC 2.7.8.3) was not inhibited. Because CPT also uses DAG as a substrate, this result implies that the regulation of DGAT might be independent of phosphatidylcholine synthesis. The investigators concluded that DGAT is regulated by short-term hormonal control which may occur via a phosphorylation-dephosphorylation mechanism. Phosphorylation/dephosphorylation of proteins is a universal signal-transduction mechanism in living systems which involves the reversible post-translational covalent modification of functional proteins (Yupsamis *et al.*, 1993). Two groups of enzymes are responsible for this mechanism: protein kinases and protein phosphatases. Protein kinases are responsible for catalyzing the transfer of a phosphoryl group from ATP to a protein whereas protein phosphatases catalyze the cleavage of the phosphoryl group from the protein (Yupsamis *et al.*, 1993).

The first report that mammalian DGAT activity was affected by a factor in the 105 000 x g supernatant was by Manley *et al.* (1974). The investigators found that DGAT activity in their preparations required the presence of the 105,000 x g

supernatant together with ATP and $MgCl_2$, and that the responsible factor was a soluble protein. Haagsman *et al.* (1982) reported that microsomal DGAT from rat liver can be inactivated and reactivated *in vitro* by a phosphorylation-dephosphorylation mechanism (Haagsman *et al.*, 1982). Unlike Manley *et al.* (1974), they found that the reversible inactivation of DGAT required the 105,000 xg supernatant, ATP and $MgCl_2$ to be present and that the reactivation would occur in the presence of the 105,000 xg supernatant if ATP and $MgCl_2$ were absent. The rate of inactivation was found to be lower in microsomes isolated in the presence of F^- (50 mM) than in microsomes isolated in the presence of Cl^- (50 mM), but both microsomes were affected in the same manner by treatment with Mg^{2+} , ATP and the 105,000 xg supernatant. Reactivation of the enzyme was found to be inhibited by F^- (50 mM). The reactivating factor was also found to be heat-labile, non-dialyzable and trypsin sensitive (Manley *et al.*, 1974; Haagsman *et al.*, 1982).

Rodriguez *et al.* (1992) reported that the inactivation of rat adipose DGAT by phosphorylation is not catalyzed by either PKC or cAMP-protein kinase but can be reversed by incubation with partially purified protein phosphatase from rat liver. They also found that the activity was inhibited by incubation with ATP and cytosol. DGAT from rat adipose tissue has since been shown to be regulated by a protein tyrosine kinase with an apparent molecular weight of 68 kDa which is also capable of inactivating GPAT in the presence of ATP (Lau and Rodriguez, 1996). In guinea pig parotid gland microsomes, DGAT has been shown to be stimulated by both calcium/calmodulin dependent PKII (CaM-PKII) and the catalytic subunit of cAMP-dependent protein kinase (Soling *et al.*, 1989). This effect was shown to occur within

the first 60 s and to be reversible, but since the effects of the two kinases were not additive it was thought that only one phosphorylation site was responsible for the activation (Söling *et al.*, 1989b). In hamster fibroblasts, DGAT has also been shown to be activated by a cAMP-dependent phosphorylation process (Mazière *et al.*, 1986). In this system DGAT activity was increased by 2 to 4-fold in the presence of dibutyl cyclic adenosine monophosphate (dbcAMP) after either short or long term incubation. In the presence of NaF, which is a phosphoprotein phosphatase inhibitor, there was a marked increase in activity suggesting that DGAT activity was activated by a cAMP-dependent phosphorylation process (Mazière *et al.*, 1986).

Since DGAT has been implicated as a rate limiting enzyme in TAG formation, the presence of a phosphorylation/dephosphorylation mechanism for its regulation may enable mammalian cells to effectively regulate storage lipid formation. Further studies on the details of the phosphorylation of the enzyme would be greatly facilitated if purified enzyme could be obtained. DGAT has not yet been purified from any mammalian system and most studies to date have used microsomal fractions containing the enzyme.

DGAT in rat liver microsomes has also been reported to be inhibited by several small organic molecules isolated from hops, *Humulus lupulus* L. (Tabata *et al.*, 1997), and soil isolates (Tomoda *et al.*, 1995). Several chalcones (xanthohumol and xanthohumol B) were isolated and characterized from hops by Tabata *et al.* (1997). The molecules were not cytotoxic to intact Raji cells from rat liver but showed preferential inhibition of DGAT activity in the cells. Another group of small organic molecules called amidepsines have also been shown to inhibit DGAT

activity in rat liver microsomes (Tomada *et al.*, 1995). Four structurally related amidepsines, one of which was identical to the known lichen tridepside 2,4-di-O-methylgyrophoric acid, have been isolated from the fermentation broth of the fungal strain FO-2942 (Tomada *et al.*, 1995). These compounds also showed specific inhibition of TAG formation in intact Raji cells, indicating that they were capable of inhibiting DGAT activity in living cells.

Characterization of Fungal DGAT

DGAT activity has been extensively characterized in the membrane fraction of the fungus *Mortierella ramanniana* var. *angulispora* by Kamisaka *et al.* (1993). The enzyme was found to have an optimum pH between 7.0 and 7.5 and the activity was blocked by thiol-reactive reagents such as NEM. The microsomal enzyme was also found to be substantially inhibited by dithiothreitol (DTT) (1mM). The detergents CHAPS (0.05%), *n*-octylglucoside (0.1%) and Tween-80 (0.1%) were found not to affect the activity of the enzyme, whereas the detergents Triton X-100 and sucrose monolaurate inhibited activity. The inhibition by Triton X-100, however, was found to be overcome by the addition of exogenous DAG. While the enzyme could be shown to be dependent on DAG in the presence of detergents, in the absence of detergents no dependence on exogenous DAG was detected. This was consistent with other studies, and Kamisaka *et al.* (1993) suggested that this may be because exogenous DAG has no access to the enzyme.

Kamisaka *et al.* (1997) recently reported the purification of DGAT from the lipid body fraction of *Mortierella ramanniana* var. *angulispora*. The investigators first

solubilized the enzyme from the lipid body fraction using 0.1% Triton X-100. The enzyme was then partially purified by chromatography on Yellow 86 agarose, Superdex-200 and Heparin-Sepharose columns. Separation via a second Superdex-200 column and a second Yellow 86 agarose column resulted in almost 5000-fold purification of the enzyme. Kamisaka *et al.* (1997) found that the enzyme had a molecular mass of 53 KDa as determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. Characterization of the purified fraction showed no GPAT, LPAT, lipase, transacylase or acyl-CoA hydrolase activities although the fraction was found to acylate 2-monolein. The purified DGAT was reported to be dependent on exogenous *sn*-1,2-diolein (200 μ M) and oleoyl-CoA(20 μ M). Anionic phospholipids were also found to activate the solubilized and purified enzymes with PA having the greatest stimulatory effect (Kamisaka and Nakahara, 1996; Kamisaka *et al.*, 1997).

Characterization of Plant DGAT and a Hypothesis Concerning Regulation of the Enzyme

Plant DGAT has been studied less extensively than mammalian DGAT and has been shown also to be an intrinsic membrane protein associated with the ER (Stymne and Stobart, 1987). DGAT has been detected in the plastid membranes of leaf tissue but there is no indication that plastidial DGAT is of any importance in oil-storing tissues. DGAT from spinach leaves has been shown to have an optimum pH of 8.0 and to be stimulated 2-fold by the addition of 20 mM Mg²⁺ or Mn²⁺. In this system, DGAT was found to be associated with the chloroplast and oil bodies

(Martin and Wilson, 1983; Martin and Wilson, 1984; Martin and Wilson, 1985). Ichihara and Noda (1982) reported, however, that in maturing seeds of safflower, DGAT activity was associated with the microsomal fraction and was suppressed by either Mg^{2+} or Ca^{2+} . The activity could be stimulated by bovine serum albumin (BSA) as well as by exogenous DAG. The investigators found that thiol-specific reagents had no effect on enzyme activity which suggested that there were no important sulphhydryl groups present which affected the activity of the enzyme. Cao and Huang (1986) found that microsomal DGAT from maturing maize scutellum had a pH optimum of 6-7 and its activity was increased by the addition of either Mg^{2+} or exogenous diolein. In maturing groundnut seeds, DGAT activity has been found to be associated mainly with the microsomal fraction and had a pH optimum of 8.0 (Sukumar and Sastry, 1987). Other studies with developing seeds, have indicated that DGAT activity is associated with both the microsomal and oil body fractions (Murphy and Mukherjee, 1987; Murphy, 1988).

A number of studies have implicated DGAT as a rate-limiting enzyme in TAG production. In one study, Ichihara *et al.* (1988) reported that DGAT activity in maturing safflower seeds was much lower than those of other enzymes in the TAG biosynthetic pathway. The lower activity of DGAT caused a build-up of DAG, the precursor to TAG. Ichihara and Noda (1982) also detected this accumulation of DAG when [^{14}C] acetate was used to follow the production of radiolabelled TAG. A similar build-up of DAG was observed by Perry and Harwood (1993) during the rapid phase of oil accumulation in maturing seeds of *B. napus* L.

The substrate specificity of DGAT has been studied extensively in several systems. In maturing safflower, the fatty acids located at the *sn*-3 position of TAG depend on the composition of the acyl-CoA pool within the cell (Ichihara and Noda, 1982; Ichihara *et al.*, 1988). DGAT was found to have no strict selectivity for acyl-CoA substrates when they were supplied as a mixture. Although DGAT did not show any strict selectivity for acyl-CoA, the relative specificity of the enzyme was found to differ among plant species (Cao and Huang, 1987; Ichihara *et al.*, 1988). Safflower (Ichihara *et al.*, 1988) and groundnut (Cao and Huang, 1986) DGAT were found to have a broad specificity whereas spinach (Martin and Wilson, 1983), maize and castor bean (Cao and Huang, 1986) DGAT exhibited specificity for 16:0-CoA and 18:2-CoA. In contrast, DGAT from spinach leaves was found to show a preference for 18:1-CoA over 16:0-CoA and 18:0-CoA (Martin and Wilson, 1983). A higher preference for 18:1-CoA over 16:0-CoA and 18:0-CoA was also found in DGAT from oil body and microsomal fractions of oil palm mesocarp (Chew and Oo, 1989; Oo and Chew, 1992). DGAT from oil body fractions in the system also required Mg^{2+} , DTT, gelatin and BSA for optimum activity. DGAT from plant species which contain unusual fatty acids such as castor bean have been shown to have a greater specificity for those acyl-CoAs than species containing the common fatty acids (Wiberg *et al.*, 1994).

The activity of DGAT in developing groundnut was shown to peak 30 days after fertilization. This peak occurred during the period of development when the groundnut seed was actively accumulating TAG (Sukumar and Sastry, 1987). This peak of DGAT activity, during the period of most active oil accumulation, has also

been observed in other oilseeds such as maize, safflower and oilseed rape (Tzen *et al.*, 1993; Weselake *et al.*, 1993). Seed storage lipids are not accumulated in a linear manner during development (Gurr, 1980). There is little or no accumulation of oil during the early stages of seed development after flowering, although an increase in membrane lipids does occur at this time. Later, there is a period of rapid oil accumulation during which most of the storage TAG is produced. When approaching maturity, the seed undergoes dehydration and little lipid is formed during this stage of development (Gurr, 1980). The study of lipid biosynthetic enzymes such as DGAT requires that the seeds be obtained during the period of rapid oil accumulation. Obtaining sufficient working material at a specific stage of growth, however, poses logistical problems. Due to the developmental process characteristic of most plants, neighboring seeds on the same plant are usually not at the same stage of development (Gurr, 1980). The seasonal availability of crop species also limits studies on fresh seeds unless considerable greenhouse or growth chamber space is available.

Several different approaches have been used to overcome the problem of obtaining seeds at the same stage of development. Wilson and Kwanyuen (1986) found that some degree of TAG biosynthesis occurred in germinating soybean cotyledons. They concluded that germinating soybean could be used as a source of enzyme which was uniform in developmental stage. Their purification of DGAT from this system is the only extensive purification of DGAT reported from a plant source (Kwanyuen and Wilson, 1986). They first solubilized DGAT from the membrane fraction using the detergent CHAPS. Further purification using Sepharose CL4B

chromatography and agarose gel electrophoresis resulted in 3000-fold purification. The enzyme, which was stable for 3 months when stored at -20°C , was found to consist of 3 different polypeptides, with molecular weights of 40.8, 28.7 and 24.5 KDa in a molar ratio of 1:2:2 (Kwanyuen and Wilson, 1990). These 5 subunits were proposed to form a subcomponent of the native enzyme which was thought to contain 10 such subcomponents. More recently, however, Wilson *et al.* (1993) reported that this DGAT preparation contained oil body proteins which suggested that interpretations regarding its structure may have been premature.

Tissue culture systems of oilseeds (Taylor and Weber, 1994) and fruits (Rutter *et al.*, 1997) have also been used to study lipid biosynthetic enzymes. These systems have the advantage in that they allow researchers to obtain tissue of the same developmental stage throughout the year without requiring extensive greenhouse or growth cabinet space. In many cases, enzyme preparations from cultures of fruits are more stable than enzyme preparations from the fruits themselves possibly because the cultures contain lower levels of phenolics and/or proteinases than the fruit (Rutter *et al.*, 1997). TAG bioassembly has been studied extensively in MD cultures of *B. napus* L. (Taylor *et al.*, 1990b; Pomeroy *et al.*, 1991; Taylor *et al.*, 1991; Taylor and Weber, 1994). Non-differentiating MD cell suspension cultures of *B. napus* L. cv Jet Neuf have been used in both the study of TAG biosynthesis and gene expression (Weselake *et al.*, 1993; Little *et al.*, 1994; Kocsis *et al.*, 1996; Weselake *et al.*, 1997; Weselake *et al.*, 1998). This cell suspension culture system of winter oilseed rape was generated in 1983 (Simmonds *et al.*, 1991) and was initially used for studies of freezing tolerance (Orr *et al.*, 1986;

Johnson-Flanagan and Singh, 1987; Johnson-Flanagan *et al.*, 1991).

DGAT has been partially purified from MD cultures of oilseed rape. Weselake *et al.* (1991) found high levels of DGAT activity in both the 1 500 to 10 000 x g and 10 000 to 100 000 x g fractions of MD embryos of *B. napus* L.. Further purification of DGAT has been carried out using the MD embryo system. The enzyme was dispersed from the microsomal fraction using the non-ionic detergent MEGA-8 (Weselake *et al.*, 1993). The presence of glycerol was found to stimulate the activity of the enzyme. Further separation was achieved using Mono Q ion exchange chromatography resulting in a fraction with a 10 to 80-fold increase in specific activity with about 40% recovery. Gel filtration chromatography on Sepharose CL4B indicated that DGAT may be part of a large complex. More recently, DGAT was solubilized from the 1 500 to 100 000 x g fraction of both MD embryos and cell suspension using 1% (w/v) MEGA-8 and 2 M NaCl (Little *et al.*, 1994).

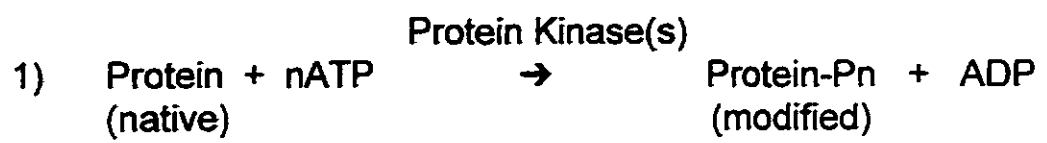
While the regulation of DGAT has been studied extensively in mammalian systems, its regulation has been much less studied in plants. DGAT has been shown to be regulated by phosphorylation in mammalian systems (Rodriguez *et al.*, 1992) but it has not been shown to be regulated by phosphorylation in plant systems. Phosphorylation, however, is a common post-translational modification for the regulation of enzyme activity in plants. It has been shown that many plant proteins undergo phosphorylation although few of them have been identified. Phosphorylation of a protein can result in conformational changes in an enzyme which can in turn result in either activation or inactivation. The process of

phosphorylation involves a minimum of three proteins and two reactions as shown in Fig. 3 (Ranjewa and Boudet, 1987). Cyclic nucleotides, Ca^{2+} and calmodulin are the major effectors of protein phosphorylation (Budde and Chollet, 1988). Phosphorylation has been found to occur primarily on serine, tyrosine and threonine residues and seems to be independent of molecular weight, pI or relative abundance. While many proteins contain serine, tyrosine and threonine residues, only a relatively small number undergo phosphorylation indicating that the process has considerable specificity (Budde and Chollet, 1988).

Salimath and Marme (1983) found that protein phosphorylation in membrane fractions from zucchini hypocotyls is stimulated by calcium and calmodulin and inhibited by fluphenazine (a calmodulin antagonist), but was not stimulated by cAMP. Polyamines have been found to promote phosphorylation of proteins *in vitro* in corn coleoptiles and spermine was found to promote phosphorylation of several membrane and soluble proteins with the process being dependent on the presence of Mg^{2+} (Veluthambi and Poovaiah, 1984). In *Arabidopsis thaliana* a receptor-like protein kinase gene has been identified which is rapidly induced by ABA, dehydration, high salt and cold treatments. This gene is thought to play a role in the signal transduction pathway of ABA and environmental stress (Hong *et al.*, 1997). Nine metal-independent protein phosphatases of germinated alfalfa seeds have also been identified (Yupsanis *et al.*, 1993).

Although many proteins in plants undergo reversible phosphorylation, only a few plant enzymes have been identified which undergo this process. The discovery

Figure 3. Reactions involved in the phosphorylation mechanism of protein regulation.



of this mechanism of enzyme regulation resulted from the observation that some crude enzyme preparations are either activated or inhibited if kept at room temperature (Ranjeva and Boudet, 1987). For example, pyruvate dehydrogenase is a latent enzyme that gradually becomes active as a function of time when crude preparations are held at room temperature. This time-dependent activation is stimulated by Mg^{2+} (a cofactor for protein phosphatase) and inhibited by F^{-} (a phosphatase inhibitor) (Rao and Randall, 1980). In contrast, quinate NAD^{+} oxidoreductase loses activity as a function of time, with Mg^{2+} stimulating and F^{-} inhibiting the process, respectively (Refeno *et al.*, 1982).

Overall, in the light of what is known about the regulation of mammalian DGAT, efforts to manipulate seed oils would be facilitated by knowledge of DGAT regulation in plants. If similar regulatory mechanisms exist for the plant enzyme it may be possible to utilize them in order to increase seed oil production. In the absence of the hormonal control which is present in mammalian systems, plants may also have novel mechanisms for the control of DGAT activity and lipid biosynthesis. The purpose of this thesis was to systematically examine the effects of various exogenous factors on DGAT activity in MD cell suspension cultures of oilseed rape and to investigate the possibility of a phosphorylation-dephosphorylation mechanism for the regulation of DGAT in the system.

MATERIALS AND METHODS

Plant Material

The MD cell suspension culture of *B. napus* L. cv Jet Neuf was provided by Dr. J. Singh of the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa and maintained essentially as described by Orr *et al.* (1986). The culture was grown on a rotating shaker at 25°C under constant light with an intensity of 36 $\mu\text{mol s}^{-1} \text{m}^{-2}$. Cells were collected following a 2 week growth period, washed with water over a nylon sieve, blotted with filter paper to remove excess water and the fresh weight determined. Cells were either used immediately or frozen in liquid N₂ and stored at -80°C until use.

Chemicals

[1-¹⁴C]Oleic acid (51 Ci mol⁻¹) was obtained from Amersham Canada Ltd., Oakville, Ontario, Canada. Merck Silica Gel 60H was from VWR Canlab, Mississauga, Ontario, Canada. Ecolite™ (+) biodegradable scintillant was from ICN Biomedicals, Inc. Irvine, California, USA. Dye reagent concentrate for protein assays was from Bio-Rad, Richmond, California, USA. HPLC-grade solvents were from BDH, Inc., Toronto, Ontario, Canada. *sn*-1,2-Diolein was from Avanti Polar-Lipids, Inc., Alabaster, Alabama, USA. Ultrafiltration membranes were from Amicon, Ltd., Oakville, Ontario, Canada. Acyl-CoA synthetase (*Pseudomonas fragi*) was from Boehringer Mannheim Canada, Laval, Quebec, Canada. Acyl-CoAs were synthesized from radiolabelled fatty acids according to Taylor *et al.* (1990a). All

other biochemicals used were of the highest purity available and were obtained from Sigma-Aldrich Chemical Company, St. Louis, Missouri, USA.

Microsome Preparation

Cells were ground in 4 volumes of grinding buffer (0.5 M sucrose, 0.2 M HEPES-NaOH, pH 7.4) per g of fresh weight using a chilled mortar and pestle. Differential centrifugation of the homogenate was performed at 4°C. The homogenate was centrifuged at 10,000 x g (Beckman J2-21M induction drive centrifuge with JA-20 34° fixed angle rotor using 50 mL polycarbonate centrifuge tubes) for 20 min and the supernatant was filtered through glass wool. The 10,000 x g supernatant was then centrifuged at 100,000 x g (Beckman model L3-50 ultracentrifuge with Type 65 23.5° fixed angle rotor using 16 mm x 76 mm Beckman Ultra-Clear tubes) for 1 h. The resulting microsomal pellet was washed with 10 mM HEPES-NaOH buffer (pH 7.4) and re-sedimented at 100,000 x g for 1 h. The washed pellet was resuspended in a volume equivalent to one-tenth of the original mass of cells using 10 mM HEPES-NaOH buffer (pH 7.4). Both the first 100,000 x g supernatant (cytosolic fraction) and the resuspended microsomes were divided into small aliquots, flash frozen with liquid N₂ and stored at -80°C.

Enzyme Assays and Protein Determination

Microsomal DGAT was assayed at 30°C for 10 min essentially as described by Little *et al.* (1994) using a 60 µL reaction mixture containing 0.2 M HEPES-NaOH (pH 7.4), 330 mM *sn*-1,2-diolein, 15 µM [1-¹⁴C]oleoyl-CoA (51 Ci mole⁻¹), 0.1 % (w/v)

Tween-20 and 6 mg BSA mL⁻¹. Reactions were initiated with the addition of 5-10 μ L resuspended microsomes. The reactions were quenched with 10 μ L 5 % (w/v) sodium dodecyl sulfate (SDS) and radiolabelled TAG was separated from 50 μ L of each reaction mixture by TLC using Silica gel 60 H preparative TLC plates and hexane:diethyl ether (80:20, v/v) as the solvent system. The co-chromatography of a triolein standard was used to establish the position of TAG on the TLC plate and the bands containing TAG from each lane were scraped into scintillation vials with 5 mL EcoliteTM (+). Radioactivity was determined using a scintillation counter. Microsomal protein introduced into the reaction mixture ranged from 20 to 90 μ g. A 120 μ L reaction volume was used to test the effect of various volumes of soluble fraction on DGAT activity in order to allow for a greater range of concentrations. Studies of other reaction components and additives were carried out under the standard conditions except that the concentrations of the component(s) of interest were varied.

Thioesterase activity was assayed at 30°C for 10 min using a 60 μ L reaction mixture containing 0.2 M Hepes-NaOH (pH 7.4), 330 mM *sn*-1,2-diolein, 15 μ M [¹⁴C]oleoyl-CoA (51 Ci mole⁻¹), 0.1 % (w/v) Tween-20 and 6 mg BSA mL⁻¹. Reactions were initiated with the addition of 5-10 μ L resuspended microsomes. The reactions were quenched with 10 μ L 5 % (w/v) sodium dodecyl sulfate (SDS) and radiolabelled fatty acid (FA) was separated from 50 μ L of each reaction mixture by TLC using Silica gel 60 H preparative TLC plates and hexane : diethyl ether : acetic acid (70:30:1, v/v) as the solvent system. The co-chromatography of an oleic acid

standard was used to establish the position of FA on the TLC plate and the bands containing FA from each lane were scraped into scintillation vials with 5 mL Ecolite™ (+). Radioactivity was determined using a scintillation counter.

The protein content of microsomes was determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure, using BSA as a standard.

Characterization of Fractions Stimulating DGAT Activity

To determine if inorganic stimulatory factors were present, samples were ashed by placing a 2 mL sample into a crucible on a steam bath and slowly adding 20 drops of concentrated HCl. An equivalent volume of buffer (0.5 M sucrose, 0.2 M HEPES-NaOH, pH 7.4) was ashed alongside the samples to serve as a control. When the samples approached dryness and were beginning to char the crucibles were placed in a cold muffle furnace and ignited gradually to 600°C overnight. Samples were then cooled to room temperature and resuspended in 2 mL of 10 mM HEPES-NaOH buffer (pH 7.4). A 5 µL aliquot of each sample was used to test for stimulation of DGAT.

A ninhydrin test on gel filtration fractions was performed by adding 12 µL of 0.1 % (w/v) ninhydrin in 95 % methanol to 200 µL of sample and placing in a boiling water bath for 10 minutes (Robyt and White, 1987). An additional 200 µL of concentrated HCl was added to each negative test tube and they were placed in a 100°C oven overnight before being retested.

Ultraviolet and visible spectrums (200 to 400 nm) of stimulatory fractions which had been filtered through a YM1 membrane were determined using a

Beckmann 65 spectrophotometer with 100 μ L cuvettes.

Acyl-CoA synthetase activity was assayed at 35°C for 3 hours using a 90 μ L reaction mixture containing 100 mM Mops-NaOH (pH 7.5), 10 mM DTT, 10 mM ATP, 5 mM CoASH, 0.05 μ Ci [14 C]18:1, 0.1 % (w/v) Triton X-100 and 2 μ L of acyl-CoA synthetase from *Pseudomonas fragi* (0.5 mg in 50 μ L 100 mM Mops-NaOH, pH 7.4). Reactions were quenched with 10 μ L 5 % (w/v) SDS and 75 μ L of each mixture was run on a silica gel H plate in *n*-butanol : water : acetic acid (5:3:2, v/v) as described by Taylor *et al.* (1990a) with oleoyl-CoA as a standard. The bands containing acyl-CoA were scraped into scintillation vials, 5 mL Ecolite™ was added and assayed for radioactivity.

Partial Purification of an Unidentified Factor Stimulating DGAT

Samples of the cytosolic fraction from differential centrifugation were dialyzed against 100 volumes of buffer (10 mM Hepes-NaOH, pH 7.4) for 4 h at 4°C using 6000-8000 MW cutoff dialysis tubing.

Ultrafiltration was carried out using an Amicon ultrafiltration cell with YM 30, YM 10 and YM 1 membranes (nominal MW cutoff of 30, 10 and 1 K, respectively). Samples of 100 000 $\times g$ supernatant were placed in the cell and pressure was applied using N₂ gas. The ultrafiltrate was collected on ice and either used immediately or flash frozen in liquid N₂ before being stored at -80°C.

Gel filtration was conducted with a Bio-gel P6 (Bio-Rad) column (25 mL). Bio-gel P6 beads (5 g) were hydrated for 48 h in distilled water. Beads were then equilibrated with 10 mM Hepes-NaOH buffer (pH 7.4) on a sintered glass funnel until

the filtrate reached pH 7.4. A column with a radius of 0.45 cm was packed with the beads, first by gravity and then with a peristaltic pump at maximum flow rate (5 mL min⁻¹), using the equilibration buffer. The flow rate was adjusted to 1 mL min⁻¹. The void volume was determined using blue dextran (2 mg mL⁻¹). Samples were loaded by gravity and 1 mL fractions were collected for analysis.

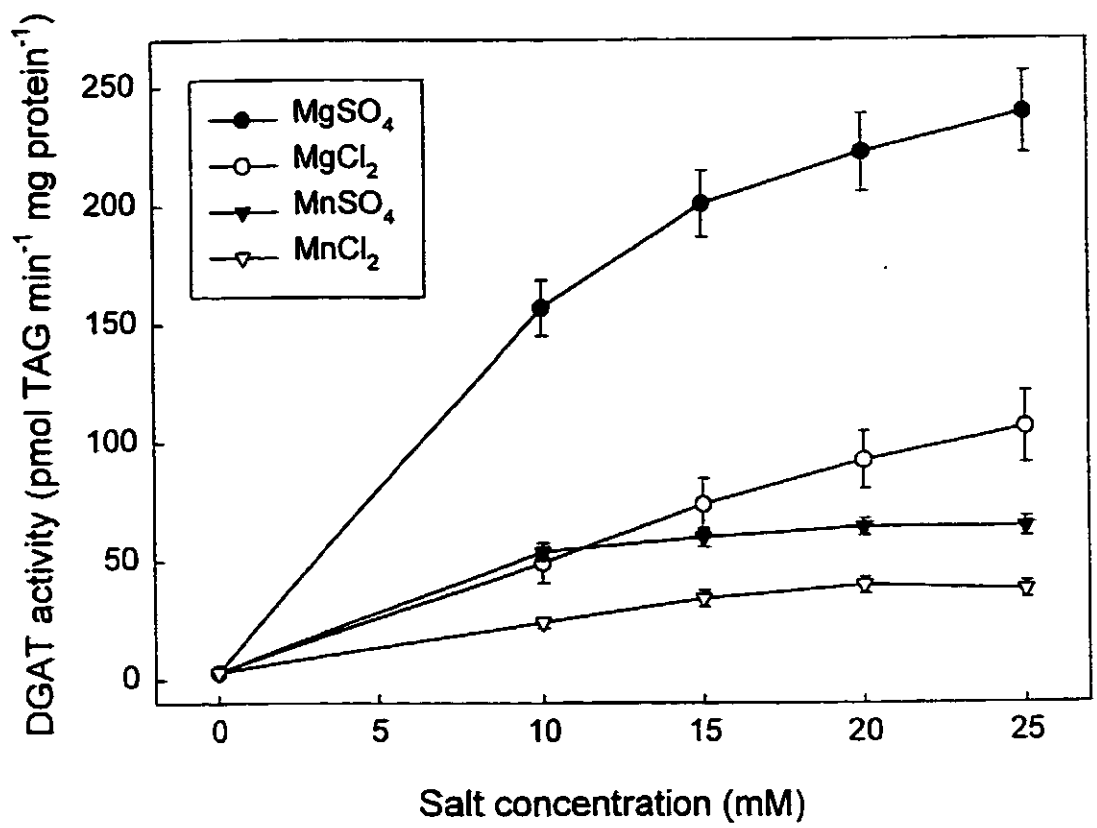
Reverse phase binding behavior was determined with a SepPak C18 cartridge washed with methanol and equilibrated with 0.5 M Hepes-NaOH buffer (pH7.4) containing 0.2 M sucrose. After sample application, the column was washed with 0.5 M Hepes-NaOH buffer (pH7.4) containing 0.2 M sucrose before being eluted first with methanol : water (1:1, v/v), and then with methanol. After removal of methanol by evaporation under N₂ the fractions were resuspended in 10 mM Hepes-NaOH (pH 7.4).

RESULTS AND DISCUSSION

Effect of Selected Salts on DGAT Activity.

In an effort to probe the regulation of DGAT activity, a systematic study of potential factors affecting the activity of the enzyme was undertaken. The study began by examining the effects of selected salts on DGAT activity. In various systems, divalent cations have been shown to alter acyltransferase activity, either negatively or positively (Martin and Wilson, 1983; Ichihara and Noda, 1982; Cao and Huang, 1990). Magnesium is sometimes included in reaction mixtures for assaying DGAT activity (Martin and Wilson, 1983; Ichihara, 1984; Cao and Huang, 1986; Oo and Chew, 1992). The effects of magnesium and manganese salts on microsomal DGAT activity from the cell suspension culture are shown in Fig. 4. Mg^{2+} salts had the greatest effect on DGAT activity with $MgSO_4$ and $MgCl_2$ at 25 mM concentrations stimulating microsomal DGAT by about 25 and 10-fold respectively. These results agree with previous findings that Mg^{2+} activated DGAT from *Mortierella ramanniana* var. *angulispora* (Kamisaka et al., 1993). Mg^{2+} may be involved in the regulation of DGAT activity in both of these systems. Cl^- did not have a significant effect on the enzyme although the results suggested that SO_4^{2-} may have an additive effect above that of Mg^{2+} or Mn^{2+} . High concentrations of Mg^{2+} have previously been shown to decrease the solubility of fatty acyl-CoAs (Constantinides and Steim, 1986). Consequently, it may be favorable to use a moderate concentration in order to balance high activity levels with substrate

Figure 4. Effect of various concentrations of MgSO₄, MgCl₂, MnSO₄ and MnCl₂ on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. Values represent the mean \pm SE (n=9).



solubility. CaCl_2 and NaCl were found to have lesser effects on DGAT in this system (Fig. 5) with stimulation being in the range of that caused by MnCl_2 . Ichihara and Noda (1982) reported that DGAT activity in microsomes from maturing safflower seeds is not stimulated by Ca^{2+} .

Effect of ATP and CoA on DGAT Activity

ATP and CoA are sometimes added to reaction mixtures for assaying TAG biosynthetic enzymes (Taylor *et al.*, 1991; Little *et al.*, 1994). The concentration dependence of microsomal DGAT activity on ATP and CoA concentration was examined. The addition of 2 mM ATP increased DGAT activity up to 2.4-fold in the absence of added Mg^{2+} (Fig. 6A) and up to 1.5-fold in the presence of 3 mM Mg^{2+} (Fig. 6B), but ATP did not have a significant effect in the presence of 25 mM Mg^{2+} (Fig. 6C). Bacterial diacylglycerol kinase activity has been shown to be dependent on the concentration of a Mg-ATP complex as well as being activated by free Mg^{2+} (Walsh and Bell, 1986). At high Mg^{2+} concentrations, the ATP may have formed a complex with Mg^{2+} which prevented activation of DGAT in our system.

CoA at a concentration of 1 mM stimulated DGAT up to 14 and 13-fold in the absence of added Mg^{2+} and in the presence of 3 mM Mg^{2+} , respectively (Fig. 7A and 7B). In the presence of 25 mM Mg^{2+} , however, the 1 mM CoA only increased DGAT activity 1.5-fold (Fig. 7C). The fact that the enzyme responded very differently to ATP and CoA in the presence of the high Mg^{2+} concentration suggested that keeping the Mg^{2+} concentration low may be desirable so as not to interfere with

Figure 5. Effect of various concentrations of CaCl_2 and NaCl_2 on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. Values represent the mean \pm SE (n=9).

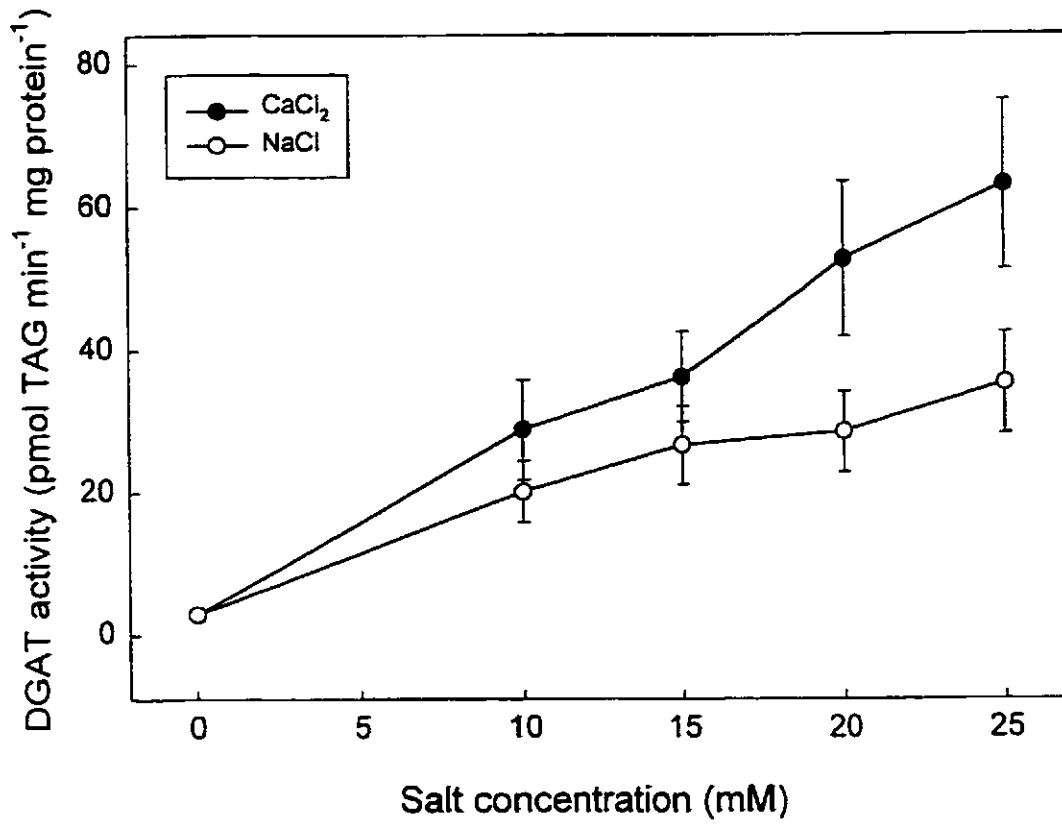


Figure 6. Effect of ATP concentration on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. **A)** no Mg^{2+} , **B)** 3 mM Mg^{2+} , **C)** 25 mM Mg^{2+} . Note the differences in scale for DGAT activity among the 3 panels. Values represent the mean \pm SE (n=9).

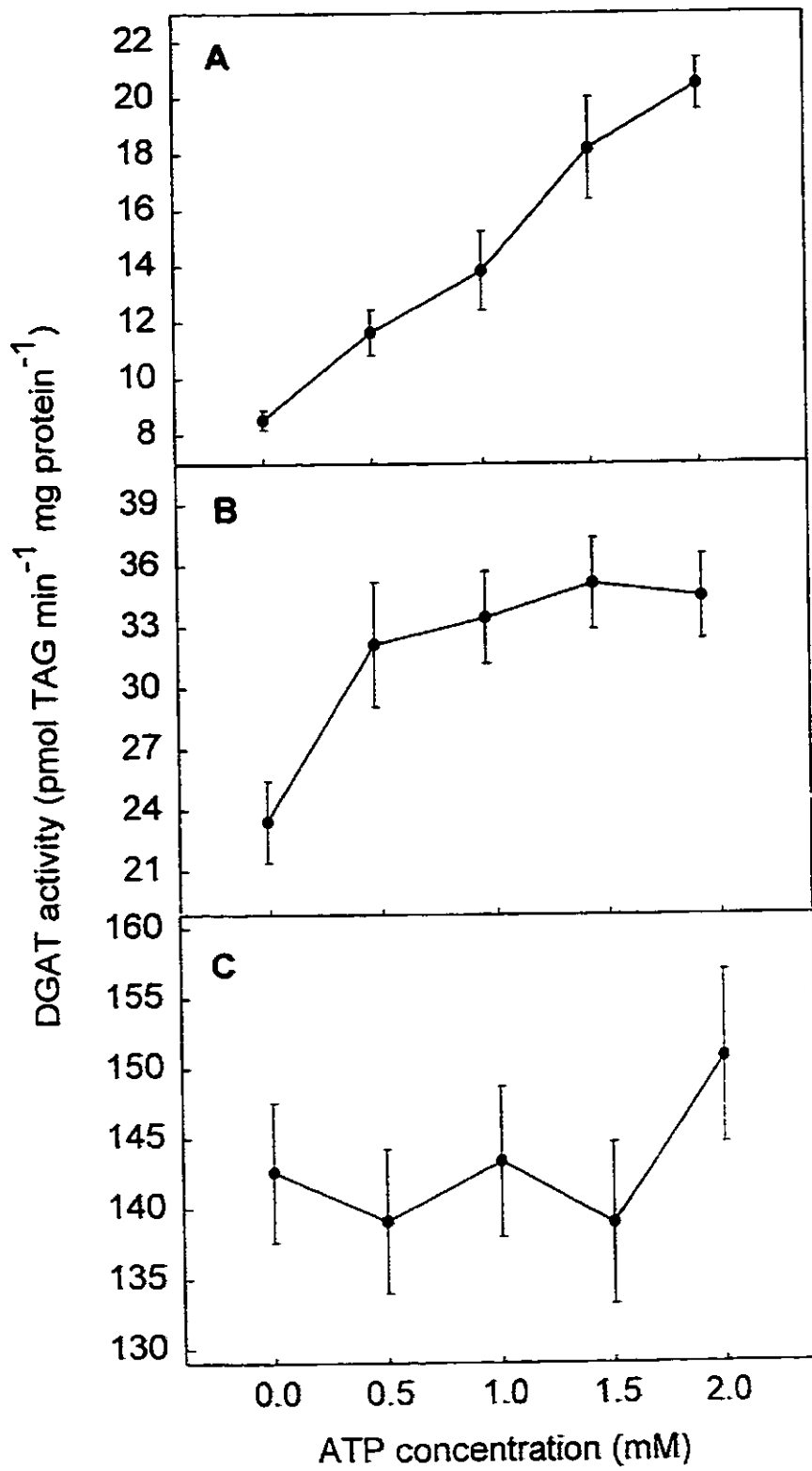
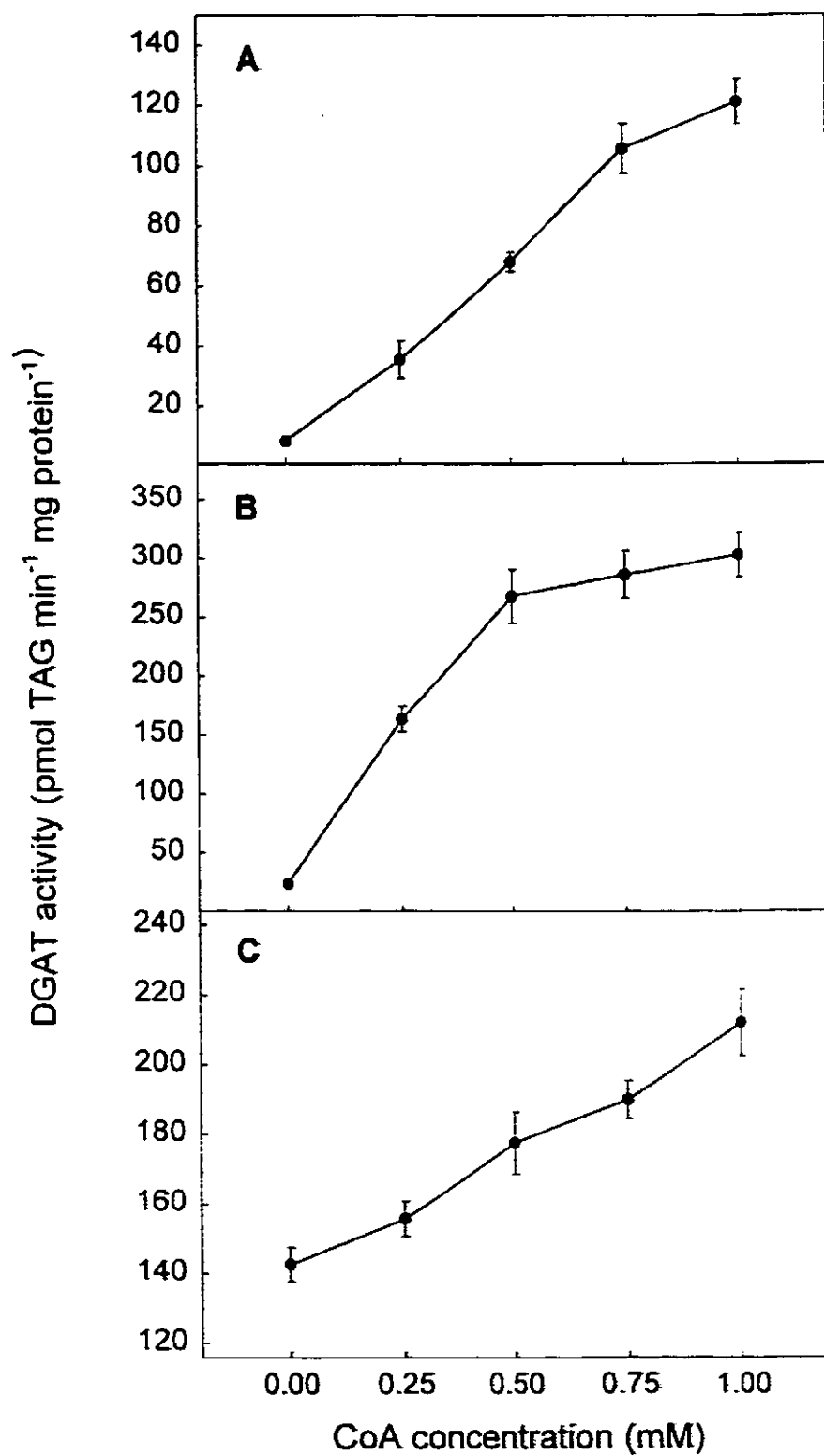


Figure 7. Effect of CoA concentration on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. **A)** no Mg^{2+} , **B)** 3 mM Mg^{2+} , **C)** 25 mM Mg^{2+} . Note the differences in the scale for DGAT activity among the 3 panels. Values represent the mean \pm SE (n=9).



other interactions occurring in the system.

It was previously reported that thioesterase activity was high enough in MD embryos of *B. napus* to interfere with the quantification of DGAT activity and that ATP and CoA were required to restore the acyl-CoA pool through the action of an endogenous acyl-CoA synthetase (Taylor *et al.*, 1990b). Endogenous thioesterase activity in the MD cell suspension culture was found to degrade only a small percentage of the radiolabelled acyl-CoA in our reaction mixtures under the same conditions used to assay DGAT activity (Table 1). This suggested that although ATP and CoA caused an increase in DGAT activity, it was not through restoration of the acyl-CoA pool. Instead, these compounds may be acting as allosteric modulators of DGAT in this system.

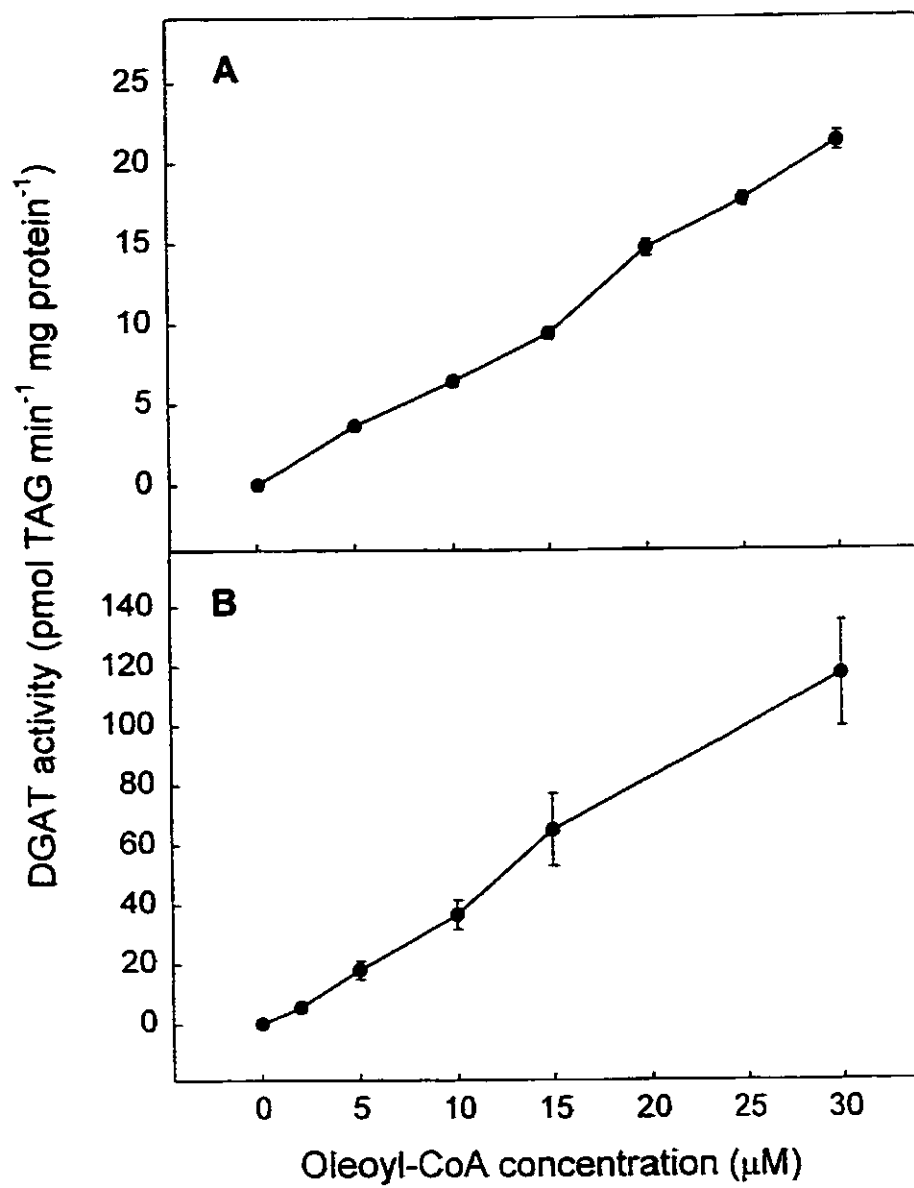
Effect of Exogenous Substrates on DGAT Activity

The dependence of DGAT activity on acyl-CoA and *sn*-1,2-diolein concentration in the presence of 3 mM and 25 mM MgCl₂ was examined. The effect of oleoyl-CoA concentration seemed to be similar in the presence of both low (Fig. 8A) and high Mg²⁺ concentration (Fig. 8B), even though the solubility of fatty acyl-CoAs has been shown to be greatly reduced in the presence of high Mg²⁺ concentrations (Constantinides and Steim, 1986). Study of the regulation of acyltransferases is complicated by the fact that long chain acyl-CoAs tend to form micelles in aqueous solution in the micromolar range. It is essential to keep the acyl-CoA substrate in its monomeric form while studying acyl-CoA utilizing

Table 1 - Thioesterase activity in microsomes prepared from MD cell suspension cultures of *B. napus* L. cv Jet Neuf.

Treatment	DGAT Activity (pmol/min/mg)	% of substrate degraded
0 mM MgCl ₂	30.1	1.3
3 mM MgCl ₂	44.6	5.0
25 mM MgCl ₂	153.5	17.1

Figure 8. Effect of oleoyl-CoA concentration on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. **A)** 3 mM Mg²⁺, **B)** 25 mM Mg²⁺. Note the differences in scale for DGAT activity among the 3 panels. Values represent the mean \pm SE (n=9).



enzymes in order for the reaction to proceed linearly. As well, long-chain acyl CoA tends to bind strongly to biological membrane fractions, as well as to hydrophobic proteins, so actual concentration of acyl-CoA in the reaction mixtures is an unknown (Constantinides and Steim, 1985). If the concentration is raised above the critical micelle concentration (CMC), many of the TAG biosynthetic enzymes may be inhibited while others may be stimulated.

The critical concentration of oleoyl-CoA for micelle formation has been reported to be approximately 32 μM (Constantinides and Steim, 1985; Smith and Powell, 1986). Excessively high concentrations of oleoyl-CoA were not used in the standard reaction mixture to avoid micelle formation. *In vivo*, the acyl-CoA concentration would never exceed the CMC (Smith and Powell, 1986) and the monomeric acyl-CoA concentration is probably kept low through reversible binding to membrane proteins and high turnover of intermediates. Regulation studies done with *in vitro* systems would be more meaningful under conditions which reflect the cellular environment, and thus, keeping substrates below the CMC is advised (Smith and Powell, 1986). BSA is known to interact with acyl-CoAs (Richards *et al.*, 1990; Trigatti and Gerber, 1995). The use of BSA in reaction mixtures allows acyl-CoA to bind reversibly to BSA, helping prevent the formation of micelles and also reducing any harmful detergent effects of the acyl-CoAs (Rutter *et al.*, 1997). BSA may also act as a blocking agent, and decrease non-specific binding of hydrophobic components. This probably mimics *in vivo* conditions to some extent and allows the enzyme a constant, yet low, supply of substrate. The use of BSA in acyl specificity

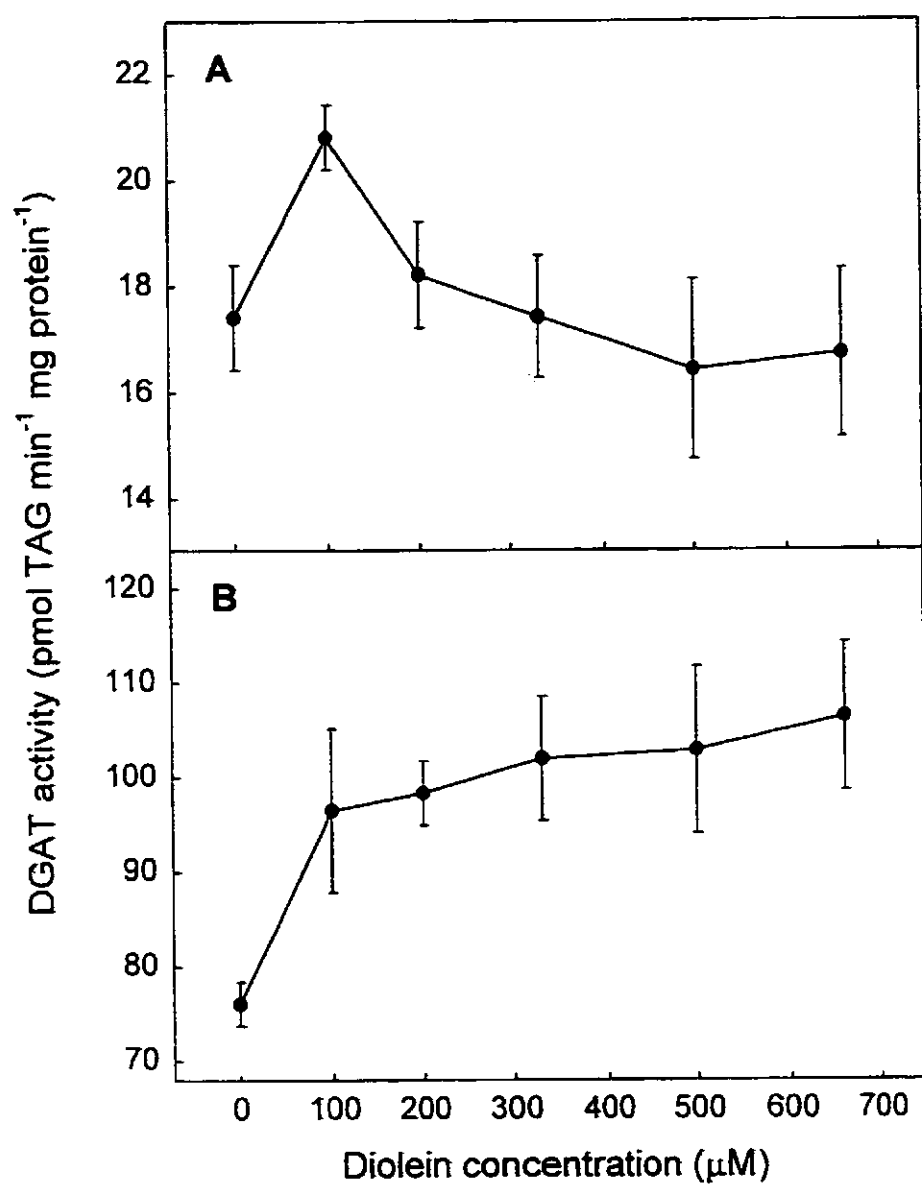
experiments could be misleading, however, if the dissociation constants for the acyl-CoA-protein complexes are different for each species of acyl-CoA (Smith and Powell, 1986).

DGAT activity was not dependent on exogenous *sn*-1,2-diolein when assayed in the presence of 3 mM Mg²⁺ (Fig. 9A), but 100 μM (bulk concentration) *sn*-1,2-diolein caused a 1.3 fold increase in activity when 25 mM Mg²⁺ was present (Fig. 9B). This may have been a result of the increased activity at high Mg²⁺ concentration which rapidly used up the endogenous substrate during the reaction. Little *et al.* (1994) previously reported that DGAT activity in fractions solubilized from microsomes of cell suspension cultures was not dependent on exogenous DAG. As well, Cao and Huang (1986) reported that endogenous DAG was present in microsomes from maturing oil seeds of maize and other species. DGAT from the fungus *Mortierella ramanniana* var. *angulispora* was found to be dependent on DAG in the presence of detergents, but not in the absence of detergents (Kamisaka *et al.*, 1993). This was consistent with other studies and suggested that exogenous DAG may have little access to the enzyme in the absence of detergents.

Effect of Phospholipids and G-3-P on DGAT Activity.

Phospholipids have been shown to affect DGAT activity in mammalian (Parthasarathy *et al.*, 1981), fungal (Kamisaka and Nakahara, 1996) and plant systems (Taylor *et al.*, 1991), and may represent allosteric modulators of the enzyme. In our study, the addition of 500 μM (bulk concentration) PA to the

Figure 9. Effect of bulk concentration of *sn*-1,2-diolein on microsomal DGAT activity in MD cell suspension cultures of *B. napus* L. cv Jet Neuf. A) 3 mM Mg²⁺, B) 25 mM Mg²⁺. Note the differences in scale for DGAT activity among the 3 panels. Values represent the mean \pm SE (n=9).



reaction mixture caused a 2-fold increase in DGAT activity (Fig. 10). Phosphatidylcholine (PC), phosphatidylserine (PS), cardiolipin (CL), and G-3-P, tested at 500 μ M, had no significant effect on DGAT activity (Fig. 10). Although PA is a precursor for DAG production, the addition of DAG to our system did not stimulate DGAT which suggested that PA may be acting as an allosteric modulator. Alternatively, the PA may have been used by microsomal PA phosphatase to produce DAG which was presented to DGAT in a manner that enabled it to be used more efficiently than exogenous DAG. This raised the possibility that substrate channeling (Roughan and Ohlogge, 1996) may have occurred between PA phosphatase and DGAT in this system. Stimulation by PA was concentration dependent with almost a 2.5-fold stimulation at 1 mM PA (Fig. 11). Although we found that G-3-P had no significant effect on DGAT activity in the cell suspension system, Taylor *et al.* (1991) reported that G-3-P resulted in increased TAG production in MD embryo microsomes and suggested that G-3-P could be a positive regulator of TAG biosynthetic enzymes. Kamisaka and Nakahara (1996) examined the effects of various phospholipids on solubilized DGAT from *Mortierella ramanniana* var. *angulispora* and also found that PA had the greatest stimulatory effect and that G-3-P had little effect on the activity of the enzyme.

Figure 10. Effect of 500 μ M bulk concentrations of PA, PC, PS, CL and G-3-P on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. Stock mixtures (0.5 mL) of PA (from egg yolk lecithin), PC (from egg yolk lecithin), PS (from bovine brain) and CL (from heart) were prepared using 10 mM HEPES-NaOH buffer, pH 7.4. The stock mixtures were sonicated in small glass vials on ice for 10 min using a Cole-Palmer Ultrasonic Cleaner, Model 8845-44 (Cole-Palmer Instrument Co., Chicago). The dispersed phospholipids were diluted into the reaction mixtures at the appropriate final bulk concentration immediately prior to assaying for enzyme activity. Bar heights represent the mean \pm SE (n=9).

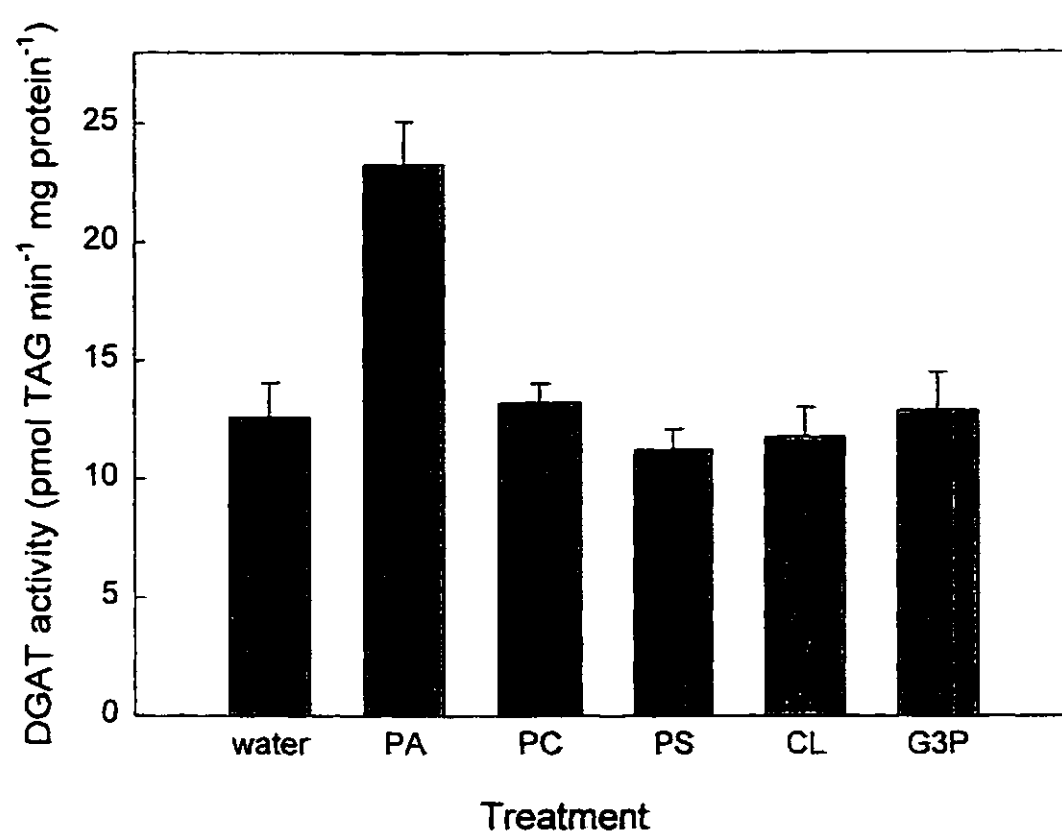
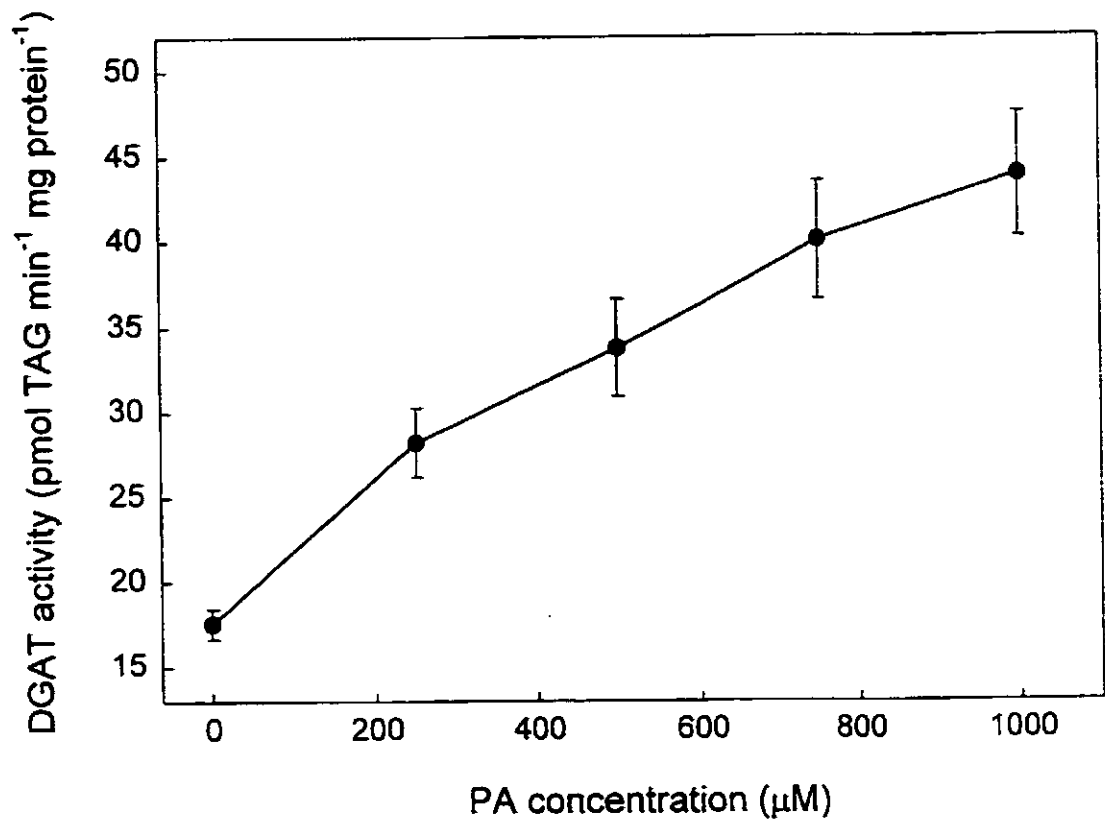


Figure 11. Effect of bulk concentration of PA on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. Stock mixture was prepared as described in the legend to Figure 10. Values represent the mean \pm SE (n=9).



Stimulation of DGAT Activity by a Cytosolic Component

In mammalian systems, DGAT may be regulated by a phosphorylation-dephosphorylation mechanism (Haagsman *et al.*, 1982). The reversible inactivation by phosphorylation was detected by incubating rat liver microsomes with an aliquot of the cytosolic fraction, plus ATP and Mg^{2+} . The resulting inactivation was reversed upon incubation of the microsomes with the cytosolic fraction in the absence of ATP.

In an attempt to determine if a similar regulatory mechanism was present in the MD cell suspension culture, an aliquot of the cytosolic fraction (100,000 x g supernatant) was added to the DGAT assay, either in the presence or absence of ATP. The cytosolic fraction stimulated DGAT activity when added back to the microsomal fraction in the presence of 3 mM Mg^{2+} , with 5 μ L of the cytosolic fraction resulting in about 2-fold stimulation of DGAT activity (Table 2). In the presence of 25 mM Mg^{2+} , however, a much lower level of stimulation by the cytosolic fraction was observed (Table 2). Unlike results from mammalian studies, no inhibition was detected under any of the conditions tested. The stimulation by the cytosolic fraction was not ATP dependent (Table 2) or affected by preincubation (Fig. 12), but was dependent on the amount of cytosolic fraction added. The degree of stimulation showed an essentially linear dependence on the quantity of cytosolic fraction added to the reaction mixture (Fig. 13). Thirty microliters of the cytosolic fraction resulted in a 6-fold stimulation of DGAT activity.

Dialysis indicated that the stimulatory factor(s) had a nominal molecular weight (MW) of less than 8000 with all of the stimulatory activity passing through a

Table 2. Effect of ATP on stimulation of microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf by 5 μ L of the cytosolic fraction. Values represent the mean \pm SE (n=9).

	Control	Cytosolic Fraction
	DGAT Activity	
	(pmol/min/mg protein)	
3 mM Mg²⁺		
0 mM ATP	18.5 \pm 1.3	40.5 \pm 3.4
1 mM ATP	50.1 \pm 4.5	99.9 \pm 8.4
2 mM ATP	52.3 \pm 4.4	103.5 \pm 7.4
25 mM Mg²⁺		
0 mM ATP	98.4 \pm 7.3	112.1 \pm 6.6
1 mM ATP	190.4 \pm 14.4	222.6 \pm 16.1
2 mM ATP	189.4 \pm 12.4	223.1 \pm 14.0

6000- 8000 MW cut-off membrane. Ultrafiltration indicated that the factor(s) causing the stimulation of DGAT had a MW less than 1000 with 80 % of the stimulation remaining in the filtrate after passage through a YM1 membrane (Table 3). Although a ninhydrin test (limit of detection 0.1 μ mol) on gel filtration fractions containing stimulatory activity did not reveal the presence of amino acids, either before or after hydrolysis, the stimulatory factor(s) may be a small peptide present in low concentration. The stimulatory factor retained most of its activity when boiled for 5 min but was largely inactivated when boiled for 30 min (Table 4). When PA was boiled for 30 min, PA stimulation of DGAT was retained (Table 4) indicating that the stimulation was not due to the presence of endogenous PA in the cytosol. The factor bound weakly to C18 resin which suggested that it had some hydrophobic character (Table 5). Ashing of the cytosolic fraction resulted in a loss of the stimulatory effect indicating that the factor was organic (Table 6). There have not been any previous reports of a small organic stimulator of DGAT in plant systems. Xanthohumols (which are chalcones), isolated and characterized from hops of *Humulus lupulus*, however, have been shown to inhibit DGAT activity in intact Raji (rat liver) cells (Tabata *et al.*, 1997).

An ultraviolet spectrum of the partially purified stimulatory factor (post YM1 filtration) resulted in a single peak with a maximum absorbance at 264 nm (Fig. 14). This corresponded to the absorbance peak of thymine (264.5 nm), indicating that there may be one, or several, nucleotides present in the stimulatory fraction. In order to determine if CoA present in the cytosolic fraction might be

Figure 12. Effect of length of preincubation on stimulation of microsomal DGAT activity by the 100,000 x *g* supernatant. Values represent the mean \pm SE (n=9).

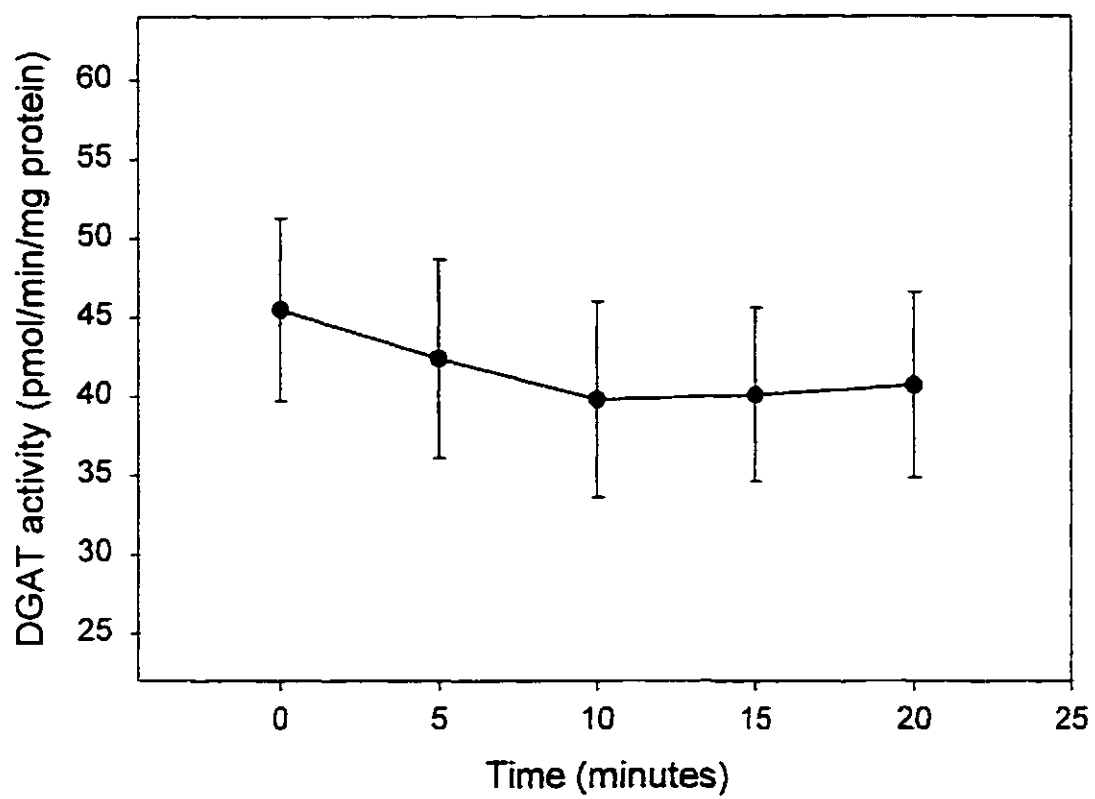


Figure 13. Effect of the volume of 100,000 x *g* supernatant on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. Values represent the mean \pm SE (n=9).

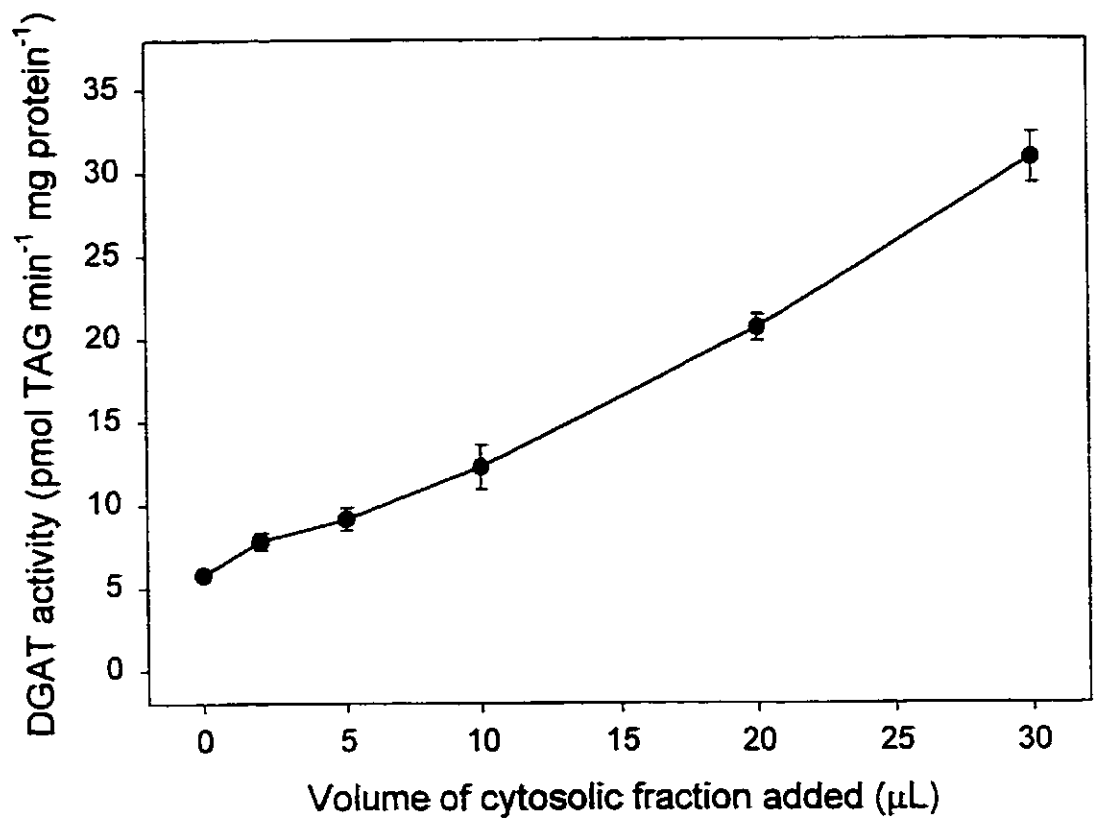
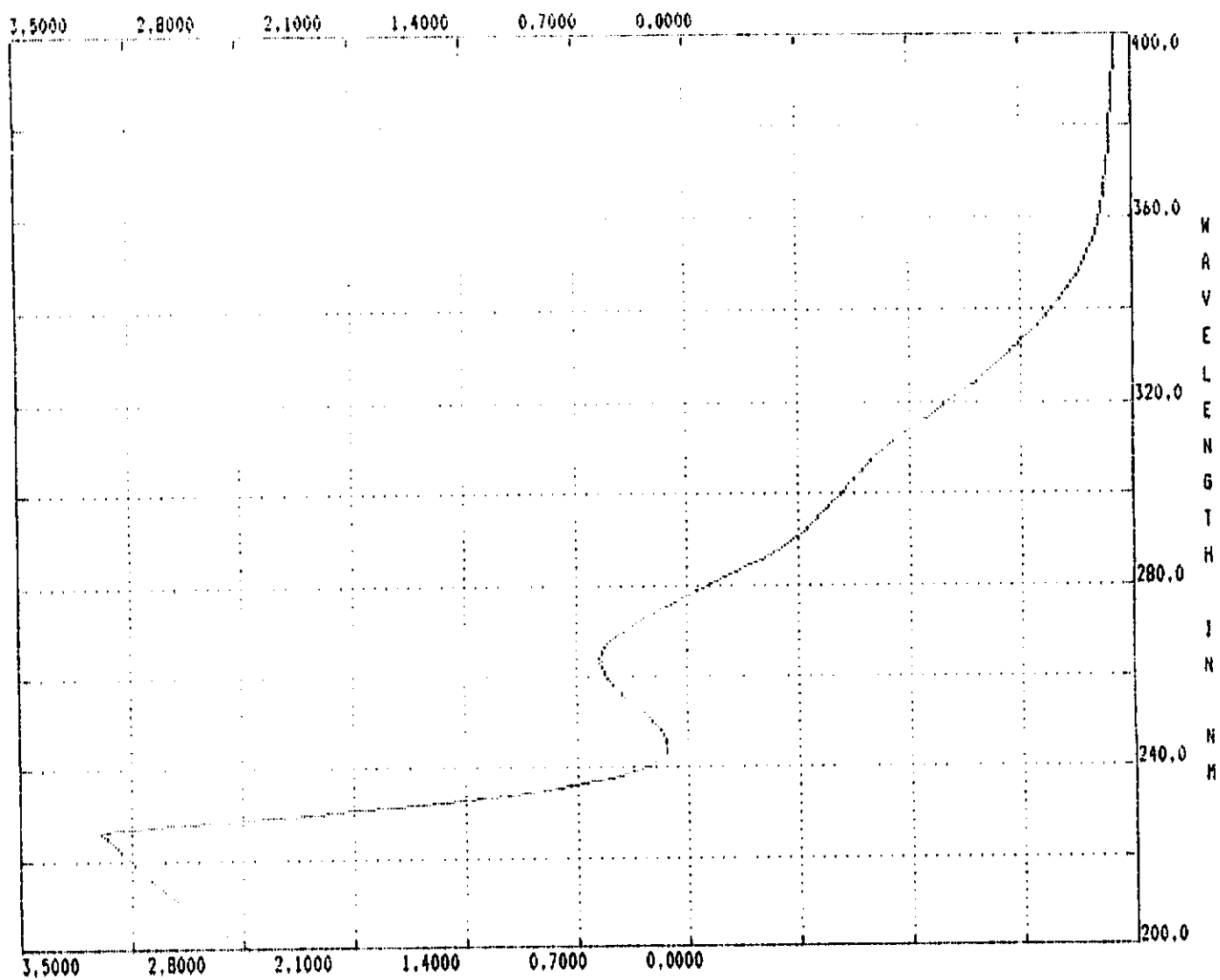


Figure 14. Ultraviolet spectrum of partially purified stimulatory factor(s).



Scan Speed: 500 nm/min

Table 3. Effect of 5 μ L of the cytosolic fraction on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf before and after ultrafiltration through YM30, YM10 and YM1 membranes.

Treatment	% Stimulation
pre-filtration	82.18
post - YM 30	88.05
post - YM 10	81.64
post - YM 1	52.61

Table 4. Effect of boiling the 100,000 x g supernatant and PA on stimulation of microsomal DGAT from cell suspension cultures of *B. napus* L. cv Jet Neuf.

Treatment	% Stimulation
Cytosolic fraction	112.79
Cytosolic fraction boiled 5 min	75.98
Cytosolic fraction boiled 30 min	38.49
PA	155.42
PA boiled 5 min	182.31
PA boiled 30 min	260.54

Table 5. Effect of fractions eluted from a SepPak C18 cartridge on microsomal DGAT activity from MD cell suspension cultures of *B. napus* L. cv Jet Neuf.

Fraction	% Stimulation
YM 10 filtrate	107
unbound	101
MeOH:H ₂ O (1:1,v/v)	41
MeOH	10

Table 6. Effect of ashing the cytosolic fraction on the stimulation of microsomal DGAT activity from cell suspension cultures of *B. napus* L. cv Jet Neuf.

Treatment	% of Control Activity
Cytosolic fraction	186
Ashed cytosolic fraction	88

responsible for the stimulation, the effects of CoA and the cytosolic fraction on acyl-CoA synthetase were compared. Acyl-CoA synthetase activity was found to be highly dependent on CoA concentration (Fig. 15), with a 4-fold increase in activity in the presence of 2 μ M CoA. In contrast, the addition of increasing volumes of the cytosolic fraction in the absence of exogenous CoA resulted in mild stimulation of acyl-CoA synthetase activity up to a volume of 30 μ L (Fig. 16). A volume of 50 μ L, however, resulted in a return to control levels. ATP was found to stimulate DGAT only up to 1.5-fold (Fig. 6) under the conditions used to test the stimulation of DGAT by the cytosolic fraction indicating that ATP alone could not be responsible for all of the stimulatory activity present in the cytosolic fraction.

Figure 15. Effect of CoA concentration on *Pseudomonas fragi* acyl-CoA synthetase activity. Values represent the mean \pm SE (n=3)

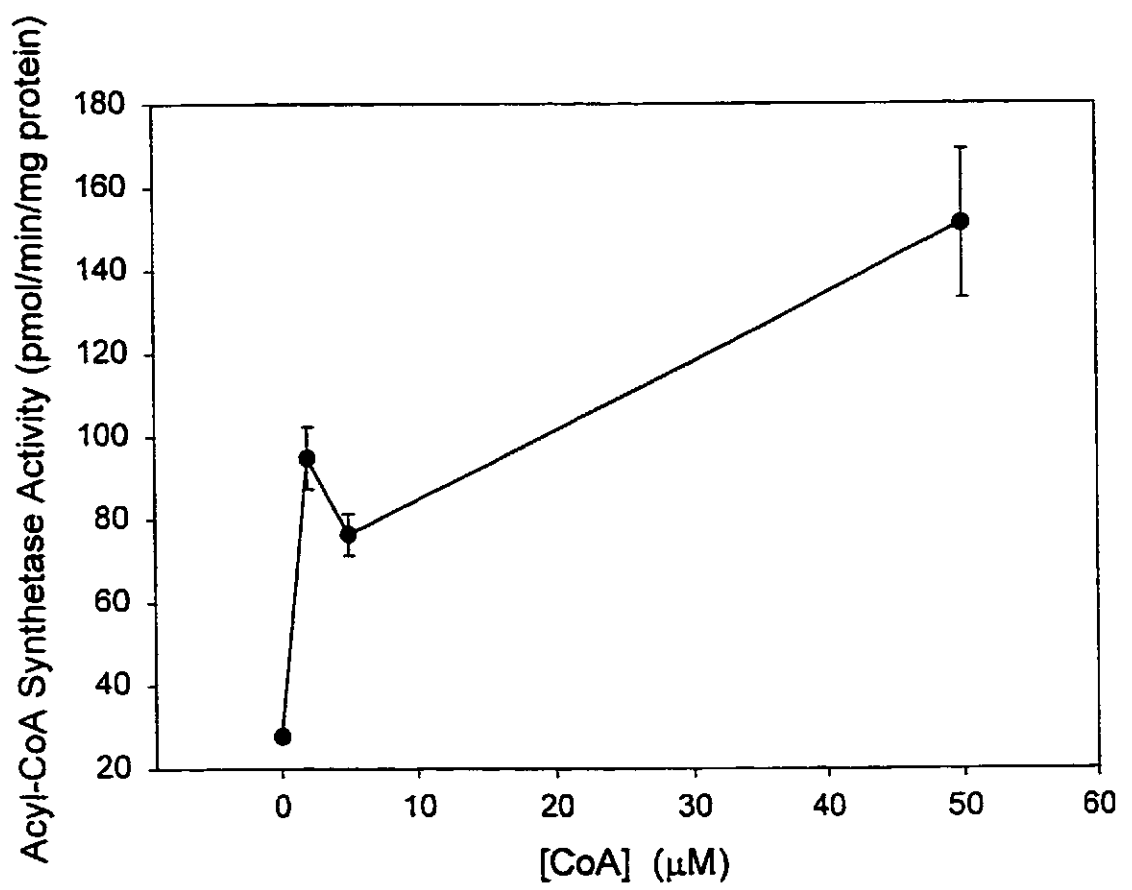
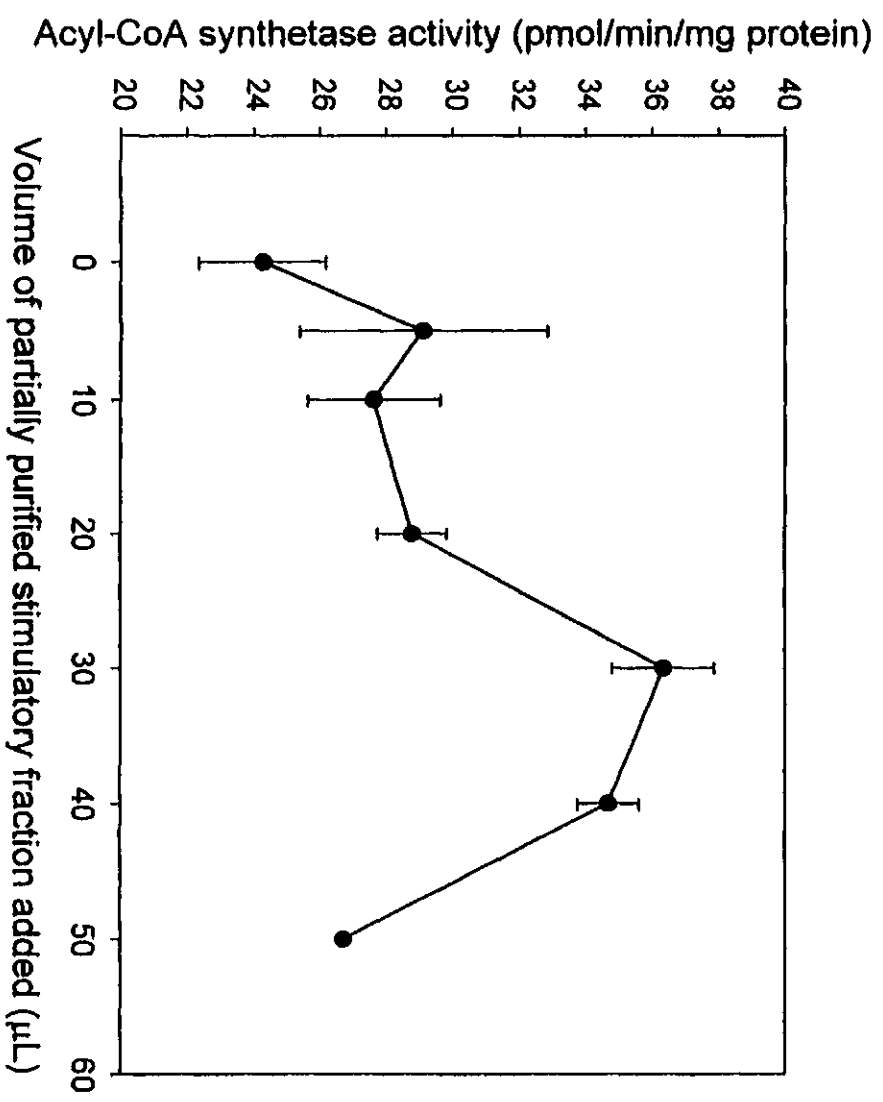


Figure 16. Effect of the volume of 100,000 x g supernatant prepared from homogenate of MD embryos of *B. napus* L. cv Jet Neuf on *Pseudomonas fragi* acyl-CoA synthetase activity. Values represent the mean \pm SE (n=3).



CONCLUSIONS AND IMPLICATIONS FOR THE FUTURE

Collectively, the results indicated that DGAT in the ER of the MD cell suspension culture of *B. napus* L. cv Jet Neuf might be regulated by a number of molecules including inorganic divalent ions, PA, CoA, ATP and an unidentified organic factor(s). Of the salts tested, Mg^{2+} was found to have the greatest effect on DGAT activity. Endogenous DAG was the preferred substrate of the enzyme because microsomal DGAT activity was independent of exogenous DAG. The stimulatory effect of PA may have involved the generation of DAG which was more readily utilized by DGAT than exogenous DAG. Thus, it is possible that substrate channeling (Roughan and Ohlogge, 1996) is involved in TAG biosynthesis in the ER. Although addition of exogenous CoA and ATP resulted in enzyme stimulation, the cytosolic fraction, prepared from cell homogenate, may have been too dilute to reflect the endogenous effects of these compounds.

Attempts to demonstrate down-regulation of DGAT activity by phosphorylation revealed the presence of a cytosolic factor(s) which stimulated the enzyme. Future identification of the stimulator(s) will provide new insight into the regulation of DGAT and may result in the development of a new affinity-based procedure for isolation of the solubilized enzyme. The hydrophobic character of the stimulator suggest that reverse phase chromatography may be useful in the purification of the factor(s). The use of HPLC may enable separation of the factor(s) and allow detection of stimulatory activity in the purified fractions. Identification by

various spectrometric and chemical methods may lead to the identification of the structure of this novel stimulatory factor. The presence of the stimulator in MD and zygotic embryos of oilseed rape, or in developing seeds or fruits of rapeseed and other oil-forming plants also remains to be established.

Although there was no evidence for the presence of a phosphorylation mechanism for the regulation of DGAT in this system, further investigation would be needed to determine whether such a mechanism is in fact absent. For example, it may prove useful to examine possible phosphorylation/dephosphorylation mechanisms in MD and zygotic embryos of oilseed rape as well as in other oilseeds. The study of other plant systems would be useful in determining whether the regulatory mechanism present in animal systems is present in any plant system.

Knowledge of the mechanisms of DGAT regulation present in oilseeds may lead to methods by which oil content or composition can be modified. If this membrane bound enzyme can be purified and sequenced it will provide a template for the preparation of primers to enable the isolation of cDNA encoding the protein. Biotechnological techniques would enable the gene to be expressed (or overexpressed) in oilseeds and may provide a means to increase oil production. Since Alberta currently produces over a third of Canada's seed oils, the development of methods to increase production would have substantial economical impact in this province. The world's demand for oilseed products will likely expand as our population increases and becomes more health conscious. If Canada hopes to continue to supply a large proportion of the global demand we will have to

continue to develop methods of increasing seed oil production and maintaining a desirable fatty acid composition.

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