DIACYLGLYCEROL ACYLTRANSFERASE IN RELATION TO INTRAMUSCULAR FAT DEPOSITION IN THE BOVINE

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ABSTRACT

Studies on the enzymology of fat formation in beef cattle may lead to the development of a marker for predicting the propensity of these animals to deposit intramuscular (i.m.) fat. Diacylglycerol acyltransferse (DGAT, EC 2.3.1.20) catalyzes the acylation of *sn*-1,2-diacylglycerol to form triacylglycerol (TAG). This thesis has focused on evaluating DGAT as a potential marker for predicting i.m. fat deposition in cattle and on characterization of the enzyme in three tissue types involved in TAG biosynthesis. Microsomes were isolated from subcutaneous (s.c.) adipose, i.m. adipose and muscle tissue of Wagyu crossbred cattle displaying a wide range of i.m. fat content (marbling). One aspect of this thesis focused on the relationship between total lipid present in meat samples from the *pars costalis diaphragmatis* (*p.c.d.*) and DGAT activity from s.c. adipose tissue, i.m. adipose tissue and muscle tissue of the *p.c.d.* Intramuscular adipose tissue DGAT activity exhibited a negative correlation with % TAG per gram wet weight *p.c.d.* ($r = -.547$; $P < .01$). There was, however, a positive correlation ($r = .735$; $P < .001$) between DGAT activity per unit wet weight i.m. adipose tissue and muscle DGAT activity per unit wet weight suggesting these two depots are coordinated in terms of TAG biosynthetic capacity. The second aspect of the thesis examined the characteristics of microsomal DGAT from the three tissue types. Microsomes from several animals were combined and were used for enzymological studies of DGAT in an attempt to define possible differences in enzymatic properties among the three tissue types. Diacylglycerol acyltransferase from s.c. adipose tissue in the presence of 15 mM $MgCl_2$ exhibited an enhanced preference for substrates containing oleoyl moieties relative to the other two

tissue types. Diacylglycerol acyltransferase from s.c. adipose tissue also differed slightly in sensitivity to *N*-ethylmaleimide and stability when compared to the other two tissues, muscle and i.m. adipose tissue.

Collectively, these results suggested that either a different isoform of DGAT is expressed in s.c. adipose tissue as compared to i.m. adipose and muscle tissue, or that the endoplasmic reticulum micro-environments are sufficiently different to account for the depot specific properties of DGAT in bovine. The data presented in this thesis suggested that DGAT was not a suitable marker for the deposition of i.m. fat in mature cattle. Perhaps an examination of i.m. fat deposition in less mature animals will yield stronger, positive correlations between DGAT activity and the amount of i.m. fat present.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Appendix Tables

List of Appendix Figures

ABBREVIATIONS

ACCase	acetyl-coenzyme A carboxylase
ASP	acylation stimulating protein
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Bis Tris Propane	1,3-bis[tris(hydroxymethyl)methylamino]propane
BSA	bovine serum albumin
CDP	cytidine diphosphate
CHAPS	3-[{3-cholamidopropyl}dimethylammonio]-1-propane sulfonate
Ci	Curie
CMC	critical micelle concentration
CoA	coenzyme A
d	day
DAG	sn-1,2-diacylglycerol
^{0}C	Celsius
DGAT	diacylglycerol acyltransferase
DHAP	dihydroxyacetone phosphate
dpm	disintegrations per minute
DTT	dithiothreitol
EDDI	ethylene-diamine-dihydroidide
EDTA	ethylenediamine tetraacetate
ER	endoplasmic reticulum
FA	fatty acid

FAMES	fatty acid methyl esters
FFA	free fatty acid
g	gram
G3P	*sn*-glycerol-3-phosphate
GLC	gas-liquid chromatography
GPAT	glycerol-3-phosphate acyltransferase
h	hour
HEPES	N-[2-hydroxyethyl]peperazine-N′-[2-ethanesulfonic acid]
HPLC	high performance liquid chromatography
HSL	hormone sensitive lipase
i.m.	intramuscular
kDa	kilo-Dalton
kg	kilogram
LPA	lysophosphatidate
LPAAT	lysophosphatidate acyltransferase
LPL	lipoprotein lipase
M	molar
MAG	monoacylglycerol
min	minute
mL	millilitre
mm	millimetre
mM	millimolar

mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
N_2	nitrogen gas
NEM	*N*-ethylmaleimide
NADP	nicotinamide adenine dinucleotide phosphate
P_i	inorganic orthophosphate
PA	phosphatidate
PC	phosphatidylcholine
p.c.d.	*pars costalis diaphragmatis*
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
pmol	picomole
s.c.	subcutaneous
SEM	standard error of the mean
SFA	saturated fatty acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEL	Soxhlet extracted lipid
sn	stereospecific numbering
TAG	triacylglycerol
TL	total lipid
TLC	thin layer chromatography
µL	microlitre

μM	micromolar
U.S.D.A.	United States Department of Agriculture
wt.	weight

INTRODUCTION

Production of beef in Canada generates revenues of over $10 billion per year for national producers. Marbling is an attribute of beef and is characterized by the presence of discrete depots of adipocytes within bovine skeletal muscle. These depots are often referred to as intramuscular (i.m.) fat. The production of beef that is well marbled, but with minimal amounts of fat that must be trimmed from the carcass, is one of the current challenges to the beef industry. The majority of beef (84 % in 1993) produced in Canada, however, exhibits less than a small to modest amount of marbling (Jones, 1993). Carcasses that fail to exhibit at least "trace" marbling receive a lower grade and price as a result.

Marbling is a production trait that can be selected for in breeding schemes. Like most other carcass traits, selection for marbling requires tremendous effort, expense and time. Progeny of a given animal must be raised to market weight and its carcass evaluated for marbling before any conclusions can be made about the animals genetic potential to deposit i.m. fat. This process takes about two years. Development of a biochemical or genetic marker for marbling is desirable, because it could reduce this selection procedure to only a few days or weeks. Thus, breeding programs designed to produce breeds or lines of cattle with high levels of carcass marbling could be accelerated. Increased levels of marbling fat will result in a greater number of carcasses receiving a higher marbling grade. Beef producers and purveyors have already stated that the demand for well marbled carcasses exceeds supply. Therefore, attaining this goal will

help increase the demand for beef in Canada, and of Canadian beef exports (e.g. U.S.A. and Japan). This in turn will lead to increased profits for the Canadian beef industry.

Candidates for a biochemical marker that determine the propensity of young cattle to marble may include triacylglycerol (TAG) biosynthetic enzymes. We hypothesized that the rates of lipid biosynthetic enzymes should be higher in cattle that marbled well as compared to cattle that marbled poorly. Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the final step of TAG biosynthesis and DGAT may also represent an important regulatory enzyme in fat formation (Hosaka *et al.*, 1977; Mayorek *et al.*, 1989). Thus, cattle that marble well may exhibit higher DGAT activities in i.m. adipose tissue than those found in cattle that marble poorly. One facet of this thesis was to examine the potential of using DGAT as a marker for predicting i.m. fat deposition. We examined the strength and significance of the correlation coefficient between activity of DGAT in three tissue types and lipid content of the skirt muscle. A second facet of this thesis was to characterize microsomal DGAT from bovine subcutaneous (s.c.) adipose, i.m. adipose and muscle tissue with the emphasis on determining differences in the characteristics of the enzyme from these three sources.

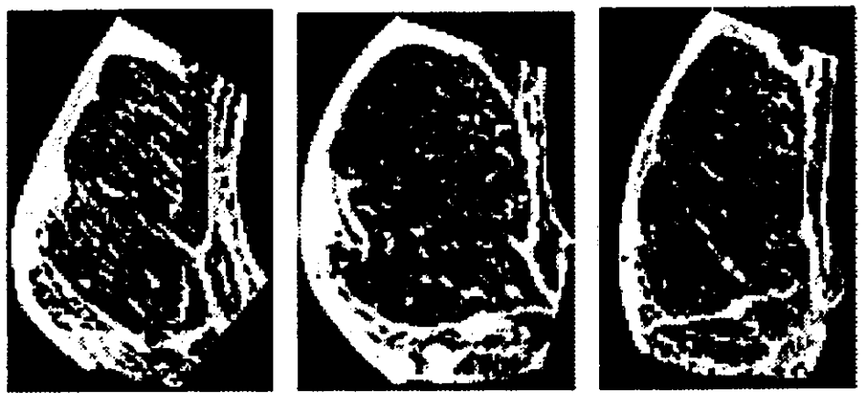
CHAPTER 1.
LITERATURE REVIEW

Economic Importance of Marbling

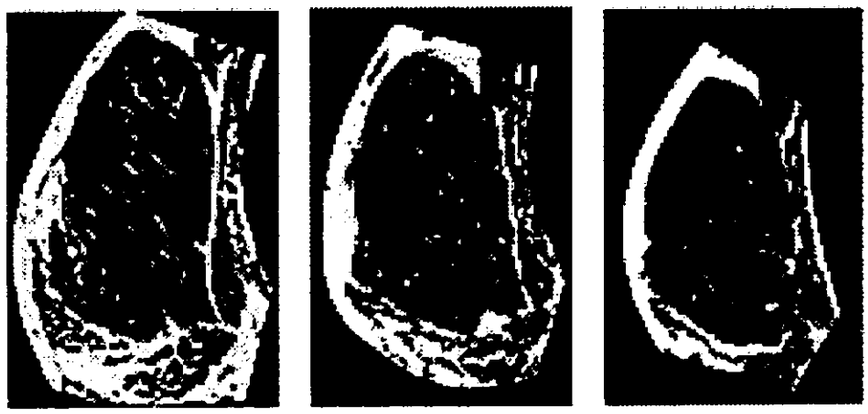
In todays economy, Canada must compete for it's market share in the domestic and export beef market. The deposition of i.m. fat in bovine skeletal muscle is known in the beef industry as marbling. Marbling has a low but positive relationship with beef palatability (Savell *et al.*, 1987; Wheeler *et al.*, 1994). Marbling is composed of white adipocytes located within bovine skeletal muscle as small discrete depots (Figure 1). Marbling is a desirable characteristic of beef as a "small" to "modest" amount of i.m. fat contributes to the taste, texture, and ease of preparation of the meat, while increasing the total caloric content only slightly (Smith *et al.*, 1984). Developing methods of producing cattle containing meat with sufficient levels of i.m. fat can give domestic producers a competitive edge. Beef with this characteristic is in high demand both in domestic and foreign markets, including Japan and the United States.

Currently, only 16 % of the beef carcasses produced in Canada exhibit a small to modest amount of marbling (Jones, 1993). Grading systems in the U.S.A. and Canada do not award top grade to a carcass unless it displays a slight to moderate amount of marbling. For example, within the U.S.A., a marbling score of "slightly abundant" is the minimum requirement for U.S.D.A. "prime". "Moderate", "modest" and "small" amounts of marbling comprise U.S.D.A. "choice". "Slight" and "trace" amounts of

Figure 1. An example of marbling in beef cattle. Ribeye cuts of beef displaying varying degrees of marbling found in standard U.S.D.A. grades. Adapted from the website at www.meatman.com.



Moderately Abundant | Slightly Abundant | Moderate



Modest | Small | Slight

marbling comprise the upper half of U.S.D.A. "select". Carcasses devoid of marbling are relegated to the lower grades and consequently have a much lower price (Savell *et al.*, 1987).

Recently, the National Cattlemen's Association of the U.S.A. solicited a study examining the quality of beef produced in the U.S.A. Producers faced a lost opportunity of $279.82 per steer or heifer slaughtered in 1991 (Smith *et al.*, 1991). Waste comprised $219.25 of the total with $111.99, $62.94, $14.85 and $29.47 accounting, respectively, for excessive external fat, excessive seam fat, for fat in excess of 20% in beef trimmings and for incorrect muscling. Taste comprised $28.81 of the total lost opportunity per animal with $2.89 for inadequate overall palatability, $21.68 for insufficient marbling, $3.80 for maturity problems (too old or young at slaughter) and $0.44 for gender problems (failure to castrate, pregnancies). Similar deficiencies were noted in the Canadian beef industry. In contrast, the U.S. National Beef Quality Audit reported that there was $137.82 in lost opportunity per steer or heifer slaughtered in 1995 (Smith *et al.*, 1995). Unfortunately, a breakdown of these more recent costs was not available. Among the most important quality concerns defined by this audit were: excessive external and seam fat, low overall palatability, low uniformity of product and insufficient marbling. When a comparison of beef quality audits was conducted including data from 1995, 1991 and 1974, a slow decline in marbling score and quality grade was apparent, in spite of efforts within the industry to reverse this trend. Even when only 1991 and 1995 were examined, a decline in animals receiving U.S. prime and choice grades was observed (55% in 1991, 48% in 1995).

Attempts to Enhance Marbling in Cattle

Due to the emphasis placed on producing carcasses displaying adequate marbling, producers have responded by over-fattening their slaughter cattle in an attempt to enhance deposition of i.m. adipose tissue. Feeding cattle on a high energy diet, typically rich in grain, for extended duration often results in animals with greater backfat thickness and pelvic fat than those fed low energy diets or for short durations. Smith and Crouse (1984) performed a study examining the differences in fat deposition between Angus cattle on high and low energy diets. The investigators found the cattle on the high energy diet had higher carcass weights, but this greater weight was composed of mostly backfat and excess seam fat. In grain-fed cattle, changes in marbling are generally less dramatic than those associated with backfat thickness (Aberle *et al.*, 1981). The disparity between the growth of the different fat depots illustrates the difficulty facing the beef industry. The industry must reduce the overall adiposity of beef carcasses in order to provide a nutritious product with a minimum of waste, while maintaining or improving the amount of i.m. fat and hence palatability and marketability of the beef.

The poor association between the deposition of i.m. fat and the deposition of fat in other depots (Aberle *et al.*, 1981; Smith and Crouse, 1984; Shackelford *et al.*, 1994) enables selection for marbling by use of breeding schemes. This process requires a large investment of time and expense because progeny of a given dam and sire must be raised to market weight and slaughtered before any conclusions can be made about the potential of the parents to transmit the trait to their progeny. Using marker assisted selection

protocols (Lande and Thompson, 1990; Meuwissen and Van Arendonk, 1992), a biochemical or genetic marker that identifies young animals with the propensity to deposit marbling fat would enable producers to more quickly select for this trait in the population.

Characteristics of Wagyu Cattle

Japanese Wagyu cattle are considered to be the world's best marbling cattle (Yamazaki, 1981). Research has shown that *longissimus lumborum* muscle trimmed of s.c. fat from Wagyu cattle may contain as much as 20 % extractable lipid (Lunt *et al.*, 1993). This amount of fat is approximately twice as much as that found in U.S.D.A. Prime (Savell *et al.*, 1986). Lunt *et al.* (1993) examined carcass characteristics of 3/4 - 7/8 Wagyu steers versus Angus steers under identical feed conditions and found that Wagyu crossbred cattle, on average, had higher marbling scores than their Angus counterparts. These results were confirmed in a study conducted by May *et al.* (1994). In addition, Wagyu adipose tissue possessed higher percentages ($P < .05$) of myristic, palmitoleic and oleic fatty acids and a lower percentage ($P < .05$) of palmitic and stearic fatty acids than corresponding tissues from Angus steers (Lunt *et al.*, 1993). Studies using sensory panels have shown that the ratio of monounsaturated fatty acids (MUFA) to saturated fatty acids (SFA) appears to affect beef palatability, with flavor being the attribute most affected (Dryden and Marchello, 1970; Melton *et al.*, 1982). American Wagyu crossbred cattle typically display a MUFA to SFA ratio of 1.5:1 or greater while domestic breeds are often closer to 1:1 (May *et al.*, 1993). Lunt *et al.* (1993) also noted

a large degree of variation in marbling score in both American crossbred Wagyu and Angus breeds. These results underscore the inherent genetic variability for the propensity to marble in the bovine and the need, even within Wagyu hybrids, to identify those sires and dams that marble well.

Assessment of Marbling

There are several methods of determining the i.m. fat content or marbling score of beef cattle. Traditionally, marbling is visually assessed by graders after the carcass has been chilled for 24 h and the *longissimus dorsi* (ribeye) cross-sectioned. The inspectors use comparison cards to assess the extent of marbling present. In Canada and the USA, the inspection site is located between the 12^{th} and 13^{th} rib at the ribeye portion of the carcass (Gaskins *et al.*, 1995). Although a subjective measurement, it provides a quick and relatively low cost method of assessing quality grade for a given carcass. In Canada, marbling is scored on an inverse 10 to 1 scale, with 10 assigned to carcasses devoid of marbling and 1 assigned to carcasses with "abundant marbling". The correlation between marbling score assigned to the carcass using this method and extractable fat present has been shown to be relatively high ($r = .7$; $P < .05$, Park *et al.*, 1994). This assessment method, however, provides carcass information only after the animal is slaughtered.

Image analysis represents an alternative method to the visual appraisal system for determination of i.m. fat. This procedure uses a 7 mm core sample taken from the *longissimus dorsi*. Digital fat images are made from source images obtained through a

microscope equipped with a high sensitivity video camera (Ishii *et al.*, 1992). The system processes the data into binary images, and then measures the area of the binary images. This method represents a more objective approach to quantifying i.m. fat. A large amount of variation has been encountered, however, depending on the location from which the core was obtained. Ishii *et al.* (1992) found that the correlations were not particularly high, especially if only one core sample was taken. Major disadvantages utilizing this method include: 1) the necessity for multiple core samples to be taken from the grading site, 2) causing damage to the carcass and 3) the technique provides carcass information only after the cattle have been slaughtered.

In contrast, ultrasonography can provide the producer with pertinent information on fat content before the animal is slaughtered. Park *et al.* (1994) investigated the use of ultrasonic A-mode which uses the velocity of sound waves to estimate the i.m. fat content present in a sample or the living animal. A difference in the velocity at which sound waves travel through fat and muscle exists (Kanis *et al.*, 1986). By measuring the time it takes the sound wave to travel through the specimen, an estimate can be made of the percentage of fat and muscle present in the sample. Correlation coefficients between actual fat present and fat predicted by ultrasound were fairly high ($r = .82$; $P < .05$). It was not possible, however, to distinguish between i.m., s.c. or seam fat (Park *et al.*, 1994). Therefore, until the technology is improved, ultrasound may best be suited to quantify backfat thickness, ribeye area and 12^{th} rib fat thickness in live animals (Gaskins *et al.*, 1995).

The most quantitative assessment of lipid content is conducted following the extraction of lipid from tissue with an organic solvent. Gravimetric analysis can be used to determine total lipid following evaporation of the organic solvent. If quantifying TAG present in the sample is the goal, chromatography can be used to resolve lipid classes such as TAG, sn-1,2-diacylglycerol (DAG), monoacylglycerol (MAG) and free fatty acids (FFA) in the total lipid extract. The resulting lipid classes can be further characterized. For example, the acyl chains of TAG can be converted to methyl esters for analysis by gas-liquid chromatography (GLC) for determination of acyl chain length and number of double bonds present (Christie, 1982). The identities of fatty acid moieties can be confirmed by co-chromatography and mass spectrometry. The procedures dealing with lipid analysis have been extensively reviewed by Christie (1982).

Characteristics of Different Fat Depots

White adipocytes are cells which specialize in the formation and storage of TAG. The TAG of adipose tissue is stored during periods of energy excess and can be mobilized to oxidizable fatty acids (FA) in periods of energy deprivation. Adipocytes comprise between 1/3 and 2/3 of the total cells in adipose tissue. The remaining cells are of the stromal-vascular type and include endothelial and blood cells, along with adipocyte precursor cells (Smas and Sul, 1995). Embryonic stem cell precursor lines have the capacity to differentiate into mesodermal cell types, which include adipocytes, chondrocytes and myocytes. Terminal differentiation from stem cells to adipocytes involves alterations in the levels of expression to over 100 proteins (Hausman *et al.*,

1993). These changes occur primarily at the transcription level, although post-transcriptional regulation occurs for some adipocyte genes (Moustaid and Sul, 1991).

White adipocytes from different depots are known to differ both morphologically and metabolically (Smith and Crouse, 1984; Pond, 1992). Differences between i.m. adipose tissue and other adipose depots such as s.c. adipose tissue have been noted in the literature. Adipocytes from i.m. tissue are substantially smaller than cells from s.c. adipose tissue. On average, i.m. and s.c. adipocytes have been shown to be 60.2 μm ± 2.7 and 71.3 μm ± 2.9 in diameter, respectively, in Angus steers (Smith and Crouse, 1984). Consistent with the smaller mean diameters exhibited by i.m. adipocytes, i.m. adipose tissue had greater numbers of cells per gram of tissue, when compared to s.c. adipose tissue (7.226×10^5 cells/g wet weight (wt) versus 6.462×10^5 cells/g wet wt in s.c. adipose tissue). May *et al.* (1994) found that both Wagyu and Angus breeds exhibited smaller i.m. adipocyte diameter, cell volume and greater numbers of cells per gram tissue when compared to s.c. adipocytes within each breed. These investigators also found significantly smaller cell diameters and volumes ($P < .05$) with both s.c. and i.m. adipocytes in the Wagyu breed when compared with the Angus breed. These results indicated that Wagyu s.c. and i.m. adipose tissues were less mature than the corresponding Angus tissues (May *et al.*, 1994).

The occurrence of a discrete population of small adipocytes in relatively fat cattle and pigs was demonstrated in an earlier study. Allen (1976) demonstrated a biphasic distribution for cell diameters in cattle with an excess of 5 mm of s.c. fat and suggested the occurrence of small adipocytes was the result of reinitiation of hyperplasia

or the result of recruitment of preadipocytes. Smas and Sul (1995) have demonstrated that the potential to make new fat cells continues throughout the lifespan of an animal. For example, the cells of the stromal vascular fraction of adipose tissue from aged rats and humans differentiate *in vitro* into adipose cells (Djian *et al*, 1983; Kirkland *et al.*, 1990).

In vitro studies of adipose tissues from mature cattle indicated that i.m. adipocytes exhibit a distinct preference for glucose as a precursor for lipogenesis, whereas adipocytes from s.c. adipose tissue exhibited a preference for acetate (Smith and Crouse, 1984; May *et al.*, 1993). Glucose provided 50 -75 % of acetyl units for lipogenesis in i.m. adipose tissue and only 1 - 10 % of the acetyl units in s.c. adipose tissue. Acetate provided 70 - 80 % of the acetyl units for lipogenesis in s.c. adipose tissue, but only 10 - 25 % in i.m. adipose tissue. Lactate was utilized equally by both tissue types. Smith and Crouse (1984) also attempted to examine the i.m. adipose tissue precursor preference in cattle that were one year old. Unfortunately, adequate amounts of i.m. adipose tissue could not be obtained from these animals to conduct *in vitro* assays. In addition, the activities of ATP-citrate lyase, NADP-malate dehydrogenase, NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate were higher in s.c. adipose tissue than in i.m. adipose tissues (Smith and Crouse, 1984).

Recently, Pond (1992) described site specific differences in rodent adipose depots. The author has referred to small depots of adipose tissue within muscle groups in small mammals as intermuscular adipose tissue. This depot shares a number of

attributes with bovine i.m. adipose tissue. The intermuscular adipose of small mammals was always small, usually accounting for 4 - 15 % total dissectable fat (Pond, 1992). The intermuscular depot displayed much higher FA / TAG cycling and lipoprotein lipase (LPL) activity than other depots suggesting that intermuscular adipose tissues differed metabolically from other adipose depots. In general, more massive fat depots such as perirenal and epididymal adipose tissue appeared less metabolically active and less responsive to changes in nutritional status than the smaller depots. The metabolic difference between small and large depots may represent an efficient way to combine the storage of large quantities of lipid with rapid, accurate response to small changes in nutritional status in animals. The capacity for a high rate of FA / TAG cycling allows the intermuscular adipose tissue to respond rapidly to changes in energy flux. This metabolic scenario, however, constitutes a futile cycle which is energetically expensive to sustain and generates significant quantities of heat (Newsholme, 1977). If these activities are confined to a few small depots, energy economy can be combined with responsiveness (Pond, 1992). Therefore, the smaller depots appear to contribute more to routine glucose and lipid homeostasis than the more massive depots, such as the s.c. or abdominal depots.

Production and Remodeling of Fatty Acids in the Rumen

In the non-ruminant animal, the processes of digestion and absorption of dietary fats occur essentially in the small intestine. As well, the FA composition of circulatory lipids are readily influenced by FA composition of the diet (Savary and

Constantin, 1967). This is not the case in ruminants. In the ruminant animal, the events that occur in the rumen environment have a profound effect on the chemical nature of lipids presented to the small intestine for digestion (Christie, 1981). The rumen is by far the largest chamber in the digestive system, and in the adult bovine can comprise up to 64 - 69% of the total weight of the four compartments (Phillipson, 1970). The rumen of the adult bovine typically contains 30 - 60 kg of material along with of a vast population of bacteria and protozoans. The rumen is the major site of pregastric microbial fermentation of cellulose and other plant polymers not normally hydrolyzed by mammalian digestive enzymes. Under normal feeding conditions, it is estimated that the rumen fluid contains approximately 10^{11} bacteria and 10^{6} protozoa per mL (Harfoot *et al.*, 1973). Two major processes that occur within the rumen have an important impact on the composition and distribution of the lipid contents of the digesta. These processes are the lipolysis of dietary lipids and biohydrogenation of the unsaturated FA constituents (Christie, 1981). Short-chain (C_2 - C_5) acyl-chains, known as volatile FAs, are generated in the rumen (Christie, 1981). Most of these acyl chains (up to 80 %) are absorbed directly from the rumen (Watson *et al.*, 1972). Unesterified FA, which under normal circumstances comprise only a minor proportion of dietary lipid intake, become the predominant fraction of the rumen. Stearic acid constitutes by far the major FA present whereas dietary linoleic and linolenic acids are reduced to minor proportions (Harfoot *et al.*, 1973).

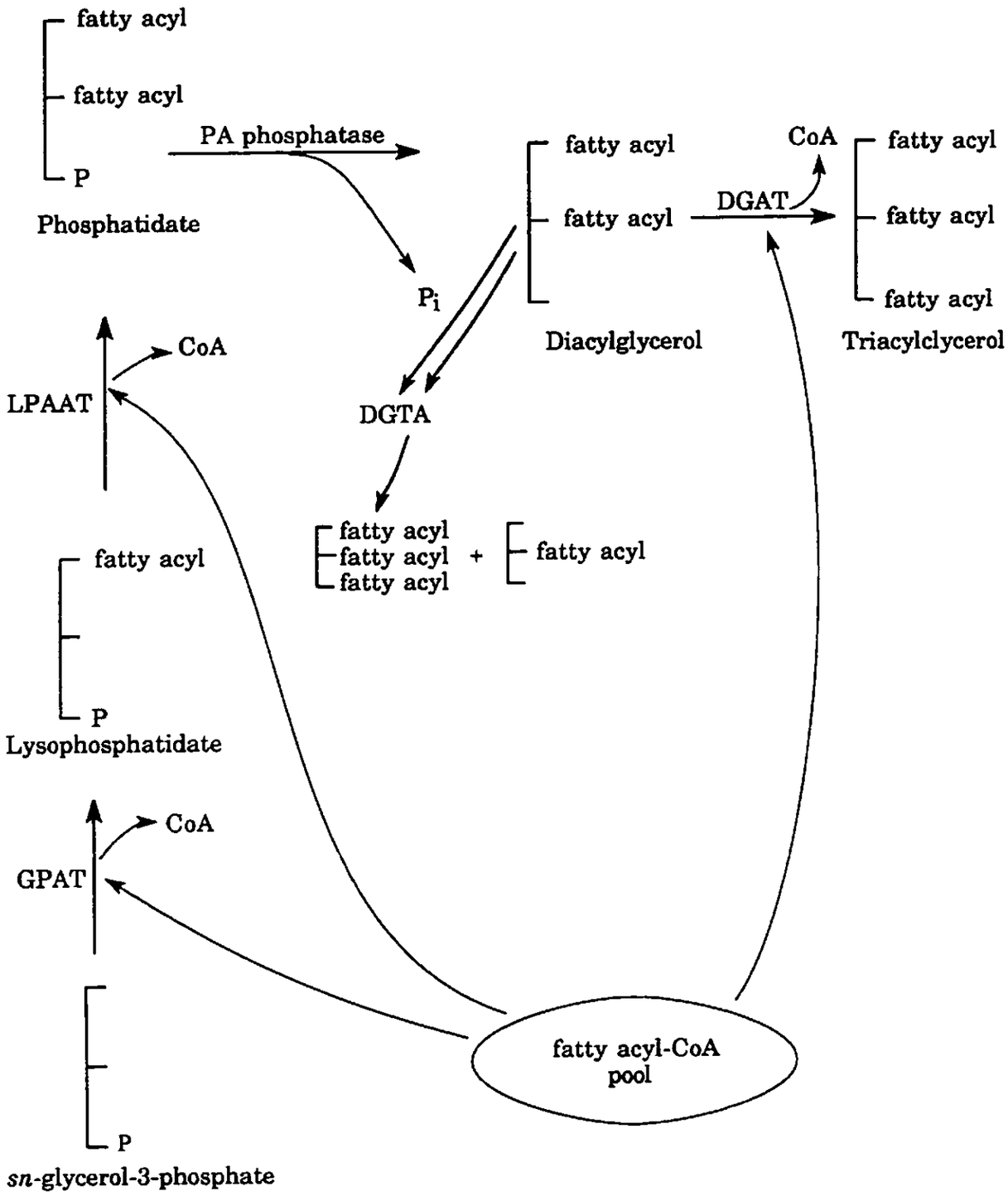
Pathways for TAG Biosynthesis in Mammalian Tissues

The basic structure of TAG consists of a glycerol backbone to which three FAs are esterified at the *sn*-1, *sn*-2 and *sn*-3 carbon positions of the glycerol molecule (Gurr and Harwood, 1991). The glycerol molecule does not exhibit rotational symmetry and thus carbon atoms are classified by stereospecific numbering (*sn*). The composition of FAs in TAG determines its fluidity. A variety of FAs, in different combinations, are commonly found in mammals. The most abundant FAs found to occur in TAG in the bovine are palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acid (Miller *et al.*, 1981; Gurr and Harwood, 1991).

Fatty acid precursors for use in TAG bioassembly are synthesized in the cytosol via the action of acetyl-CoA carboxylase (ACCase) and the FA synthase complex. Acetyl-CoAs are utilized as substrates for FA biosynthesis. The initial step is catalyzed by ACCase (Bloch and Vance, 1977). The carboxylation reaction utilizes acetyl-CoA and HCO_3^- resulting in malonyl-CoA as a product. Malonyl-CoA is then utilized by FA synthase as a carbon donor for the elongation of the FA chain. The acyl chain is lengthened 2 carbons at a time and termination in mammalian systems typically results in free palmitic acid (Gurr and Harwood, 1991). The acyl chain may be further lengthened to form stearoyl-CoA via elongase action (Wakil *et al.*, 1983). A desaturase may catalyze the introduction of a double bond into the acyl chain, typically between the 9^{th} and 10^{th} carbons in mammals to generate for example, oleoyl-CoA (Gurr and Harwood, 1991).

The main pathway of TAG biosynthesis follows the stepwise acylation of *sn*-glycerol-3-phosphate (G3P) to produce TAG as shown in Figure 2 (Kennedy, 1957;

Figure 2. Schematic depicting mammalian TAG biosynthesis. Adapted from Kocsis and Weselake (1996) and Lehner and Kuksis (1993).


fatty acyl
fatty acyl
P
Phosphatidate
PA phosphatase
Pi
fatty acyl
fatty acyl
Diacylglycerol
CoA
DGAT
fatty acyl
fatty acyl
fatty acyl
Triacylclycerol
CoA
LPAAT
DGTA
fatty acyl
fatty acyl
fatty acyl
+
fatty acyl
fatty acyl
P
Lysophosphatidate
CoA
GPAT
P
sn-glycerol-3-phosphate
fatty acyl-CoA
pool

Kennedy, 1961) although some TAG biosynthesis occurs by the esterification of 2-monoacylglycerol. The glycerol backbone of the TAG molecule is derived from G3P which is considered to be generated in the cytosol via the reduction of dihydroxyacetone phosphate (DHAP) or from the direct phosphorylation of glycerol catalyzed by the enzyme glycerol kinase (Gurr and Harwood, 1991). The four main enzymes which catalyze the reactions of TAG biosynthesis (the Kennedy pathway) is located primarily on the cytosolic side of the endoplasmic reticulum (ER) membrane (Kennedy, 1961; Coleman and Bell, 1978).

The first enzyme, G3P acyltransferase (GPAT, EC 2.3.1.15), catalyzes the acylation of G3P at the *sn*-1 position forming lysophosphatidate (LPA). In adipose tissue, GPAT is primarily involved in TAG production, while in the liver both TAG and phospholipids are major products (Rule, 1994). GPAT was purified from *E. coli* membranes, resulting in a 83 kDa protein (Scheidler and Bell, 1986). The purified enzyme was found to be inactive unless reconstituted with phospholipid, specifically cardiolipin and phosphatidylglycerol (PG). An 85 kDa GPAT was recently solubilized and purified from rat liver mitochondria (Vancura and Haldar, 1994). This enzyme also required reconstitution with phospholipid for activity.

Lysophosphatidate acyltransferase (LPAAT, EC 2.3.1.51) catalyzes the acylation of LPA at the *sn*-2 position to produce phosphatidate (PA). Yamashita *et al.* (1981) reported on a successful solubilization and partial purification of LPAAT (7.5-fold) from rat liver microsomes using Triton X-100. These investigators were also successful in

resolving the LPAAT from other lipogenic enzymes. Rat liver LPAAT exhibited enhanced specificity for unsaturated FA such as oleoyl-CoA and linoleoyl-CoA over stearoyl-CoA (Yamashita *et al.*, 1981).

Phosphatidate phosphatase (PA phosphatase; EC 3.1.3.4) catalyzes the dephosphorylation of PA to produce inorganic orthophosphate (P_i) and DAG. Two forms of PA phosphatase are believed to be present in mammals. The *N*-ethylmaleimide (NEM) sensitive form requires Mg^{2+} for activity and is postulated to participate in TAG biosynthesis (Brindley, 1984). The enzyme is present in both the soluble and ER fractions. Diacylglycerol production is hypothesized to be regulated by insulin, glucagon, cyclic AMP and the FA induced translocation of the enzyme from the cytosol to the ER (Brindley, 1984). This form of PA phosphatase has not been purified. A NEM-insensitive PA phosphatase, with no requirement for Mg^{2+}, was recently purified 2700-fold from the plasma membrane of rat liver (Waggoner *et al.*, 1995). This form of PA phosphatase, however, is believed to be involved in signal transduction rather than TAG biosynthesis.

Diacylglycerol acyltransferase (EC 2.3.1.20) catalyzes the final step of TAG bioassembly by acylation of DAG at the *sn*-3 position to yield TAG. Diacylglycerol acyltransferase, however, may not be the only enzyme solely committed to TAG bioassembly in mammals. Recently a diacylglycerol transacylase from rat intestine was shown to catalyze a transesterification reaction using two molecules of DAG to form TAG and MAG (Lehner and Kuksis, 1993). Although some progress has been made in the solubilization and partial purification of the Kennedy pathway enzymes, further studies with purified enzymes and isolated genes may be required to elucidate regulatory

mechanisms underlying TAG biosynthesis. The properties of mammalian DGAT will be discussed in more detail in a later section.

Glycerolipids function not only as a storage source for fuel in the form of TAG, but they also serve as the major constituents of biological membranes (Bretscher and Raff, 1975). The biosynthesis of TAG and phospholipids occurs through a common intermediate, PA. For example, membrane phospholipids typically contain a glycerol backbone and FA esterified to the *sn*-1 and *sn*-2 positions. Phospholipids are distinguished from TAG by a polar head group at the *sn*-3 position (Bretscher and Raff, 1975). As in TAG biosynthesis, phospholipid bioassembly begins with the stepwise acylation of G3P at the *sn*-1 and *sn*-2 position of the G3P (Kennedy, 1961). PA, via cytidine diphosphate (CDP)-diacylglycerol, is a key intermediate in the biosynthesis of PG and phosphatidylinositol (PI) (Brindley and Sturton, 1982). It is also the intermediate for the phospholipase D catalyzed hydrolysis reaction which yields PA; PA can then be dephosphorylated by PA phosphatase to produce DAG (Gurr and Harwood, 1991).

Diacylglycerol is required for the formation of important membrane phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Other membrane constituents include sphingolipids, moieties built on the long chain amino alcohol sphingosine instead of glycerol (Fishman and Brady, 1976). Sphingomyelins, the most common sphingolipids, are ceramides having either a phosphocholine or PE moiety. The production of phospholipids represents a branch

point of the Kennedy pathway and thus can affect the production of TAG (Sampath *et al.*, 1981; Tijburg *et al.*, 1989). A progression into the Kennedy pathway, however, would lead to the formation of TAG catalyzed by DGAT.

Properties of Mammalian DGAT

Diacylglycerol acyltransferase catalyzes the last step of TAG biosynthesis in the G3P pathway (Kennedy, 1961). The reaction involves the acyl-CoA dependent acylation of DAG supplied either by esterification of 2-monoaclyglycerol or by the hydrolysis of PA (Lehner and Kuksis, 1996). In addition to being intermediates in TAG biosynthesis, DAGs also serve as metabolic signaling molecules. For example, during signal transduction, DAG can be produced from PC by phospholipase C (Irving and Exton, 1987). Diacylglycerol second messengers become inactivated by conversion to TAG, PA, or PI, or lipolysis to MAG (Lehner and Kuksis, 1996).

In general, DGAT activity displayed a broad optimum pH near neutrality in microsomes from isolated rat adipose cells (Coleman and Bell, 1976) and in rat muscle myotubes (Sauro and Strickland, 1990). Magnesium had a 20-fold stimulatory effect on DGAT activity in rat liver microsomes (Andersson *et al.*, 1994), and a lesser effect in rat adipose microsomes (2 fold) (Coleman and Bell, 1976). The enzyme was also found to be labile at room temperature. Coleman and Bell (1978) reported a 50 % drop in DGAT activity in microsomes from rat adipocytes at 37^{0}C over 10 min. Activity was abolished by boiling.

Diacylglycerol acyltransferase activity of microsome from rat adipose tissue was shown to be dependent on acyl-CoA concentration (Coleman and Bell, 1976). Maximal activation of rat adipose tissue DGAT occurred at 40 μM (Coleman and Bell, 1976) and at 200μM in rat liver (Andersson *et al.*, 1994). Microsomal DGAT from rat adipose and liver tissue was found to display a broad acyl-donor specificity utilizing both saturated and unsaturated acyl-CoAs effectively, except stearoyl-CoA which was utilized at a rate considerably lower than the others tested (Coleman and Bell, 1976; Hosaka *et al.*, 1977).

Ide and Murata (1993) examined the DAG specificity of DGAT from rat liver microsome and found that the degree of unsaturation of the DAG species employed is a determinant of activity. *sn*-1,2-Dioleoylglycerol was utilized preferentially followed sequentially by 1-palmitoyl-2-oleoyl-sn-glycerol, *sn*-1,2-dilinoleoylglycerol (18:2) and 1,2-di-linoleneoylglycerol (18:3). Saturation by DAG occurred at approximately 250 μM (Ide and Murata, 1993), at 100 μM (Coleman and Bell, 1976), and 1 - 2 mM (Yashiro *et al.*, 1996) depending on assay method employed. Mammalian DGAT was highly dependent on exogenous DAG, being stimulated 3-fold in rat parotid gland microsome (Yashiro *et al.*, 1996), 3-fold in rat myotube preparations (Sauro and Strickland, 1990) and 10 to 30 fold (depending on DAG type) in rat adipose microsome (Coleman and Bell, 1976).

Mammalian DGAT has been shown to be sensitive to thiol specific reagents, being inhibited 70 % in isolated rat adipose cells (Coleman and Bell, 1976) and 64 % in rat myotubes (Sauro and Strickland, 1990) by the addition of 1 mM NEM. The addition

of a reducing agent such as dithiothreitol (DTT), at 4 mM stimulated DGAT activity by 3-fold (Hosaka *et al.*, 1977) and could reduce the effect of NEM, suggesting a thiol group is near the active site (Sauro and Strickland, 1990).

The functional size, *in situ*, of rat liver DGAT has been investigated by radiation inactivation (Ozasa *et al.*, 1989). The loss of enzymatic activity from frozen rat liver microsomes was measured following periods of irradiation. The rate of loss was directly related to the mass of the molecules that were involved in the activity or function measured. It was determined that a single unit of about 72 kDa was required for expression of DGAT activity.

In early studies with rat liver microsomes, DGAT was partially purified following solubilization with Triton X-100 and separation by gel filtration chromatography and sucrose density gradient centrifugation (Hosaka *et al.*, 1977). This DGAT was highly sensitive to sulfhydryl-binding reagents, and was stimulated over 4-fold with the addition of 4 mM DTT when compared to preparations without DTT. The enzyme from rat liver microsome also displayed a broad specificity to acyl-CoA substrates utilizing myristoyl-CoA, palmitoyl-CoA, oleoyl-CoA and arachidonyl-CoA efficiently. The broad acyl-donor specificity exhibited by DGAT from rat liver suggested the FA composition at the *sn*-3 position was dependent on the endogenous acyl-CoA pool. Polokoff and Bell (1980) partially purified DGAT from rat liver microsomes following solubilization with cholate. DGAT was purified about 9-fold following chromatography on Sepharose 4B and separation by sucrose density gradient centrifugation. The partially purified enzyme

was stimulated 5-fold by the addition of phospholipids and was strongly dependent on Mg^{2+}. Lehner and Kuksis (1995) isolated an acyl-CoA acyltransferase from a TAG synthetase complex containing acyl-CoA ligase, acyl-CoA acyltransferase, monoacylglycerol acyltransferase and DGAT in rat liver. The complex was purified following solubilization with 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and separation on a Cibacron blue 3GA-agarose affinity column. Polypeptides ranging from 52 - 72 kDa were observed following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

There is only one report, however, on the purification of mammalian DGAT to near homogeneity. DGAT was purified 415-fold from rat liver microsomes following solubilization with CHAPS and purification by gel filtration, Q-Sepharose and immunoaffinity chromatography (Andersson *et al.*, 1994). A fraction of rat liver microsomes prepared by chromatography on Q-Sepharose and Sephacryl S-200 HR was enriched in proteins approximately 70 kDa in size as determined by SDS-PAGE. This was similar to the size of DGAT which was determined by Ozasa *et al.* (1989) following radiation inactivation studies. Thus, the proteins were subsequently used for immunizations and development of monoclonal antibodies. Western blot analysis indicated that one clone reacted with the DGAT-containing fraction from gel filtration chromatography as well as with a 60 kDa protein present in the solubilized microsomal fraction. The monoclonal antibody was used to further purify native DGAT by immunoaffinity chromatography. Following immunoaffinity chromatography, 60 kDa and 77 kDa proteins were identified by SDS-PAGE. The monoclonal antibody reacted

with the 60 kDa protein and not to the 77 kDa protein as determined by Western blot analysis.

Regulation of DGAT activity in Mammalian Adipose Tissue

The majority of TAG biosynthesis in mammals occurs through the Kennedy pathway (Tijburg *et al.*, 1989). There is some debate about which enzyme of this pathway is rate limiting. Brindley (1984) proposed that PA phosphatase is the rate limiting enzyme in the G3P pathway. This theory is supported by observations that PA accumulation varied inversely with TAG biosynthesis and, in general, changes in PA phosphatase activity have been shown to be much greater than those noted by other glycerolipid synthetic enzymes (Brindley and Sturton, 1982; Glenny and Brindley, 1978). Additionally, vasopressin and FA stimulated PA phosphatase activity in the short term causing the enzyme to translocate from the cytosol to the ER (Pollard and Brindley, 1984). Over the long term, the synthesis of PA phosphatase was stimulated by glucocorticoids and inhibited by insulin (Lawson *et al.*, 1982).

Mayorek and Bar-Tana (1985) reported inhibition of DGAT in rat hepatocytes using the inhibitor 2-bromooctanoate. Inhibition of DGAT resulted in an increase in DAG at the expense of TAG synthesis. Later Mayorek *et al.* (1989) conducted a study measuring the inhibition of overall synthetic flux of TAG in intact and permeabilized rat hepatocytes. Utilizing the same inhibitor, under saturating glycerol and oleate substrate conditions, the investigators demonstrated the flux-control coefficient to be 0.76 and

0.99 in intact and permeabilized rat hepatocytes, respectively. Thus, the investigators concluded that for rat liver, under the experimental conditions employed, DGAT is the rate-limiting enzyme for TAG synthesis in the G3P pathway. The debate over which enzyme is rate limiting in TAG biosynthesis, however, is ongoing.

Lipids may modulate DGAT activity *in vivo*. Haagsman *et al.*, (1981) observed a 3.5 fold increase in DGAT activity when rat liver microsomes were preincubated with 200 μM oleate and 200 μM palmitate. Hillmar *et al.* (1983) incubated rat liver microsomes with various FAs (500 μM) for 72 hours. Each FA had a stimulatory effect. In decreasing order, the stimulatory effects were oleate 191%, linoleate 161%, linolenate 148 %, stearate 124%, palmitate 116 % and arachidonate 114 % over control values. Lysophosphatidylcholine was shown to have a 2-fold stimulatory effect on DGAT at 75 μM, but inhibited enzyme activity at higher concentrations with complete inhibition at 500 μM (Sampath *et al.*, 1981).

Treatment of adipocyte microsomes with cytosolic fractions resulted in reduction of DGAT activities by 30 - 40 % (Rodriguez *et al.*, 1992; Haagsman *et al.*, 1982) similar to that observed for GPAT from rat adipose tissue when incubated with adeneine tri-phosphate (ATP) (Walsh *et al.*, 1989). The inhibitory effect of the cytosolic factors on DGAT was diminished by heat treatment and required both Mg^{2+} and ATP. Diacylglycerol acyltransferase activity could be restored to control levels by incubation with partially purified protein phosphatase from rat liver (Rodriguez *et al.*, 1992). Further investigation using SDS-PAGE determined the apparent molecular weight of the

inactivating activity to be 68 kDa (Lau and Rodriguez, 1996). The investigators also noted that DGAT inactivation did not occur when ATP was substituted with β,γ-methylene-ATP. As well, the activity was removed with washing of the microsome and was sensitive to tyrosine kinase inhibitors. In addition, the activity could not be replaced by different preparations of protein kinase C and protein kinase A. Collectively, these results suggested that the inactivating activity was a protein kinase and both GPAT and DGAT were regulated by a phosphorylation / dephosphorylation mechanism. Evidence for a similar mechanism in human adipose tissue has been found, and partial purification of the putative cytosolic kinase was achieved with multidimensional chromatography resulting in enrichment of a band at 97 kDa (Baldo *et al.*, 1996).

Diacylglycerols are able to move rapidly across membranes (Gurr and Harwood, 1991) and this has prompted investigators to localize the active sites of DAG utilizing enzymes present in the ER. Previous work indicated that the active site of DGAT was located on the cytoplasmic side of the ER, based upon susceptibility to proteases and non-permeable inhibitors (Coleman and Bell, 1978). More recent work with rat liver membranes has suggested that both lumenal and cytoplasmic sides of rat liver ER have DGAT activity (Owen *et al.*, 1997). The microsomal membrane was permeabilized with taurocholate which resulted in a doubling of DGAT activity, indicating that DGAT activities of approximately equal magnitude exist on either side of the membrane (Owen *et al.*, 1997). The existence of separate pools for TAG and DAG, and multiple sites of TAG synthesis within the ER has been established for some time from kinetic studies on the metabolism of FA *in vivo* and in isolated liver preparations (Fukuda *et al.*, 1982).

Owen *et al.* (1997) postulated that compartmentalization of DGAT represented an added level of control in TAG biosynthesis, separating DAG destined for TAG synthesis and substrate cycling (cytosolic DGAT I) from TAG destined for export via very-low-density lipoproteins (lumenal DGAT II). It should be noted, however, that DGAT is the only Kennedy pathway enzyme that has been postulated to be active on both the lumenal and cytosolic sides of the ER (Owen *et al.*, 1997).

Diacylglycerol Acyltransferase in Muscle Tissue

Traditionally, carbohydrate was long considered to be the predominant fuel utilized by skeletal muscle during work. It is now known, however, that FFAs contribute a significant portion of substrate for oxidation by muscle (Holloszy and Booth, 1976) and that TAG is stored within the muscle cell (Pearsall and Palmer, 1990). The enzymatic regulation of muscle lipid metabolism has not been studied extensively, but some progress has been made. Dyck *et al.* (1997) examined differences in lipid metabolism in skeletal muscle of various fiber types in the rat including fast glycolytic and slow oxidative muscle. Using pulse-chase studies, the investigators showed that incorporation of palmitate into TAG was similar in all types of muscle when expressed as a percentage of total uptake, despite differing muscle oxidative potentials. These results suggested that there are a number of similarities in lipid metabolism among different muscle types within the same animal. Correlative studies have indicated that an intracellular form of LPL may have a role in cardiac and skeletal muscle TAG hydrolysis (Palmer *et al.*, 1987). Evidence for this was provided by the high negative correlation between TAG

content and intracellular LPL activity and sensitivity of LPL to hormonal control (Palmer *et al.*, 1987). Presumably, the role of DGAT in muscle lipid metabolism would be the esterification of excess FFA imported into the muscle cell, and the conversion of signal messenger DAG into TAG (Bocckino *et al.*, 1987; Irving and Exton, 1987). Some researchers have hypothesized that before plasma FFAs can be oxidized by muscle, they must first be esterified into the intracellular TAG pool, then after cytosolic TAG hydrolysis, oxidation of FFAs could occur (Zierler, 1976). This requirement to reesterify FFA before use, along with the need to quickly reesterify second messenger DAG would likely account for the relatively high DGAT activity exhibited in muscle tissue, but this remains to be proven.

Sauro and Strickland (1990) reported DGAT activity in rat myoblasts and myotubes derived from a permanent line of L_6 myoblasts from rat skeletal muscle. This represents one of the few reports on DGAT activity in muscle in the literature. In common with the reports of DGAT activity in adipose tissue, muscle DGAT exhibited a pH optimum near neutrality, preferentially utilized oleoyl-CoA, was stimulated by the addition of exogenous FA, showed similar saturations of acyl-CoA and DAG substrates and showed inhibition to thiol specific reagents and solubilization by nonionic detergents such as Triton X-100 (Sauro and Strickland, 1990). In contrast to adipose tissue DGAT, muscle DGAT from rat myotubes showed only marginal stimulation in response to Mg^{2+} and utilized didecanoylglycerol at a rate equal to dioleoylglycerol.

CHAPTER 2.
GENERAL MATERIALS AND METHODS

Chemicals

[1-^{14}C]Lauric acid 59 (Curie/mole) (Ci/mol), [1-^{14}C]palmitic acid (8.4 Ci/mol), [1-^{14}C]stearic acid (58 Ci/mol), [1-^{14}C]oleic acid (50-56 Ci/mol) and [1-^{14}C]erucic acid (57 Ci/mol) were obtained from NEN-Dupont (Mississauga, ON, Canada). [1-^{14}C]acyl-CoAs were synthesized from radiolabeled fatty acids as described by Taylor *et al.* (1990). Silica gel 60 H was from VWR Canlab (Mississauga, ON, Canada). High performance thin layer chromatography (HPTLC)-Fertigplatten Kieselgel plates were from E Merck, Darmstadt Germany. HPLC-grade solvents were from BDH Inc. (Toronto, ON, Canada). Ecolite biodegradable scintillant was from ICN Biomedicals (Irvine, CA, USA). Authentic methyl esters of straight chain fatty acids, fatty acid free bovine serum albumin, *sn*-1,2-dioleoylglycerol, 1,3-bis [tris(hydroxymethyl)methylamino]propane (Bis Tris Propane), *N*-ethylmaleimide (NEM), succinic acid, *sn*-1,2-dilauroylglycerol, *sn*-1,2-dimyristoylglycerol, *sn*-1,2-dipalmitoylglycerol, *sn*-1,2 -dierucin and all other biochemicals were from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Animals and Preparation of Microsome

Samples of adipose tissue and muscle tissue were obtained from either 50% Wagyu x Angus or 75% Wagyu x Angus, Wagyu x Holstein or Wagyu x Simmental cattle. The correlational study was comprised of 11 males and 11 females raised at the Agriculture and Agri-Food Centre in Lethbridge. Tissues from additional animals were

used in characterization studies. Microsomes for these studies represented composites of four or more animals. All animals were maintained on the same diet and reared in individual pens. The finishing diet consisted of 79% rolled barley and 20% barley silage on a dry matter basis, with 1% mineral mix. The composition of the mineral mix per 100 kg was as follows: white salt 47.7 kg; dicalcium phosphate 15.0 kg; limestone 15.0 kg; dynamate 10.0 kg; zinc sulphate 1.3 kg; manganese sulphate 1.5 kg; copper sulphate 492 g; ethylene-diamine-dihydroiodide (EDDI) (80%) 7.8 g; sodium selenite 5.5 g; colbalt sulphate 6.0 g; vitamins A, D and E 9.0 kg. The vitamin concentrations were: A = 10 x 10^6 IU/kg, D = 1 x 10^6 IU/kg, E = 100,000 IU/kg. Time of slaughter was determined by a combination of liveweight (75% Wagyu > 370 kg; 50% Wagyu > 400 kg) and backfat thickness as detected by ultrasonography (> 10 mm). Animals were slaughtered between the ages of 509 and 560 d.

Subcutaneous adipose tissue from the brisket and samples of the *pars costalis diaphragmatis (p.c.d.)* muscle were obtained within 30 min of exsanguination and frozen with liquid N_2 to preserve enzyme activity. In addition, *longissimus* muscle was obtained from the 7^{th} to 9^{th} rib section of the carcass 24 h postmortem for the measurement of solvent extractable fat. Tissues were thawed immediately prior to preparation of microsome fractions, and procedures carried out at 4^0C. The *p.c.d.* muscle sample was carefully dissected to separate the muscle and i.m. adipose tissue components. Subcutaneous adipose tissue, i.m. adipose tissue and muscle tissue were all homogenized in three volumes of ice cold buffer containing 250 mM sucrose, 10 mM potassium phosphate pH 7.0, 1 mM DTT, and 1 mM disodium ethylenediamine tetraacetate

(EDTA) (Lozeman *et al.*, 1995). Tissue was homogenized using a Kinematica homogenizer, model PT-10-35 (Brinkmann Inst., Rexdale, ON, Canada) with a 2-cm blade at setting 5 for 30 s and at full speed for a further 30 s. The homogenate was centrifuged at 500 x *g* for 15 min and the supernatant was filtered through four layers of cheesecloth. The supernatant was recentrifuged at 17,300 x *g* for 30 min and again filtered through four layers of cheesecloth. The final centrifugation was at 100,000 x *g* for 1 h. The supernatant was discarded and the pellet was routinely suspended in approximately 0.1 volume (volume/wet wt. of tissue) fresh homogenization buffer, frozen immediately in liquid N_2, and stored at -20°C.

Lipid Analyses

Lipids were extracted from samples of the *p.c.d.* muscle according to the method of Hara and Radin (1978). Approximately 1 g of tissue was ground and extracted with solvent (hexane: isopropanol, 7:2), then evaporated under a stream of N_2 gas to constant weight at 40°C. This value was used to determine total lipid (TL) content of the tissue. The lipid was resuspended in 3.0 mL of hexane and stored at -20°C until further analysis.

Determination of TAG in the *p.c.d.* lipid extract was performed according to the method of Pomeroy et al. (1991) and Weselake et al. (1993). This involved the separation of TAG from other lipid classes by thin-layer chromatography (TLC), followed by methylation, and analysis of the FAME by flame ionization gas-liquid chromatography (GLC). Triacylglycerol content was calculated from total FAME recovered from the GLC

analysis. Separation of FAME was performed on a flame ionization GLC (Hewlett Packard, Mississauga, ON) equipped with a 30 m DB-23 Megabore column (Chromatographic Specialties, Brockville, ON) and using helium as the carrier at a flow rate of 12 mL/min. Temperatures were 180^0 C for the initial 5 min, gradually increased to 230^0 C at 2^0C / min. Peaks were assigned by comparing retention times to FAME standards.

The TL content of the 7th to 9th rib *longissimus* muscle samples were determined in a different lab (Agriculture and Agri-Food Canada, Lacombe, AB). These samples were extracted by the soxhlet extraction method (AOAC 1984) using petroleum ether as the solvent, and TL determined gravimetrically after evaporating the extracting solvent.

DGAT Assay and Protein Determination

Enzyme assays were performed according to Lozeman *et al.* (1995) with some modifications. For the correlational study, 15 µL of microsome were added to 12 x 75 mm disposable culture tubes and desiccated under vacuum at 4^0C. For the characterization of DGAT, 10 µL (2 - 8 µg/µL) of microsome were added to disposable culture tubes and desiccated under vacuum at 4^0C. The desiccated residue was then mixed with 50 µL of benzene containing 1.5 mg *sn*-1,2-dioleoylglycerol/ mL unless otherwise indicated. The benzene was evaporated under a stream of N_2 until completely dry. Thirty five µL stock assay buffer (0.34 M HEPES (pH 7.0), 30 mM $MgCl_2$, 10.0 mg BSA/ mL) was combined with 20 µL H_2O and the mixture sonicated for 10 min. The

reaction was initiated with 5 μL 180 μM [1- ^{14}C] 18:1 -CoA (50 - 56 Ci/mol). Unless otherwise indicated, the reaction was allowed to proceed for 10 min at 30^{0}C. The reaction was terminated by the addition of 10 μL 5% sodium dodecyl sulfate (SDS). Triacylglycerol was resolved from other lipids by TLC on silica gel H, using one ascension of hexane/ether (80:20, v/v). The trioleoylglycerol standard was visualized by iodine vapour and corresponding sections containing TAG were scraped into scintillation vials, 5 mL of scintillation cocktail was added and analyzed for radioactivity with a Philips PW 4700 liquid scintillation counter (Philips Export B.V., Eindhoven, Netherlands). Enzyme assays were performed in triplicate. Protein concentrations of bovine tissue extracts were determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure using BSA as the standard.

Statistical Analyses

Generally the data from the correlational study were not normally distributed, and thus the Spearman Rho nonparametric test of association was used to assess possible relationships between variables. A log transformation was applied to the data so that parametric statistics (Pearson correlation) could be applied. There were no substantial differences found between the two types of analyses (data not shown), subsequently the Spearman Rho results are reported. There were no significant differences in enzymatic activities between samples from male and female cattle, so these results were pooled. Enzyme activity and total lipid data were analyzed using JMP IN program, version 3.1.7© SAS 1996 according to Sall and Lehman (1996).

Chapter 3.

LIPID ENZYMATIC ACTIVITIES AND MARBLING IN CATTLE

Evaluation of Diacylglycerol Acyltransferase as an Indicator of Intramuscular Fat Content in Beef Cattle

Abstract

Improvement of the marbling trait (i.m. fat) is limited by the long period required to raise cattle before marbling can be evaluated. Thus, development of a biochemical or molecular marker for predicting the propensity for cattle to deposit i.m. fat would reduce the time required to improve this trait. In the current study, we investigated diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) as a potential biochemical marker for predicting the deposition of i.m. fat. Diacylglycerol acyltransferase catalyzes the acylation of *sn*-1,2-diacylglycerol to generate TAG. Both the specific activity of DGAT and its activity per unit wet wt. of tissue were determined in microsomal fractions obtained from s.c. adipose tissue, i.m. adipose tissue and muscle tissue of Wagyu cross cattle at slaughter. Muscle tissue and i.m. adipose tissue were obtained from the *p.c.d.* These cattle displayed a wide range of marbling scores ranging from 1 to 8 (Canadian grade). They also displayed a wide variation of TL (gravimetric) at two different anatomical sites. The TL of the *p.c.d.* muscle correlated with the SEL of the *longissimus* muscle ($r = .508$; $P < .05$). Inverse correlations between *p.c.d.* i.m. adipose tissue

DGAT specific activity and % TAG per gram wet wt. ($r = -.547$; $P < .01$) and TL *p.c.d.* (gravimetric) ($r = -.503$; $P < .05$) were found. There was a strong correlation ($r = .735$; $P < .001$) between DGAT activity per unit wet wt. of i.m. adipose tissue and muscle DGAT activity per unit wet wt. suggesting these two depots were related in terms of TAG biosynthetic capacity.

Introduction

Marbling refers to the presence of visually discernible depots of i.m. fat in beef. Marbling has a low but positive relationship with beef palatability (Savell *et al.*, 1987; Wheeler *et al.*, 1994), and grading systems (e.g. U.S.A. and Canada) assign higher quality grades to carcasses exhibiting higher marbling. Attempts to achieve higher levels of marbling often result in the production of beef that is overfat in other adipose tissues, such as s.c. and intermuscular (seam fat) depots. Recently, the U.S. National Beef Quality Audit (1995) reported that there was $137.82 in lost opportunity per steer or heifer slaughtered in 1995 (Smith *et al.*, 1995). Among most important quality concerns defined by this audit were: excessive external and seam fat, low overall palatability, low uniformity of product and insufficient marbling. Therefore, the challenge facing the beef industry is to consistently produce sufficiently marbled beef that is not overfat in other depots.

Marbling is a highly heritable trait in cattle (Shackelford *et al.*, 1994), but improvement for this trait is limited by the long growth periods before marbling can be evaluated. Using marker assisted selection protocols (Lande and Thompson, 1990; Meuwissen and Van Arendonk, 1992), a biochemical or genetic marker that identifies young animals with the potential to deposit marbling fat would enable producers to more quickly select for this trait in the population. Possible markers include lipid biosynthetic enzymes present in i.m. adipocytes. Several studies have attempted to correlate the

activity of fatty acyl synthetic enzymes or binding proteins, with breed type or marbling score (Miller *et al.*, 1991; Moore *et al.*, 1991; May *et al.*, 1994), but did not observe any strong associations. Further studies with different lipogenic enzymes may reveal a potential marker of i.m. fat deposition.

Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the final step in TAG biosynthesis. The reaction involves the acyl-CoA dependent acylation of DAG supplied either by esterification of 2-monoacylglycerol or by hydrolysis of phosphatidate (G3P pathway) (Lehner and Kuksis, 1996). Diacylglycerol acyltransferase represents the only step committed to TAG biosynthesis in the G3P pathway. Furthermore, studies with the inhibitor, 2-bromooctanoate, have suggested that DGAT is a rate limiting step in the synthesis of TAG (Mayorek and Bar-Tana, 1985). In the current study, DGAT activity was investigated as a potential biochemical marker for predicting the deposition of i.m. fat. Tissues from cattle at slaughter were sampled and assayed for DGAT activity. The relationship between DGAT activity of i.m. adipose tissue and other tissues, and the quantity of i.m. fat was explored.

Materials and Methods

Chemicals

[1-^{14}C]Oleic acid (50-56 Ci/mol) was obtained from NEN-Dupont (Mississauga, ON, Canada). [1-^{14}C]Oleoyl-CoA was synthesized from radiolabeled FAs as described by Taylor *et al.* (1990). Silica gel 60 H was from VWR Canlab (Mississauga, ON,

Canada). HPTLC-Fertigplatten Kieselgel plates were from E Merck, Darmstadt Germany. HPLC-grade solvents were from BDH Inc.(Toronto, ON, Canada). Ecolite biodegradable scintillant was from ICN Biomedicals (Irvine, CA, USA). Authentic methyl esters of straight-chain fatty acids, bovine serum albumin, *sn*-1,2-dioleoylglycerol and all other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals and Preparation of Microsome

Samples of adipose tissue and muscle tissue were obtained from either 50% Wagyu x Angus or 75% Wagyu x Angus, Wagyu x Holstein or Wagyu x Simmental cattle. The study was comprised of 11 males and 11 females. All animals were maintained on the same diet and reared in individual pens. The finishing diet consisted of 79% rolled barley and 20% barley silage on a dry matter basis, with 1% mineral mix. The composition of the mineral mix per 100 kg was as follows: white salt 47.7 kg; dicalcium phosphate 15.0 kg; limestone 15.0 kg; dynamate 10.0 kg; zinc sulphate 1.3 kg; manganese sulphate 1.5 kg; copper sulphate 492 g; EDDI (80%) 7.8 g; sodium selenite 5.5 g; colbalt sulphate 6.0 g; vitamins A, D and E 9.0 kg. The vitamin concentrations were: A = 10 x 10^6 IU/kg, D = 1 x 10^6 IU/kg, E = 100,000 IU/kg. Time of slaughter was determined by a combination of liveweight (75% Wagyu > 370 kg; 50% Wagyu > 400 kg) and backfat thickness as detected by ultrasonography (> 10 mm) (Park *et al.*, 1994). Animals were slaughtered between the ages of 509 and 560 d.

Subcutaneous adipose tissue from the brisket and samples of the *p.c.d.* muscle were obtained within 30 min of exsanguination and frozen with liquid N_2 to preserve

enzyme activity. In addition, *longissimus* muscle was obtained from the 7th to 9th rib section of the carcass 24 h postmortem for the measurement of SEL. Tissues were thawed immediately prior to preparation of microsome fractions, and procedures carried out at 4^{0}C. The *p.c.d.* sample was carefully dissected to separate the muscle and i.m. adipose tissue components. Subcutaneous adipose, i.m. adipose and muscle tissue were all homogenized in three volumes of ice cold buffer containing 250 mM sucrose, 10 mM potassium phosphate pH 7.0, 1 mM DTT, and 1 mM EDTA (Lozeman *et al.*, 1995). Tissue was homogenized using a Kinematica homogenizer, model PT-10-35 (Brinkmann Inst., Rexdale, ON, Canada) with a 2-cm blade at setting 5 for 30 s and at full speed for a further 30 s. The homogenate was centrifuged at 500 x *g* for 15 min and the supernatant was filtered through four layers of cheesecloth. The supernatant was recentrifuged at 17,300 x *g* for 30 min and again filtered through four layers of cheesecloth. The final centrifugation was at 100,000 x *g* for 1 h. The supernatant was discarded and the pellet was routinely suspended in approximately 0.1 volume (volume/wet wt. of tissue) fresh homogenization buffer, frozen immediately in liquid N_2, and stored at -20^{0}C.

Lipid Analyses

Lipids were extracted from samples of the *p.c.d.* muscle according to the method from Hara and Radin (1978). Total lipid (TL) from approximately 1 g of tissue was extracted and determined gravimetrically. After the hexane was evaporated under a stream of N_2 gas to constant weight at 40^{0}C, the lipid was resuspended in 3.0 mL of hexane and stored at -20^{0}C until further analysis.

Determination of TAG was performed according to the method of Pomeroy *et al.* (1991) and Weselake *et al.* (1993). Triacylglycerol content was calculated from total FAs recovered from the GLC analysis. Temperatures were 180°C for the initial 5 min, gradually increased to 230° C at 2°C/ min. Lipid samples from the *longissimus* muscle were extracted with the Soxhlet extraction method (AOAC, 1984), and SEL determined gravimetrically. Several *longissimus* muscle samples were also assayed by the Hara and Radin (1978) extraction method, which resulted in the same TL values ($r = .98; P < .05$) as by the soxhlet extraction method (data not shown).

DGAT Assay and Protein Determination

Enzyme assays were performed according to Lozeman *et al.* (1995) with some modifications. In the current assay, 15 µL (1.5 - 8 µg/µL) of microsome were added to 12 x 75 mm disposable culture tubes and desiccated under vacuum at 4°C. The desiccated residue was then mixed with 50 µL of benzene containing 1.5 mg *sn*-1,2-dioleoylglycerol/ mL. The benzene was evaporated under a stream of N_2 until completely dry. Thirty five µL assay buffer (0.34 M HEPES (pH 7.0), 30 mM $MgCl_2$, 10.0 mg BSA/mL) were combined with 20 µL H_2O and the reaction was initiated with 5 µL 180 µM [1- ^{14}C] 18:1 -CoA. Unless otherwise indicated the reaction was allowed to proceed for 10 min at 30°C. The reaction was terminated by the addition of 10 µL 5% SDS. Triacylglycerol was resolved from other lipids by TLC on silica gel H, using one ascension of hexane/ether (80:20, v/v). The silica gel was scraped into scintillation vials, 5 mL of scintillation cocktail added and analyzed for radioactivity with a Philips PW

4700 liquid scintillation counter (Philips Export B.V., Eindhoven, Netherlands). Enzyme assays were performed in triplicate. Protein concentrations of bovine tissue extracts were determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure using BSA as the standard.

Statistical Analyses

Generally the data were not normally distributed, and thus the Spearman Rho nonparametric test of association was used. A log transformation was applied to the data so that parametric statistics (Pearson correlation) could be applied. There were no substantial differences found between the two types of analyses (data not shown), subsequently the Spearman Rho results are reported. There were no significant differences (Table 1) in enzymatic activities between samples from male and female cattle, and thus these results were pooled. Enzyme activities and TL were analyzed using JMP IN program, version 3.1.7© SAS 1996 according to Sa-ll and Lehman (1996).

Results and Discussion

The means ± SE of TL and SEL content, TAG content and DGAT activity are presented in Tables 2 and 3. Marbling scores were assessed by Canadian federal graders based upon visual assessment of the *longissimus* muscle at the $12^{th}/13^{th}$ rib interface after carcass was chilled for 24 h. The possible scores range from no marbling (score =10), to abundant marbling (score = 1).

Table 1. Wilcoxon rank sum test of DGAT activities versus sex

Tissue type	n	Z	P- value
s.c. pmol/min/mg	22	1.050	.2934
s.c. wet wt.	22	.952	.3408
i.m. pmol/min/mg	22	.066	.9215
i.m. wet wt.	22	-.098	.9215
mus pmol/min/mg	22	.591	.5545
mus wet wt.	22	1.08	.2782

TAG = triacylglycerol

DGAT = diacylglycerol acyltransferase

s.c. pmol/min/mg = s.c. microsome DGAT activity (pmol TAG/min/mg)

s.c. wet wt. = s.c. microsome DGAT activity (pmol TAG/min/ g wet wt.)

i.m. pmol/min x mg = i.m. microsome DGAT activity (pmol TAG/min /mg)

i.m. wet wt. = i.m. microsome DGAT activity (pmol TAG/min/ g wet wt.)

mus pmol/min/mg = muscle microsome DGAT activity (pmol TAG/min /mg)

mus wet wt. = muscle microsome DGAT activity (pmol TAG/min/ g wet wt).

Table 2. Summary of lipid analysis from the *pars costalis diaphragmatis* and *longissimus* muscle

Variable	Percent lipid per unit wet wt.	SE	n
Total lipid (*p.c.d.*)	18.23 %	1.26	22
SEL (*longissimus*)	8.82 %	.83	21
TAG (*p.c.d.*)	15.61 %	1.28	22

TAG = triacylglycerol

SE = standard error

p.c.d. = *pars costalis diaphragmatis* muscle

SEL = Soxhlet extracted lipid

Table 3. Summary of mean diacylglycerol acyltransferase enzymatic activities from bovine subcutaneous adipose, intramuscular adipose and muscle microsome.

	s.c. microsome	i.m. microsome	mus microsome
DGAT activity pmol TAG/ min/mg	138.40[a]	111.46[b]	57.70[c]
SE	21.2	24.4	8.7
DGAT activity pmol TAG/ min/g wet wt.	5.74[a]	19.12[b]	14.00[b]
SE	1.1	3.5	2.2
n	22	22	22

[a b] enzymatic activities in the same row with a different superscript differ (Wilcoxon rank sum $P < .05$ Bonfaroni corrected)

TAG = triacylglycerol

DGAT = diacylglycerol acyltransferase

SE = standard error

ranged from a minimum score of 8 to a maximum of 1. Wagyu crossbred cattle were used for this study, and several of these cattle exhibited marbling exceeding that typically seen in slaughter steers and heifers in North America. Wagyu cattle are noted for their marbling ability (Yamazaki, 1981; Lunt *et al.*, 1993). There was, however, a large degree of variation in marbling observed within this group of cattle. A similar variation in the SEL and TL for both *longissimus* muscle and *p.c.d.* muscle was also observed. Total lipid served as an objective estimate of the amount of i.m. fat present in the sample, even though membrane lipids and lipids stored in cells other than adipocytes (e.g. lipids stored in muscle cells) were extracted in the assay.

Soxhlet extracted lipid of *longissimus* muscle ranged from 3.9 % to 22.5 % with a mean of 8.8 % (Table 2). These values agree well with previous estimates of bovine *longissimus* muscle lipid (Savell *et al.*, 1987; Gaskins *et al.*, 1995). In comparison, TL of the *p.c.d.* muscle ranged from 9.2 % to 31.1 % with a mean of 18.2 % (Table 2). The lipid content of *p.c.d.* and *longissimus* muscle samples were determined by different methods (see Materials and Methods), but analysis of some *longissimus* muscle samples by both methods resulted in essentially the same TL values ($r = 0.98$, $P < 0.001$; data not shown). This close agreement is consistent with previous observations that ether extraction or chloroform:methanol extraction of bovine longissimus muscle resulted in very similar TL content values (Marchello et al. 1968). Hara and Radin (1978) stated that the efficiency of lipid extraction using their procedure was very similar to extraction with chloroform:methanol.

The average and SE of DGAT activities isolated from microsomal fractions of

i.m. fat (dissected free of muscle tissue), s.c. fat and *p.c.d.* muscle (dissected free of any fat) are presented in Table 3. DGAT activities are presented on a per mg of microsomal protein (specific activity) or per g wet wt. of tissue (total activity) basis. There was substantial DGAT activity in muscle from the *p.c.d.* (Table 3) relative to that found in s.c. and i.m. adipose tissue. Diacylglycerol acyltransferase activity has been previously reported in skeletal myotubes from rats (Sauro and Strickland, 1990), but the regulation of lipid metabolism in muscle has yet to be elucidated. As expected, there was substantial DGAT activity in the two depots of adipose tissue investigated. Total DGAT activity of i.m. fat on a per g wet wt. basis was higher than that found in s.c. fat (Wilcoxon test; $P < .01$). This may have been indicative of adipocyte size from the respective depots; s.c. adipocytes are generally larger than i.m. adipocytes (Smith and Crouse, 1984; Miller *et al.*, 1991). The activity of DGAT was measured in the s.c. adipose depot and the i.m. depot in order to assess the potential of DGAT as a biochemical marker of i.m. fat deposition, and not of general fat deposition.

Correlation analysis was conducted and results are presented in Table 4. In Canada, as marbling increases, a lower marbling score is assigned to each carcass, and therefore TL was inversely proportional to marbling score. As expected, the correlation between *longissimus* muscle TL and marbling score was highly significant ($r = -.797$; $P < .001$). Sampling occurred between the 7^{th} and 9^{th} rib of the *longissimus* muscle, which was several inches away from the grading site (at the 12^{th} / 13^{th} rib interface). Gaskins *et al.* (1995) reported that correlation values of marbling scores within *longissimus* muscle

Table 4. Correlations of diacylglycerol acyltransferase activities, % total lipid, % triacylglycerol and marbling score

	i.m. w.wt [B]	mus w.wt[C]	s.c. /mg[D]	i.m. /mg[E]	mus /mg[F]	% TL (p)[G]	% SEL (l)[H]	% TAG (p)[I]
s.c. wwt.[A]	.356 (22)	.240 (22)	.675*** (22)	.273 (22)	.103 (22)	-.171 (22)	-.351 (21)	-.139 (22)
i.m. wwt.		.735*** (22)	.336 (22)	.805*** (22)	.575** (22)	-.266 (22)	-.239 (21)	-.245 (22)
mus wwt.			.205 (22)	.504* (22)	.738*** (22)	-.099 (22)	-.299 (21)	-.066 (22)
s.c. /mg				.385 (22)	.223 (22)	.065 (22)	-.252 (21)	.074 (22)
i.m. /mg					.313 (22)	-.503* (22)	-.336 (21)	-.547** (22)
mus /mg						.147 (22)	.023 (21)	.185 (22)
% TL (p)							.508* (21)	.963*** (22)
% SEL (l)								.533* (21)

Table 4 footnote

[A]s.c. wwt. = s.c. DGAT activity (pmol TAG/min/ g wet weight tissue)

[B]i.m. wwt. = i.m. DGAT activity (pmol TAG/min/ g wet weight tissue)

[C]mus wwt. = muscle DGAT activity (pmol TAG/ min/ g wet weight tissue)

[D]s.c. /mg = s.c. DGAT specific activity (pmol TAG/min/ mg protein)

[E]i.m. /mg = i.m. DGAT specific activity (pmol TAG/min/ mg protein)

[F]mus /mg = muscle DGAT specific activity (pmol TAG/min/ mg protein)

[G]% TL *(p)* = Percent total lipid from the *pars costalis diaphragmatis* muscle

[H]% SEL *(l)* = Percent total lipid from the *longissimus* muscle

[I]% TAG *(p)* = Percent triacylglycerol from the *pars costalis diaphragmatis* muscle

DGAT = diacylglycerol acyltransferase

*P < .05 ** P < .01 *** P < .001

(22) = number of observations

tend to decrease as the sampling site is further removed from the grading site. The *p.c.d.* muscle TL was also negatively correlated with marbling score ($r = -.590$; $P < .01$) and positively correlated with *longissimus* muscle TL ($r = .508$; $P < .05$). In the current study, the *p.c.d.*. muscle was selected as a sampling site for DGAT analysis because it was accessible shortly after exsanguination and damage to the carcass was minimized. Since the correlation coefficient revealed that marbling in the *p.c.d.* muscle was significantly related to that in the *longissimus* sample, the *p.c.d.* muscle may serve as an acceptable muscle for studying the biochemistry of i.m. fat, while maintaining a relationship to marbling at the grading site.

Within tissue type, correlations ($r = .675$, .805, and .738; $P < .001$) were found for DGAT activities per mg microsomal protein versus per gram wet wt. (Table 4) from s.c., i.m. and muscle tissue, respectively. The recovery of microsomal protein from the respective tissue sources was generally similar for all animals, which accounted for the significant association within tissue types. There was a significant correlation observed between DGAT activity from i.m. fat versus DGAT activity from muscle tissue. The correlation coefficient was strongest when comparing DGAT activity on a per g wet wt. basis ($r = .735$; $P < .001$) in the two tissues. The significant correlation in DGAT activity implies a coordinated physiological control of DGAT in i.m. fat and the muscle surrounding i.m. depots. This coordinated regulation, however, was not extended to s.c. adipose tissue, as suggested by the low correlation ($r = .24$; $P > .05$) of DGAT activity in s.c. fat compared to either i.m. fat or muscle. When examined on a wet wt. basis both i.m. and muscle activities were higher with average activities (19.1 and 14.0 pmol /min /

g wet wt., respectively) than those assayed from the s.c. depot (5.74 pmol/min / g wet wt.; Table 2). Other studies have suggested that i.m. and s.c. adipocytes represent two distinct adipose cell types (Smith and Crouse, 1984; May *et al.*, 1994).

The amount of marbling present at slaughter may be related to the activity of DGAT from i.m. fat. The *p.c.d.* TL and percentage TAG per unit wet weight were compared to the activity of DGAT from the i.m. fat of the *p.c.d.* and resulted in weak negative correlations of $r = -.503$; $P < .05$ and $r = -.547$; $P < .01$, respectively (Table 4). This suggested that cattle exhibiting higher marbling levels expressed lower DGAT activity in the i.m. depot. This is in contradiction to the original hypothesis that high DGAT activity in i.m. depots would be significantly correlated to greater levels of marbling. The negative correlation suggested that finished animals have already filled the i.m. depot and the DGAT activity may have been subsequently down-regulated. An examination of cattle at earlier stages of finish would address this issue. The *p.c.d.* muscle, however, would not be accessible in live animals. Examining DGAT activity in younger animals would necessitate biopsies of skeletal muscle as opposed to utilization of *p.c.d.* Assessment of DGAT activity from the skeletal muscle in younger animals may result in a positive correlation between enzyme activity and level of i.m. fat at slaughter.

Implications

This study examined the association of the quantity of i.m. fat present at two different sites, and the activity of a key lipogenic enzyme, DGAT. Total lipid

(gravimetric) from the *p.c.d.* muscle correlated with SEL from the *longissimus* ($r = .508$; $P < .05$). A strong correlation was found ($r = .735$; $P < .001$) between DGAT activity per unit wet wt. from i.m. adipose tissue and muscle DGAT activity per unit wet wt., suggesting these two depots are coordinated in terms of TAG biosynthetic capacity. The negative correlation between i.m. DGAT activity and TL from the *p.c.d.* muscle ($r = -.503$; $P < .05$), however, suggested that DGAT may not serve as a marker for marbling in finished cattle. Perhaps analysis of tissue from young animals before the i.m. adipose tissue accumulates fat would result in a stronger, positive correlation between DGAT activity and TL content. As well, examination of other enzymes of lipid synthesis and mobilization might yield significant correlations with the amount of marbling or TL / TAG content in a given animal.

Chapter 4.
LIPID ENZYMATIC ACTIVITIES IN BEEF CATTLE

Characterization of Diacylglycerol Acyltransferase in Subcutaneous, Intramuscular and Muscle Tissue in Beef Cattle

Abstract

Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the acylation of DAG to generate TAG. Microsomal DGATs from bovine s.c. adipose, i.m. adipose and muscle tissue were partially characterized and compared in order to determine the existence of possible isoforms of DGAT. Diacylglycerol acyltransferase activity from the three tissues exhibited pH optima near neutrality and similar dependence on magnesium ion concentration (40 mM). Diacylglycerol acyltransferase from s.c. adipose tissue, however, exhibited an enhanced preference for substrates containing oleoyl moieties as compared to the other two tissues. Sensitivity to NEM and temperature lability were also examined, with slight differences noted between DGAT activity from s.c. adipose versus i.m. adipose and muscle tissue. The data suggested that either a different isoform of DGAT is expressed in s.c. adipose tissue as compared to the other two tissues or that ER membrane micro-environments are sufficiently different to account for the different properties of DGAT.

Introduction

Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the final step in TAG biosynthesis. The reaction involves the acyl-CoA dependent acylation of DAG which is supplied either by esterification of 2-monoacylglycerol, or by hydrolysis of phosphatidate (G3P pathway) (Lehner and Kuksis, 1996). Diacylglycerol acyltransferase represents the only step committed to TAG biosynthesis in the G3P pathway. There have been conflicting reports about the regulatory roles of DGAT and phosphatidate phosphatase. Researchers have postulated that phosphatidate phosphatase is rate-limiting in TAG biosynthesis (Brindley, 1984). In contrast, flux studies with the inhibitor, 2-bromooctanoate, have suggested that DGAT is a rate limiting step in the synthesis of TAG (Mayorek *et al.*, 1989). DGAT has been studied in animals (Tijburg *et al.*, 1989), higher plants (Little *et al.*, 1994) and in microorganisms (Christiansen, 1979) but little is known about its molecular structure and regulation.

Diacylglycerol acyltransferase may contribute to the FA composition of TAG based on its selectivity for acyl donors. For example, under certain conditions, DGAT from seeds of oilseed rape is more active with erucoyl-CoA than oleoyl-CoA (Cao and Huang, 1987). In contrast, DGAT from rat liver microsomes has been shown to have a broad acyl-donor specificity, such that the fatty acid incorporated at the *sn*-3 position was largely dependent on the fatty acyl pool available to the enzyme (Hosaka *et al.*, 1977).

The quality of beef as a food is partly determined by both the amount and the

composition of fat (May *et al.*, 1993). Excess fat is undesirable and costly. The presence of a small to modest amount of i.m. fat (marbling), however, enhances the taste and juiciness of beef (Wheeler *et al.*, 1994). Diacylglycerol acyltransferase may serve as a biochemical marker for the propensity to deposit i.m. fat in the bovine because DGAT is postulated to have a rate limiting role in TAG biosynthesis. Perhaps those animals that deposit greater amounts of i.m. fat, do so because they express greater amounts of DGAT in i.m fat cells or the DGAT isoform found in i.m. adipocytes is more active.

In the current study, microsomal DGAT from s.c. adipose and i.m. adipose tissue was characterized and compared to determine if isoforms of DGAT exist in these two distinct adipose tissue depots. Muscle tissue surrounding the i.m. adipose tissue was also investigated, since muscle often has significant TAG bioassembly activity (Sauro and Strickland, 1990).

Materials and Methods

Chemicals

[1-^{14}C]Lauric acid (59 Ci/mol), [1-^{14}C]palmitic acid (8.4 Ci/mol), [1-^{14}C]stearic acid (58 Ci/mol), [1-^{14}C]oleic acid (50-56 Ci/mol) and [1-^{14}C]erucic acid (57 Ci/mol) were obtained from NEN-Dupont (Mississauga, ON, Canada). [1-^{14}C]acyl-CoA was synthesized from radiolabeled FAs as described by Taylor *et al.* (1990). Silica gel 60 H was from VWR Canlab (Mississauga, ON, Canada). HPLC-grade solvents were from BDH Inc. (Toronto, ON, Canada). Ecolite biodegradable scintillant was from ICN

Biomedicals (Irvine, CA, USA). Bovine serum albumin, Bis Tris Propane, NEM, succinic acid, *sn*-1,2-dioleoylglycerol, *sn*-1,2-dilauroylglycerol, *sn*-1,2-dimyristoylglycerol, *sn*-1,2-dipalmitoylglycerol, *sn*-1,2 dierucin and all other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals and Preparation of Microsome

Samples of adipose and muscle tissue were obtained from a herd of Wagyu crossbred cattle. In general, Wagyu cattle deposit more i.m. fat in proportion to fat in other depots (Lunt *et al.*, 1993), as compared to other breeds common in North America. All animals were maintained on the same diet and reared in individual pens. Age of slaughter was determined by a combination of liveweight (370 - 400 kg) and backfat thickness as detected by ultrasonography (> 10 mm) (Park *et al.*, 1994). Animals were slaughtered between the ages of 509 and 560 d. Subcutaneous adipose tissue from the brisket, (a large s.c. depot located on the underside of the neck) and samples of the *p.c.d.* muscle were obtained within 30 min of exsanguination and frozen with liquid N_2 to preserve enzyme activity. Tissues were thawed immediately prior to preparation of microsomal fractions. Additional procedures were carried out at 4^0C. Samples from four or more animals were combined as a source for microsomes. The *p.c.d.*. sample was carefully dissected to separate the muscle and i.m. adipose tissue components. Subcutaneous adipose tissue, i.m. adipose tissue and muscle tissue were all homogenized in three volumes of ice cold buffer consisting of 250 mM sucrose, 10 mM potassium phosphate buffer pH 7.0, 1 mM DTT, and 1 mM EDTA (Lozeman *et al.*, 1995). Tissue

was homogenized using a Kinematica homogenizer, model PT-10-35 (Brinkmann Inst., Rexdale, ON, Canada) with a 2-cm blade at setting 5 for 30 s and at full speed for a further 30 s. The homogenate was centrifuged at 500 x *g* for 15 min and the supernatant was filtered through four layers of cheesecloth. The supernatant was recentrifuged at 17,300 x *g* for 30 min and again filtered through four layers of cheesecloth. The final centrifugation was at 100,000 x *g* for 1 h. The supernatant was discarded and the pellet was resuspended in approximately 0.1 volume (volume/wet weight of tissue) fresh homogenization buffer, frozen immediately in liquid N_2 and stored at -20^{0}C.

DGAT Assay and Protein Determination

Enzyme assays were performed according to Lozeman *et al.* (1995) with some modifications. In the standard assay, 10 µL of microsome (2 - 8 mg protein/mL) were added to 12 x 75 mm disposable culture tubes and desiccated under vacuum at 4^{0}C. The desiccated residue was then mixed with 50 µL of benzene containing 1.5 mg *sn*-1,2-dioleoylglycerol / mL. The benzene was evaporated under a stream of N_2 until completely dry. Thirty five µL stock assay buffer (0.34 M HEPES, pH 7.0, 30 mM $MgCl_2$, 10 mg BSA / mL) were combined with 20 µL H_2O and sonicated for 10 minutes. The reaction was initiated with 5 µL 180 µM [1- ^{14}C] 18:1-CoA (50 - 56 Ci/ mol). Unless otherwise noted the reaction was allowed to proceed for 10 min at 30^{0}C. The reaction was terminated by the addition of 10 µL 5% SDS. Enzyme assays were performed in triplicate. In testing the effect of NEM, microsomes were preincubated at 30^{0}C for 5 min prior to initiation of the reaction with acyl-CoA. TAG was resolved from

other lipids in the reaction mixture by TLC on silica gel H plates, using one ascension of hexane/ether (80:20, v/v) (Little *et al.*, 1994). After drying, the trioleoylglycerol standard was located with iodine vapor and the corresponding sections containing TAG were scraped into scintillation vials, 5 mL of scintillation cocktail added and analyzed for radioactivity with a Philips PW 4700 liquid scintillation counter (Philips Export B.V., Eindhoven, Netherlands). Activity was determined by quantifying the amount of radioactive product formed (TAG) over a set time period per mg of microsomal protein or per g wet weight of bovine tissue. Protein concentrations of bovine tissue extracts were determined using the Bio-Rad protein microassay (Richmond, CA, U.S.A.) based on the Bradford (1976) procedure using BSA as the standard.

Results

Initially, experiments were performed to determine optimal conditions for the assay of bovine DGAT activity. Assays were conducted with the 100,000 x *g* particulate fraction of homogenized bovine s.c. adipose, i.m. adipose and muscle tissue. Dependence of DGAT activity on protein content of the reaction mixture is shown in Figure 3. The activity followed a linear relationship over a protein content of 20 - 80 μg. Therefore assays were conducted with 20 - 80 μg of particulate protein. The time course of TAG catalyzed by DGAT from s.c. adipose, i.m. adipose and muscle microsome was linear for 10 min (Figure 4). Thus 10 min assays were routinely used in all experiments.

Temperature stability of DGAT in microsomal preparations was tested at 4^0 and 30^0C. Enzyme activities from all three tissues were stable for over 12 h at either 4^0

Figure 3. Dependence of DGAT on microsomal protein (mg) from s.c. adipose, i.m. adipose and muscle tissue. Results are given as means, ± SE, n = 6.

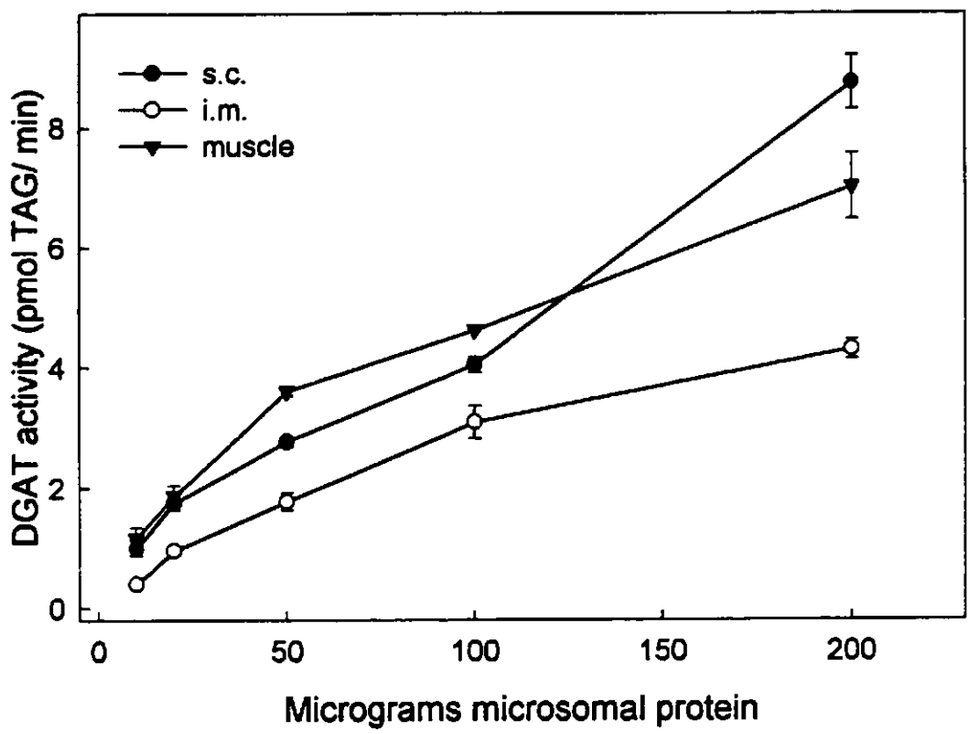
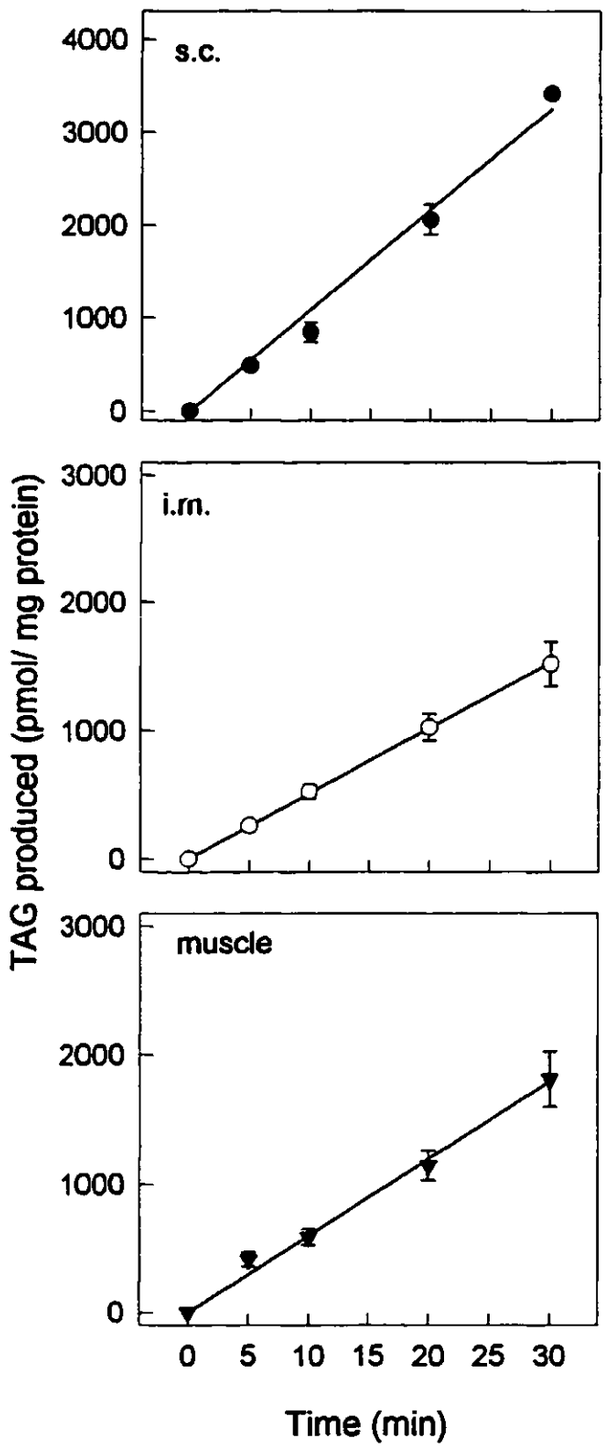

s.c.
i.m.
muscle
DGAT activity (pmol TAG/ min)
0
2
4
6
8
0
50
100
150
200
Micrograms microsomal protein

Figure 4. Time course for the production of TAG catalyzed by microsomal DGAT from s.c. adipose, i.m. adipose and muscle tissue. Results are given as means, ± SE, n = 3.


s.c.
i.m.
muscle
TAG produced (pmol/ mg protein)
Time (min)
4000
3000
2000
1000
0
0 5 10 15 20 25 30

or 30°C (Figure 5) when incubated in assay buffer prior to dessication. The DGAT activity of s.c. adipose tissue decreased markedly after 24 h at 30°C, which was in contrast to the stability of the DGAT activity from i.m. adipose and muscle tissue. Since there was little effect of temperature over the short duration of the assay (10 min), a temperature of 30°C was adopted for all assays.

Optimal DAG bulk concentrations were determined using *sn*-1,2-dioleoylglycerol. In the assay, DAG was dissolved in benzene and added to the desiccated microsome (Lozeman *et al.*, 1995). Immediately following solvent removal, microsomes were resuspended by sonication in DGAT assay buffer. A range of 0 to 5 mM *sn*-1,2-dioleoylglycerol was tested and maximum activity was found at approximately 2 mM (Figure 6). Therefore the bulk concentration of DAG was maintained at 2 mM in other experiments.

The optimal acyl-CoA concentration was determined using oleoyl-CoA. A range of 5 to 100 μM acyl-CoA was tested. Microsomes from the s.c. adipose, i.m. adipose and muscle tissue displayed optimal activity over a concentration range of 60 to 100 μM acyl-CoA (Figure 7). The standard reaction mixture, however, contained 15 μM acyl-CoA. The concentration of acyl-CoA used in our experiments was below the critical micelle concentration of 32 μM for oleoyl-CoA (Smith and Powell, 1985). Presumably a concentration of 15 μM oleoyl-CoA avoided micelle formation and its possible confounding effects on substrate availability.

Figure 5. Stability of microsmal DGAT from s.c. adipose, i.m. adipose and muscle tissue. Microsomes were stored in homogenizing buffer. Results are given as means, n = 5.

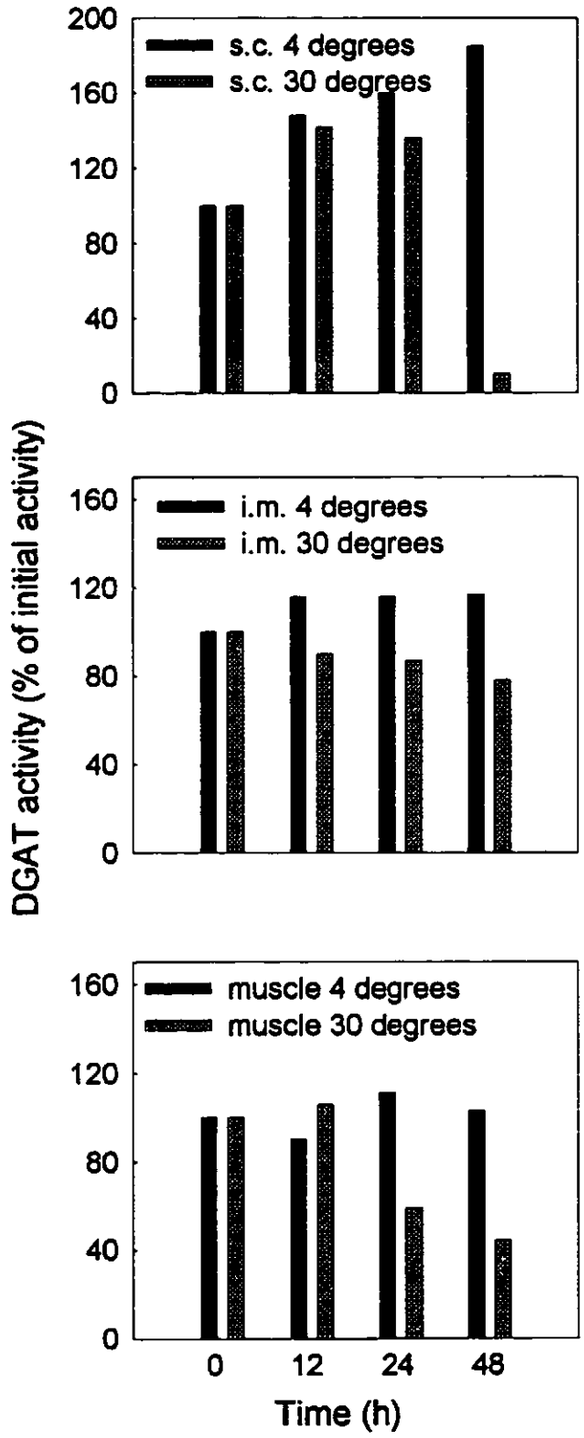

200
160
120
80
40
0
s.c. 4 degrees
s.c. 30 degrees
160
120
80
40
0
i.m. 4 degrees
i.m. 30 degrees
160
120
80
40
0
muscle 4 degrees
muscle 30 degrees
0
12
24
48
Time (h)
DGAT activity (% of initial activity)

Figure 6. Dependence of microsomal DGAT activity from s.c. adipose, i.m. adipose and muscle tissue on bulk DAG concentration (mM). Results are given as means, n = 3.

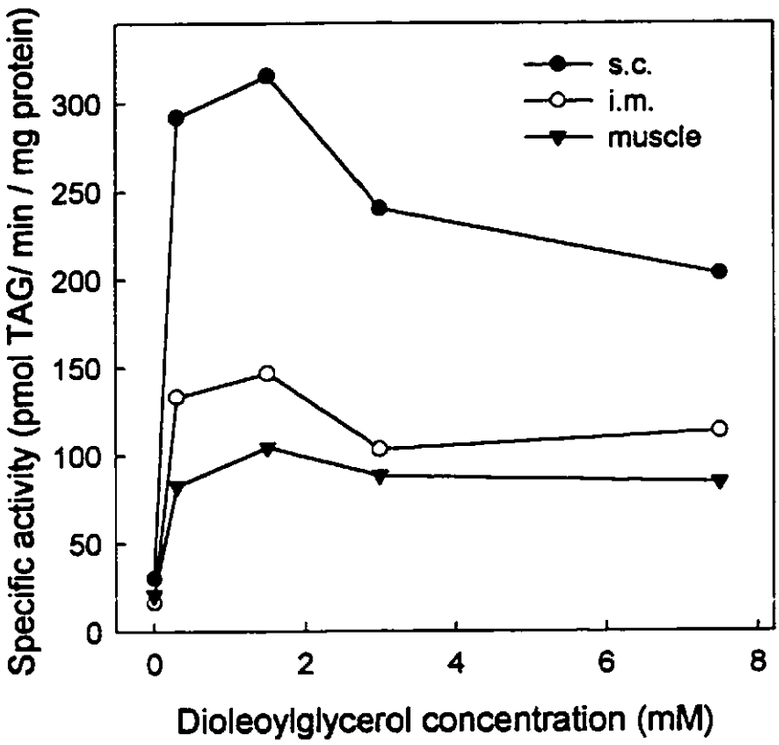
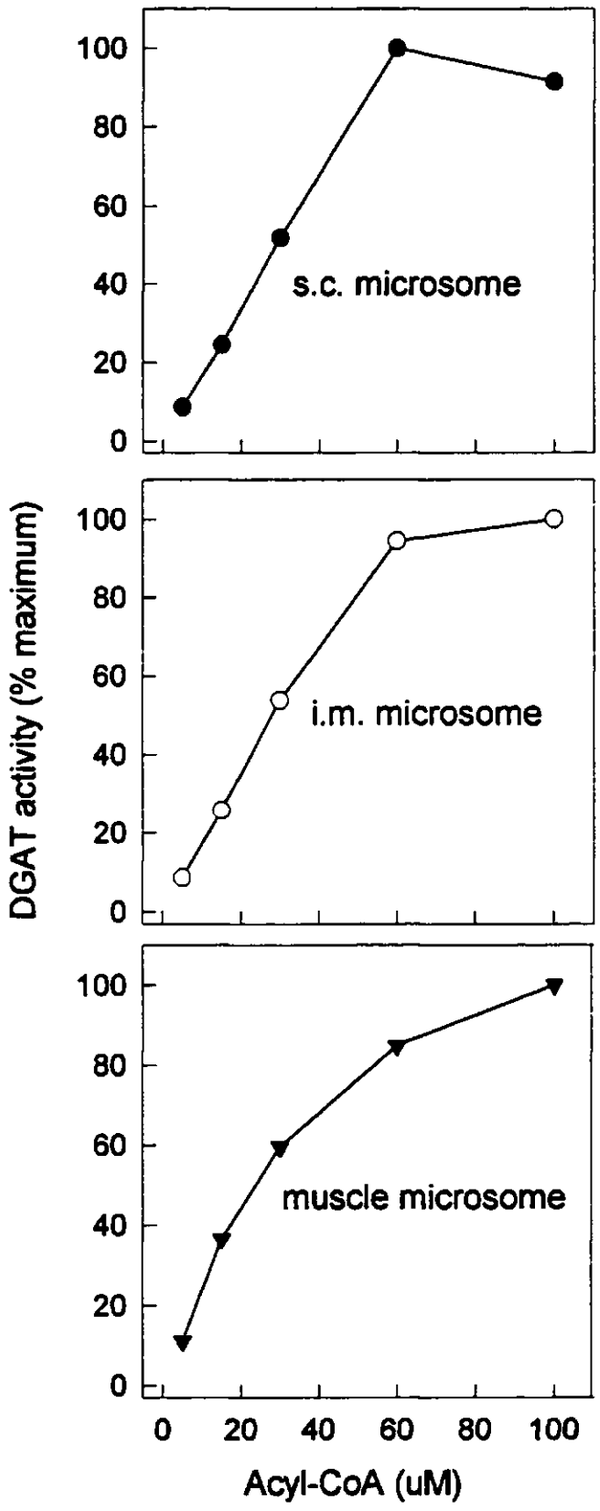

Specific activity (pmol TAG/ min / mg protein)
0
50
100
150
200
250
300
0
2
4
6
8
Dioleoylglycerol concentration (mM)
s.c.
i.m.
muscle

Figure 7. Dependence of DGAT activity from s.c. adipose, i.m. adipose and muscle tissue on the concentration of acyl-CoA (μM). Results are given as means, n = 3.


DGAT activity (% maximum)
100
80
60
40
20
0
s.c. microsome
i.m. microsome
muscle microsome
0
20
40
60
80
100
Acyl-CoA (uM)

Magnesium dependence was examined over a concentration range of 0 to 60 mM. Magnesium ions were most effective in stimulating DGAT activity at a concentration of 40 mM (Figure 8). The response of DGAT activity to Mg^{2+} concentration was similar for the microsomal enzyme from all three tissues. All DGATs showed a strong dependence on Mg^{2+} because activity was stimulated at least 20-fold upon addition of the divalent ion over reaction mixture with no Mg^{2+} added.

The thiol reactive reagent, NEM, had a strong inactivating effect on DGAT activity from all three tissues (Figure 9). Concentrations of NEM tested ranged from 0 to 40 mM. At 5 mM NEM, DGAT activity was reduced 50 to 70 % depending on enzyme source. Results from s.c. adipose, i.m. adipose and muscle microsome gave similar profiles following a 5 min preincubation with NEM, but at NEM concentrations above 20 mM, DGAT activity of the s.c. adipose microsome was not inhibited to the same extent as the i.m. adipose and muscle DGAT activities. At 40 mM NEM, microsome from all tissue types were almost completely inactivated with less than 5 % of control activity remaining.

The pH optima for DGAT was determined using both succinate and Bis-Tris-Propane buffers in order to test a wider range of pH values and to determine if there were any buffer specific effects. Optimal DGAT activity was near neutral pH for all three tissue types (Figure 10).

Acyl-CoA preference was tested with bovine s.c. adipose, i.m. adipose and muscle tissue microsomes using lauroyl-(12:0), palmitoyl-(16:0), stearoyl-(18:0), oleoyl-(18:1) and erucoyl-(22:1) CoA. The results of specificity studies conducted in the

Figure 8. Dependence of microsomal DGAT activity from s.c. adipose, i.m. adipose and muscle tissue on magnesium ion concentration (mM). Results are given as means, ± SE, n = 3.

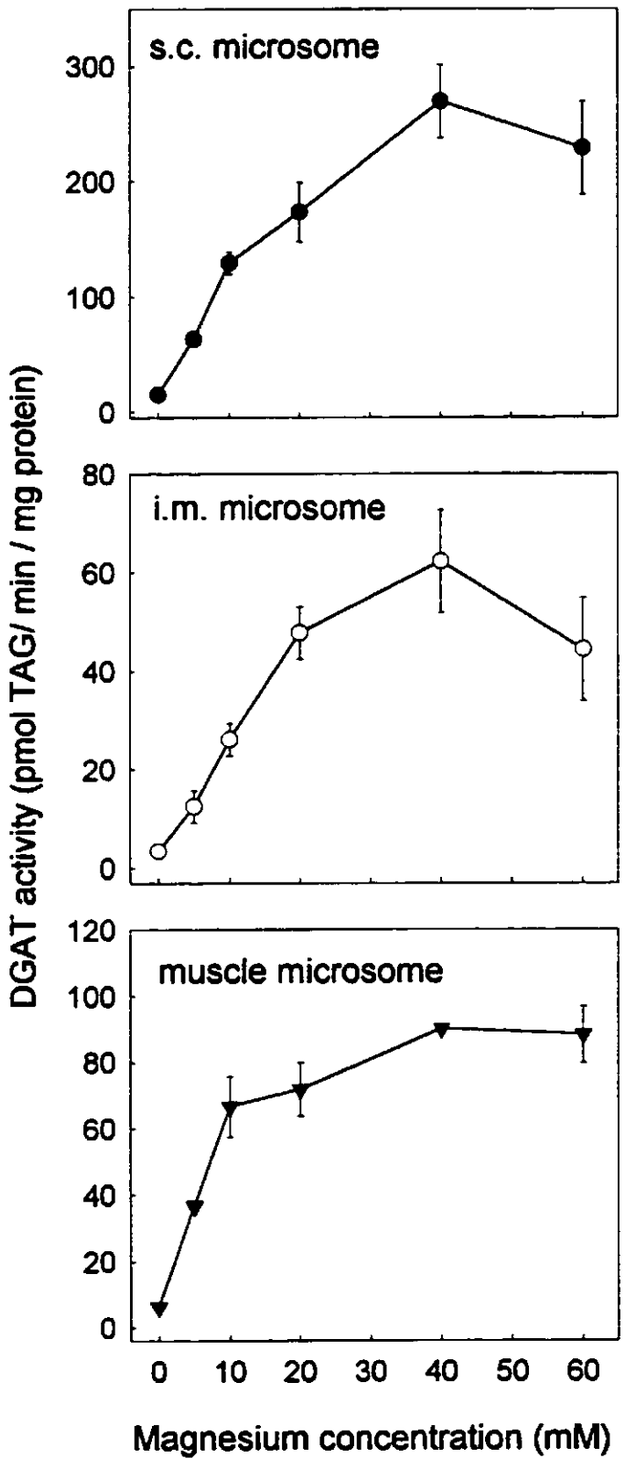

s.c. microsome
i.m. microsome
muscle microsome
DGAT activity (pmol TAG/ min / mg protein)
Magnesium concentration (mM)
0
100
200
300
20
40
60
80
120
10
30
50

Figure 9. Inhibition of DGAT activity from s.c. adipose, i.m. adipose and muscle tissue. Microsomes were preincubated for 5 min with NEM at 30^0C prior to assay. Results are given as means, n = 3.

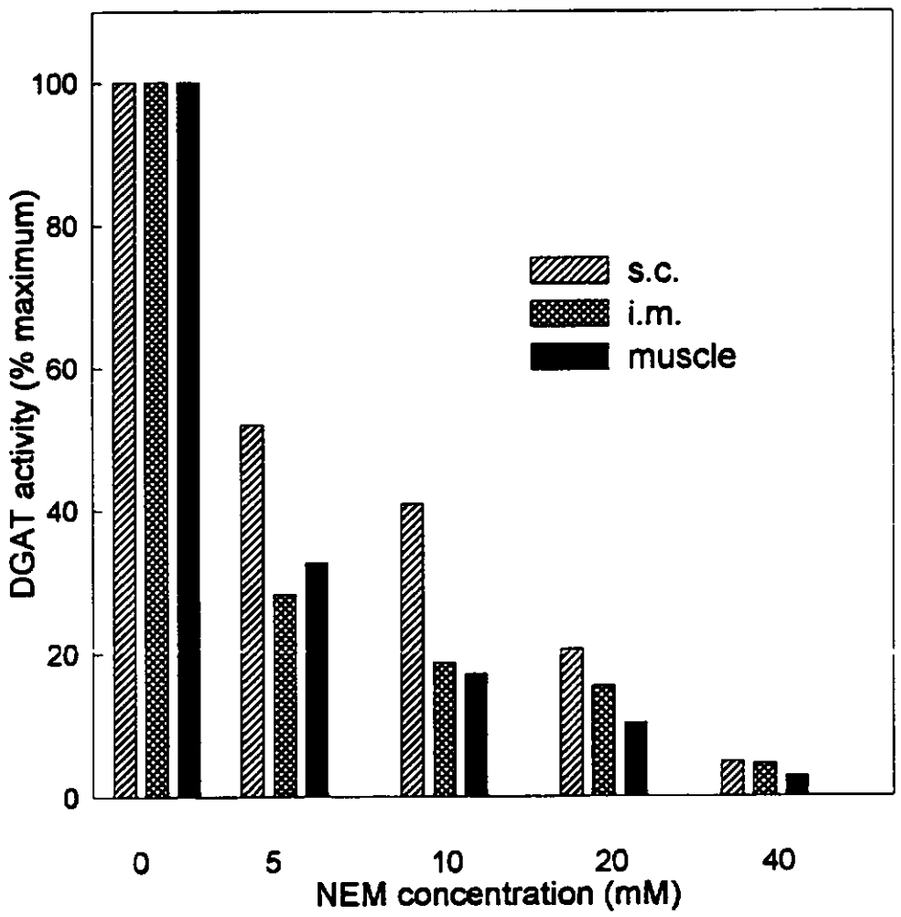
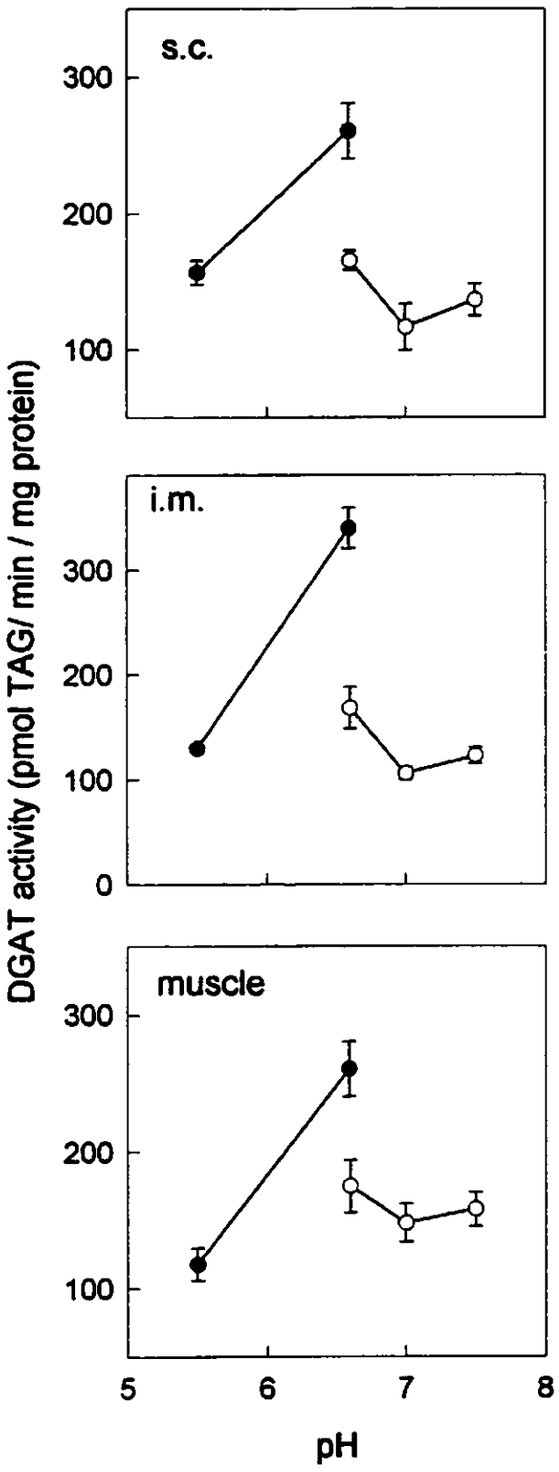

DGAT activity (% maximum)
100
80
60
40
20
0
s.c.
i.m.
muscle
0
5
10
20
40
NEM concentration (mM)

Figure 10. Dependence of microsomal DGAT activity from s.c. adipose, i.m. adipose and muscle tissue on the pH of the assay buffer. ● = succinate buffer (200 mM). ○ = Bis Tris Propane buffer (200 mM). Results are given as means, ± SE, n = 3.


s.c.
i.m.
muscle
DGAT activity (pmol TAG/ min / mg protein)
300
200
100
0
5
6
7
8
pH

presence of 15 mM Mg^{2+} are shown in Figure 11A. In general, DGAT utilized all the acyl-CoAs presented, but DGAT activity was lower in the presence of stearoyl-CoA and erucoyl-CoA. The relatively low specificity for stearoyl-CoA (18:0) was unexpected considering stearic acid is a major constituent of TAG in beef tissues (Miller *et al.*, 1981). There was also a striking tissue specific observation. Diacylglycerol acyltransferase activity from s.c. adipose tissue showed a preference for oleoyl-CoA in the presence of 15 mM Mg^{2+}, a characteristic not seen in either of the other two tissues. When 40 mM Mg^{2+} was used in the assay buffer, the preference for oleoyl-CoA was diminished and lauroyl-CoA was utilized preferentially by DGAT in microsomes from s.c. adipose tissue. Oleoyl-CoA and pamitoyl-CoA were utilized at approximately 70 % of the rate observed with lauroyl-CoA (Figure 11B).

Diacylglycerol specificity of DGAT was also examined using various molecular species of DAG. The DAGs tested were dilauroyl, dimyristoyl, dipalmitoyl, dioleoyl and dierucoylglycerol. The microsomal DGAT from s.c. adipose tissue displayed an enhanced preference for dioleoylglycerol, utilizing this DAG at twice the rate of any others tested (Figure 12). The microsomal DGAT from i.m. adipose tissue utilized dilauroylglycerol at an equal rate to dioleoylglycerol.

Discussion

Utilizing methodology adapted from Slack *et al.* (1985) and Vogel and Browse (1995a), we have optimized a sensitive assay for DGAT activity in the bovine. DGAT

Figure 11 (A). Dependence of microsomal DGAT activity from s.c. adipose, i.m. adipose and muscle tissue on acyl-CoA species. Assays were performed with 15 μM acyl-CoA in the presence of 15 mM Mg^{2+}. Results are given as means, ± SE, n = 5.

B) Dependence of microsomal DGAT activity from s.c. adipose i.m. adipose and muscle tissue on acyl-CoA species. Assays were performed with 15 μM acyl-CoA in the presence of 40 mM Mg^{2+}. Results are given as means, ± SE, n = 5.

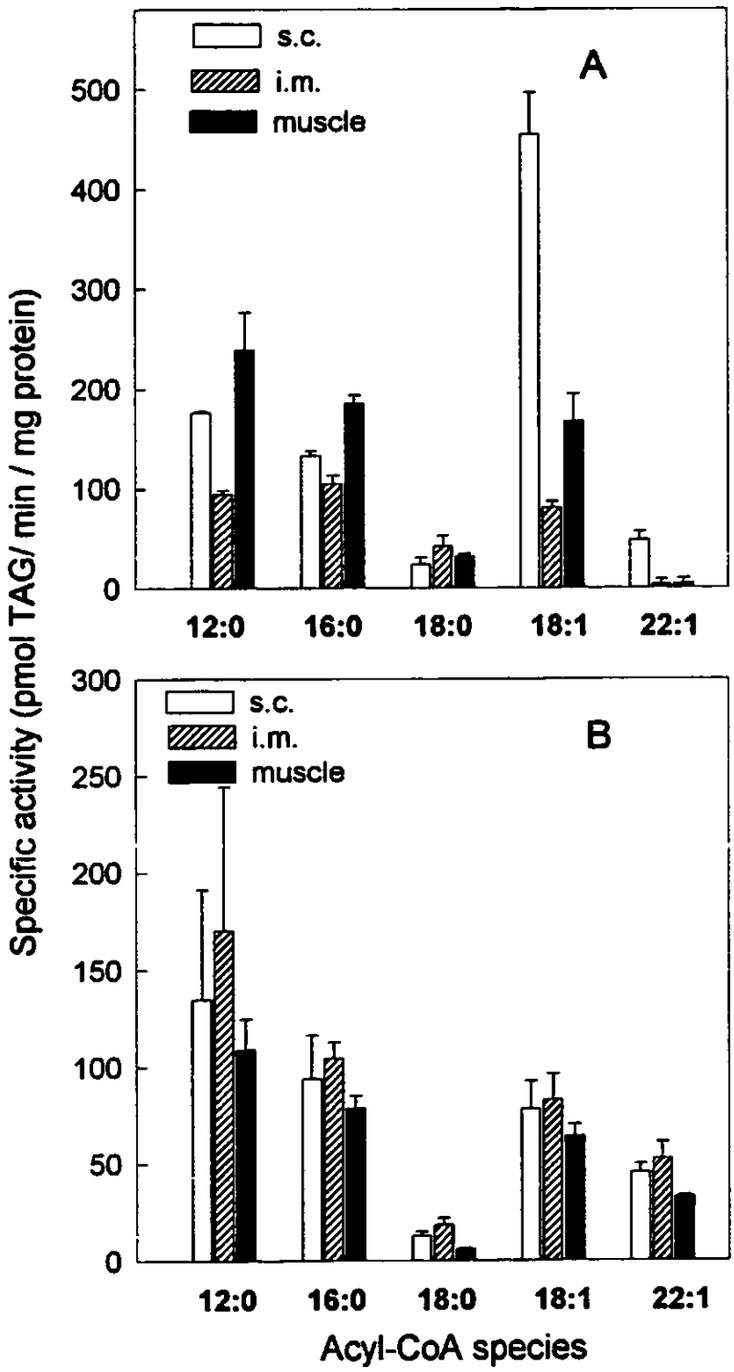
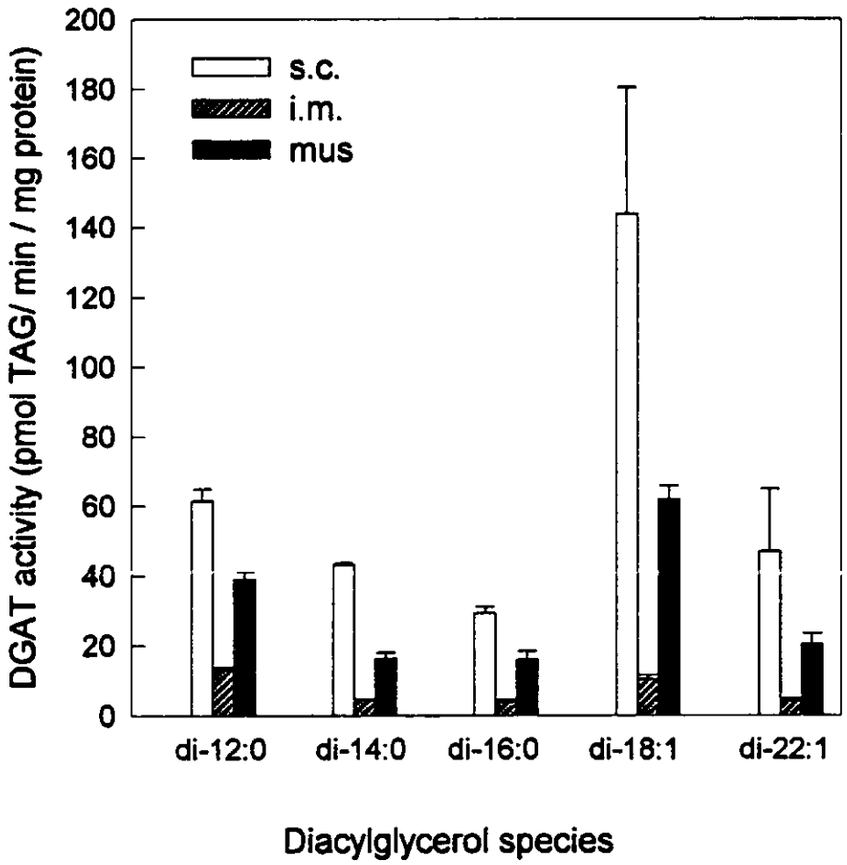

s.c.
i.m.
muscle
A
500
400
300
200
100
0
12:0
16:0
18:0
18:1
22:1
s.c.
i.m.
muscle
B
300
250
200
150
100
50
0
12:0
16:0
18:0
18:1
22:1
Specific activity (pmol TAG/ min / mg protein)
Acyl-CoA species

Figure 12. Dependence of DGAT activity on DAG acceptor species used. Two mM dioleoylglycerol used. Results are given as means, ± SE, n = 3.


DGAT activity (pmol TAG/ min / mg protein)
200
180
160
140
120
100
80
60
40
20
0
s.c.
i.m.
mus
di-12:0
di-14:0
di-16:0
di-18:1
di-22:1
Diacylglycerol species

activity can easily be detected with a low concentration of [^{14}C]oleoyl-CoA and a small assay volume (60 μL) subsequently resulting in a cost-effective assay. This assay was originally developed for the assay of cholinephosphotransferase and introduces DAG dissolved in benzene. The assay gave a strong signal (2000-10,000 dpm) over a low background (60 - 120 dpm) and was relatively simple to perform. Previous use of this assay involved freeze-drying the microsomal preparation before the addition of DAG in benzene solution (Lozeman *et al.*, 1995). In this study, microsomes were desiccated under vacuum at 4^{0}C. Tests performed with s.c. and i.m microsome indicated a 30 - 50 % decline in activity by freeze-drying the microsome as compared to desiccation (data not shown).

Activity from muscle tissue microsomes was relatively high in comparison to that exhibited by microsomes from s.c. and i.m. adipose tissues. Because the dissection of muscle from the i.m fat in tissue samples was methodical, it is unlikely that the activity values seen with muscle microsome were a result of contamination with i.m adipose tissue. Lipid in the form of FFA, is a primary fuel for skeletal muscle during submaximal work (Dyck *et al.*, 1997). Some researchers have hypothesized that before plasma FFA can be oxidized by muscle, it must first be esterified into the intracellular TAG pool. Then after cytosolic TAG hydrolysis, oxidation of FFA would occur (Zierler, 1976). This reesterification of FFA may account for the relatively high DGAT activity noted in muscle tissue. There is, however, relatively little information about the metabolic fate of FFA in muscle. It has been proposed that intracellular stores of FFA are supplied by

plasma FFA derived from chylomicron and very-low-density lipoproteins, TAG from adipose tissue and intracellular TAG (Palmer, 1983).

The response of DGAT activity to several different conditions (including pH, Mg^{2+} concentration and DAG concentration) were similar for all three tissues studied. Bovine DGAT activity was optimal near neutral pH. These values agree with those reported by Coleman and Bell (1976) for DGAT activity in rat adipose tissue and Sauro and Strickland (1990) for DGAT activity in rat myotubes.

The amount of DAG required to saturate the bovine enzyme was considerably higher than what others have reported in the literature using adipose or intestinal tissue from rats (100-300 μM), but the DAG was delivered in either ethanol (Ide and Murata, 1993), acetone (Rodriguez *et al.*, 1992) or CHAPS (Lehner and Kuksis, 1995), and not desiccated before assaying. Enzyme activity was found to be highly dependent on exogenous DAG with only 9 to 19 % of DGAT activity remaining at 0 mM exogenous DAG compared to 2 mM (bulk concentration) exogenous DAG. Ide and Murata (1993) found that dioleoylglycerol was utilized preferentially in rat liver, while other long chain unsaturated DAGs (18:2, 18:3) were utilized at slightly lower rates. Unlike the results obtained by Ide and Murata (1993) saturated medium chain DAGs were utilized adequately by all three DGATs included in this study, with dilauroylglycerol and dimyristoylglycerol being utilized at approximately 40 - 60 % of the rate observed for dioleoylglycerol. Dierucoylglycerol was utilized at a rate comparable to that seen for the saturated DAG. Acyl-CoA acceptor specificity in the bovine was similar to that found in

rat liver (Ide and Murata, 1993) and in rat adipose tissue (Coleman and Bell, 1976), with dioleoylglycerol resulting in maximal DGAT activities under saturating DAG conditions (2 mM bulk concentration). Our data suggested that bovine DGAT has a broad DAG specificity, but microsomes from s.c. adipose tissue displays an enhanced preference for dioleoylglycerol.

Bovine DGAT was also dependent on exogenous Mg^{2+}, with at least 20 fold stimulation in DGAT activity at a 40 mM Mg^{2+} concentration as compared to DGAT activity with no exogenous Mg^{2+}. These results are in agreement with Andersson *et al.*, (1994), who found the optimal concentration of Mg^{2+} to be 50 mM for rat liver DGAT.

Hosaka *et al.* (1977) found that microsomal DGAT from rat liver possessed a broad specificity for acyl-CoAs. This was in contrast to the strict acyl donor specificities of glycerolphosphate acyltransferase and 1-acylglycerolphosphate acyltransferase (Yamishita *et al.*, 1975). Our data suggested DGAT in the bovine also possesses a broad acyl-donor specificity. The pattern of acyl-CoA preference for 12:0-, 16:0-, 18:0- and 22:1-CoA was similar among DGAT from the three tissue types. Diacylglycerol acyltransferase isolated from microsomes of s.c. adipose tissue, however, displayed an enhanced preference for oleoyl-CoA relative to the DGAT from i.m and muscle tissue when 15 mM Mg^{2+} was present. This suggested that s.c. adipose tissue may contain a DGAT isoform that was distinct from the enzymes in i.m. adipose tissue and muscle tissue, or the membrane environment in microsomes from s.c. adipose tissue differed from i.m. adipose and muscle tissue.

When the same experiment was performed with 40 mM Mg^{2+}, the enhanced preference for oleoyl-CoA was diminished and lauroyl-CoA was utilized preferentially, with oleoyl-CoA and palmitoyl-CoA utilized at approximately 70 % of the rate of lauroyl-CoA (Figure 11B). It would appear that the abundance of oleic acid in muscle and i.m. adipose tissue at the *sn* - 3 position (Miller *et al.*, 1981) cannot be accounted for by the acyl-donor specificity of bovine DGAT because the enzyme utilizes several chain lengths adequately. Therefore, it appears that the fatty acid composition at the *sn*-3 position of the TAG in these tissues may be dependent upon the acyl-CoA pool presented to the enzyme. In bovine s.c. adipose tissue, oleic acid is the predominant fatty acid located at the *sn*-3 position of TAG (Christie, 1981). This is consistent with our findings of an enhanced preference of s.c. adipose tissue DGAT for oleoyl-CoA as a substrate.

Sensitivity to NEM also suggested that DGAT from s.c. microsome was slightly different in its properties when compared to either i.m. adipose or muscle tissue microsome. Microsomal DGAT from s.c. adipose tissue was inhibited less by NEM than microsomal DGAT from the other two tissues. In studies with rat skeletal L_6 myotubes, Sauro and Strickland (1990) reported 64 % inactivation with 1 mM NEM. Ditthiothrietol, however, was not present in the reaction mixture. The investigators also noted that the addition of a reducing agent such as DTT stimulated enzyme activity above control levels and could reduce the effect of NEM suggesting the existance of a thiol group near the active site of DGAT. The concentration of approximately 0.1 - 0.3 mM DTT present in our assay was due to carry over of DTT present in the buffer used

to resuspend microsomes. This may explain the need for higher concentrations of NEM to obtain significant enzyme inactivation. Kamisaka *et al.* (1993) reported a 40 % decrease in DGAT activity from *Mortierella ramanniana* fungus membrane fraction at 1 mM NEM, whereas DGAT from the lipid body fraction was stimulated 25 % over control values (Kamisaka and Nakahara, 1994).

In microsomes of s.c. adipose tissue, DGAT activity was consistently stimulated following storage at 4^0C for 12, 24 and 48 h, while the microsome from i.m. adipose and muscle tissue did not display this storage-dependent stimulation to the same extent (Figure 5). In addition, DGAT from s.c. adipose tissue microsome was more labile to higher temperatures than the enzyme in either i.m adipose or muscle microsome. At 30^0C, enzyme activity in microsomes of s.c. adipose tissue declined rapidly between 24 and 48 h, retaining less than 10 % of the original activity after 48 h, while the enzymes in microsome from i.m. adipose and muscle tissue retained 50 % of the initial activity. Coleman and Bell (1976) reported that in rat adipose tissue DGAT activity was unstable at temperatures over 28^0C.

The above observations suggest either the existence of isoforms of DGAT and/or different ER micro-environments for DGAT from s.c. adipose tissue, resulting in altered properties. The activities of enzymes from i.m. adipose and muscle tissue appeared to exhibit similar properties when compared with microsomal DGAT from s.c. adipose tissue. This was in agreement with another study (see Chapter 3) where DGAT activity levels from i.m. fat and its surrounding muscle tissue were highly correlated ($r = .735$; $P < .001$), while the correlation with s.c. adipose tissue was not significant ($r =$

.356; P >.10). These results suggested that there was coordinated control of DGAT from i.m. adipose and surrounding muscle tissue.

Pond (1992) recently summarized some properties of major adipose depots in rodents. Small adipose depots found amongst muscle fibers ("intermuscular" depots) in rodents tended to have much higher FA / TAG cycling rates, and higher lipoprotein lipase, phosphofructokinase and hexokinase activities when compared to other depots such as the mesenteric and epididymal adipose tissue depots. The data suggested that the small intermuscular adipose tissues are different when compared to other adipose tissue types. The main function of intermuscular adipose tissue was to supply fuel to the muscle during exercise while other depots likely functioned in the uptake of dietary lipids (Pond, 1992).

Recently, other researchers have proposed the existence of DGAT isoforms. Owen *et al.* (1997) proposed that there are DGAT activities found on the lumenal as well as the cytosolic side of the ER in rat liver. They have tentatively named these isoforms DGAT I (cytosolic) and DGAT II (lumenal). The authors proposed that this compartmentalization of DGAT activity may serve to add a level of control to hepatic TAG secretion, physically separating TAG synthesis destined for cytosolic storage and substrate cycling and TAG synthesis destined for export as very-low-density lipoprotein.

In summary, bovine DGAT from s.c., i.m. and muscle tissue exhibited optimal activity near neutral pH, could utilize a range of DAG and acyl-CoA substrates containing various FA moieties and had similar requirements for Mg^{2+}. Microsomal

DGAT from s.c. adipose tissue displayed an enhanced specificity for substrates containing oleoyl moieties and was less sensitive to NEM and less stable than the enzymes from the other two tissues. The difference in apparent enzymatic properties may be attributable to the existence of different micro-environments in the ER membrane, or isoforms of DGAT.

Chapter 5.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Investigation of TAG metabolism in tissue depositing i.m. fat may lead to the development of markers for predicting the propensity of cattle to deposit i.m. fat. Diacylglycerol acyltransferase may play a rate limiting role in TAG biosynthesis in mammalian systems (Mayorek *et al.*, 1989; Tijberg *et al.*, 1989), and thus the enzyme may represent a potential marker for predicting i.m. fat deposition in cattle. This thesis has focused on evaluating DGAT as a potential marker for predicting i.m. fat deposition in cattle and on characterization of the enzyme in three tissue types involved in TAG biosynthesis. In our original hypothesis, we assumed that cattle with superiour marbling characteristics would be associated with elevated i.m. DGAT activities. Studies were conducted with Wagyu crossbred cattle displaying a wide range of marbling scores (1 to 8) with an average score of 5.3 and a standard deviation of 1.9 (n = 22).

Microsomal DGAT activity was separately examined in i.m. adipose and muscle tissue procured from the *p.c.d.* (skirt) muscle. Diacylglycerol acyltransferase activity was also examined in s.c. adipose tissue, sampled from the brisket region, to evaluate any association of the amount of marbling with DGAT activity in an alternate adipose tissue depot. Diacylglycerol acyltransferase activity from the three depots did not show positive correlations with the lipid content of the skirt muscle. In fact, microsomal DGAT specific activity of i.m. adipose tissue showed a negative correlation with the lipid

content of the skirt muscle. Our results indicated the amount of i.m. fat present at slaughter was inversely related to the activity of DGAT from i.m. tissue ($r = -.503$; $P = .017$). Microsomal i.m. adipose DGAT activity was reduced with greater amounts of i.m. adipose tissue.

The results suggest that, in cattle displaying superiour marbling characterstics, the filling of i.m. fat depots may result in a decrease of TAG biosynthesis, possibly accomplished by down-regulation of DGAT activity. Perhaps DGAT activity in younger animals, with i.m. depots not yet filled, may show a positive correlation with the lipid content of muscle. Experiments using younger animals would require a sampling site accessible by biopsy, such as a muscle core sample similar to that used by Ishii *et al.* (1992).

Intramuscular adipose tissue may perform a different function compared to other adipose depots, such as the s.c. adipose depot. Pond (1992) suggested the main function of smaller adipocyte depots located within skeletal muscle (termed intermuscular in rat) was to respond quickly to changes in energy flux. Intermuscular adipose tissue accomplished this via a high FA / TAG cycling ratio and high sensitivity to noradrenalin. By confining these attributes to smaller adipose depots, mammals are able to combine energy responsiveness with economy, thus avoiding metabolically wasteful cycles in the larger adipose depots (Pond, 1992). As well, Smith and Crouse (1984) and May *et al.* (1994) investigated the relative insensitivity of marbling scores and lipogenic activities in i.m. adipose tissue to changes in diet. Collectively, these data indicate that the potential

exists to manipulate fat deposition in other depots without adversely affecting marbling scores and palatability.

One might argue that the TAG metabolism in *p.c.d.* muscle does not reflect events in the rib-eye area or grading site. We did, however, show a positive correlation between lipid content of these two tissues. The total lipid present in the *p.c.d.* correlated with the SEL from *longissimus* ($r = .508$; $P = .0188$; Figure 13, Appendix) suggesting that the *p.c.d.* was an acceptable alternative tissue source reflecting marbling at the grading site. Dyck *et al.* (1997) suggested various skeletal muscle types within rats share some common attributes in lipid metabolism. It is possible, however, that the developmental profiles for lipid accumulation in these tissues were different. Nonetheless, effective sampling from the rib-eye area was not possible due to restrictions imposed by grading and health regulations. Any sampling of the ribeye would neccessitate the purchase of the side of beef and cost of the study would escalate rapidly.

A strong correlation was found ($r = .735$; $P < .001$; Figure 14, Appendix) between DGAT activity per unit wet weight from i.m. adipose tissue and muscle tissue. These results suggested that these two depots are related in terms of TAG biosynthetic capacity. This coordinated regulation, however, was not extended to the s.c. adipose depot as suggested by the lack of correlation ($P > .05$) of DGAT activity from s.c. adipose tissue when compared to either i.m. adipose or muscle tissue. The strong correlation between the level of DGAT activity in i.m. adipose and muscle tissue and the lack of correlation with s.c. adipose tissue suggested that there may be differences in the

regulation of DGAT in these compartments and/or the involvement of different isoforms of the enzyme. Thus, the biochemical properties of microsomal DGAT from three depots were examined.

The s.c. adipose DGAT displayed an enhanced preference for oleoyl moieities, was more sensitive to inhibition by NEM, and lost activity more rapidly at 30^0C than the DGAT from i.m. adipose and muscle tissue. The enhanced preference of s.c. adipose tissue for oleoyl-CoA at 15 mM Mg^{2+} was the most striking example of these differences. The enhanced preference of oleoyl moieties suggests that s.c. adipose tissue contained a DGAT isoform that is distinct from those found in i.m. adipose or muscle tissue. When 40 mM Mg^{2+} was present in the assay, however, the s.c. adipose tissue DGAT preference for oleoyl-CoA was replaced by a less distinct preference for lauroyl-CoA and palmitoyl-CoA. It remains unclear why this alteration of specificity occurred. Perhaps the Mg^{2+} concentration affected the solubility of oleoyl-CoA in the reaction mixture. Constantinides and Steim (1986) demonstrated that palmitoyl-CoA solubility decreased rapidly with increasing Mg^{2+} concentration. The change in specificity of s.c. adipose DGAT may be, in part the result of lower oleoyl-CoA solubility and subsequently less substrate is presented to the enzyme. It should be noted that both palmitoyl-CoA (42 μM) and lauroyl-CoA (1130 μM) have higher CMC's than that for oleoyl-CoA (32 μM). Constantinides and Steim (1986) also noted that different buffers, BSA concentration and salt concentration could affect the solubility of palmitoyl-CoA. Due to the possible

confounding effects of Mg^{2+}, BSA, salt concentration and buffer type we chose to conduct our assays at sub-optimal conditions (15 μM acyl-CoA and 15 mM Mg^{2+}).

In conclusion, our results indicate that DGAT activity from tissue of mature beef cattle may not be a suitable marker for i.m. fat deposition in the bovine, despite the negative relationship between i.m. DGAT activity and the amount of i.m. fat present. The study does represent a good starting point for the identification and development of a molecular marker for marbling. To date, much of the mammalian DGAT research has been performed with laboratory rats and a paucity of information existed on DGAT characteristics in the bovine. We have utilized a new, sensitive technique to assay DGAT in beef cattle. The utilization of an alternative tissue site to the ribeye area and freezing of tissue samples allowed us to conduct correlation analysis for a sizable group of cattle in a timely and cost effective manner. It is hoped these techniques can be used in future research for the development of a marker for i.m. fat deposition in beef cattle. We have shown a close association between muscle and i.m. DGAT activities and this may negate the need to carefully dissect these two tissue types in future enzymological studies.

Future studies on other enzymes of lipid metabolism may yield a more suitable marker for i.m. fat deposition. For example, hormone sensitive lipase catalyzes the rate-limiting step in the deacylation of TAG. Perhaps cattle that display high levels of i.m. fat deposition, have a lower level of expression of this enzyme within the i.m. adipose depot or conditions in the i.m. adipose depot result in lower activities of hormone sensitive lipase. Fatty acid composition of the TAG produced in the i.m. depot represents another

potentially useful marker. Preliminary research has indicated that the proportion of certain FAs from bovine skeletal muscle are highly correlated with marbling score. Other researchers within our laboratory are currently examining these possibilities, in the hope of identifying factors associated with i.m. fat deposition in cattle. The fatty composition of lipid extracted from selected Wagyu crossbred cattle is presented in Table 5 (Appendix) as an example.

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APPENDIX

Table 5. Fatty composition of bovine *pars costalis diaphragmatis* muscle from selected cattle

Animal	*140*	*141*	*142*	*146*	*157*
Marbling score	1	4	6	8	3
Acyl moiety	% of total lipid	% of total lipid	% of total lipid	% of total lipid	% of total lipid
14:0	2.65	2.01	2.79	1.59	1.80
14:1	0.36	0.37	0.35	trace	trace
15:0	trace	trace	0.37	trace	trace
16:0	28.6	25.28	27.29	25.52	25.43
16:1	2.17	2.65	2.49	2.09	2.54
17:0	0.95	0.84	1.23	1.07	0.88
18:0	17.56	15.47	18.09	18.65	14.61
18:1 t 11	0.93	0.63	1.49	trace	1.09
18:1 c 9	43.09	49.05	41.17	48.3	48.87
18:1 c 11	1.27	1.51	1.46	1.45	1.74
18:2 c9 c12	0.86	1.18	1.3	1.31	1.12
18:2 t 9 t12	trace	trace	0.28	trace	0.47

Figure 13. Total lipid from the *pars costalis diaphragmatis* muscle versus SEL from *longissimus* muscle, n = 21.

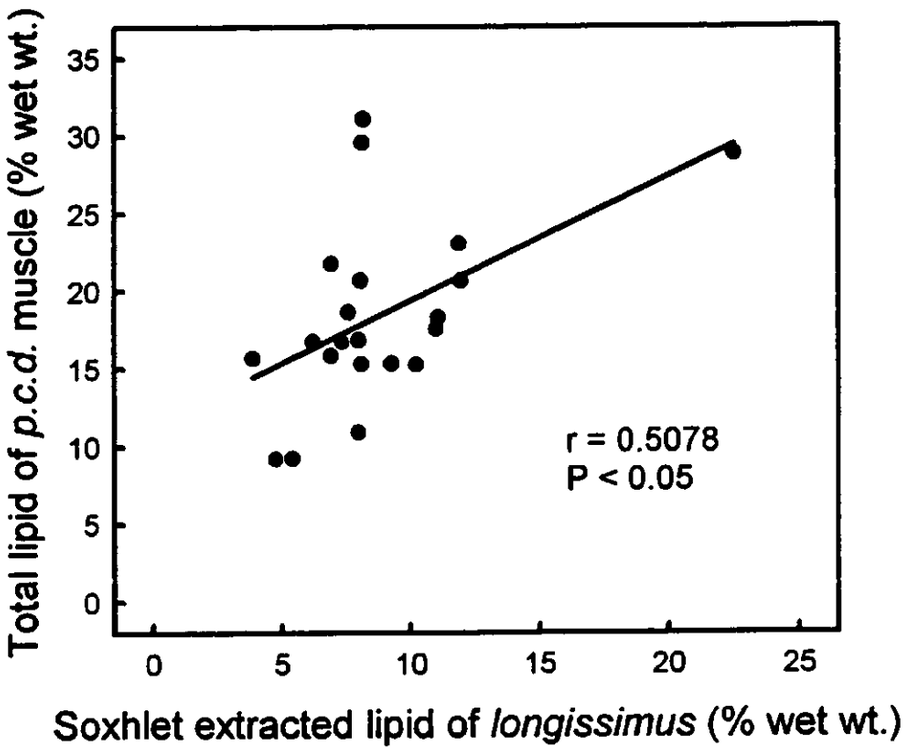
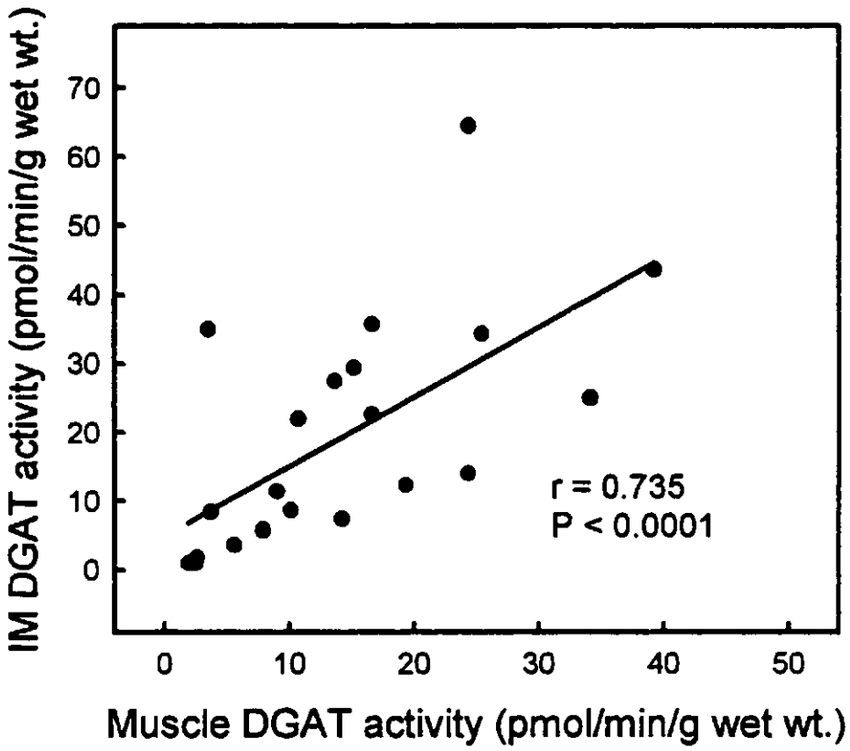

Total lipid of *p.c.d.* muscle (% wet wt.)
35
30
25
20
15
10
5
0
0
5
10
15
20
25
r = 0.5078
P < 0.05
Soxhlet extracted lipid of *longissimus* (% wet wt.)

Figure 14. Intramuscular DGAT activity per unit wet weight versus muscle DGAT activity per unit wet weight, n = 22.


IM DGAT activity (pmol/min/g wet wt.)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
Muscle DGAT activity (pmol/min/g wet wt.)
r = 0.735
P < 0.0001