Regulation of Mammary Stearoyl-CoA Desaturase and the Effects on Milk Fat Composition in Lactating Mice

by

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Errata

Page xi,

'...Bicichoninic acid ...' should read '...Bicinhoninic acid...'

insert

sed is standard error of the mean.

Page 1, paragraph 2, line 5

'...much more...' should read '...made more...'

'...54.' should read '...24.'

Page 3, paragraph 3 line 1

'...random glycerol distribution...' should read '...random distribution...'

Page 13, paragraph 3, Line 1

'...clofibrates...' should read '...clofibrate's...'

Page 22, penultimate line

'...heirachy...' should read 'hierachy..'

Page 23 line 6

'....in the EPA... ' should read '...in that EPA...'

Page 27, line 6

'...prolifelators...' should read '...proliferators...'

Page 28 paragraph 2, line 8

'...half-life of SCD...' should read '... half-life of SCD mRNA...'

Page 29 paragraph 2, line2

'...discountered...' should read '...discounted..'

Page 33, line 5 and paragraph 3 line 7; page 52, line 3

'...by manufacturere...' should read '...by the manufacturer...'

Page 36 line 5

'...for at 480...' should read '...at 480...'

Page 37, paragraph , line 4

'...a further for...' should read '...a further...'

Page 37 last line; 43, last line; caption to Table 3.6; page 52 , line 10

'...data was...' should read '...data were...'

Caption Table 3.6, last line

'...was...' should read '...were...'

Line 6

'...agreement to...' should read '...agreement with...'

Page 68, Line 6

'...avtivity...' should read '...activity...'

Table 5.6, column heading

'Mammary... ' should read 'Liver...'

page 85, Beaulieu reference

'...effcets...' should read '...effects...'

page 86,penultimate line

'...fatyy...' should read '...fatty...'

Page 97, line 13

'...press...' Should read '...pressure...'

Abstract

The research described in this thesis tested, in the lactating mammary gland of female Swiss mice, a model for the control of lipogenesis developed for the liver of male mice. In male mice feeding a fat free diet, or a diet containing 0.5% w/w clofibrate, induces hepatic stearoyl CoA desaturase (SCD) mRNA transcription, which increases SCD activity and the amount of oleate incorporated into membrane phospholipids and the triacylglycerols of liver lipoprotein.

In a preliminary trial, SCD mRNA in liver and mammary gland and fatty acids (FA) in the liver, mammary gland and milk fat were measured in three groups (n=3) of lactating mice fed either a control diet or the control diet with added clofibrate (0.05% w/w) or a fat free diet. Concentrations of SCD mRNA in liver and mammary gland and proportions of individual FA in liver, mammary gland and milk were not significantly different between the control and clofibrate groups. There were, however, positive linear correlations between liver SCD mRNA and hepatic 16:1/16:0 FA ratio ($r = 0.495$, $P<0.05$), 18:1/18:0 FA ratio ($r = 0.520$, $P<0.05$) and milk 16:1/16:0 FA ratio ($r = 0.552$, $P<0.05$).

In a second trial, four groups (n=6) of lactating Swiss mice were used to compare the effect of clofibrate ingestion (control diet v. diet containing clofibrate (0.05% w/w)) and clofibrate injection (olive oil vehicle subcutaneously v. 15 mg clofibrate/100g LW in olive oil subcutaneously) for 7 days. Mammary SCD mRNA, but not liver SCD mRNA, was induced by ingested and injected clofibrate ($P<0.05$), compared to their control treatments. FA composition of liver, mammary gland and milk was not affected by either treatment. Correlations between mammary SCD mRNA and mammary tissue 16:1/16:0 FA ratio ($r = 0.660$, $P<0.05$), and 18:1/18:0 FA ratio ($r = 0.59$, $P<0.05$) were significant in the group ingesting clofibrate. Liver SCD mRNA for both treatments and mammary SCD mRNA for the injected group were not significantly correlated with FA composition. It was concluded that female mice that are lactating may be less sensitive to the effects of clofibrate than male mice.

In the preliminary trial, SCD mRNA transcription was induced ($P<0.05$) 2.1 fold in the mammary gland and 5.3 fold in the liver ($P<0.05$) of the mice fed the fat free diet over the control treatment. Induction of transcription was not transmitted to an effect on the FA composition of the liver, mammary gland or milk. However, there was a trend ($P<0.10$) for

milk 16:1/16:0 FA ratio to be increased in the fat free treatment over the control treatment. Liver SCD mRNA was correlated ($r = 0.552$, $P<0.05$) with milk 16:1/16:0 FA ratio, liver 18:1/18:0 FA ratio ($r = 0.520$, $P<0.05$) and liver 16:1/16:0 FA ratio ($r = 0.61$, $P<0.05$). In a third trial, lactating Swiss mice were allocated to three groups (6 mice/group) which were either fed a fat free diet, a safflower oil diet (25% w/w) or an olive oil diet (25% w/w) over a 7 day period. The safflower oil diet was included because polyunsaturated FA inhibit SCD activity in the liver while a fat free diet stimulates its activity. The olive oil treatment was included as a reference point with which to compare the responses to the other treatments. In the event, the intake of polyunsaturated FA by the mice on this diet may have been sufficient to inhibit the induction of SCD mRNA so that only relative responses between the various diets could be considered.

Mammary SCD and liver mRNA transcription levels were greater in the fat free treatment ($P<0.05$), compared with the olive and safflower oil treatments. Mammary SCD enzyme activity was not significantly affected by treatment., The fat free treatment increased liver 16:1/16:0 FA ratio and 18:1/18:0 FA ratio ($P=0.05$) and the mammary 16:1/16:0 FA ratio ($P<0.05$) but not the 18:1/18:0 FA ratio compared with the other two diets. The olive oil treatment increased palmitoleate and oleate concentration in the liver, mammary gland and milk ($P<0.05$). The increase in the concentration of oleate reflected the composition of the olive oil in the diet. Similarly, dietary intake influenced the significantly greater proportion of linoleate in the milk of the safflower treatment ($P<0.05$). The oleate concentration, 16:1/16:0 and 18:1/18:0 FA ratios were greater ($P<0.05$) in the milk of the group fed the fat free diet than those in the milk of the group on the safflower oil diet. An accumulation of stearate ($P<0.10$), indicating SCD inhibition, was present in the milk of the safflower oil treatment compared to the fat free treatment. The proportion of saturated fatty acids from octanoate to palmitate was greater in the milk from the mice on the fat free diet compared with those on the safflower oil treatment. The proportions of long chain fatty acids of molecular weight greater than linoleate were higher in the milk from the mice fed the diets containing the oils.

Acknowledgements

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Throughout the experience of gaining this qualification, I have met and been befriended by many different people. Most will be remembered for different reasons, but one person who stands out and deserves special mention, is Megan Callaghan. Megan, you have made integration into the lab and Ruakura social life easy. You listened to all my problems, mostly 'non-scientific', gave good advice and even chewed my ear out when it was needed. I hold our friendship in high regard.

Finally, I wish to thank my family. Thank you Mum and Dad for listening to my ceaseless drivel when it seemed all my existence was just about writing this thesis. To my brother, sister-in-law and little Chloe, you guys being over here for Christmas gave me the tonic I needed to finish.

Development of chronic disease depends on genetic responses to the environment for which the diet is a major influence (Paisley et al. 1996). Within the diet, the type of dietary fat has the potential to alter body weight and composition and also influence the onset and progression of various chronic diseases in human beings (Clarke and Jump 1996: Waters et al. 1997). Dietary fat affects membrane composition and fluidity as well as increasing cell metabolism and division rates (Ntambi 1995). The nutritional functionality of fats is influenced by its chain length, the degree of unsaturation, the type of isomer (cis/trans) and the position of the FA on the glycerol backbone (Kaylegian 1995). Saturated FA in the sn-1 and sn-3 positions of TAG can exhibit different metabolic patterns due to their low absorptivity. This means dietary fats with saturated FA in the sn-1 and sn-3 positions (cocoa butter and oil, palm oil) can have different biological consequences than those fats (milk fat) in which the saturated FAs are primarily in the sn-2 position (Decker 1996).

While there is a positive relationship between saturated FA intake and adverse lipoprotein cholesterol concentrations in humans (German et al. 1997; Vanden Havel 1997), this relationship is not seen with mono-unsaturated FA. Therefore, a reduction in palmitate, myristate and laurate while increasing mono-unsaturated FA (eg. oleate) would make milk products, like butter, a more nutritionally attractive product. Unlike other saturated FA, stearates lack of artherogenic properties has been attributed to its rapid conversion to oleate (Decker 1996).

Nutritional guidelines recommend the consumption of 30% or less of dietary energy as fat and less than 10% of dietary energy from saturated FA (Vanden Havel 1997), of which only 20% of New Zealand people meet these recommendations (Metcalf et al. 1998). For this reason butter, with high total and saturated fat content, has suffered strong criticism from the medical community (Jimenez-Flores 1997). Much of this criticism is not warranted. When butter provides 20-40% of dietary energy it is unquestionably hypercholesterolaemic. However, all products (not just butter) based on milk fat, provide only about 5% of dietary energy in a typical Western diet (Fumeron et al. 1991).

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List of Abbreviations

ACC	Acetyl-CoA carboxylase
ACO	Acetyl-CoA oxidase
ANOVA	Analysis of variance
bp	Base pairs
BCA	Bicichoninic acid
CLA	Conjugated linoleic acid
dATP	Deoxyadenine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DEPC	Diethyl pyrocarbonate
DHA	Docosahexanoic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EPA	Eicosapentanoic acid
FA	Fatty acid
FAS	Fatty acid synthase
FABP	Fatty acid binding protein
FAMES	Fatty acid methyl esters
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
LPL	Lipoprotein lipase
LT	Leukotrienes
ME	Malic enzyme
MF	Milk fat
MFGM	Milk fat globular membrane
mRNA	Messenger ribosomal nucleic acid
MOPS	3-[N-morpholino]propane-sulfonic acid
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PG	Prostaglandins
PP	Peroxisomal proliferator

PPAR	Peroxisomal proliferator activator receptor
SCD	Stearoyl-CoA desaturase
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride, sodium citrate buffer
TAG	Triacylglycerol
TCA	Tricarboxylic acid cycle
Thio II	Thioesterase II
UV	Ultra-violet

CHAPTER ONE. INTRODUCTION.

1.1.Dairy Fat.

1.1.1. Milk fat Composition.

Bovine milk is about 10-15% solids of which about a third is milk fat (MF) (Mansbridge and Blake 1997). The variation in the solids content of liquid milk is mainly due to the variability of the fat component of milk. Even though greater variation is shown between species, eg. the cow (about 4% MF), the mouse (10-12% MF) and the rabbit (about 35% MF), the proportion of the different classes of lipids (Table 1.1.) remains similar between species (Dils 1983).

Table 1.1. Classes of Lipids in Whole Bovine Milk.

Lipid	Percent weight
Triacylglycerols	97-98
Diacylglycerols	0.28-0.59
Unesterified sterols	0.22-0.41
Phospholipids	0.2-1.0
Unesterified fatty acid	0.1-0.44
Monoacylglycerols	0.02-0.04
Sterol esters	trace
Hydrocarbons	trace

Adapted from Dils (1983).

The phospholipids and sterols of milk are mainly associated with the milk fat globular membrane (MFGM). The majority of glycerols and fatty acids (FAs) are bound by MFGM (Decker 1996; Griinari et al. 1997). While lipid classes may not vary between species, types of FA do show inter-species variation (Table 1.2). The FAs in milk fat range in carbon chain lengths from 4-54. The composition of FAs is much more complicated by the many isomers that include mono- and multibranched, dienes and trienes, hydroxy and cyclic FAs. This provides over 400 FAs to be available for triacylglyceride (TAG) synthesis (Jimenez-Flores 1997). However, due to the stereospecificity of FA esterification to glycerol, the range of TAG or phospholipids is reduced relative to the potential number if they were distributed at random (Mizuguchi et al. 1996).

In comparing the cow and mouse milk FA profiles, one similarity between the two species (Table 1.2) is that oleate is the most common unsaturated FAs present (German et al.

1997; Kaylegian 1994). This is due to an intra-mammary influence that is described in Section 1.2.3.

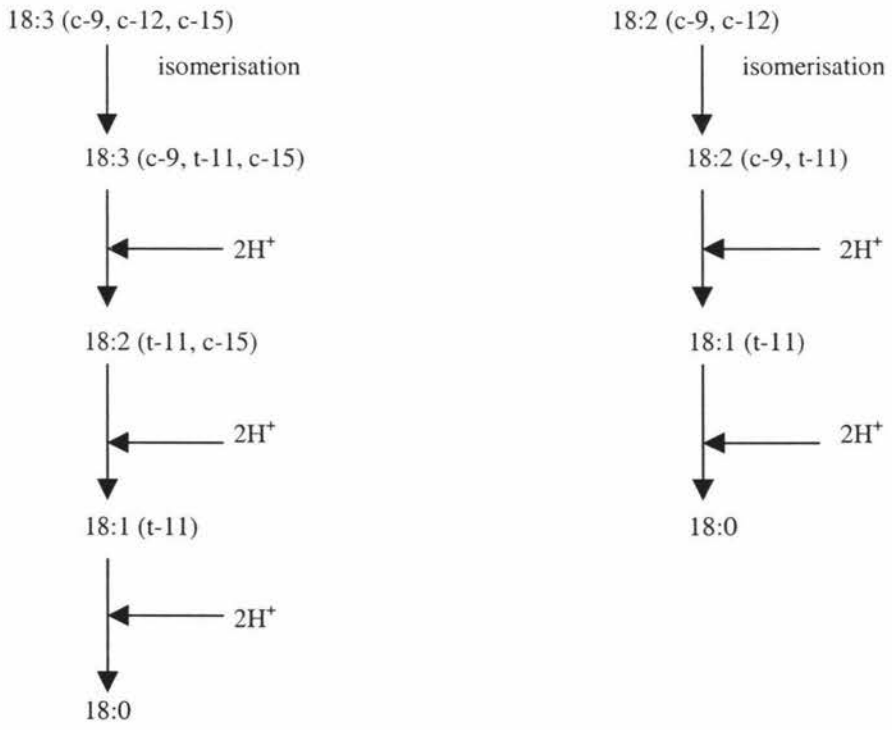
Table 1.2. A comparison of milk composition between the mouse and the cow for some of the main FAs of milk. Values are expressed as mol% of total FAs.

	4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
mice	-	-	trace	6	9	13	33	5	2	29	9	-
cow	12	4	2	4	4	10	24	2	12	24	2	1

Adapted from Dils (1983).

The obvious differences between the two species are the cow has much higher butyrate and hexanoate concentrations; and the mouse has a higher amount of medium chain FAs. These reflect intra-mammary differences in the types of enzyme present (thioesterase II) and types of enzyme reactions (terminating transferase activity) which are outlined in section 1.2. A non-mammary influence, which is seen in Table 1.2, is the high saturated FA and low polyunsaturated FA content of milk in the cow. This is a reflection of rumen hydrogenation.

Figure 1.1. The process of hydrogenation of linoleate (18:2n6) and linolenate (18:3n3) to stearate (18:0), while in the rumen.



While polyunsaturated FA content in the cow is low due to hydrogenation, most of it is linoleate and linolenate, which reflects the high content of these FAs in grass (M^{c}Donald et al. 1996). Within the polyunsaturated FA content of cows' milk the ratio of n-6 to n-3 is 2:1 (Mansbridge and Blake 1997). Several steps of hydrogenation depending on the degree of desaturation (Figure 1.1.) follow the initial step of isomerisation. The last unsaturated bond to be hydrogenated is the trans bond created in the first reaction. Small amounts of FA are not fully hydrogenated so these trans FAs end up in the milk at 1-3%, by weight (Kaylegian 1995; Mansbridge and Blake 1997).

1.1.2. Butter.

The properties of TAGs and phospholipids vary a great deal, depending on their constituent FAs. Plant glycerides tend to be relatively liquid at room temperature due to the preponderance of mono- and polyunsaturated FAs, as well as shorter chain FAs than those usually found in animal glycerides. Partial hydrogenation of vegetable oils, as in the production of margarine, raises the melting temperature by saturating the double bonds (Linder 1991). Functional attributes that are commonly associated with traditional sources of milk fat (milk, cream, butter and anhydrous milk fat) include flavour and flavour potential; and physical properties such as structure formation, hardness, spreadability, layering, shortening and lubricity (Kaylegian et al. 1993). While world-wide butter consumption is steadily declining, total replacement of butter has not occurred. This is mainly due to the combination of flavour and physical properties of milk fat that are not able to be duplicated (Jimenez-Flores 1997). Developments within the market are fuelled both by the need for new, novel and better quality milk products and by increasing consumer awareness of the interaction between diet and health (Mansbridge and Blake 1997). The ability to expand the use of milk lipid components to new areas of application is related to the ability to modify the functionality of components to meet the specifications of the application (Kaylegian 1995).

The FA composition and their non-random glycerol distribution on the glycerol moiety in milk fat is important in its functionality to dairy products, such as butter (German et al. 1997). An example of this is that the high level of saturated FA means butter will not spread when taken from the refrigerator (Mansbridge and Blake 1997). The physical properties of milk fat ingredients are greatly influenced by the inherent chemical composition of the milk

fat, and can be further influenced by processing conditions, including tempering, fractionation and texturisation (Kaylegian 1995). Milk fat is liquid above +40°C and solid below - 40°C, while in between it is a mix of solid fat and liquid oil. Thus milk fat has a melting range (Table 1.3) rather than a single melting point, which is due to the wide array of FAs present. The more desaturated a FA becomes, the lower its melting point (M^{c}Donald et al. 1996). Longer chain saturated FAs increase the melting point, while shorter chain saturated FAs tend to decrease the melting point of milk fat (German et al. 1997). The physical functionality of milk fat is often dependent upon the relative amounts of solid and liquid phases present at any given time and the rate at which this proportion changes (Kaylegian 1995). Long chain saturated FAs that increase the melting range are also responsible for the plasticity of the milk fat. This is a property that enables milk fat to form a self-standing structure at room temperature. Long chain saturated FAs form crystalline arrays that support a much larger volume of liquid oil. While only a minor fraction of milk fat TAG, a decrease in long chain saturated FAs (<18 carbons) would soften milk fat (German et al. 1997), as would any increase in short chain FAs (Holmes and Wilson 1987).

Table 1.3. Melting points of some FAs found in milk fat.

Fatty acid	Melting point (°C)
1) Saturated Fatty-acids	
Butyric (C4:0)	-7.9
Caproic (C6:0)	-3.4
Caprylic (C8:0)	16.7
Capric (C10:0)	31.4
Lauric (C12:0)	44.0
Myristic (C14:0)	58.5
Palmitic (C16:0)	63.0
Stearic (C18:0)	69.6
Arachidic (C20)	75.5
2) Unsaturated Fatty-acids	
Palmitoleic (C16:1n-7)	0
Oleic (C18:1n-9)	4.0
Linoleic (18:2n-6)	-12.0
Linolenic (18:3n-3)	-14.5
Arachidonic (C20:4n-6)	-49.5

Adapted from M^{c}Donald et al. (1996)

The spreadability of butter is another property of milk fat that determines its functionality. The ability of milk fat as butter, to yield to deformation at relatively low shear rates is based on the relative proportion of solid crystals in the network lattice (Palmquist et al. 1993). Although both solid and liquid phases may be present in milk fat, many physical

properties of fats are dictated by the presence of the solid phase. The solid phase of milk fat is present in the form of crystals, and the functionality is dependent upon the polymorphic form, size of the crystals, and the amount of crystals present (ie. solid fat content). Also impacting on the physical properties are the point at which the solid crystals become liquid, and the speed of melting of the crystals (Kaylegian 1995). Impacting on spreadability are the melting properties of milk fat (Palmquist et al. 1993). This is evidenced in the production of easy spread butter where fats of intermediate melting points are removed. The presence of the high melting point fat in the butter means it retains its shape at room temperature and the greater proportion of fats which are still liquid at 4°C means the butter is soft when removed from the refrigerator (Holmes and Wilson 1987).

Modifications of milk lipid components to meet functionality requirements of selected applications are illustrated by the production of high melting point milk fat fractions for pastries, the production of low melting point fractions for cold-spreadable butter and the production of monoglycerides for use as emulsifiers in baked goods (Kaylegian 1995). The major fatty acids contributing to the variation in melting properties that can be manipulated are palmitate and oleate (Palmquist et al. 1993). Milk fat with higher oleate and lower palmitate concentrations should yield a softer butter (German et al. 1997). However, it is necessary to understand in greater detail the function of the mammary gland in TAG synthesis to be able to regulate fat quality (Jimenez-Flores 1997). Butters from particular regions exhibit distinctive textures. An example is Californian butter, which is hard and spreads poorly at room temperature. Paradoxically, Californian butter has a high oleate and reciprocally lower palmitate level, leading to the assumption that Californian butter is soft. However, a higher than usual amount of long chain saturated FAs overrides this effect rendering the butter hard (German et al. 1997).

1.2. Mammary Lipogenesis.

The outline of mammary lipogenesis in Figure 1.2 provides an overview that shows the production of TAG and phospholipids with points throughout the process that can be manipulated to alter the FA composition of milk fat.

1.2.1 Fatty Acid Synthesis.

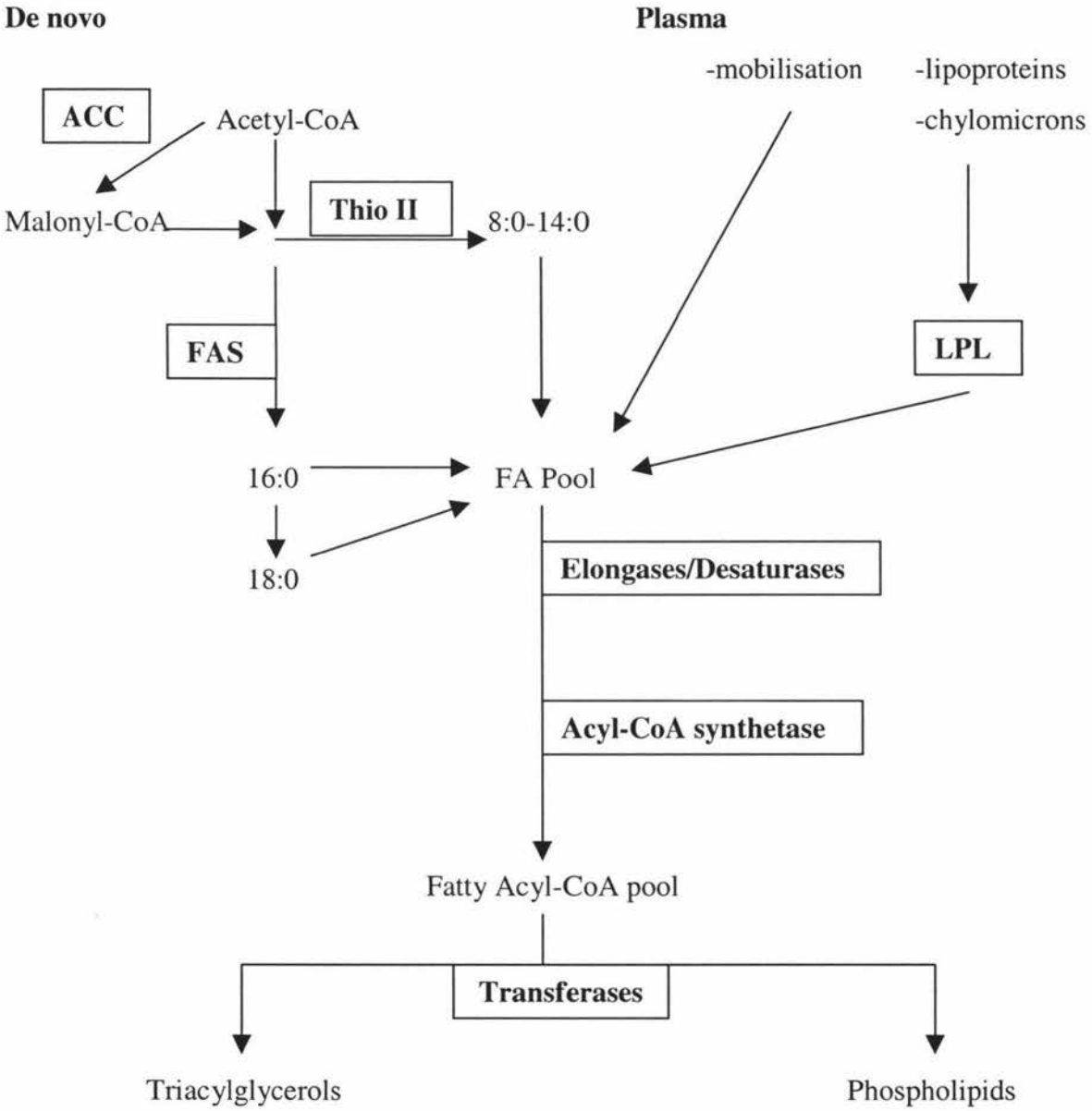
About half the FAs are synthesised in the mammary gland and half are delivered preformed in the plasma (German et al. 1997), although both sources of fats for milk fat are ultimately derived from the diet (Mansbridge and Blake 1997). FAs in the plasma include those mobilised from adipocytes, FAs in the TAG of chylomicrons, and TAG released with the hepatic lipoproteins (German et al. 1997).

Lipoprotein lipase (LPL) is produced by the secretory epithelium but is released into the interstitial space and becomes bound to the membrane on the luminal surface of the mammary gland capillary endothelium. LPL facilitates the transport of free FAs, monoacylglycerides and glycerol after hydrolysis of TAG at the *sn*-1 and *sn*-2 in chylomicrons. This also occurs for very low and low-density lipoproteins (Jensen et al. 1994; Liesman et al. 1988). Uptake of FAs into the mammary gland from high-density lipoproteins by LPL is poor. Within the lipoproteins, polyunsaturated FAs tend to be concentrated in the phospholipids and cholesterol esters of high density lipoprotein. The low utilisation of the FAs in HDL may also explain the low levels of polyunsaturated FA in cows' milk (Mansbridge and Blake 1997).

For *de novo* mammary FA synthesis, acetate and β–hydroxybutyrate are precursors in the ruminant (Griinari et al. 1997), while glucose is the precursor in the monogastric species (Linder 1991). In *de novo* FA synthesis acetyl-CoA is the 'primer' of malonyl-CoA for the stepwise elongation of the FAs by fatty acid synthase (FAS). The formation of malonyl-CoA from acetyl-CoA is a rate limiting step in *de novo* fat synthesis because acetyl-CoA carboxylase can be inhibited and stimulated (Holmes and Wilson 1987). FAS consists of two subunits in close proximity, with the partial activities of the two polypeptide chains in a head-to-tail arrangement. A shuttle system has been suggested whereby the growing acyl chain is transferred from the acyl carrier protein moiety of one chain to that on the other chain each time a two carbon unit is added from the malonyl-CoA. The final reaction of FAS is the termination of chain elongation so that palmitate is the predominant product (Dils 1983).

Mammary *de novo* FA synthesis produces the majority of the saturated FA from butyrate to myristate, and about half the palmitate (Mansbridge and Blake 1997). The high butyrate and hexanoate in bovine milk has evolved from the 'terminating transferase activity' of FAS that is the reverse of the 'loading transferase reaction' for the priming of FA synthesis.

Figure 1.2. A general overview of mammary lipogenesis in the mouse. This diagram shows the two sources of lipids that combine to provide the precursors for milk fat synthesis. Throughout the schematic diagram are enzymes that are able to be manipulated and cause a change in milk fat composition. ACC= Acetyl-CoA carboxylase; Thio II= thioesterase II; FAS= Fatty acid synthase; LPL= lipoprotein lipase.



Bovine milk also contains other short chain and hydroxylated FAs important to its flavour. In many non-ruminants a separate thioesterase II catalyses the hydrolysis of FA from FAS at the same position in the FA as that of thioesterase I. However, the action of thioesterase II produces a lot of medium chain FA (octanoate-laurate) that is common in non-ruminant milk (German et al. 1997).

The mechanism of FA release from FAS is via an interaction between a hydrophobic binding site on the thioesterase 1 (or FAS) and the long acyl chain of the substrate. Until the growing chain reaches this length it will be insufficiently hydrophobic to interact with the hydrophobic site of the terminating thioesterase. Comparatively, thioesterase II has a more hydrophilic binding site and therefore interacts with, and cleaves FAs with a medium chain length, octanoate to laurate from FAS (Dils 1983).

1.2.2 Synthesis of Triacylglycerols and Phospholipids.

FAs are acylated during their synthesis and go on to form TAG or phospholipids.. Aside from energy, TAG can be converted to membrane phospholipids, cholesterol and other lipids when metabolically required (German et al. 1997).

Phospholipids and cholesterol principally function in the formation of all cell membranes. They provide a semi-fluid matrix within which, float various membrane proteins (Linder 1991). The phospholipids are unique in containing a polar end, and thus have detergent properties, with negative and positive charges from phosphate and additional substituents (Linder 1991). Many of the properties of cell membranes are reflections of their polar lipid content (Lehninger 1982) and presence of specific polyunsaturated FAs (Linder 1991). These include arachidonate and eicosatrienoates that are important precursors for eicosanoids while docosahexanoic acid (DHA) is required for myelination in many developing young animals (Kawashima et al. 1990).

Phospholipids are required for the MFGM and are produced *de novo* by the mammary gland rather than received from lipoproteins (Dils 1983). Phospholipids contain two FAs for which there are many enzymes involved in positional esterification and maintenance of the acyl composition (Mizuguchi et al. 1996). The specificity of some of these enzymes for saturated as opposed to unsaturated FAs, varies between rodent and bovine species (German et al. 1997). However, certain dietary influences (Sections 1.3 and 1.4) in both species up-regulate some of these transferases and change the FA composition of the phospholipid. For

example, under normal dietary conditions oleate is evenly distributed on the *sn*-1 and *sn*-2 positions of phosphatidylcholine (Mizuguchi et al. 1996). Fat free diets and ingested clofibrate up-regulate stearoyl-CoA desaturase and the *sn*-2 positional enzyme, 1-acyl-GPC-acytransferase. Stearoyl-CoA desaturase produces more oleate (Chapter 1.2.1) and the 1-acyl-GPC-acytransferase esterifies proportionately more oleate at the *sn*-2 position of phospholipids (Millar and Ntambi 1996; Mizuguchi et al. 1996).

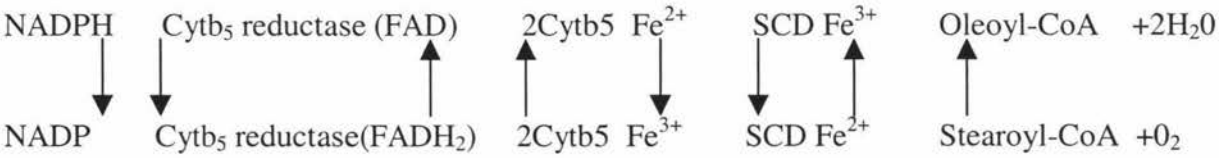
Three pathways achieve the synthesis of TAG, with the relative importance of each, differing between species. The primary route in the bovine mammary gland is the phosphatidic pathway. In this pathway two acyl-CoA esters are esterified to glycerol-3-phosphate to form phosphatidate which is subsequently converted to TAG by esterification of the third fatty acyl-CoA. The monoacylglycerol pathway involves the esterification of two acyl-CoA esters to 2-monoacylglycerol. In the lactating mouse mammary gland, TAG synthesis can also be achieved from the dihydroxyacetone phosphate pathway; although this pathway contributes less to TAG synthesis than the phosphatidic pathway (Dils 1983).
In stereo-chemical terms the three positions on glycerol-3-phosphate are not identical and are readily distinguishable by their respective enzymes (M^{c}Donald et al. 1996). This leads to the pattern of fatty acyl-CoA attachment to glycerol-3-phosphate being non-random. The pattern of esterification can lead to the identification of some different species (Decker 1996).

A distinctive feature of bovine milk fat compositional is the predominance of *sn*-2 being occupied by a saturated FA. This feature of FA positioning, also has a hierarchy of FA preference in myristate over laurate, and palmitate over stearate (Decker 1996; German et al. 1997). Short and medium chain FAs are esterified almost exclusively to the *sn*-3 position which is unique to the ruminant. This is not due to an increased specificity of microsomal diacylglycerol transferase. Rather, it just reflects the intracellular concentration of these short chain FAs caused by the terminating transferase activity of FAS (Dils 1983: M^{c}Donald et al. 1996).

1.2.3. Stearoyl-CoA Desaturase.

Stearoyl-CoA desaturase (SCD) oxidises palmitoyl-CoA and stearoyl-CoA (the preferred substrate) to form the monounsaturates, palmitoeyl-CoA and oleoyl-CoA, respectively (Millar and Ntambi 1996; Shimomura et al. 1998; Ward et al. 1998). The desaturation reaction is catalysed by a complex of three membrane-bound enzymes (Stryer 1988), with the terminal enzyme being SCD (Ntambi 1995).

Figure 1.3. Diagrammatic representation of the three enzymes involved in Δ^9 desaturation of stearoyl-CoA to oleoyl-CoA (adapted from Ntambi 1995).



It is a mixed oxygenase reaction with the oxidation reaction catalysed by the iron containing SCD enzyme (Ntambi 1995). The non-heme iron of SCD is converted to the Fe^{2+}, which enables it to interact with oxygen and the saturated FA (Figure 1.3) to form water. Two electrons come from NADPH and two electrons from the single bond of the fatty acyl substrate in the formation of water (Shimomura et al. 1998). A double bond is placed nine carbons from the carboxylic acid to produce palmitoleate (16:1n-7) and oleate (18:1n-9) from their respective saturated FA precursors (German et al. 1997; Jeffcoat and James 1984). By using labelled stearate, it was shown that only the D-hydrogens were removed from carbons 9 and 10 (Jeffcoat and James 1984). Isotope rate effects indicate that the hydrogen removal is the rate-limiting step in Δ9 desaturation (Enoch et al. 1976). It is suggested that as a result of substrate interaction with the enzyme, the linear stearoyl-CoA is twisted about carbons 9 and 10 to take on a 'pseudo-oleic acid' conformation with the D-hydrogens on the same side as the molecule. This type of interaction between substrate and enzyme is a common feature in desaturation and occurs in the synthesis of linoleate (Jeffcoat and James 1984).

In the rat liver, SCD mRNA transcripts are translated at soluble cytoplasmic polysomes (Theide and Strittmatter 1985) and do not undergo post-translational modification (Theide et al. 1986). Enzymes responsible for processing FAs are malonyl-CoA dependent elongases and desaturases. Both are located in the endoplasmic reticulum (Giron et al. 1996). SCD is a membrane protein so it is assumed that SCD is assembled into the endoplasmic reticulum membrane by a co-translational mechanism. In inserting SCD into the endoplasmic reticulum membrane, the mRNA/ribosome complex is in close proximity to the endoplasmic reticulum membrane surface (Gonzalez and Martin 1996). The 3-enzyme system is held in a functionally correct orientation within the membrane (Jeffcoat and James 1984).

Desaturation of stearate to oleate reduces the melting point. This allows for either further metabolism or blood transport as oleate has a melting point below body temperature.

The Δ^9 desaturation appears to be primarily involved in the maintenance of physiochemical properties of the membrane (Garg et al. 1988) such as fluidity. Enzymes of Δ^6 and Δ^5 desaturation provide FAs for membrane structure, from their precursors, linoleate and linolenate. These enzymes also produce arachidonic acid for eicosanoid synthesis (Garg et al. 1988; Jeffcoat and James 1984).

SCD is the predominant, if not exclusive, enzyme for the desaturation of saturated FA in liver, mammary gland, brain, testes, and adipose tissue (Madsen et al. 1997). Regulation of the SCD enzyme level is primarily achieved at the level of gene transcription of the two SCD genes, SCD1 and SCD2 (Jones et al. 1996; Sessler et al. 1996). SCD1 and SCD2 are expressed and regulated differently in various tissues (Landau et al. 1997; Madsen et al. 1997). SCD1 is constitutively expressed in the hepatocyte and adipocyte, but inducible only in the liver. SCD1 is slightly induced in the kidney and lung, and absent in the brain, heart and spleen. In many ways SCD2 mirrors SCD1 in its expression patterns. SCD2 is constitutively expressed in the brain and inducible in the kidney, lung and adipose tissue. There is no hepatic expression of SCD2, and low levels in the heart and spleen (Landau et al. 1997; Ward et al. 1998). Species differences in expression also occur. For example, ovine SCD mRNA is more widely expressed than rodent SCD mRNA. This may compensate for the fact that sheep have only one SCD gene and rodents have two SCD genes (Ward et al. 1998). SCD1 mRNA encodes a protein with more than 87% sequence homology to SCD2 mRNA (Landau et al. 1997). Other comparisons of amino-acid sequences within the SCD gene family, show a highly conserved carboxyl terminal half of the enzyme molecule, suggesting a common catalytic region (Kaestner et al. 1989). Not all of the nucleotides of SCD mRNA encode for the enzyme (Theide and Strittmatter 1985). A long 3'-noncoding sequence may play a role in SCD expression regulation (Ntambi et al. 1988). There are also indications that some of the non-coding region may have a role in a recognition sequence that inserts SCD into the membrane with the correct orientation (Theide and Strittmatter 1985).

In rodents, hepatic SCD, like a number of lipogenic enzymes, responds to many internal and external stimuli (Jeffcoat and James 1984; Waters and Ntambi 1997). SCD is a key enzyme in the production of unsaturated FA synthesis, which is mostly regulated hormonally or by dietary means (Giron et al. 1996). SCD is induced by high carbohydrate diets and inhibited by starvation and diabetes (Waters and Ntambi 1997).

Upon starvation FAs are mobilised as energy sources and under these conditions SCD activity in the liver drops to only 2% of the level found in fed animals (Ntambi et al. 1988). Mediating the effects of starvation and refeeding on hepatic lipogenic enzymes are glucagon

and insulin, respectively. Glucagon, which is elevated in the blood of starved animals, inhibits the synthesis of lipogenic enzymes (Ntambi 1992). SCD inhibition appears to be mediated by the glucagon activation of cAMP dependent protein kinases (Waters and Ntambi 1997). However, the decrease in SCD1 mRNA in the liver during starvation could also be due to repression by polyunsaturated FAs in mobilised fat that is released in response to elevated cAMP and a drop in insulin levels (Ntambi 1992).

As insulin is an antagonist of glucagon in many physiological reactions (de Gomez Dumm et al. 1975), it could be expected that insulin would increase SCD activity. In meal feeding of mice, SCD levels exhibit a diurnal variation that coincides with food intake (Jeffcoat and James 1984). In diabetics with low insulin levels, the activity of SCD is markedly decreased but restored by insulin injection. However, changes in insulin secretion within the physiological range are not the primary regulator of enzyme activity (Weekes et al. 1986). Insulin's effect may be explained by its effect on glycolysis, as some of the glycolytic intermediates enhance SCD (de Gomez Dumm et al. 1975; Waters and Ntambi 1997).

Nutrient induced hormonal changes are responsible for altering SCD gene expression, while nutrients themselves can act as ligands for transcriptional factors (Paisley et al. 1996). Examples are dietary polyunsaturated FAs (Section 1.4) inhibiting SCD transcription whereas dietary copper, zinc and selenium have shown positive regulation of SCD and other desaturases (Jeffcoat and James 1984; Ntambi 1995). Dietary cholesterol induces hepatic SCD but not in adipose tissue, heart or kidney. Cholesterol at 1% dietary inclusion even over-rides the inhibitory effect of dietary corn oil at 10% inclusion. The mechanism proposed was that dietary cholesterol decreases the membrane fluidity, therefore stabilising the components of the SCD complex and increasing its activity. However, as SCD is induced before membrane composition changes occurred, this does not seem plausible. It is possible that dietary cholesterol positively regulates SCD mRNA stability (Landau et al. 1997).

Studies, mainly in rodent liver, show SCD activity is sensitive to many complex control mechanisms. Whether these control mechanisms apply to mammary SCD is unknown, especially as SCD exhibits expression levels that vary between tissues (Kaestner et al. 1989; Ntambi 1995). Alteration of the FA composition in order to produce a softer milk fat may be dependent on manipulation of mammary SCD gene expression (Jones et al. 1996; Ward et al. 1998). However, mammary SCD regulation is poorly described given the potential it has in producing softer butter (German et al. 1997). Peroxisomal proliferators (PPs) and dietary polyunsaturated FAs have opposing effects on hepatic SCD gene expression (Jones et al.

1996). They then offer themselves as tools for the manipulation of mammary SCD so that a change is seen in the milk fat FA composition that better favours production of a softer butter.

1.3 Effects of Clofibric Acid on Lipid Metabolism.

Peroxisomal proliferators (PPs) include amphipathic carboxylates, pesticides (Millar and Ntambi 1996), phenoxy acid herbicides and azole antifungal drugs (Gibson 1993) as well as α-aryloxyisobutyric acids such as clofibrate (Pennacchiotti et al. 1996). Clofibrate's effects show differences in its regulation of lipogenic gene expression between species and tissue (Section 1.3.4.), but the effect of clofibrate occurs in a co-ordinated manner within an animal. Clofibrate influences the equilibrium of the FA pool by changes in three major pathways; that of peroxisomal β-oxidation, FA biosynthesis and phospholipid biosynthesis (Millar and Ntambi 1996). This influence leads to a reduction in the proportion of the shorter, more saturated FA, and an increase in longer more unsaturated FA being incorporated into phospholipid.

Clofibrates effects can be divided into peroxisomal (Section 1.3.1.) and extra-peroxisomal (Section1.4.2.). Peroxisomes contain many enzymes that use molecular oxygen to oxidise substances in the process of detoxification. Detoxification is primarily seen in the liver and kidneys, and the effect of clofibrate on peroxisome effect is mainly exhibited in these tissues (Devlin 1986; Lidner 1991). The many extra-peroxisomal effects in liver and other tissues from clofibrate, include both stimulation and inhibition of mitochondrial and cystolic enzymes. These responses may be directly or indirectly related to the peroxisome effect (Youssef and Badr 1998).

Clofibrate causes hypolipidaemia in mice, rats, humans and other animals (Millar and Ntambi 1996), and as such, PPs are used therapeutically for human hyperlipoproteinemias that are resistant to diet manipulation, weight reduction and exercise (Pennachiotti et al. 1996). Clofibrate acts as a hypolipidaemic drug by depressing FA synthesis and esterification into glycerolipids, while increasing FA oxidation (Kawashima et al. 1982). While used therapeutically in humans, these hypolipidaemic drugs act at multiple loci and have been associated with undesirable effects in some animals (Pennachiotti et al. 1996).

In rodents, clofibrate induces a stereotypical pleiotropic response which is tissue specific with most effects occurring in the liver (Lee et al. 1995). PPs, like clofibrate, increase hepatic peroxisome number while being carcinogenic but not genotoxic in rodents (Gibson

1993; Muerhoff et al. 1992). PPs decrease the rate of programmed cell death so altering the balance between mitosis and apoptosis, a key mechanism in carcinogenesis. Correlating with the carcinogenicity of PPs, is their ability to induce the oncogenes *c-Ha-ras, jun* and *c myc* (Vanden Heuvel 1999). Some evidence suggests, however, that PPs are not carcinogenic and in fact may be valuable antitumor agents in humans (Youssef and Badr 1998). Phenylacetate and phenylbutyrate are plasma components that have antitumor activity by inducing cytostasis and differentiation of various hematopoietic and solid tumors. Structurally these compounds share the aromatic nucleus and carboxylic acid features of "fibrate" hypolipidemic drugs like clofibrate (Pineau et al. 1996).

1.3.1. Peroxisomal Effects of Clofibrate.

Clofibrate induces cell organelle proliferation that is best characterised by increased peroxisomal numbers and their size (Millar and Ntmabi 1996). Other organelles, such as endoplasmic reticulum and mitochondria, proliferate along with peroxisomes (Pennachiotti et al. 1996) but the increase is not as marked as peroxisome proliferation (Kawashima et al. 1990). Associated with organelle proliferation is an increase in the transcription of their enzymes (Diczfalusy et al. 1995; Flatmark et al. 1988; Kawashima et al. 1983). Mitochondrial synthetases and dehydrogenases are induced by peroxisome proliferator activated receptor (PPAR) which is activated by clofibrate (Pineau et al. 1996). However, like organelle proliferation the mitochondrial enzymes are not induced as much as peroxisomal enzymes. Increased mitochondrial protein can account for up to 30% of the increase of total protein in the liver with clofibrate treatment (Reinhart et al. 1993). Concomitant with organelle proliferation is general liver hypertrophy and hyperplasia (Millar and Ntambi 1996; Pennachiotti et al. 1996).

Under normal circumstances, peroxisomal β-oxidation is only a minor pathway for FA oxidation relative to the mitochondrial system. However, peroxisomal enzymes become up regulated when an animal is subject to a lipid overload such as that when stimulated with clofibrate and high fat diets (Lee et al. 1995). Clofibrate via PPAR activation (Section 1.4.3.) induces the transcription of acyl-CoA oxidase (Green 1995; Madsen et al. 1997). Other peroxisomal β-oxidation enzymes up regulated by PPAR activation include the hydrolysis by enol-CoA hydratase; the oxidative 3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme and thiolytic cleavage by β-ketothiolase (Pineau et al. 1996). Enzymes of the peroxisomal

cytochrome P450-dependent mono-oxygenase family are also up regulated (Braissant et al. 1996; Silva et al. 1988). These mono-oxygenases are involved in FA ω-hydroxylation and are all induced by activated PPARs (Pineau et al. 1996).

Many of the reactions catalysed by these peroxisomal enzymes produce hydrogen peroxide, an example being the reaction catalysed by acyl-CoA oxidase (Lazarow et al. 1982). Catalase, which is the marker enzyme for peroxisomes (Reddy and Kumar 1979), uses hydrogen peroxide to oxidise potentially dangerous organic compounds. As for the other peroxisomal enzymes, catalase is induced when clofibrate activates PPARs (Osumi and Hashimoto 1978; Reddy and Kumar 1979). The products of peroxisomal β-oxidation are either converted to ketone bodies or oxidised further in the tricarboxylic acid cycle (Lidner 1991). Clofibrate also induces hydrolases, but of the two studied by Kawashima et al. (1983a), the effects seem to be mediated via different mechanisms that may involve hormone attenuation (Kawashima et al. 1982).

Peroxisomal FA oxidation differs from mitochondrial FA oxidation, in that peroxisomes of the rat liver have the capacity to shorten FAs by a different β-oxidation mechanism. Peroxisomal oxidation seems to be particularly important in the metabolism of very long chain FAs, such as isomers of 22:1, which are poorly oxidised by the mitochondria (Flatmark et al. 1988; Kawashima et al. 1989). Very long chain FA oxidation, stimulated by PPs, explains the observations of decreased levels of 24 carbon polyunsaturated FA in the liver of clofibrate treated mice (Pennachiotti et al. 1996). While decreasing concentrations of 22:1 and 24 carbon polyunsaturated FAs reflect PP induced FA oxidation, other long chain FAs, such as oleate and linoleate are not good indicators of clofibrate induced β-oxidation. These FAs are oxidised at almost the same rate whether there has been clofibrate induction, or not (Kawashima et al. 1984).

1.3.2. Extra-peroxisomal Effects of Clofibrate.

Clofibrates influence on lipid metabolism results in a reduction in the shorter, more saturated FA in phospholipids. This is accomplished by clofibrate repression of lipogenic proteins like ACC, FAS and S14. Alternatively, clofibrate increases the incorporation of longer more unsaturated FA into phospholipids. This is accomplished by clofibrate inducing elongation/desaturation enzymes to provide the long chain polyunsaturated FA; and clofibrate

inducing the enzymes that provide transport for FA, activate FA, and incorporate them into phospholipids (Millar and Ntambi 1996)

Palmitoyl-CoA elongation, SCD, Δ^5 and Δ^6 desaturation activities are induced by administration of PP (Millar and Ntambi 1996). This is evidenced by the increase in liver oleate concentrations and increased conversion of ^{14}C labelled linoleate, γ-linolenate, 7,11,14-eicosatrienoate to more desaturated and elongated metabolites (Kawashima et al. 1990; Pennachiotti et al 1996). Increased palmitoyl-CoA elongation and desaturation of stearoyl-CoA by SCD increases the amount of oleate and lowers the relative proportion of linoleate in the microsomal free FA pool (Kawashima et al. 1984). Other influences of clofibrate on liver FA concentrations include increases in 20:3n-6 and 22:5n-3 (Pennachiotti et al. 1996). As a consequence of clofibrate induction, there is an increased concentration of oleate and a decrease in stearate in hepatocyte phospholipids, especially phosphatidylcholine (Kawashima et al. 1990). By affecting this ratio, long term use of PPs induce hepatic tumors in mice and rats (Millar and Ntambi 1996). The major effects of the induction of SCD on lipid metabolism are an increased amount of phospholipid enriched with oleate, and a decrease in that of plasma TAG; changes that are consistent with clofibrate being a hypolipidaemic drug (Pennachiotti et al.1996).

While transcription of some lipogenic genes such as S14 and FAS are repressed (Millar and Ntambi 1996) many lipogenic genes are induced in organelles other than peroxisomes (Kawashima et al. 1983; Pennachiotti et al. 1996). For example, the activity of microsomal acyl-CoA synthetase (Figure 1.2) is induced in livers of rats treated with clofibrate (Kawashima et al. 1984; Pennachiotti et al. 1996). The induced microsomal acyl-CoA synthetase shows the same substrate specificity for oleate and linoleate as that of control microsomal acyl-CoA synthetase, proving that the induced form of this enzyme is functionally the same as the non-induced form (Kawashima et al. 1984). Clofibrate induces many FA transferases (Figure 1.2) (Pennachiotti et al. 1996); both peroxisomal and mitochondrial (Kamashima et al. 1990). Many of the FA transferases are important in regulating acyl composition of phosphatidylcholine (Kawashima et al. 1984). Fatty-acid intracellular transport by FA binding proteins (FABPs) is also up regulated with the use of clofibrate (Mandla et al. 1996). Hepatic concentrations of FABP are increased by clofibrate treatment (Kawashima et al. 1984a; Motojima et al. 1998) which results in a concomitant increase in the rate of FA uptake by perfused rat livers (Reinhart et al. 1993).

1.3.3. Mechanism of Regulation by Clofibrate.

The diverse effects of PPs, such as clofibrate, appear to be mediated by PPARs which belong to the steroid/thyroid super-family of ligand-activated transcription factors (Diczfaluzy et al. 1995; Mandla et al. 1996). With activation of cell signal transduction pathways, PPARs bind to PPAR-response elements in association with retinoid X receptors so activating organelle proliferation and enzyme induction (Latruffe and Vamecq 1997; Millar and Ntambi 1996). Transcriptional regulation of genes involved in peroxisomal and mitochondrial lipid metabolism pathways are the best-characterised function of PPARs. Clofibrate's carboxylic acid is required for receptor activation, as blocking this moiety inactivates transcriptional regulation (Pineau et al. 1996). Some types of cancer show a tight correlation between the potency of a PP as an inhibitor of tumor cell proliferation and degree of PPAR activation (Pineau et al. 1996). This is due to PPARs being involved in differentiation of several cell types (Figure 1.4), and is often referred to as "master regulators of differentiation" (Vanden Heuval 1999).

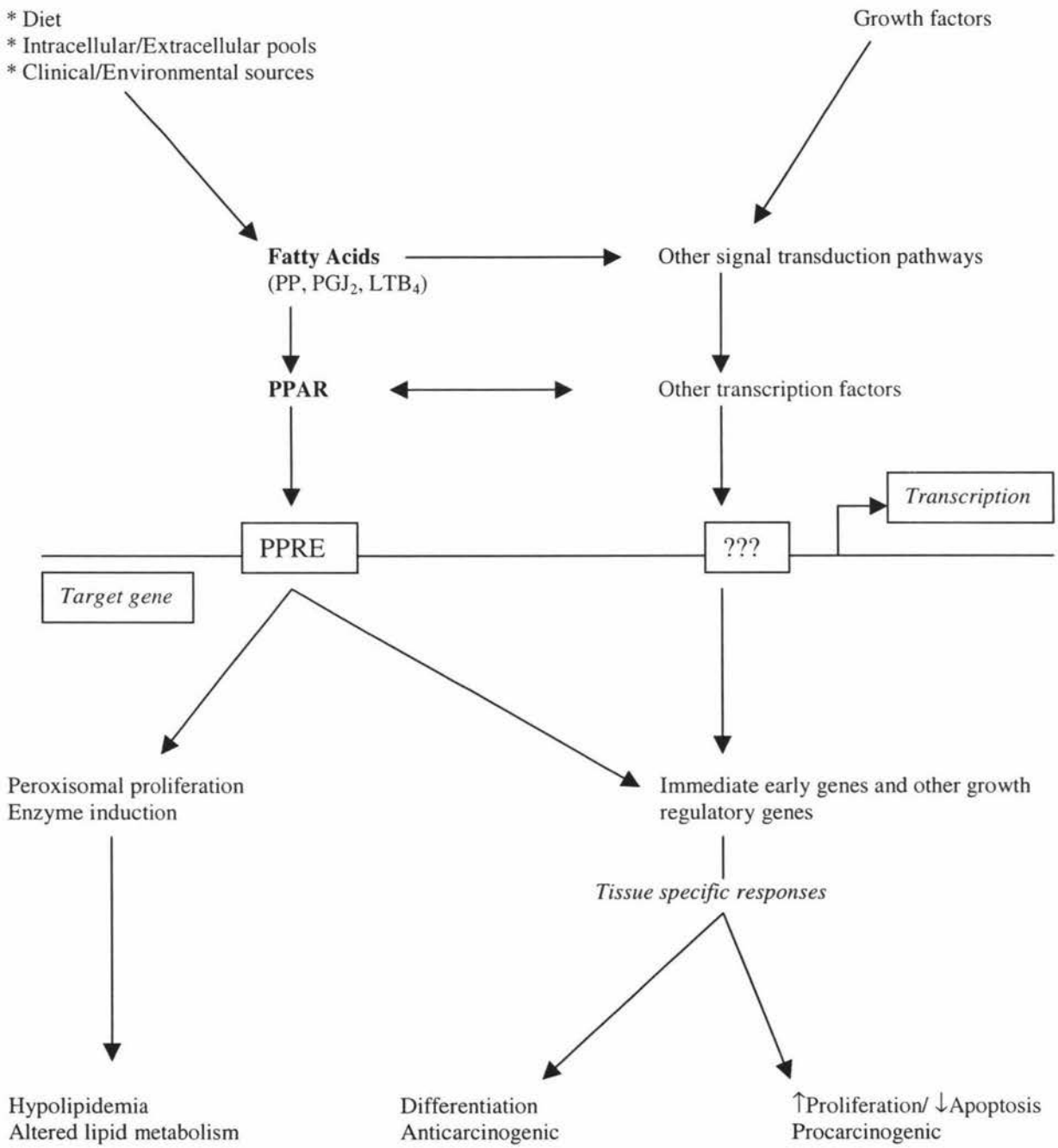
There is still some doubt as to aspects of the above hypothesis. It still remains to be determined whether FAs and PPs bind directly to any PPAR (Green 1995). A nuclear protein receptor that is activated by PPs, which is likely to mediate proliferator induced gene expression has been cloned from rodents, although its natural ligand has yet to be identified (Pennacchiotti et al. 1996). Considering the unphysiological levels needed it is unlikely that the aromatic PPs act as ligands of the receptor. One possibility is that PPs displace FA from FABPs which then go on to activate PPARs. Alternatively, receptor activation maybe due to modifications such as phosphorylation, or changes in cAMP levels (Pineau et al. 1996).

While the mechanism for peroxisomal and mitochondrial enzyme induction to some extent seems established, the mechanism for the induction of SCD by clofibrate use is not (Kawashima et al. 1982). SCD induction does not appear to be correlated to the induction of peroxisomal β-oxidation enzymes (Kawashima et al. 1989; Madsen et al. 1997). This is seen in the substantial induction of peroxisomal β-oxidation enzymes in 1-2hrs whereas significant SCD induction with clofibrate is seen in 24-30hrs (Millar and Ntambi 1996). This suggests some other indirect mechanism may function in the induction of SCD (Kawashima et al. 1989). Such a mechanism may involve the induction of SCD to compensate for a deficiency in polyunsaturated FA (Millar and Ntambi 1996).

For rapid organelle proliferation and liver enlargement, hepatocytes need to increase the supply of phospholipids, as the constituent of biologic membranes (Kawashima et al. 1990).

PPs cause an influx of plasma TAG into the liver providing an immediate supply of polyunsaturated FAs for this production of organelle membranes. However, polyunsaturated FAs are preferentially catabolised by peroxisomal β-oxidation causing polyunsaturated FA

Figure 1.4. The basic mechanism of action of peroxisome proliferator-activated receptors (PPAR).The steroid hormone receptor, PPAR, is activated by a variety of ligands and binds to the PPAR-response element (PPRE), driving the transcription of target genes. The resultant change in gene expression is responsible for the effects on lipid metabolism as well as growth regulation. Depending on the cell type being examined, PPAR activation and regulation of growth regulatory and immediate early genes result in proliferation, apoptosis or differentiation. Abbreviations: ACO, fatty acyl-CoA oxidase; LT, leukotriene; PG, prostaglandin; PP, peroxisomal proliferators. Adapted from Vanden Heuval (1999).



deficiency. Polyunsaturated FA deficiencies in turn activate SCD to catalyse the synthesis of unsaturated FA to maintain membrane fluidity (Millar and Ntambi 1996). The purpose of this catabolic FA oxidation is to fuel the energy demands, which increase dramatically, of the anabolic process of phospholipid formation (Pennachiotti et al. 1996).

While the above mechanism seems plausible, co-transfection has shown that PPARα is directly involved in SCD transcriptional induction (Millar and Ntambi 1996), making the induction of SCD by PPARs still a possible mechanism (Diczfalusy et al. 1995). Nuclear run-on transcription assays show an increased rate of SCD gene transcription with clofibrate use; and that this induction is inhibited by cyclohexamide indicating ongoing protein synthesis is necessary for induction. Time course studies show SCD mRNA induction is apparent within 6hrs and can reach a maximum of 22-fold after 30hrs. This lag time to maximum mRNA induction further supports protein synthesis as a requirement in increasing the concentration of SCD. A candidate protein could be one of the two PPAR isoforms expressed in the liver of mice, however, the endogenous PPAR ligand has not been identified in the induction of SCD by clofibrate (Millar and Ntambi 1996). Multiple signalling pathways can exist in the induction of SCD by PPARs (Figure 1.4), which is shown up by differences between tissue and species. Differential regulation of SCD is possible due to different PPAR isoforms, varied heterodimerization with other steroid hormones, or the effect of some unknown protein on PPAR binding that could result in SCD induction (Millar and Ntambi 1996).

To summarise, the clofibrate induction of peroxisomal FA oxidation, plasma TAG reduction and phospholipid formation are related and can be thought of as the direct effects of clofibrate (Osumi and Hashimoto 1978). These direct effects are brought about by the activation of PPARs by clofibrate. To what extent the induction of SCD holds to this PPAR activation theory has yet to be determined. SCD could alternatively be induced indirectly as a consequence of these direct actions of clofibrate.

1.3.4. Attenuation of Lipid Metabolism by Clofibrate.

In contrast to rats, clofibrate does not induce hepatomegaly in pigs and humans (Reinhart et al. 1993). Similarly, PPs that induce peroxisome proliferation in rat livers fail to show this induction in human livers (Pineau et al. 1996; Reinhart et al. 1993). Clofibrate induces hepatic peroxisomal β-oxidation in chickens (Reinhart et al. 1993), mice and rats but has a greatly reduced action in the liver of guinea-pigs (Kawashima et al. 1984a). Consistent with overall catabolic induction is the increases in activity of hepatic mitochondrial acyl-CoA

dehydrogenase and long chain acyl-CoA hydrolases in pigs (Reinhart et al. 1993). Both of these types of enzymes show species differences in that they are induced in the rat and mouse but not in the guinea-pig liver (Kawashima et al. 1984a).

Peroxisome proliferators effects are not just felt hepatically, as seen by SCD induction by clofibrate in other tissues (Kawashima et al. 1983). In some cases induction by PPs can occur differentially between regions of the same tissue. For example, in response to dietary clofibrate in the weaning pig, there are marked differences in FABP activity in different regions of the small intestine. The proximal small intestine FABP activity is unaffected whereas the distil small intestine showed induction of FABP activity. Interestingly, in the weaning pig, functional FABP activity in the liver is unaffected (Reinhart et al. 1993). Intra-tissue differences can also be seen in adipose tissue. Basal levels of FABPs in intestinal white adipose tissue are less affected by PP regulation than those in subcutaneous white adipose tissue (Motojima et al. 1998).

The potency of various PPs to activate PPARs is subtype specific and the expression of PPARα, β and γ varies widely from tissue to tissue (Vanden Heuvel 1999). PPARα mediates fibrate and dietary PUFA induction that is most pronounced in the liver (Motojima et al. 1999) but is also evident in cardiomyocytes, enterocytes and proximal tubule cells of the kidney (Vanden Heuvel 1999). PPAR is strongly expressed in cells with high FA catabolic rates and high peroxisomal metabolism (Braissant et al. 1996). PPARγ is expressed predominantly in adipocytes and the immune system, and exists as two distinct forms - γ_1 and γ_2 (Vanden Heuvel 1999). While effects are mostly seen in adipose tissue, there are differences between fat type and regions. Inter-intestinal, subcutaneous (both white fat) and brown adipose tissue exhibit different levels of mRNA when subjected to PPs. Brown adipose tissue did not respond to PPARα and γ activators compared with the other two fat types (Motojima et al. 1998). PPARβ is expressed more ubiquitously and often at higher levels than PPARα and γ when PPs are administered (Vanden Heuvel 1999).

Both PPAR β and γ isoforms have been implicated in attenuating the action of the PPARα isoform (Braissant et al. 1996). With co-expression of PPAR isoforms, the ratio between them and their ligands provides a means of differential tissue expression of specific target genes (Kliewer et al. 1994). PPAR isoforms are co-expressed with different levels of expression in many tissue types. In addition, the capacity of PPARs to bind identical response elements suggests specific roles for these receptors in the regulation of a similar set of genes. Thus, variations in tissue expression of the various PPAR isoforms, in concert with variations

in the distribution of their specific ligands, would lead to multiple possible combinations of stimulation or repression of target genes (Braissant et al. 1996).

Cross species comparisons may be complicated by a divergence of PPARs throughout evolution. This divergence can be seen in the varying abilities of members of the steroid nuclear receptor family to form homodimer and heterodimers (Pineau et al. 1996). There are often differences in the types of PPAR isoforms between species. Three PPAR isoforms are detected in the Xenopus laevis, two have been identified in humans, one in the rat and six isoforms in mice. Within species differences for responsiveness to PPs there are also differences in level of expression at different stages of development (Lee et al. 1995).

1.4.0. Manipulation of Lipid Metabolism by Dietary Fat.

Rats and mice have been the animal models most commonly used to study the effect of dietary fat on liver and adipose lipogenesis (Clarke et al. 1977). In this section, the use of dietary fat to manipulate SCD (Section 1.4.2.) and soften the milk fat of mice is discussed (Section 1.2.). However, dietary fat also influences other aspects (Section 1.4.2.) of lipid metabolism that will also effect the softness of the milk fat. As well as an effect on milk fat, dietary fat also influences the onset and progression of various disease states by exerting its effects at two levels; altering the FA composition of membrane phospholipids and regulating nuclear events that govern gene transcription. The battery of genes regulated by dietary fat are critical to the effect of the fat on lipid metabolism, cellular differentiation and ultimately cancer (Clarke and Jump 1996; Waters et al. 1997).

A fat component of murine chow diets is responsible for inhibition of SCD expression in the liver, since SCD gene transcription is induced upon switching to a fat free diet (Ntambi 1995). It has been found that polyunsaturated FAs, rather than saturated FA or monounsturated FA, are inhibitors of hepatic lipogenesis (Clarke and Jump 1996; Jones et al. 1996). Repression is related to the length of the FA along with the number of double bonds it contains (Ntambi 1992). Of the n-6 or n-3 polyunsaturated FAs, it is the n-3 fats of 20 to 22 carbons that display the greatest inhibitory effect (Clarke et al. 1990). Menhaden oil (high in n-3 FA) is 2-4 times more effective at hepatic lipogenic suppression than corn oil (high in n-6 FA). Products of Δ^6 desaturase activity (eg. 20:4n6 and 20:5n3) are more potent suppressers of FAS activity than the respective FA precursors (Jump et al. 1994). Many of the studies used intakes at 4-5 times

the dietary requirement for that class of lipid (Clarke and Jump 1996), which is often as high as 48% of dietary energy intake (Jones et al 1996).

Polyunsaturated FAs inhibit the expression of enzymes involved in glycolysis, *de novo* lipogenesis, fatty acyl-CoA desaturation and elongation, TAG synthesis and transport; while diverting FA into mitochondrial and peroxisomal oxidation (Clarke and Jump 1994; Jones et al. 1996; Waters et al. 1997). These actions are also accompanied by a fall in plasma TAG concentration and increased excretion of bile acids. Also associated with polyunsaturated FA regulation is the modification of acyl positioning in TAGs with an increase in polyunsaturated FA incorporated into the TAGs of lipoproteins (Baudet et al. 1984).

1.4.1. Peroxisomal Effects of Dietary Polyunsaturated FA.

The feeding of polyunsaturated FAs gives a response that closely mimics that of feeding clofibrate (Section 1.3.1). Briefly, these include dose dependent increases in peroxisomal β-oxidation and mitochondrial ω-hydroxylation (Flatmark et al. 1988). Included in these increases is the rate limiting enzyme of peroxisomal β-oxidation, acyl-CoA oxidase (Dreyer et al. 1992).

Many enzymes of peroxisomal β-oxidation and mitochondrial ω-hydroxylation have genes with peroxisomal proliferator response elements that can be up regulated by polyunsaturated FA activated PPARs. The enzymes regulated by dietary polyunsaturated FA include acyl-CoA oxidase and the bifunctional enzyme, cytochrome P450 FA ω-hydroxylase and intracellular FABPs (Vanden Heuvel 1999). With higher amounts of dietary polyunsaturated FA, plasma TAG concentrations decrease, as peroxisomal organelle proliferation and liver hepatomegaly increase (Pennachiotti et al. 1996). This means there is a tiered pattern of induction in that at lower FA concentrations only peroxisomal and mitochondrial enzymes are induced, and at higher dietary fat concentrations morphological changes occur along with the enzymatic changes.

Polyunsaturated FA's peroxisomal effects are made complex by the many metabolic interactions possible for a FA. Dietary FAs undergo metabolism that creates metabolites that also have regulatory powers. Added to this, is that polyunsaturated FAs display a heirachy according to their potency to regulate lipid metabolism enzymes (Section 1.4.3).

Different metabolic properties of the n-3 polyunsaturated FAs, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), give differing effects *in vivo* and *in vitro*. The two n-3 fish oil FAs affect mitochondrial or peroxisomal oxidation differentially. EPA increases peroxisomal and mitochondrial FA oxidation, while DHA only effects peroxisomal oxidation. EPA or its metabolites from peroxisomal oxidation can up-regulate carnitine palmitoyltransferase, the rate limiting enzyme of mitochondrial oxidation. This differential effect between EPA and DHA on FA oxidation enzymes, holds true for organelle proliferation, in the EPA increases mitochondrial number, not DHA. DHA is a stronger peroxisomal enzyme proliferator than EPA, as seen in its greater induction of acyl-CoA oxidase. However, because DHA is poorly oxidised in the peroxisome compared to EPA and is diverted to membrane phospholipid, its function may be more related to membrane structure. EPA in up-regulating two FA β-oxidation systems is more metabolically active than DHA which only up-regulates one FA β-oxidation system. The increased metabolic activity of EPA over DHA is also seen in the levels of plasma TAG being lowered by EPA and not DHA. (Madsen et al. 1999).

Animal, especially human studies are difficult to interpret due to variation between tissues in the basal concentration of FA, retro-conversion of DHA to EPA, and the differential release of EPA and DHA from membrane phospholipids. Most of these complicating factors are excluded when cultured hepatocytes are used to study the effects of fish oil FAs (Madsen et al. 1999).

1.4.2. Extra-peroxisomal Effects of Dietary Polyunsaturated FA.

Transcription of hepatic lipogenic enzymes such as ACC and FAS are inhibited by dietary polyunsaturated FA and induced by feeding fat free diets (Jones et al. 1996; Nelson et al. 1987; Waters et al.1997). FAS is suppressed more than ACC when polyunsaturated FAs in the diet are increased (Clandinin et al. 1996). Most studies looking at the effects of dietary polyunsaturated FAs on regulation of gene expression feed n-6 or n-3 polyunsaturated FAs over extended periods. However, feeding a single n-3 enriched meal after a fat free dietary regime inhibits S14 and FAS only hours after the ingestion of that meal (Jump et al. 1994), for example a 60-80% inhibition of S14 and FAS can be observed within 3hrs of adding polyunsaturated FA to a fat free diet (Clarke and Jump 1996). Polyunsaturated FA inhibits

hepatic *de novo* FA synthesis, as indicated by a decline in the mRNA for the FAS enzyme (Jump et al. 1994), and a reduction in the fatty acyl pool of butyrate to palmitate (Figure 1.2).

Hepatic expression has been the model most widely used to study dietary polyunsaturated FA inhibition of SCD transcription (Jones et al. 1996). Expression of SCD is inhibited by polyunsaturated FA at the level of gene transcription (Weekes et al. 1986). Polyunsaturated FA repression of SCD activity can also over-ride the induction of SCD by insulin and fructose. Both trilinolenin and triarachidonin repress insulin induced SCD when diabetic mice are given a physiological dose of insulin. This suggests that polyunsaturated FA repression of SCD is through a mechanism it shares with insulin (Waters and Ntambi 1996).

Of the n-3 fish oil FA, it is EPA that is thought to down-regulate SCD (Waters and Ntambi 1996). Some studies do show slight down-regulation of SCD with DHA but this effect is thought to be due to the retro-conversion of DHA to EPA (Madsen et al. 1999). FA repression of SCD by n-6 FA is seen in feeding a mixture of conjugated linoleic acid (CLA) isomers which decreases desaturation of myristate, palmitate and stearate (Chouinard et al. 1999). There may be some divergence of specificity within the isomers of linoleate. It is suggested that 9c,11t-18:2 CLA alters the Δ^6 desaturation of linoleate, and 10t,12c-18:2 decreases the Δ^9 desaturation of stearate (Bretillon et al. 1999). Regulation of SCD is supposedly greater in using n-3 FA over n-6 FA (Clarke et al. 1990), however, the n-6 arachidonic acid, as 3% dietary arachidonin, inhibits SCD activity by 95% (Waters and Ntambi 1996). Conversely, in shifting from a natural diet to a fat free diet, murine SCD levels increase 40 fold and this increase is paralleled by an increase in SCD mRNA (Ntambi 1995).

In suppressing n-9 FA production, polyunsaturated FAs also reduce the incorporation of n-9 FA into the phospholipids of plasma membranes. This ensures polyunsaturated FAs (n-3 or n-6) and their eicosanoid metabolites are incorporated into membrane phospholipids (Clarke and Jump 1996). In support of this, hepatic SCD activity is inversely related to the proportion of linoleic acid in tissue lipid of mice (Weekes et al. 1986). The greater the duration of dietary intake of CLA, the higher the concentration of CLA in the membranes. As the CLA content of the membrane increases, the negative correlation between linoleate and SCD becomes more significant (Yang et al. 1999). This relationship is also seen in TAG of adipose tissue of cattle fed bypass polyunsaturated FA. In this circumstance the low oleate concentration and high stearate concentration in the FA pool reflects SCD inhibition. The influence on the intracellular FA pool with polyunsaturated FA inhibition of SCD is reflected in the acyl positioning on TAG as well. Due to its increased concentration, stearate is

increasingly esterified into the *sn*-1, and *sn*-3 position over the usual *sn*-2 position (Yang et al. 1999).

This modulation of FA composition with SCD inhibition by polyunsaturated FA can also be seen in the FA composition of the milk fat of goats. Milk FA are normally about 72% saturated but feeding sterculic acid increased the saturation to 84% by inhibiting SCD (Bickerstaff and Johnson 1972). Dietary polyunsaturated FA regulation in the liver influences FA composition and reduces total TAG formation by decreasing the activity of diacylglycerol acyltransferase (Madsen et al. 1999). In feeding a fat free diet, the inhibition on diacylglycerol acyltransferase activity and decreased total TAG formation, is released. Oleate formed in the mammary gland by SCD is not released into circulation (Bickerstaff and Johnson 1972), so with increased SCD activity, the increased oleate concentration in the FA pool could be incorporated into increased milk fat TAG production.

Glucose-6-phosphate dehydrogenase and malic enzyme activities are associated with the production of NADPH, the reducing equivalent in many reactions in lipid metabolism. With the reduction in lipid synthesis induced by polyunsaturated FA the demand for NADPH decreases and the enzymes in the pathways producing NADPH, glucose-6-phosphate dehydrogenase and the malic enzyme are also decreased (Clarke et al. 1976; Jump et al. 1994; Waters and Ntambi 1996).

1.4.3 Dietary fat inhibitory Mechanisms.

It was thought that polyunsaturated FAs exerted their effects by interfering with the insulin-signalling cascade (Clarke and Jump 1996). As membrane phospholipids become enriched with long chain n-3 or n-6 FA the cellular responsiveness to hormonal stimuli that inhibit or stimulate lipogenesis (eg. glucagon vs insulin) are increased or decreased, respectively. However, the rapid induction of S14 and FAS following the removal of polyunsaturated FA from the diet does not support the membrane composition hypothesis. That is, 3hrs is likely to be too short a time to develop changes in membrane FA composition that would be sufficient to alter cellular hormone responses and reverse the n-3 FA suppression of S14 and FAS (Clarke et al. 1990). Polyunsaturated FA inhibition within this time frame is explicable if the polyunsaturated FA or its metabolite(s) act to control transcription and enzyme stability (Clarke et al. 1990) and not reduced enzyme catalytic efficiency (Clarke and Jump 1996).

While FA are essential biological components, they can cause chronic disease when their concentrations in the circulation are elevated. Recent studies show that FAs can rapidly modulate the transcription of genes involved in their own metabolism, and in doing so maintain the levels of FAs within physiological limits (Kliewer et al. 1997). Lack of evidence for a differential action of n-6 and n-3 FA on hepatic gene expression might suggest that polyunsaturated FAs do not utilise a prostanoid pathway to generate reactive intermediaries affecting gene expression (Jump et al. 1994). The involvement of a prostanoid seems unlikely because, a) the action of linoleate is not blocked when the prostanoid pathway is blocked, b) 5-trans,9,12-cis 18:3 inhibits FAS synthesis but does not give rise to a prostanoid precursor, and c) prostanoids derived from 20-carbon n-3 FAs are usually of lower potency compared with prostanoids derived from n-6 FA precursors, yet n-3 FAs are more antilipogenic than n-6 FA (Clarke et al. 1990).

The discovery of the novel steroid hormone receptor that was activated by peroxisome proliferators led to the development of the PPAR theory of enzyme regulation. The receptors were named PPAR because their initial characterisation involved the use of xenobiotics, but they could have easily been labelled FA receptors since they are activated by both PPs and endogenous FAs (Figure 1.4). PPAR, sometimes described as "master regulators of differentiation" are grouped with genes that specify the fate of a particular cell, such as transcription factors that are capable of activating the program of differentiation (Vanden Heuvel 1999).

PPAR are ligand activated transcription factors that control gene expression by interacting with specific DNA response elements, peroxisome proliferator response elements, located upstream from the responsive genes. There has been some debate as to whether PPs and FAs activate PPARs directly. However, most receptor activators do bind to PPAR subtypes with reasonable affinity (Vanden Heuvel 1999). Identifying a natural ligand of PPARα has been hindered by the lack of a high affinity PPARα radio-ligand. There is now a novel compound that functions as a high affinity ligand for both PPARα and γ. In using this compound *in vivo* it was shown that oleate, linoleate, linolenate and arachidonate can function as ligands for these PPAR isomers (Kliewer et al. 1997).

While various FAs can function as PPARα and γ ligands at micromolar concentrations, it does not exclude the possibility of the existence of either a higher affinity or more sub-type selective PPAR ligands such as eicosanoids. As receptors for various FAs and eicosanoids, the PPARs appear to be much more active in their interactions with ligands, than other

nuclear receptor-ligand interactions. The degree of activation of PPARs *in vivo*, may not be determined simply through interactions with a single high affinity ligand, but may instead be the summation of the effect of a variety of FAs and FA metabolites that can interact with the receptors (Kliewer et al. 1997).

Many of the lipogenic, glycolytic and FA oxidation genes are similarly regulated by insulin, dietary carbohydrate and polyunsaturated FAs, and peroxisomal prolifelators, which suggest a common response element targeting targeted by the same transcription factors (Clarke et al. 1990; Jump et al. 1994; Kliewer et al. 1997). Such response elements have been identified in the promoter regions of S14, FAS, ACC and protein kinase genes (Clarke and Jump 1996; Jones et al. 1996; Jump et al. 1994). In the regulation of SCD expression, polyunsaturated FAs have a distinct genetic mechanism. Polyunsaturated FAs may be ligands for specific nuclear transcription factors (PPARs) that function as a dominant-negative inhibition of gene transcription (Clarke and Jump 1996; Waters et al. 1997). The responsive elements for PPARs have been identified in the 5'-flanking region of the SCD1 gene (Diczfalusy et al. 1995). The hepatic actions of polyunsaturated FA are presumably mediated through this polyunsaturated FA response element in the SCD promoter, although the specific regulatory element remains undefined (Jones et al. 1996).

Waters and Ntambi (1996) believe that the transcriptional induction by activation of PPAR is not the mechanism of regulation for some of the responses seen with dietary polyunsaturated FA. PPARα plays a key role in hepatic FA catabolism (Kliewer et al. 1997), as shown by its mediation of the induction, by the polyunsaturated FA, of transcription of peroxisomal β-oxidation enzymes (Lee et al. 1995). However, the polyunsaturated FA mediated suppression of FAS does not require the polyunsaturated FA activation of PPARα (Madsen et al. 1999).

In mice the slower regulation of SCD is different from that of S14 and FAS, whose transcriptional rates increase rapidly in response to feeding a fat free diet. The slow kinetic induction of SCD gene transcription suggests the molecular basis for the dietary regulation of various hepatic lipogenic genes differs (Ntambi 1995). While SCD has slower transcriptional rates, it is more sensitive to polyunsaturated FA than FAS. Thus, the immediate effect of polyunsaturated FA is to regulate SCD transcription which results in the accumulation of the saturated FAs palmitate and stearate, which in turn feed back and inhibit ACC and FAS (Ntambi 1995; Waters et al. 1997). SCD could be the only gene regulated by polyunsaturated FAs, which then goes on to influence ACC, FAS, S14 and pyruvate kinase as secondary

effects. This could explain the difficulty in identifying any molecular mechanisms for the activation of ACC, FAS, S14 and pyruvate kinase (Waters et al. 1997). Product feedback inhibition of lipogenic enzymes by exogenous FAs may occur. However, livers in animals fed fat free diets synthesised amounts of some of these FAs in excess of that reported to inhibit lipogenesis by dietary fats, yet did not inhibit fat synthesis. Alternatively, the liver may distinguish synthesised from absorbed FAs, but such a mechanism has not been described. However, the absorption process in the intestines might provide appropriate signals to the liver. For example the apoproteins, such as apoB-48 synthesised in the intestines and incorporated into chylomicrons and very low density lipoproteins during fat absorption may provide the means to identify exogenous fats (Nelsen et al. 1987a).

Differences occur in the time course for down regulation of SCD between hepatocytes and adipocytes. This suggests that the molecular mechanism responsible for the inhibition of SCD by dietary polyunsaturated FAs, in adipocytes may differ from that in the liver (Jones et al. 1996). Post-translational regulation is not a major factor in polyunsaturated FA mediated SCD repression because the observed reduction in SCD activity (60%) can be completely accounted for by decreases in SCD mRNA levels (80%). As opposed to hepatocytes, changes in mRNA stability are the major determinants of SCD mRNA abundance in adipocytes (Sessler et al 1996). The half-life of SCD *in vitro* is 10 +/- 1.5 minutes, but the addition of polyunsaturated FA to the media decreases the half-life 3-fold to about 2.5 minutes. Destabilisation of SCD mRNA in adipocytes maybe regulated through mRNA sequences in the 3' untranslated region. This region contains several structural motifs (AUUUA) characteristic of mRNA destabilisation sequences (Sessler et al. 1996).

SCD mRNA transcript levels in adipocytes are also regulated by a second post-transcriptional mechanism involving mRNA stability. The mRNA stability controlling system also responds to unsaturated FA that does not effect the transcription of the SCD gene (Gonzalez and Martin 1996). Treatment of 3T3-L1 adipocytes with arachidonic acid also caused a 3-fold decrease in the half-life of SCD1 mRNA and no apparent decrease in SCD1 gene transcription. The pre-translational regulation of the SCD1 gene expression by polyunsaturated FAs in adipocytes seems then to result primarily from the decrease of mRNA stability (Sessler et al. 1996).

Overall, between hepatocytes and adipocytes, the decrease in SCD activity from dietary polyunsaturated FA is achieved by decreased SCD mRNA transcription and increased SCD mRNA degradation. This means that there are two independent regulatory systems that respond to the same stimuli, that of dietary polyunsaturated FA (Gonzalez and Martin 1996).

The generally accepted mechanism put forward for lipid metabolism regulation by dietary polyunsaturated FA is given by the PPAR hypothesis. The PPAR hypothesis is confirmed as the regulatory mechanism in the induction of genes encoding for peroxisomal and mitochondrial FA oxidation enzymes. However, a number of recent studies cast doubt on the this mechanism of regulation for all the effects on lipid metabolism of dietary polyunsaturated FA, and suggest dietary polyunsaturated FA effects are a sum of many pathways (Millar and Ntambi 1996). Polyunsaturated FA control of mRNA stability could potentially be a component of systems that regulate the activity of other enzymes involved in the formation of membrane lipids such as desaturases other than SCD, elongases and transferases (Gonzalez and Martin 1996). This could also be the case for FAS, ACC and malic enzyme for which the mechanism by which dietary polyunsaturated FA inhibits these enzymes has been difficult to elucidate (Waters et al. 1997).

In the event that the PPAR hypothesis for the regulation of FAS, ACC and ME is discountered, then it has been proposed that n-6/n-3 FAs that inhibit gene transcription, are transported through the plasma membrane and bind to cytosolic FABPs. It is further hypothesised that FABP shuttles the 18 carbon FAs to Δ^6 desaturase. FABPs then carry FA products to the nucleus where the metabolites of Δ^6 desaturation are transferred to a specific nuclear FABP. This nuclear FABP then binds to a specific cis-acting element, which operates to govern gene transcription (Clandinin et al. 1996).

The induction of peroxisomal and mitochondrial, and inhibition of lipogenic and glycolytic genes suggests independent mechanisms (Millar and Ntambi 1996). In conclusion there is a wide variety of responses to dietary polyunsaturated FAs, including the induction of SCD. One mechanism can not fully explain the nuclear actions of polyunsaturated FAs. The difficulty in having a unifying hypothesis stems from the many metabolic routes taken by polyunsaturated FA upon entering the hepatocyte.

1.4.4. Differential Aspects of Dietary Polyunsaturated FA Action in Lipid Metabolism.

There is no evidence of similar rapid polyunsaturated FA-mediated control of S14 and FAS in white adipose tissue (Jump et al. 1994). The rapid effects of polyunsaturated FA on gene expression is liver specific as it seems not to suppress adipose and lung lipogenic rates (Clarke and Jump 1994; Jump et al. 1994; Kouba and Morout 1998). The applicability of the hepatic model to adipose tissue is further questioned when taking into account the conflicting

results of past research. Conflicting results reported in the literature may reflect the variation in the length of the experiments. Intervention of more than two weeks generally causes the suppression of lipogenesis, whereas intervention of less than one week produces no response in adipose tissue (Jones et al. 1996). As adipose tissue holds the vast majority of fat within the animal, the duration of intervention needs to be considered when attempting to alter the lipid composition in this tissue (Jones et al. 1996).

Analysis of mRNA in the liver and other tissues from mice fed chow or fat free diets show selective tissue expression and induction patterns for SCD1 and SCD2 (Diczfalusy et al. 1995). In mature fat cells polyunsaturated FA has down regulated SCD1 mRNA (Jones et al. 1996) by as much as 75% repression (Waters et al. 1997). This PUFA inhibitory regulation is also reflected in the liver content of SCD1 mRNA (Ntambi 1992). However, SCD2 mRNA is absent in the liver under any dietary condition, but is induced in white adipose tissue, kidney and lung tissue by feeding fat free diets (Kaestner et al. 1989). Feeding a fat free diet slightly down regulated SCD1 and SCD2 mRNA in brown adipose tissue (BAT). Thus, the regulation in BAT differs from white adipose tissue, where at least SCD2 is induced by feeding fat free diets (Diczfalusy et al. 1995). Differences in tissue-specific and type specific dietary induction of SCD1 and SCD2 must be bought about by distinct control mechanisms (Kaestner et al. 1989). Tissue and species specific regulation of SCD gene expression can be inhibitory or inductive (Ntambi 1995) and as such may explain the numerous beneficial and detrimental effects attributed to the various dietary fats (Clarke and Jump 1996).

Other variables such as species and age of the animals used may contribute to the inconsistent results seen in studying the polyunsaturated FA effects on lipid metabolism (Jones et al. 1996). These factors plus sex, diet and calorific intake all affect the site of FA synthesis. Therefore, considerable caution must be exercised in assigning *de novo* FA synthesis rates to the various tissues (Nelson et al. 1987). Lipid metabolism relationships within and between various tissues is also affected by feeding regimes, with meal feeding often negating the effects of dietary polyunsaturated FAs (Jones et al. 1996; Nelson et al. 1987).

CHAPTER TWO. MATERIALS AND METHODS.

2.1 Materials.

Materials used are listed according to the manufacturer they were sourced from.

The Random prime labelling system, hybond N, *redi*prime™II, and the radiochemical [α^{32}P] deoxy-CTP used in mRNA analysis were purchased from Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England.

Through FMC Bioproducts (www.bioproducts.com, Rockland, Maine. USA) the Agarose gel powder (electrophoresis grade, SeaKem LE) was purchased.

From the Sigma Chemical Company (PO Box 14508, St. Louis. MO 63178 USA) the [1-^{14}C]-palmitoyl-CoA ; casein (sodium salt) from bovine milk; and the reducing equivalent NADH (disodium salt) for the enzyme activity assay were purchased. The Xylene cyanol FF used in the analysis of mRNA was also bought from this company.

The WALLAC (an EG&G company. Wallac UK, Milton Keyes, UK.) product, Scintilation fluid- Optiphase high safe, was used in the quantification of enzyme activity.

The TRIzol reagent used in isolating total mRNA for Northern analysis was purchased from Life Technologies (PO Box 35, Inchinnan Business Park. Paisley PA4 9RF Scotland)

In separating the FA fractions after conversion of palmitate to palmitoleate by SCD the Varian (24201 Frampton Avenue, Harbour City, CA90710 USA.) product Bond Elut™ SCX solid-phase extraction columns were used.

All chemicals used throughout the procedures of sections 2.1, 2.2, 2.3 and 2.4 were purchased from BDH (BDH Laboratory Supplies. Poole BH15 1TD, England.), and are listed below. Potassium carbonate; Sodium acetate; EDTA; Formamide; Formaldehyde; Glycerol; Bromophenol blue; Sodium chloride; Sodium citrate; Methylene blue; Potassium dihydrogen

phosphate; Sodium phosphate; Sucrose; Methanol; Hydrochloric acid; Chloroform; Dichloromethane; Silver nitrate; Acetonitrile; Acetone; Sodium methoxide.

Animals and experimental procedures are described in the section on Experimental Design in each of chapters 3.1, 4.1 and 5.1.

2.2. Stearoyl-CoA Desaturase mRNA Analysis.

2.2.1. Preparation of Total RNA.

Total RNA was isolated using TRIzol reagent as described by the manufacturer (Life Technologies). Liver and mammary tissues (approximately 100 mg) were homogenised in 1.5 ml TRIzol reagent (a monophasic solution of phenol and guanidine isothiocyanate) using an UltraTurrex T25 homogeniser (Janke and Kunkle, IKA laboratories) at full speed for 10-20 sec. The homogenates were centrifuged at 12,000 x g for 10 min at 4 °C to remove insoluble material. The resulting supernatants were transferred to fresh tubes and following the addition of 300 µl chloroform, vigorous shaking for 15 sec, the samples were incubated at room temperature for 2-3 min. The samples were then centrifuged at 12,000 x g for 15 min at 4 °C and the upper aqueous phase was transferred to a fresh tube. Precipitation of RNA was carried out by incubating the aqueous phases with 750 µl isopropyl alcohol for 10 min at room temperature and centrifugation at 12,000 x g for 10 min at 4 °C. The resulting pellets were washed once by adding 1.5 ml 75% ethanol and vortexed. The tubes were then centrifuged at 12,000 x g for 10 min at 4 °C and the resulting pellets were resuspended in 100 µl DEPC water. Total RNA was quantified by measuring UV light absorbance at 260 nm (1 A_{260} U = 40 µg/ml RNA).

2.2.2. cDNA Probes.

The rat SCD cDNA probe was kindly provided by Dr. Stephen B. Smith, Department of Animal Science, Texas A&M University, College Station, Texas (Cameron et al. 1994). The human GAPDH cDNA probe was kindly provided by (Fort et al. 1985). The isolated

fragments were then labelled with [α^{32}P] deoxy-CTP using the random prime labelling kit and Northern blot analysis was carried out.

2.2.3. Random Prime Labelling of DNA Probes.

The random prime labelling system, *redi*prime™II, was used to radiolabel the SCD and GAPDH isolated fragments as described by manufacturer (Amersham Pharmacia Biotech). The DNA, 25 ng in 45 µl of buffer was denatured by heating for 5 min at 100 °C and then added to the reaction tube containing buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primers. The [α^{32}P] deoxy-CTP, 5 µl, was added and incubation was carried out at 37 °C for at least 1 hour. The incorporation of [α^{32}P] deoxy-CTP was measured by spotting 2 x 4.5 µl of 1:10 dilution of the reaction on 2 pieces of Whatman filter paper. One filter was measured for total counts and the second filter was washed 2 x 10 min in 5 % TCA and counts incorporated into DNA measured using a Wallac 1409 liquid scintillation counter (Sci Tech).

2.2.4. Northern Hybridisation.

RNA samples, 2.5 µg, were denatured in MOPS buffer pH 7.0 (10 x MOPS contains 0.2 M MOPS, 80 mM sodium acetate, 10 mM EDTA) containing 50 % formamide and 17.5% formaldehyde and 10 % (v/v) loading dye (containing 50 % glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF). The samples were placed immediately on ice and the RNA was electrophoresed on 1 % (w/v) agarose gel containing 2.2 M formaldyhyde (Lehrach et al 1977) and blotted onto Hybond N nylon membrane as described by manufacturers (Amersham). Following UV cross-linking, the membrane was rinsed in a 2 x SSC (20 x SSC contains 3 M NaCl and 0.3 M sodium citrate) for 5 min and methylene blue (0.02 % in 0.3 M sodium acetate, pH 5.5) was used to stain the 28 S and 18 S ribosomal RNA bands to check for loading.

The membranes were pre-hybridised in buffer (500 mM sodium phosphate, pH 7.2, 1 mM EDTA, 7 % SDS, Church and Gilbert 1984) at 65 °C for 30 min, and then probed by adding [α^{32}P] deoxy-CTP labelled probes. Hybridisation was carried out at 65 °C overnight. The membranes were then washed with increasing stringent conditions for 10 min each of (4 x

SSC, 0.1 % SDS; 2 x SSC, 0.1 % SDS; 1 x SSC, 0.1 % SDS and 0.5 % SSC, 0.1 % SDS). The membranes were exposed to XAR film (Eastman Kodak Company, Rochester, NY, USA) for auto-radiography. Densitometric quantification was carried out using a Molecular Dynamics laser scanning densitometer.

2.3. SCD Enzyme Assay.

The Δ^9 desaturase activity was determined by the extent of conversion of labelled saturated palmitic acid to the unsaturated palmitoyl-CoA.

2.3.1. Tissue Preparation.

Mammary gland aliquots obtained from experimental trials as described in experimental chapters were removed immediately after slaughter and placed on tissue paper to remove excess milk. Aliquots were snap-frozen in liquid nitrogen and stored at -70 °C until required for desaturase assay determination. The mammary gland samples (0.3-1.1g) were homogenised in 5 volumes of buffer containing 0.25 M sucrose, 10 mM Tris HCl, pH 7.4 at full speed using an UltraTurrex T25 homogeniser for 10-20 sec. The buffer was at 4 °C. The homogenate was centrifuged at 5,000 x g for 10 min and the resulting supernatant was centrifuged at 10,000 x g for 15 min. The protein concentration was determined as described below, snap-frozen and stored at -70 °C until required for subsequent analysis.

2.3.2. Protein Determination.

Protein was measured using the BCA system as described by the manufacturer (PIERCE). Bovine serum albumin, provided in the kit, was used as the standard.

2.3.3. SCD Δ^9 Enzyme Assay.

The assay was carried out as described by St John et al. (1991). The reaction mixture consisted of 100 mM Tris HCl pH 7.4, 2 mM NADH, 50 μM palmitoyl Coenzyme A, 50,000 dpm [1-^{14}C]- palmitoyl Coenzyme A per incubation, 1 mg/ml casein in a total volume of 1.0 ml. The reaction mixture was pre-incubated at 37 °C in a shaking waterbath. The enzyme

fraction was thawed at 37 °C and 2 mg was added to the reaction mixture for the assay to begin. Prior to the addition of the enzyme fraction, 1.0 ml chloroform and 3.0 ml 5 % methanolic HCl was added to the blanks. The assay was stopped after a 10 min incubation by the addition of 1.0 ml chloroform and 3.0 ml 5 % methanolic HCl.

2.3.4. Methylation of Assayed Samples.

The samples were methylated twice according to Sukhija and Palmquist (1988) as described in Section 2.4.1. The dry film of methyl fatty acid esters (FAMES) resulting after the second methylation step was re-dissolved in 500 µl dichloromethane and subjected to silver ion chromatography for separation, and measurement of saturated and unsaturated fatty acids.

2.3.5. Quantification of the SCD Assay.

Silver ion chromatography using solid-phase extraction columns packed with a bonded-sulfonic acid phase (Christie, 1989) was used for separation of saturated and unsaturated fatty acids. The Bond Elut™ SCX solid-phase extraction columns were primed by allowing silver nitrate, 20 mg in 0.25 ml acetonitrile-water 10:1 (v/v) to percolate through the column, followed by flush steps with acetonitrile, acetone and lastly dichloromethane. The FAME sample was applied to the primed column. Elution of saturated FAMEs was carried out with 5 ml dichloromethane followed by elution of the monounsaturated FAMEs with 5 ml dichloromethane:acetone. The solvents were allowed to flow under gravity. A stream of nitrogen was used to evaporate the solvents and the FAMES were re-dissolved in 400 µl hexane and 2 x 200 µl were transferred to scintillation vials and radioactivity was determined using a Wallac 1409 liquid scintillation counter (Sci Tech). The rate of conversion for each sample was corrected for by subtracting the value obtained from a blank incubation.

2.4. Chromatographic Analysis of Fatty Acid Composition.

2.4.1. Sample Preparation: Acid catalysed methylation.

In the preliminary study (Chapter 3.0) mammary gland and liver (approximately 500 mg), and milk (approximately 1 ml) samples were analysed for total fatty acid composition using the one-step extraction and methylation procedure of Sukhija and Palmquist (1988). This method was initially used because processing times are shorter and losses that can be incurred during multi-step procedures of extraction, esterification and purification are less compared to other methods.

The one-step extraction produces 10 – 50 mg of fatty acids. Following the addition of 1ml chloroform and 3 ml 5% methanolic HCL the samples were homogenised, vials capped and incubated in a water bath at 70 °C for 2 hrs for transesterification and methylation. The samples were cooled to room temperature, and 5 ml of 6% potassium carbonate and 4ml of chloroform were added. The samples were vortexed and centrifuged for at 480 x g for 10 min. The lower chloroform layer was recovered and the remaining solution was re-extracted with 4 ml chloroform. The sample of combined chloroform layers was evaporated to a film in a preweighed auto-sample vial. The vial was weighed to obtain the weight of the FA residue. The sample was reconstituted in 1ml hexane and capped with nitrogen.

2.4.2. Sample Preparations: Base Catalysed Methylation.

The base catalyst procedure, as described in Appendix 1, was adopted for milk fat extractions in studies described in Chapters 4.0 and 5.0 following the preliminary study (Chapter 3.0). Justification for, and development of, the new procedure is also outlined in Appendix 1.

Mouse milk (100 µl) was used, to which 0.9 ml of chloroform/methanol (1:2 v/v) was added. After vortexing, 0.5 ml chloroform and 0.5 ml water were added to the sample and vortexed again. The resulting lower chloroform layer was recovered and the remaining sample was re-extracted with 0.75 ml chloroform. The sample of combined chloroform layers was evaporated to a film in a pre-weighed auto-sample vial. In contrast to the one-step extraction and methylation procedure, Section 2.4.1, this evaporation step does not result in FA loss by

volatilisation since the majority of FA in tissue and milk are in the TAG or phospholipid form which have not yet been methylated.

The method for fat extraction from tissue was as described by Kates (1982). Liver and mammary gland (10g) was homogenised for 2 min in 30 ml methanol/chloroform (2:1 v/v). Following centrifugation at 480 x g for 10 minutes at 480g, the resulting supernatant was recovered and the pellet was re-extracted with 39 ml methanol/chloroform/water (2:1:0.9 v/v). The 2 chloroform aliquots were combined and diluted with 20 ml chloroform and 20 ml water. This was centrifuged at 480 x g for 10 min. The lower chloroform phase was recovered and evaporated by a nitrogen gas stream. All aliquots, whether tissue or chemical, can be uniformly scaled according to the amount of sample.

To methylate the FA residues from both milk and tissue fat extracted above, 200 µl sodium methoxide was added to the samples. The vials were capped with a gas stream of nitrogen, vortexed and incubated for 10 min at room temperature. On addition of 0.5ml hexane, the vials were re-capped with nitrogen, mixed well and incubated for a further for 10 min at room temperature. The methylation reaction was stopped by adding 200 µl neutralising solution, which was prepared by dissolving 100g potassium dihydrogen phosphate (KH_2PO_4) and 150g sodium chloride in 1.0*l* of MQ water. The vials were capped with nitrogen and vortexed. The sample was centrifuged at 480 x g for 10 mins. For fat isolated from milk, 0.5 ml of the resulting upper layer was made up to 1.0 ml with hexane in an auto-sampler vial. For tissue samples, the 0.5 ml of the upper layer was applied to a silica gel column (0.3 g of silica gel in a Pasteur pipette) and eluted into an auto-sampler vial with 1.5 ml of hexane. All samples are then ready to apply to the GC.

2.5. Statistical analysis.

Data were analysed using Minitab ANOVA , Balanced one way. If patterns on the residual vs fits graph showed there could be a case to suspect outliers the data were re-analysed with ANOVA, General Linear Model to identify the animal with the suspect data. If no reason was found to omit the data, the data was log transformed where appropriate and re-analysed in

ANOVA, General Linear Model. The generated Least Squares Difference was then used to find significance between treatment means. Mammary and liver SCD mRNA was analysed for significant relationships with each other and the FA composition (16 and 18 ratio) of tissues and milk. These relationships were derived using Minitab, Simple Linear Correlations.

CHAPTER THREE - PRELIMINARY TRIAL.

3.1. Introduction.

Bovine milk fat contains small amounts of unsaturated fatty acids and a large proportion of saturated FAs, which are implicated as being adverse for human health. The degree of desaturation also influences milk fat hardness thus altering the spreadability of butter. Thus, an ability to modify the FA composition of bovine milk fat could be of major benefit to the dairy industry and human health.

Therefore techniques for increasing the synthesis of unsaturated FAs should be investigated. SCD is an enzyme that catalyses the Δ^9-desaturation of palmitoyl-CoA (16:0) and stearoyl-CoA (18:0), converting these to palmitoleoyl-CoA (16:1) and oleoyl-CoA (18:1), respectively (Ntambi 1995). It is the terminal enzyme in a multi component system bound to microsomal membranes and is involved in regulation of the system.

SCD is the predominant desaturation enzyme of the mammary gland and other tissues such as liver, adipose, brain and testes (Madsen et al. 1997). In the liver of male mice, SCD expression is induced up to 40-fold by feeding a fat free, high carbohydrate diet (Ntambi 1995), and up to 22-fold by feeding a diet supplemented with clofibrate (Millar and Ntambi 1996). Clofibrate is a hypolipidaemic drug causing peroxisomal organelle and enzyme induction. It has multiple effects on lipid metabolism by depressing FA synthesis and esterification, and increasing FA oxidation (Pennachiotti et al. 1996). Positive regulation by a fat free diet, and negative regulation by a polyunsaturated FA diet (Section 1.4) on SCD gene expression have both been shown in different tissues of non-lactating rodents (Ntambi 1995).

There have been no reports of SCD gene expression and its manipulation in the lactating mammary gland. This study focuses on understanding the influence of a fat free/ high carbohydrate diet and dietary clofibrate on SCD mRNA expression and, milk fat synthesis in the mammary gland of lactating mice.

3.2. Experimental Design.

Female mice were obtained from the Small Animal Colony at Ruakura, AgResearch and the trial was granted approval by the Ruakura Animal Ethics committee. The mice were housed in banked individual cages with a light/dark cycle of 14/10hrs.

Lactating mice, ranging from 3 days of lactation to 8 days of lactation were randomly divided into 6 groups of 3 mice each. All groups were fed a control diet for 2 days prior to fasting for 24hrs. The groups were then fed either a control diet, a fat-free/high carbohydrate diet or a control diet supplemented with clofibrate (0.5% w/w) for 24 or 48hrs. Non-lactating female mice, randomly divided into 3 groups of 3 mice each were fasted for 24hrs, then fed the control and test diets for 24hrs. Dietary composition data are set out in Appendix 2.

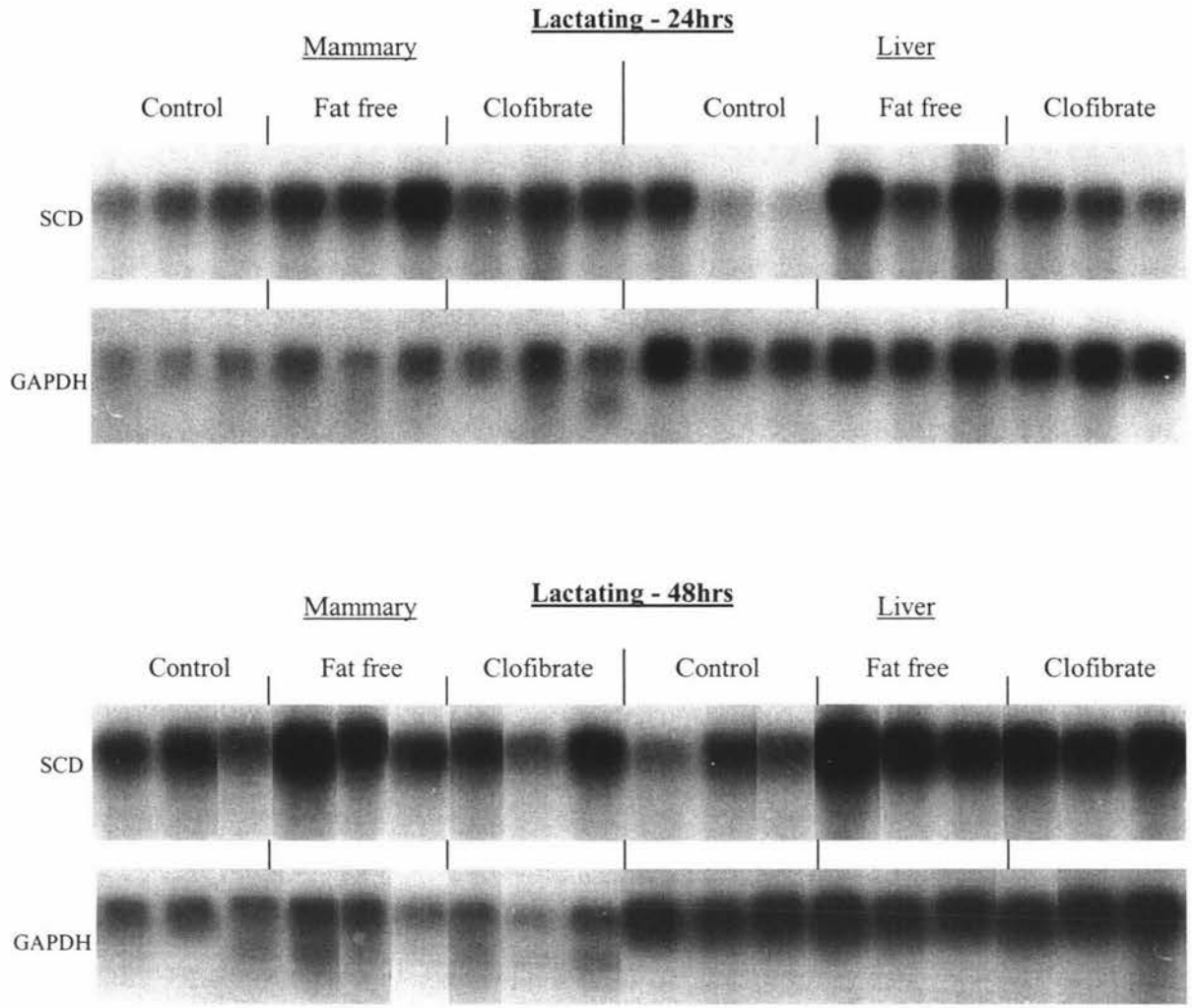
Milk was collected from the lactating mice in the Small Animal Colony. The pups were separated from their dams 3-4 hrs before slaughter to facilitate the collection of a larger milk sample. The mice were first lightly anaesthetised with a 150µl intraperitoneal injection of Hypnorm/Hypnovel, followed by an intraperitoneal injection of 0.15ml (1iu/ml) of oxytocin in saline. Milk was removed from the mammary gland by gentle manual pressure into Pasteur pipettes and then transferred to Eppendorf tubes. These were snap frozen in liquid nitrogen and stored at –70 °C until analysed for fatty acid composition.

After milking, the mice were sacrificed by cervical dislocation. Aliquots of liver and mammary gland were collected immediately and also snap-frozen in liquid nitrogen and stored at –70 °C until analysed for FA composition and SCD mRNA content.

Fatty acid composition of milk, mammary gland and liver was determined by a one-step extraction–transesterification procedure as described by Sukhija and Palmquist (1988) (Section 2.4. and Appendix 1) followed by gas chromatography. The ratios of 16:1/ 16:0 and 18:1/ 18:0 were calculated as measures of the changes in desaturase activity. SCD mRNA analysis was carried out by extracting total RNA from the tissues using TRIzol reagent as described by the manufacturers (Section 2.2.). Northern blot analysis was carried out using 2.5 µg total RNA as described in section 2.2. Transferred RNA was detected by staining with methylene blue (data not shown). The rat SCD cDNA probe was random prime labelled using ^{32}P-dCTP and hybridisation of the membrane was carried out as described in section 2.1. The membrane was washed and densitometry was carried out to obtain arbitrary values for quantitative analysis. Data were normalised to GAPDH mRNA levels because no statistical difference was seen between the treatment groups for this mRNA. Data were analysed (Section 2.5) by Minitab, ANOVA- Balanced and General Linear model. Relationships were determined by Minitab, Correlations (Pearson).

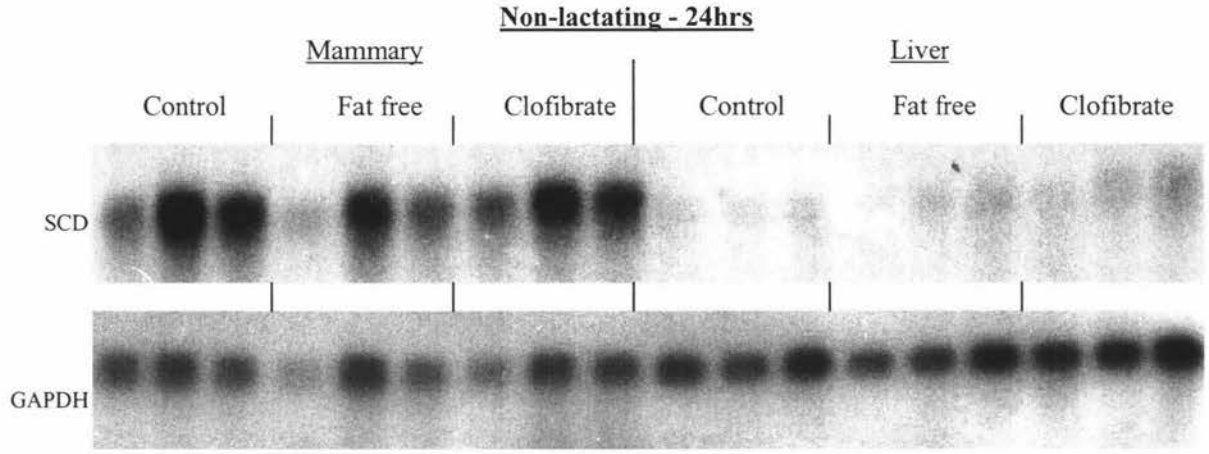
3.3. Results.

Figure 3.1. Northern blot of SCD mRNA levels in liver and mammary gland of lactating mice fed control, fat free/high carbohydrate and clofibrate diets.



Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out with 2.5ug total RNA for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Transferred RNA was stained with methylene blue (data not shown).

Figure 3.2. Northern blot of SCD mRNA levels in liver and mammary gland of non-lactating mice fed control, fat free/high carbohydrate and clofibrate diets.



Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out 2.5ug total RNA for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Transferred RNA was stained with methylene blue (data not shown).

A 5.1kb transcript was detected for SCD mRNA and a 1.3kb transcript for GAPDH (Figure 3.1 and 3.2). SCD mRNA was barely detectable in the livers of non-lactating female mice (Figure 3.2).

Table 3.1. The effect of control, fat free and clofibrate diets on stearoyl-CoA desaturase mRNA levels in liver and mammary gland of lactating mice.

Tissue	Control	Fat free	Clofibrate	Sed
Liver	0.26	1.38*	0.65	0.30
Mammary	1.70	3.52*	2.82	0.68

* Treatment is significantly increased from the control treatment at $P<0.05$.

Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out on SCD mRNA and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values. The SCD data were normalised to GAPDH mRNA as there were no significant treatment effects.

Effects of diet on mammary gland SCD mRNA levels of lactating mice.

No significant differences were detected in the mRNA levels for SCD between 24 and 48hrs samples for any of the treatments, allowing the values for these time points to be combined for further analysis.

Mammary SCD mRNA levels were significantly increased compared to the control treatment by 2.1-fold ($P<0.05$) by feeding the fat free/ high carbohydrate diet, whereas the clofibrate diet resulted in an apparent 1.6-fold increase that was not statistically significant (Table 3.1).

Effects of diet on liver SCD mRNA levels in lactating mice.

The levels of liver SCD mRNA from the lactating mice were significantly increased, in this case 5-fold, by feeding the fat free/ high carbohydrate diet ($P<0.05$, Table 3.1). Feeding the clofibrate diet did not result in significant differences at 24hrs, however, at 48hrs the clofibrate levels were significantly greater than those for the mice fed the control diet (0.94 vs 0.30 respectively, Sed 0.3, $P<0.05$).

Effects of diet on mammary gland and liver SCD mRNA levels in non-lactating mice.

In contrast to the livers from the lactating mice, no effects of either diet were detected in the non-lactating mice. No differences were detected in the mammary gland of non-lactating mice fed the experimental diets (Table 3.2).

Table 3.2. The effect of control, fat free and clofibrate diets on stearoyl-CoA desaturase mRNA levels in mammary gland and liver of non-lactating mice.

Tissue	Control	Fat free	Clofibrate	Sed
Liver	0.003	0.004	0.009	0.03
Mammary	1.36	0.54	1.16	0.30

Total RNA was extracted from liver and mammary gland of non-lactating mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values. The SCD data was normalised to GAPDH mRNA as there was no significant treatment effects.

Table 3.3. The effect of lactation on stearoyl-CoA desaturase mRNA levels in livers of mice fed the control diet.

Tissue	Non-lactating	Lactating	Sed
Liver	0.021	0.212	0.25

Total RNA was extracted from liver non-lactating and lactating mice fed the control diet and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values. The SCD data were normalised to GAPDH mRNA as there were no significant treatment effects.

No statistically significant differences were detected in the levels of SCD mRNA between the livers of non-lactating and lactating mice fed the control diet (Table 3.3).

Table 3.4. The effect of control, fat free/high carbohydrate and clofibrate diets on the milk, mammary gland and liver FA composition of lactating mice.

Time/Sample	FA ratio	Control	Fat free	Clofibrate	Sed
Milk	16	0.13	0.19^{+}	0.13	0.05
	18	9.34	8.69	9.50	0.60
Mammary	16	0.13	0.11	0.16	0.03
	18	5.30	5.35	5.08	0.82
Liver	16	0.11	0.10	0.11	0.02
	18	2.50	2.06	3.07	0.74

+ = P<0.10

Fatty acid composition of milk, mammary gland and liver was determined by a one-step extraction–transesterification procedure as described in methods (section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity.

Effects of diet on milk 16:1/16:0 and 18:1/18:0 FA ratios of lactating mice.

No significant differences were detected in the 16:1/16:0 and 18:1/18:0 FA ratios between 24 and 48hrs samples for any of the treatments, allowing the values for these time points to be combined for further analysis.

There were no significant differences between treatment in milk or either tissue for 16:1/16:0 and 18:1/18:0 FA ratios (Table 3.4), although the fat free /high carbohydrate diet tended to increase the milk 16:1/16:0 ratio (P<0.10).

Table 3.5. The effect of control, fat free/high carbohydrate and clofibrate diets on mammary gland and liver FA composition of non-lactating mice.

Tissue	FA ratio	Control	Fat free	Clofibrate	Sed
Mammary	16	0.29	0.27	0.34	0.05
	18	10.10	11.18	9.93	1.20
Liver	16	0.07	0.07	0.12	0.03
	18	2.53	2.42	5.14*	0.44

* Treatment is significantly increased from the control treatment at $P<0.05$.

Fatty acid composition of mammary gland and liver was determined by a one-step extraction–transesterification procedure as described in methods (section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity.

Effects of diet on mammary gland ratios of 16:1/16:0 and 18:1/18:0 FA ratios of non-lactating mice.

Mammary 16:1/16:0 and 18:1/18:0 FA ratios were unchanged by feeding either the fat free/high carbohydrate or clofibrate diets compared to the control treatment in the non-lactating mice (Table 3.5).

Effects of diet on liver ratios of 16:1/16:0 and 18:1/18:0 FA ratios of non-lactating mice.

In the liver of non-lactating mice, although no differences relating to diet could be detected in the 16:1/16:0 ratio, the 18:1/18:0 FA ratio was significantly ($P<0.05$) increased by the clofibrate diet compared to the control diet (Table 3.5).

Table 3.6. The correlation of mammary gland SCD mRNA, with liver SCD mRNA and the 16:1/16:0 and 18:1/18:0 FA ratios for milk, liver and mammary gland tissue in lactating mice.

Mammary SCD mRNA associations with:	FA ratio	r	P
Milk	16	0.20	0.42
	18	-0.22	0.37
Mammary	16	0.39	0.11
	18	0.32	0.19
Liver	16	-0.15	0.55
	18	0.59	0.01
Liver SCD mRNA		0.25	0.32

Fatty acid composition of mammary gland and liver was determined by a one-step extraction–transesterification procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of non-lactating mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values. The SCD data was normalised to GAPDH mRNA as there was no significant treatment effects.

Relationships between mammary SCD mRNA, and tissue and milk FA composition.

There were no significant relationships between mammary SCD mRNA and the other parameters listed in Table 3.6.

Relationships between liver SCD mRNA, and tissue and milk FA composition.

Milk 16:1/16:0 ratio was positively correlated (r= 0.558, P= 0.016) with liver SCD mRNA but no other ratios were significantly correlated to tissue SCD mRNA levels (Table 3.7).

Table 3.7. The correlation of liver SCD mRNA with milk, liver and mammary gland tissue 16:1/16:0 and 18:1/18:0 FA ratios, in lactating mice.

Liver SCD mRNA associations with:	FA ratio	r	P
Milk	16	0.552	0.017
	18	-0.265	0.288
Mammary	16	0.610	0.007
	18	0.241	0.336
Liver	16	0.495	0.037
	18	0.520	0.027

Fatty acid composition of mammary gland and liver was determined by a one-step extraction–transesterification procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity.

3.4. Discussion.

This preliminary study examined the effects of a fat-free diet and a clofibrate supplemented diet on the expression of SCD mRNA in mammary gland and liver of the lactating mouse and the possible relationship to changes in the fatty acid composition of these tissues and milkfat. The results (Figure 3.1; Table 3) show that the expression of SCD in both the mammary gland and liver in lactating mice can be enhanced by feeding a fat-free diet, suggesting regulation at the transcriptional level in both tissues. This is in agreement to earlier reports in male mice, where hepatic SCD mRNA levels are induced (Ntambi 1995). In contrast, clofibrate caused no significant changes to SCD mRNA levels in either tissue.

While the fat free/high carbohydrate diet induced tissue SCD mRNA, there were no associated changes seen in tissue and milk FA ratios. However, the fat free/high carbohydrate diet tended to increase the milk 16:1/16:0 ratio (Table 3.4) compared with the control and clofibrate treatments. Furthermore, although the milk 16:1/16:0 ratio was not correlated to mammary SCD mRNA (Table 3.6), it was correlated ($P<0.05$) to liver SCD mRNA (Table 3.7). Past studies in the liver of male mice have shown increases in these ratios in feeding a

fat free diet. In contrast, when male mice are fed dietary polyunsaturated FAs these ratios are reported to decrease (de Antueno et al. 1993; Nelson et al. 1987).

The addition of clofibrate to the diet did not effect the FA ratios in the milk or the mammary gland of the lactating mice which is consistent with a lack of effect on SCD mRNA (Table 3.4). This is in contrast with previous reports in which clofibrate modifies the FA composition of various phospholipid fractions and TAG in the liver of male mice (Pennachiotti et al. 1996). The reason for this discrepancy is not apparent, although there is a possible effect of sex difference having an influence (Section 4.3)

The FA ratios in the liver were correlated ($P<0.05$) with the SCD mRNA in the liver (Table 3.7) which is consistent with the results from other studies (Ntambi 1995; Pennachiotti et al. 1996). There were also significant correlations between liver SCD mRNA and the 16:1/16:0 FA ratio in the mammary gland ($P<0.01$) and milk ($P<0.05$). This, coupled with the lack of significant correlations between SCD mRNA in the mammary gland and the 16:1/16:0 FA ratio (Table 3.6) implies that the liver has a stronger influence in determining the 16:1/16:0 FA ratio in milk fat.

In the livers of non-lactating mice, neither SCD expression nor the fatty acid composition were affected by the dietary manipulations. This was unexpected as previous studies have shown up to 40-fold increases by feeding a fat-free high carbohydrate diet after a period of fasting (Ntambi 1992) and up to 20-fold increases by dietary supplied clofibrate (Miller and Ntambi 1996). There is no obvious explanation for this apart from the observation that these previous studies were carried out in male mice in contrast to the present study. The expression of SCD mRNA has been reported to be dependent on gender, with levels in livers of female mice 5-fold those in male mice (Lee et al. 1996). Ratios of palmitoleate:palmitate and oleate:stearate were used to represent a desaturation index that was also significantly greater in female mice. It was hypothesised that this gender difference may be due to differences in levels of hormones such as estrogen and testosterone (Lee et al., 1996).

The experience gained in running this preliminary study and the results obtained were used to design studies described in Chapters 4.0 and 5.0. For example, in the subsequent studies, the effects of a fat free diet and clofibrate supplemented diet were examined in separate trials with increased numbers per group. The treatment groups were also balanced for pup number and day of lactation. In the preliminary trial the treatment groups were not balanced for pup number, and day of lactation at the start of the trial ranged from day 3 to day 8. In both the subsequent trials the starvation period was omitted so that any manipulation seen would be from the effects of a fat free diet or clofibrate use. Starvation is a

physiologically altered state that reduces the levels of SCD mRNA (Mizuguchi et al. 1996) and thus may attenuate the response of SCD mRNA to treatments. In both the subsequent trials, the feeding of experimental diets was carried out for a longer time period. While the logistics of running a trial for several weeks (Mandla et al. 1996) may be too complicated, a trial duration of 6-8 days commonly sees induction of liver SCD mRNA in male mice (Diczfalusy et al. 1995; Kawashima et al. 1990). The main aim of this preliminary trial was to accomplish an induction in SCD mRNA levels and consequent alteration of FA composition in milk. For this reason another route of clofibrate administration was proposed. Peters et al. (1996) used a saturating dose by intraperitoneal injection of clofibrate to induce peroxisomal organelle proliferation. The clofibrate was injected in a corn oil vehicle at 15mg/100g of body weight. In the subsequent dietary fat trial (Chapter 5.0) the high carbohydrate diet approach was omitted, as just like starvation, high carbohydrate also has its effects on SCD mRNA that could confound SCD mRNA attenuation by dietary fat. In looking at the diet composition data in Appendix 2, it is also doubtful that the concentration of carbohydrate in the fat free/ high carbohydrate is significantly higher than the carbohydrate concentration in the control diet. The main emphasis of this research is to investigate if changes in SCD are related to possible alterations in milk fat composition. For this reason the subsequent trials described in the following chapters did not include non-lactating mice. The analysis of the FA composition of milk fat was expanded to the 32 FAs found in the GLC-85 standard (Appendix 1) as this may also reflect alterations in enymes other than SCD that produce certain FAs found in milk. An example being the *de novo* production of octanoate to palmitate by FAS.

CHAPTER FOUR - CLOFIBRATE INDUCTION OF SCD.

4.1. Introduction.

Many steps in lipid metabolism are affected by clofibrate in a co-ordinated manner. This leads to a reduction in the incorporation of shorter more saturated FAs and an increase in longer more unsaturated FAs in phospholipids (Millar and Ntambi 1996).

The main responses to clofibrate are an increase in the proliferation of peroxisomal organelles and an induction of liver hepatomegaly. Associated with these changes are an induction of mitochondrial, and particularly peroxisomal enzymes, in the β-oxidation pathway. Peroxisomal β-oxidation differs from mitochondrial β-oxidation in its greater ability to oxidise long chain polyunsaturated FAs (Millar and Ntambi 1996). In addition, clofibrate is a peroxisomal proliferator with many extra-peroxisomal effects in liver and other tissues. Some of these effects include the stimulation and inhibition of mitochondrial and cystolic enzymes, that may be directly or indirectly related to the peroxisomal effect (Yousseff and Badr 1998). For example clofibrate inhibits FAS (Pennachiotti et al. 1996), which is reflected in the decrease of short chain saturated FAs incorporation into phospholipids (Kawashima et al. 1990).

The increased peroxisomal FA oxidation leads to a shortfall in long chain FA for incorporation into phospholipids. In response, elongation and desaturation enzymes, including SCD, are induced (Millar and Ntambi 1996). There is some debate over whether SCD is induced directly by the activation of PPARs by clofibrate, or whether SCD induction is a secondary consequence of polyunsaturated FA deficiency. Irrespective of the mechanism, the increased activity of SCD leads to an increased incorporation of oleate into phospholipid (Kawashima et al. 1990; Pennachiotti et al. 1996). The enzymes involved in FA incorporation into phospholipid and TAG (acyl-CoA synthetase and many transferases) are similarly induced by clofibrate, so facilitating the increased incorporation of oleate into phospholipids (Kawashima et al. 1990).

An improved understanding of the control of these various aspects of FA metabolism may allow the manipulation of the FA composition of milk fat of ruminants. Thus, the objective of the experiment described in this chapter was to increase our understanding of mammary FA desaturation by studying the effects of clofibrate treatment on SCD mRNA levels and changes in the milk FA composition.

4.2. Experimental Design.

The experimental design was developed using the data and experience obtained in the preliminary trial (Chapter 3.0). The Ruakura Animal Ethics committee approved the experimental protocol, used in this experiment. Twenty-four Brown Swiss mice were supplied by the Small Animal Colony, Ruakura. The mice were housed individually in plastic cages in a room maintained at 21.0 +/- 1.0 °C and a 14:10 hour light: dark cycle. All mice were assigned on the 7th day of lactation to one of the four treatments and pup numbers were balanced across treatments. These groups were:

- Control treatment group (n=6) fed the base diet only;
- Ingested Clofibrate treatment group (n=6) fed the base diet to which 0.5% w/w of clofibrate was added;
- Injected blank treatment group (n=6) fed the base diet and injected daily with the olive oil vehicle;
- Injected clofibrate treatment group (n=6) fed the base diet and injected with clofibrate (15mg/100g bodyweight) in an olive oil vehicle.

Injections were given subcutaneously, immediately anterior to the abdominal mammary glands. Details of the composition of the basal diet and calculations for dose of clofibrate are presented in Appendix 2. All treatments were applied for 7 days.

On the 7th day of treatment the pups were separated from their dams for 3-4 hrs, and milk samples were collected. In the collecting of milk, the mice were lightly anaesthetised with 150 µl Hypnorm/Hypnovel (IP), followed by a 0.15 ml IP injection of 1iu/ml oxytocin in saline. Milk was removed from the mammary gland by gentle manual pressure into Pasteur pipettes and then transferred to Eppendorf tubes and snap frozen in liquid nitrogen and stored at –70 °C until required for fatty acid composition analysis.

Animals were then sacrificed by cervical dislocation. Aliquots of liver and mammary gland were collected immediately and also snap-frozen in liquid nitrogen and stored at –70 °C until required for FA composition and SCD and GAPDH mRNA analysis.

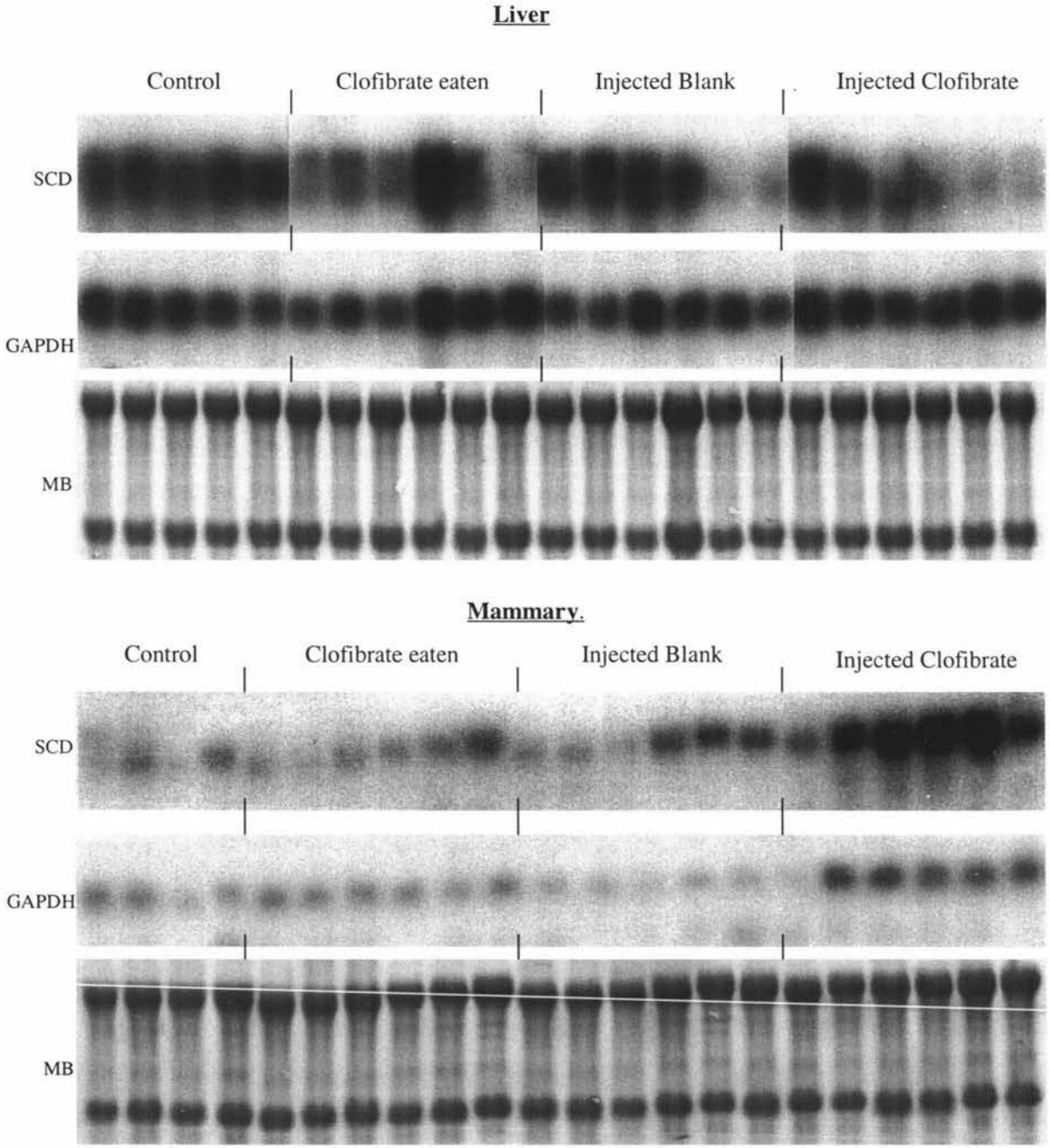
Fatty acid composition of milk, mammary gland and liver were determined by methods outlined in Chapter 2. The methylation procedure was modified as described in Appendix 1. The 16:1/16:0 and 18:1/18:0 FA ratios were calculated as measures of the desaturase activity. An increase in these ratios reflects an increase in Δ^9desaturation (Lee et al. 1996). A wider range of 32 FAs was also measured in the milk to detect any other effects of clofibrate on

lipid metabolism (Millar and Ntambi 1996). The level of SCD mRNA in the liver and mammary gland was estimated following the extraction of the total RNA from the tissues using TRIzol reagent as described by manufacturers (Section 2.2.). Northern blot analysis was carried out using 2.5 μg total RNA as described in section 2.2. The RNA was subjected to electrophoresis on a 1.2% formaldehyde agarose gel and transferred to Hybond N membranes. Transferred RNA was detected by staining with methylene blue (data not shown). Rat's SCD cDNA probe was random prime labelled using ^{32}P-dCTP and hybridisation of the membrane was carried out as described in section 2.2. The membrane was washed and densitometry was carried out to obtain arbitrary values for quantitative analysis. Data were analysed (Section 2.5) by Minitab, ANOVA- Balanced and General Linear model. Data was log transformed if required. With the subsequent generation of the least square difference value, means between treatments were assigned significance. Correlation analysis was determined by Minitab, Correlations (Pearson).

4.3. Results.

mRNA results.

Figure 4.1. Northern blots of SCD mRNA levels in the liver and mammary gland of lactating mice fed or being injected with clofibrate, and their matching control treatments.



Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out with 2.5ug total RNA for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Transferred RNA was stained with methylene blue (MB).

Table 4.1. Northern blot of SCD mRNA levels in the liver and mammary gland of lactating mice, either ingesting or being injected with clofibrate, and with their respective controls.

Tissue	RNA	Control	Clo eaten	Inj Blk	Inj Clo	Sed
Liver	SCD	2.84	2.79	2.71	2.61	0.20
Liver	GAPDH	2.96	3.01	2.90	3.04	0.10
Mammary	SCD	-0.29	1.63*	1.94*	2.55+	0.37
Mammary	GAPDH	2.11	2.12	1.80	2.46*	0.15

* Treatment is significantly increased from the control treatment at $P<0.05$.
+ Treatment is significantly greater over any other treatments at $P<0.05$.

Total RNA was extracted from liver and mammary gland of lactating mice given the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values. The means and Sed for the treatments are log transformed data.

Liver GAPDH mRNA levels (Table 4.1) were not affected by treatment, but were significantly greater in the injected clofibrate treatment in the mammary gland. For this reason the SCD mRNA levels were not normalised to GAPDH mRNA levels. The methylene blue stain was included in Figure 4.1 to show that equivalent amounts of total mRNA were analysed. The SCD mRNA values showed that the treatment ingesting clofibrate and the treatments injected (with or without clofibrate) were significantly greater than the control treatment. Within these significant treatments the injected clofibrate treatment was significantly greater than the other two treatments.

FA composition results.

Table 4.2. The effect of clofibrate, ingested or injected, and their respective control treatments on milk, mammary gland and liver FA composition of lactating mice.

Tissue	FA ratio	Control	Clo eaten	Inj Blank	Inj Clo	Sed
Liver	16	0.05	0.07	0.06	0.06	0.01
	18	1.11	1.69	1.26	1.34	0.22
Mammary	16	0.08	0.10	0.09	0.12	0.02
	18	6.20	7.97	7.44	10.30	1.56

Fatty acid composition of milk, mammary gland and liver was determined by the base catalysed methylation procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The calculated 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity.

The ingested and injected clofibrate treatments had no effect on the 16:1/16:0 and 18:1/18:0 FA ratios of milk (Table 4.3), liver or mammary gland (Table 4.2).

Table 4.3. The effect of clofibrate, ingested or injected and respective control treatments on milk FA composition of lactating mice

Fatty acid	Formula	Control	Clo eaten	Inj Blank	Inj Clo	Sed
Butyrate	4:0	0.04	0.06	0.06	0.06	0.019
Hexanoate	6:0	0.05	0.06	0.08	0.08	0.019
Octanoate	8:0	0.37	0.41	0.44	0.42	0.051
Docanoate	10:0	7.12	6.92	7.63	6.83	0.712
Undecanoate	11:0	0.002	0	0	0	0.002
Laurate	12:0	11.70	11.45	12.38	11.18	0.923
Tridecanoate	13:0	0	0	0	0	0
Myristate	14:0	15.50	15.21	15.65	14.47	0.732
Myristoleate	14:1	0.12	0.16	0.18	0.20	0.032
Pentadecanoate	15:0	0.04	0.04	0.04	0.04	0.004
10-Pentadecanoate	15:1	0.012	0	0.014	0.017	0.002
Palmitate	16:0	24.35	24.43	22.81	23.08	1.152
Palmitoleate	16:1	1.83	2.24	1.93	2.47	0.321
Heptadecanoate	17:0	0.03	0.03	0.03	0.03	0.002
10- Heptadecanoate	17:1	0.03	0	0.04	0.05	0.007
Stearate	18:0	1.69	1.58	1.54	1.54	0.153
Oleate	18:1n9c	29.50	29.60	28.36	30.51	1.529
Elaidate	18:1n9t	1.86	1.91	1.76	1.97	0.147
Linoleate	18:2n6c	2.84	2.75	2.90	3.21	0.193
gamma-Linolenate	18:3n6	0.01	0.02	0.03	0.04	0.011
alpha- Linolenate	18:3n3	0.08	0.06	0.11	0.12	0.025
Arachidate	20:0	0.0278	0.03	0.05	0.05	0.012
11-Eicosanoate	20:1n9	0.69	0.67	0.65	0.69	0.073
11,14-Eicosadienoate	20:2n6	0.10	0.10	0.09	0.13	0.027
7,11,14-Eicosatrienoate	20:3n6	0.15	0.17	0.14	0.18	0.027
11,14.17-Eicosatrienoate	20:3n3	0.16	0.22	0.15	0.19	0.039
Arachidonate	20:4n6	0	0.001	0	0	0.001
Behenate	22:0	0.004	0.009	0.048	0.014	0.028
Erucate	22:1n9	0.049	0.038	0.061	0.070	0.016
Docosadienate	22:2n6	0.009	0.027	0.008	0.017	0.011
Docosahexanoate	22:6n3	0.014	0.024	0.029	0.031	0.013
Nervonate	24:1	0.021	0.029	0.108	0.047	0.046
16		0.08	0.09	0.08	0.10	0.012
18		18.04	19.63	18.57	19.96	2.04

Fatty acid composition of milk was determined by the base catalysed methylation procedure, described in methods (section2.3) followed by gas chromatographic analysis to obtain percentage of total FA values for the 32 FA of the GC standard. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity.

Correlations analysis.

Table 4.4. The correlation of mammary gland SCD mRNA, with liver SCD mRNA and the 16:1/16:0 and 18:1/18:0 FA ratios for milk, liver and mammary gland tissue of lactating mice within the injected treatments.

Mammary SCD mRNA associations with:	FA ratio	r	P
Milk	16	0.52	0.08
	18	0.1	0.65
Mammary	16	0.21	0.51
	18	0.47	0.12
Liver	16	-0.20	0.54
	18	-0.06	0.85
Liver SCD mRNA		-0.55	0.06

Fatty acid composition of mammary gland and liver was determined by the base catalysed procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The calculated 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2).

No significant relationships exist between mammary SCD mRNA levels and the variables in Table 4.4.

Table 4.5. The correlation of liver SCD mRNA and the 16:1/16:0 and 18:1/18:0 FA ratios for milk, liver and mammary gland tissue of lactating mice within the injected treatments.

Liver SCD mRNA associations with:	FA ratio	r	P
Milk	16	0.02	0.95
	18	0.03	0.94
Mammary	16	-0.09	0.79
	18	-0.34	0.28
Liver	16	0.07	0.82
	18	0.15	0.64

Fatty acid composition of mammary gland and liver was determined by the base catalysed procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The calculated 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2).

Liver SCD mRNA had no significant relationships to the 16:1/16:0 and 18:1/18:0 FA ratios of milk or either tissue (Table 4.5).

Table 4.6. The correlation of mammary SCD mRNA with liver SCD mRNA and the 16:1/16:0 and 18:1/18:0 FA ratios of milk, liver and mammary gland tissue of lactating mice, within the ingested treatments.

Mammary SCD mRNA associations with:	FA ratio	r	P
Milk	16	-0.30	0.41
	18	0.02	0.96
Mammary	16	0.66	0.04
	18	0.59	0.07
Liver	16	0.77	0.01
	18	0.91	0.00
Liver SCD mRNA		-0.12	0.75

Fatty acid composition of mammary gland and liver was determined by the base catalysed procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The calculated 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2).

In mice eating clofibrate, mammary SCD mRNA showed strong positive linear relationships with liver FA 16:1/16:0 and 18:1/18:0 FA ratios (Table 4.6). There was also a strong positive linear relationship between mammary SCD mRNA with mammary 16:1/16:0 ratio, while the mammary 18:1/18:0 FA ratio was only trending towards significance.

Table 4.7. The correlation of liver SCD mRNA, with mammary gland SCD mRNA and the 16:1/16:0 and 18:1/18:0 FA ratios for milk, liver and mammary gland tissue of lactating mice within the ingested treatments.

Liver SCD mRNA associations with:	FA ratio	r	P
Milk	16	0.019	0.953
	18	0.025	0.940
Mammary	16	-0.085	0.793
	18	-0.337	0.284
Liver	16	0.073	0.821
	18	0.153	0.635

Fatty acid composition of mammary gland and liver was determined by the base catalysed procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The calculated 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2).

No significant relationships were seen for liver SCD mRNA and other variables in Table 4.7.

4.4. Discussion.

This trial examined the effects of clofibrate on SCD mRNA and FA composition in the liver, mammary gland and milk. In attempting to alter SCD mRNA levels and FA composition, two routes of clofibrate administration have been used, these being ingestion and injection. The calculations of intake levels between the two clofibrate treatments show the ingested clofibrate treatment received about 60mg/day, compared to the injected clofibrate treatment being injected with 5-6mg/day, depending on weight.

Liver SCD mRNA was not up regulated by either of the clofibrate treatments (Figure 4.1; Table 4.1) and nor was there an effect on the FA composition of the liver (Table 4.2). In contrast, mammary SCD mRNA expression was significantly increased when injecting or ingesting clofibrate, relative to their controls (Table 4.1).

Mammary SCD mRNA expression was also greater ($P<0.05$) in the mice injected with the olive oil vehicle in comparison with the control treatment (Table 4.1). The increase in

SCD gene transcription may be due to an increase in the availability of saturated FAs to the mammary gland. Both treatments were on the same basal diet, but the olive oil vehicle, was injected next to the mammary gland. This would provide a source of palmitate and stearate to the gland and through substrate induced up-regulation increase mammary SCD mRNA transcription.

When clofibrate was ingested, there was a significant correlation ($P<0.04$) between mammary SCD mRNA and mammary tissue 16:1/16:0 FA ratio, and a correlation that was approaching significance in the 18:1/18:0 FA ratio the mammary gland (Table 4.6). When clofibrate was injected, these relationships were not significant (Table 4.4), even though the induction of SCD mRNA transcription was greater following injection than ingestion (Table 4.1). Some of the induction of SCD mRNA following the injection of clofibrate is attributable to the olive oil vehicle (Table 4.1). Therefore, the real transcriptional induction by clofibrate, in the injected clofibrate treatment, was when the injected blank influence was subtracted from the injected clofibrate influence. It can then be seen that the induction by injected clofibrate was not as great as ingested clofibrate (Table 4.1). This suggestion is further supported by mammary SCD mRNA induction by ingested clofibrate being significantly correlated with mammary FA composition, but not with milk FA composition (Table 4.6). The injected clofibrate treatment, while inducing mammary SCD, had no significant correlations between mammary SCD mRNA and FA composition of tissue or milk. This implies that when clofibrate was ingested it was more effective than when clofibrate was injected, but it was not high enough to affect the FA composition of the milk.

Up regulation of SCD in response to clofibrate has been observed in the liver of male mice (Diczfalusy et al. 1995; Millar and Ntambi 1996). The lack of response in SCD mRNA in the liver to clofibrate in the present experiment (Table 4.1) may be due to the use of female mice. There could be differences between the sexes in absorption, metabolism and excretion that have influenced these results. For example, the renal elimination rate of clofibrate has been shown to be 10× faster in female mice than in male mice, and correspondingly the plasma concentration of clofibrate was higher in males (Kawashima et al. 1989b).

The mice ingesting clofibrate (64mg/day) had a 10× greater intake of clofibrate than the injected treatment (6mg/day). While the injected treatment received a smaller dose of clofibrate, it may still be still effective enough to induce SCD mRNA transcription. This is because the clofibrate may have diffused through membranes into the mammary gland, rather than reaching the mammary gland through the general circulation. This diffusion may also explain the SCD mRNA induction seen by the injected blank (Table 4.1). In diffusion being

the mode of delivery to the mammary gland, a lower dose is needed as it avoids the influence of increased excretion rate seen in the female. The ingested clofibrate treatment, while resulting in a greater intake of clofibrate, delivers the clofibrate in a way that may have more of the dose excreted before having an effect. Both intake levels may have caused significant transcriptional increases in SCD mRNA but the levels still may have been too low to cause significant FA compositional changes in milk (Table 4.3). An alternative explanation is that at this rate of effective dose, more time was needed for the change in FA compositions to occur.

The basal hepatic expression of SCD mRNA is five times higher in female mice than male mice. Observed in tandem with the greater SCD expression, palmitoleate and oleate concentrations in liver and plasma were higher in female mice than male mice (Lee et al. 1996). Fat accumulation was associated with increased SCD activity, so the increased expression in females explains some of the differences in body composition between males and females (Lee et al. 1996).

Sex related differences, in response to similar intakes of PPs, occur at many steps throughout rodent lipid metabolism. Peroxisomal organelle proliferation and liver hepatomegaly are less pronounced in female rodents. Associated peroxisomal enzymes like transferases and acyl-CoA oxidase are all up-regulated more in males in response to PPs; as are the cystolic hydrolases (Kawashima et al. 1989a; Kawashima et al. 1989b; Lazarow et al.1982; Lee et al.1996).

Clofibrate effects, at a certain intake level, can induce biochemical changes in both sexes but the morphological changes at this concentration (0.5-0.75% w/w) of clofibrate are often only seen in males (Lazarow et al. 1982; Reddy and Kumar 1979). Higher PP concentrations are required for PPAR activation in the female rodent (Pineau et al. 1996), as illustrated by both genders of the rat showing the same alterations in metabolism when the female receives five times more PP (Mitchel et al. 1985). Interestingly, it is the females that express basal SCD mRNA five times higher than males (Lee et al. 1996). A clofibrate intake at 0.25-0.5% (w/w) does not elicit a SCD induction response in females but does so in males (Kawashima et al. 1989b). However, at 1-2% clofibrate a response is elicited in females, which is equivalent to males receiving 0.25-0.5% w/w clofibrate (Reddy and Kumar 1979).

The sex steroids probably influence the difference between the sexes in response to clofibrate. Thus in castrated rats, testosterone stimulates the induction by PPs while oestradiol greatly reduces it (Kawashima et al. 1989b; Reddy and Kumar 1979). The induction of SCD by PPs follows a similar pattern between the sexes to that of other lipid metabolism enzymes. The level of induction was strongly dependent on the level of testosterone (Kawashima et al.

1989a; Lee et al. 1996). Compared to other lipid metabolism enzymes, however, the differential induction between the sexes for SCD was not as marked (Kawashima et al. 1989a).

Thus the differences between the sexes mean that the effective dose of clofibrate is higher for females than males. Furthermore, since SCD expression is influenced by sex hormones, the possibility that in this trial that it is also affected by the hormones present during lactation should also considered.

There are lower levels of circulating oestrogen in lactating rather than in non-lactating female mice (Mohla et al. 1981). In lactation the mammary gland becomes insensitive to oestrogen dependent regulation. Fendrick et al. (1998) reported oestrogen receptor concentration in pregnant mice at 1023 *f*mol/mg DNA, in lactating mice at 787 *f*mol/mg DNA, and in non-lactating/non-pregnant mice to be 1733 *f*mol/mg DNA. This refractory state is reversed in involution (Fendrick et al. 1998). While oestrogen responsiveness is not always coupled to the presence of the oestrogen receptor, the low oestrogen receptor concentration and low circulating oestrogen levels indicate oestrogen may not influence the effective dose of clofibrate, in lactating female mice.

Interspecies comparisons show various aspects of lipid metabolism in various tissues to be attenuated by the physiological state of lactation. In ovine adipose tissue, lactation decreases the expression of ACC, SCD, LPL, transferases and enzymes involved in esterification. The major factors responsible seem to be an insulin decrease and a growth hormone increase in lactation. In pigs and rats, elevated adipose SCD expression is more likely to be achieved by the absence of negatively acting hormones, such as growth hormone which has been implicated in repressing ACC and FAS expression (Ward et al. 1998).

To what extent lactogenic hormones attenuate clofibrate induced mammary response in the mouse, is an area of further study. Lactogenic hormones could increase or decrease the discrepancy in response to clofibrate between the sexes. Indications are that, during lactation, the attenuation of clofibrate's effects by oestrogen is not as strong as that in non-lactating mice (Fendrick et al. 1998). This may decrease the differences in effective dose seen by the different response between the sexes. However, lactogenic hormones could attenuate the response of clofibrate induction, just like that seen with oestrogen in non-lactating mice. Lactogenic hormones may then increase the effective dose of clofibrate needed, not only to increase SCD mRNA transcription, but also to affect a change in the FA composition of milk.

CHAPTER FIVE - DIETARY FAT.

5.1. Introduction.

The objective of the research described in this thesis is to develop methods of producing milk fat with a FA profile that enables the production of a butter, which is spreadable when taken from the refrigerator. On theoretical grounds the manipulation that would have the greatest effect in decreasing the melting point is the induction of mammary SCD mRNA transcription. An increased SCD transcription and hence increased SCD activity leads to a greater concentration of palmitoleate and oleate, and a decreased amount of palmitate and stearate incorporated into TAG and phospholipids (Ntambi 1995). Oleate has a melting point of 4°C (Dils 1983), so a greater proportion of milk fat as oleate will decrease the melting range of milk fat (German et al. 1997).

Chapter 4.0 outlines the attempt made to increase SCD activity and hence alter milk FA composition by administering clofibrate to lactating mice. While an increase in mammary SCD mRNA was induced, there was no consequent alteration of milk FA composition. This is contrary to that seen in the hepatic male mouse model (Pennachiotti et al 1996). The influences of sex and the lactational state are thought to have combined to increase the clofibrate dose required to elicit the response seen in the liver of male mice (Section 4.3).

An alternative means to manipulate mammary SCD mRNA transcription is by dietary means. A component of normal rodent chow diets is seen to inhibit hepatic SCD expression in male mice. Upon switching to a fat free diet, the inhibition was removed and SCD transcription increased (Ntambi 1995). A decrease in palmitate and oleate concentration, and an increase in palmitoleate and oleate concentrations in the fatty acyl-CoA pool indicate SCD induction. The ratios of 16:1/16:1 and 18:1/18:0, as an index of desaturation, will therefore increase (Lee et al. 1996).

Polyunsaturated rather than saturated or monounsaturated FAs are the regulators of lipid metabolism (Clarke and Jump 1996; Jones et al. 1996). Polyunsaturated FAs repress FA synthesis and this repression is hierarchical, in that it is related to length and number of double bonds in the FA (Ntambi 1992). Polyunsaturated FAs also inhibit the expression of enzymes involved in glycolysis, *de novo* lipogenesis, fatty acyl-CoA desaturation and elongation, TAG acyl positioning and synthesis and transport. At the same time diverting FA

into mitochondrial and peroxisomal oxidation (Clarke and Jump 1994; Jones et al. 1996; Waters et al. 1997). Mitochondrial and peroxisomal enzymes are up regulated by polyunsaturated FAs including acyl-CoA oxidase and the bifunctional enzyme, cytochrome P450 FA ω-hydroxylase and intracellular FABPs (Vanden Heuvel 1999). Dietary polyunsaturated FAs down-regulate the lipogenic enzymes ACC, FAS, SCD, desaturases other than SCD, elongases and esterification enzymes (Clark and Jump 1996). The attenuation of these enzymes of lipid metabolism reduces the endogenous synthesis of FAs and the concentration of these FAs in the fatty acyl-CoA pool (Jump et al. 1994). This then results in alterations in the composition of TAGs and membrane phospholipids (Yang et al. 1999). Conversely, a fat free diet induces all of these enzymes of lipid metabolism (Ntambi 1995) with the corresponding effects seen on the fatty acyl-CoA pool, and TAG and phospholipid composition (Bickerstaff and Johnson 1972).

The inhibitory attenuation by dietary polyunsaturated FA can also be via different routes, depending on the tissue (Clarke and Jump 1994). For example, in adipocytes the regulation of SCD is by alterations in the stability of mRNA so reducing the half-life (Sessler et al. 1996), not increased transcription as in hepatocytes (Millar and Ntambi 1996). In mice, SCD regulation (Section 1.4) is complicated by tissue differential expression of SCD 1 and 2. This is further complicated by the various isoforms of PPAR that also show tissue differential expression. Recent studies cast doubt as to the unified lipid enzyme regulation by PPARs, and suggest that polyunsaturated FA effects are expressed through a number of pathways. One mechanism cannot fully explain the nuclear actions of polyunsaturated FA, which are due to many metabolic routes being open to the polyunsaturated FA, upon entering the cell (Millar and Ntambi 1996).

The applicability of the hepatic model to other tissue is questioned by conflicting results from past research caused by trial duration and the differential induction patterns of the SCD and PPAR isoforms (Diczfalusy et al.1995; Kouba and Morout 1998). Other variables such as species, sex or age, and calorific intake may also contribute to the inconsistent results of past research (Jones et al. 1996). Relationships within or between various tissues is also affected by feeding regimes, with meal feeding often negating the effects of polyunsaturated FAs (Jones et al. 1996; Nelson et al. 1987). The objective of this trial was to test the hepatic model of SCD regulation by polyunsaturated FAs that applies in male mice, in the mammary gland of lactating females. If the model applies in the mammary gland of lactating mice, then it may be a useful model for seeking ways of altering the FA composition of milk fat in other species, such as the cow.

5.2. Experimental Design.

The Ruakura Animal Ethics committee approved the experimental protocol, used in this experiment. Eighteen Swiss mice were supplied by the Small Animal Colony, Ruakura. The mice were housed individually in plastic cages in a room maintained at 21.0 +/- 1.0 °C and a 14:10 hour light: dark cycle. All mice were assigned on the 7th day of lactation to one of the following three treatments and pup numbers were balanced across treatments. Treatment diets consisted of a fat free diet, an olive oil based diet (25% w/w) and a safflower oil based diet (25% w/w). Dietary composition data are set out in Appendix 2.

For milk collection, the mice were first lightly anaesthetised with 150 µl Hypnorm/Hypnovel (IP), followed by a 0.15 ml IP injection of 1iu/ml oxytocin in saline.

Milk collection was carried out within the Small Animal Colony at Ruakura, AgResearch. The pups were separated from the dams 3-4 hrs before milking to facilitate a larger milk sample on collection. Milk was removed from the mammary gland by gentle manual pressure into Pasteur pipettes and then transferred to Eppendorf tubes. These were then snap frozen in liquid nitrogen and stored at –70 °C until required for fatty acid composition analysis.

Animals were then sacrificed by cervical dislocation. Aliquots of liver and mammary gland were collected immediately and also snap-frozen in liquid nitrogen and stored at –70 °C until required for FA composition and SCD mRNA analysis.

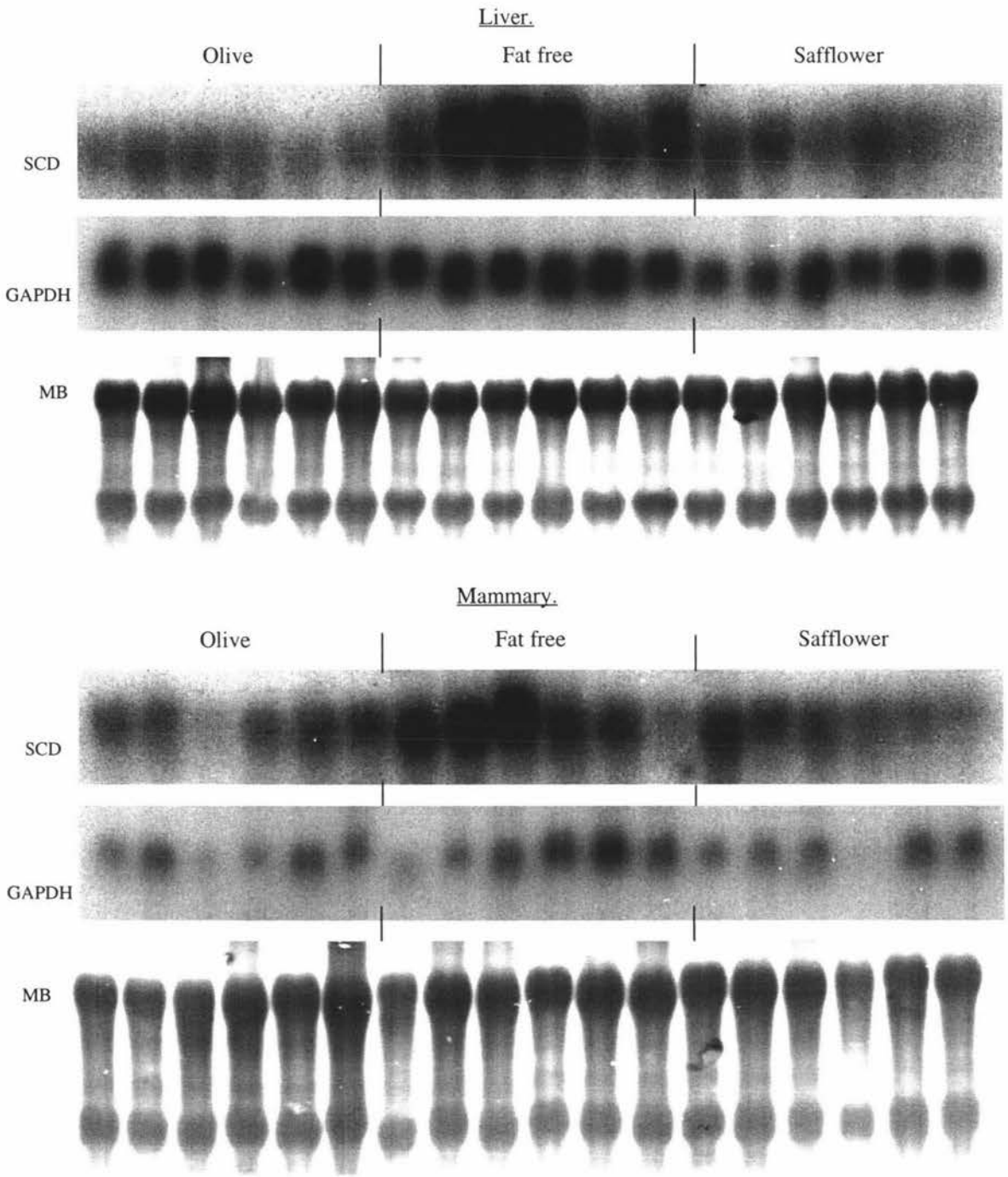
Fatty acid composition of milk, mammary gland and liver was determined by the base catalysed methylation procedure as described in section 2.4 and Appendix 1, followed by gas chromatographic analysis to obtain the concentration of relevant FAs as a percentage of total FAs. The 16:1/ 16:0 and 18:1/ 18:0 ratios were calculated to reflect changes in desaturase activity (Lee et al. 1996). Total RNA was extracted from the tissues using TRIzol reagent as described by manufacturers (Section 2.2.) and Northern blot analysis was carried out for SCD mRNA using 2.5 µg total RNA as described in section 2.2. Transferred RNA was detected by staining with methylene blue. The rat SCD cDNA probe and human GAPDH cDNA probe were random prime labelled using ^{32}P-dCTP and hybridisation of the membrane was carried out as described in section 2.2. The membrane was washed, exposed to XAR film, and scanned in a densitometer for quantitative analysis. Data were not normalised to GAPDH mRNA for reasons described in section 5.3. The activity of the mammary SCD enzyme was determined using the methods described in section 2.3. Results of the enzyme assay (Table

5.2) are expressed as the percentage conversion of [1-^{14}C] palmitoyl-CoA to [1-^{14}C] palmitoleoyl-CoA (16:1/(16:0+16:1)*100). Data were analysed (Section 2.5) by Minitab, ANOVA- Balanced and General Linear model. The generated least squares differences identifies the means significantly different from each other. Correlations were determined by Minitab, Correlations (Pearson).

5.3. Results.

mRNA results.

Figure 5.1. Northern blot of SCD mRNA levels in the liver and mammary gland of lactating mice fed a fat free, olive oil (25% w/w) and safflower oil (25% w/w) diets.



Total RNA was extracted from the liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis c was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Transferred RNA was stained with methylene blue (MB).

A 5.1kb transcript was detected for SCD mRNA and a 1.3kb transcript for GAPDH (Figure 5.1). SCD is greatly induced by the fat free treatment in the liver and mammary gland. The liver GAPDH transcripts show an even intensity across the samples. However, the density of the mammary GAPDH transcripts varied in intensities within treatments. Therefore, SCD mRNA was not normalised to GAPDH mRNA. The methylene blue stain showed even loading and staining of the rRNA 28s and 18s bands between the treatments (Figure 5.1).

Table 5.1. The effect of fat free, safflower (25% w/w) and olive oil (25% w/w) diets on Stearoyl-CoA desaturase mRNA levels in liver and mammary gland of lactating mice.

Tissue	RNA	Fat free	Olive	Safflower	Sed
Liver	SCD	401.5[a]	211.0	149.4	39.4
	GAPDH	737.9	748.8	555.5	95.5
Mammary	SCD	341.0[a]	219.5	187.8	50.4
	GAPDH	262.5	184.5	206.0	60.9

a = Treatment is significantly increased over other treatments ($P<0.05$)

Total RNA was extracted from liver and mammary gland of lactating mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values.

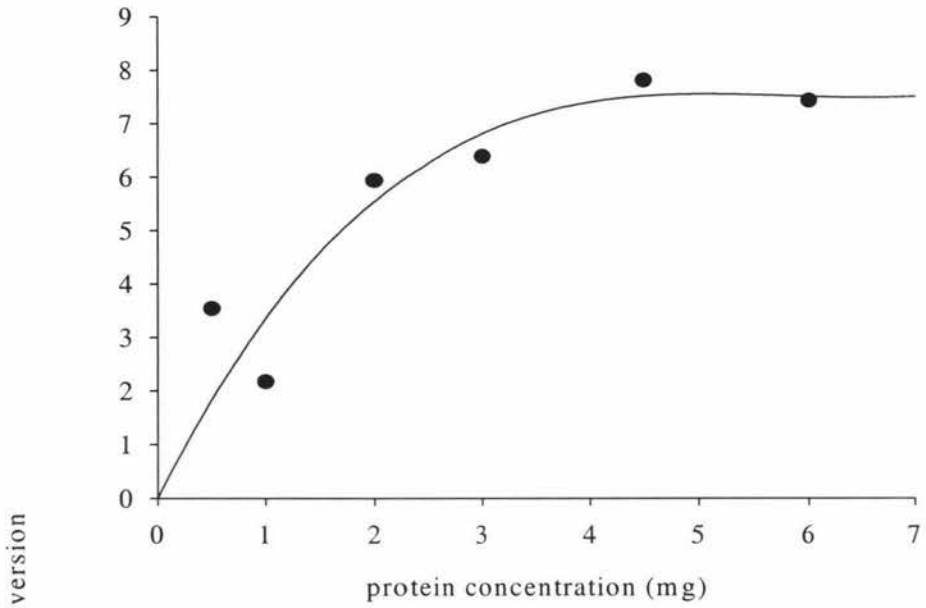
In both the liver and mammary gland (Table 5.1; Figure 5.1), SCD mRNA transcription was up regulated in only the mice fed the fat free diet compared to the other two treatments.

SCD enzyme activity in the Mammary gland.

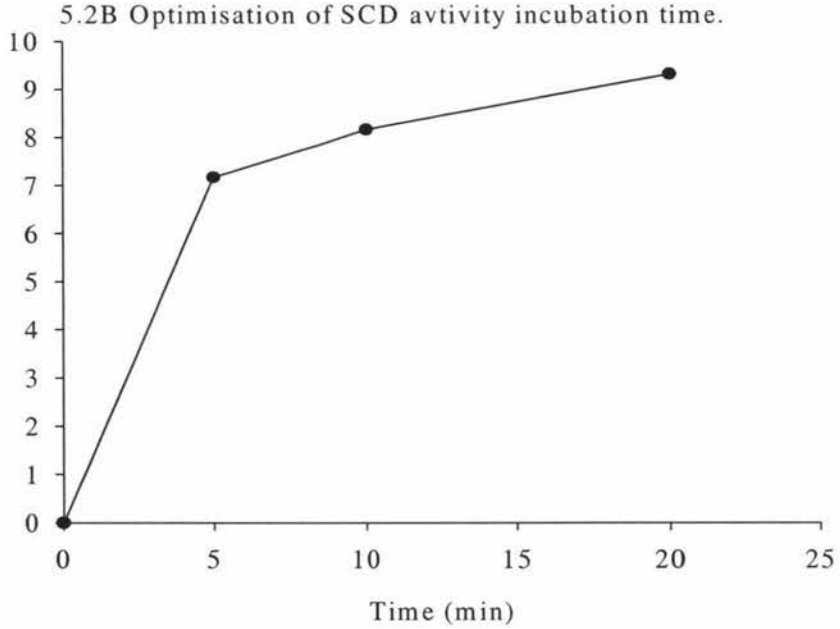
SCD enzyme assay validation.

Figure 5.2 Optimisation of SCD enzyme activity assay.

5.2A. Optimisation of the protein concentration required in the enzyme assay.



5.2B Optimisation of SCD avtivity incubation time.



Assay Validation.

a) SCD activity in the mouse mammary gland as a function of protein. The assays were incubated for 10 minutes

b) SCD activity in mouse mammary gland as a function of time. The assays were conducted with 2mg protein Data were expressed as the percentage conversion of 1-^{14}C palmitate to 1-^{14}C palmitoleate, described in section 2.3.

Sample preparations from 1 mouse were used to optimise the SCD activity assay for the mammary gland. The activity was linear between 1*u*g/ml and 3*u*g/ml of protein (Figure 5.2a) and for up to five minutes (Figure 5.2b). The SCD activity was measured in the following assays (Table 5.2), containing 2mg/ml of sample protein and for 10 minutes incubation time.

SCD enzyme assay results.

Table 5.2. The effect of Fat free, Safflower (25% w/w) and Olive oil (25% w/w) diets on Stearoyl-CoA desaturase activity levels in the mammary gland of lactating mice.

Fat Free	Olive	Safflower	Sed
1.17^{+}	0.74	0.71	0.23

+ P=0.11

The SCD activity assay was carried out in the mammary gland of lactating mice fed the experimental diets, as described in the methods (Section 2.3). The assays were conducted with 2mg/ml protein for 10 minute incubations and the data were expressed as the percentage conversion of 1-^{14}C palmitate to 1-^{14}C palmitoleate.

The fat free diet tended to increase SCD enzyme activity in the mammary gland compared to the olive and safflower oil treatments.

FA composition results.

Table 5.3. The effect of fat free, safflower (25% w/w) and olive oil (25% w/w) diets on Liver and Mammary gland tissue FA composition.

Tissue	FA ratio	Fat Free	Olive	Safflower	Sed
Liver	16	0.15^{a}	0.01	0.01	0.01
	18	1.84^{a}	1.02^{b}	0.36^{c}	0.22
Mammary	16	0.18^{a}	0.08^{b}	0.04^{c}	0.04
	18	2.80	4.62	3.24	1.91

a,b,c Within rows, means with unlike superscripts are significantly different (P<0.05)

Fatty acid composition of milk, mammary gland and liver was determined by a one-step extraction–transesterification procedure as described in methods (section2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity.

In the liver, the fat free diet had significantly higher 16:1/16:0 and 18:1/18:0 FA ratios, compared with the other treatments (Table 5.3). The olive oil diet a significantly greater 18:1/18:0 FA ratio compared to the safflower oil diet. In the mammary gland, the 16 ratio was greatest in the fat free treatment, intermediate in the olive oil treatment and least for safflower oil treatment with each significantly different from the other ($P<0.05$). The 18:1/18:0 FA ratio in the mammary gland was unaffected by treatment.

Table 5.4. The effect of fat free, safflower (25% w/w) and olive oil (25% w/w) diets on milk FA composition.

Fatty acid	Formula	Fat free	Olive	Safflower	Sed
Butyrate	4:0	0.03	0.03	0.04	0.01
Hexanoate	6:0	0.06	0.02	0.03	0.02
Octanoate	8:0	0.59[a]	0.24	0.27	0.05
Docanoate	10:0	9.30[a]	3.22	3.82	0.59
Undecanoate	11:0	0	0	0	0
Laurate	12:0	14.35[a]	3.41	3.97	0.77
Tridecanoate	13:0	0	0	0	0
Myristate	14:0	16.94[a]	2.86	3.62	0.90
Myristoleate	14:1	0.27[a]	0.01	0.01	0.03
Pentadecanoate	15:0	0.05[a]	0.03	0.03	0.01
10-Pentadecanoate	15:1	0.03[a]	0	0	0.01
Palmitate	16:0	22.88[a]	13.78[b]	10.13[c]	1.20
Palmitoleate	16:1	4.33[a]	1.20	0.28	0.60
Heptadecanoate	17:0	0.02	0.06	0.04	0.01
10- Heptadecanoate	17:1	0[c]	0.05[a]	0.01[b]	0.003
Stearate	18:0	1.46	1.84	3.12	0.77
Oleate	18:1n9c	18.30[b]	54.34[a]	11.74[c]	1.45
Elaidate	18:1n9t	5.03[a]	2.47[b]	0.68[c]	0.19
Linoleate	18:2n6c	1.30[c]	9.28[b]	56.43[a]	1.10
gamma-Linolenate	18:3n6	0	0.08	0.40[a]	0.06
alpha- Linolenate	18:3n3	0.02[c]	0.19[a]	0.07[b]	0.01
Arachidate	20:0	0.02[c]	0.11[a]	0.08[b]	0.01
11-Eicosanoate	20:1n9	0.88	1.24	0.18[c]	0.21
11,14-Eicosadienoate	20:2n6	0.07	0.39	1.14[a]	0.18
7,11,14-Eicosatrienoate	20:3n6	0.08[c]	0.24[b]	0.55[a]	0.06
11,14.17-Eicosatrienoate	20:3n3	0.18[c]	0.52[b]	0.85[a]	0.10
Arachidonate	20:4n6	0	0	0	0
Behenate	22:0	0.03	0.03	0.04	0.02
Erucate	22:1n9	0.07	0.10	0.02[c]	0.02
Docosadienate	22:2n6	0.08	0.01[c]	0.06	0.02
Docosahexanoate	22:6n3	0.07	0.09	0.06	0.02
Nervonate	24:1	0.04	0.02	0.03	0.02
16		0.19[a]	0.09[b]	0.03[c]	0.02
18		12.88[b]	31.40[a]	4.78[c]	3.16

a = Treatment is significantly increased over other treatments (P= 0.05)
b = Treatment is significantly increased over 'c' treatments (P= 0.05)
& treatment is significantly decreased over 'a' treatments (P= 0.05)
c = Treatment is significantly decreased over other treatments (P= 0.05)

Fatty acid composition of milk was determined by the base catalysed methylation procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for the FA listed above. The 16:1/16:0 (16) and 18:1/18:0 (18) FA ratios were calculated and analysed to reflect changes in desaturase activity.

The short chain saturated FAs (4:0 and 6:0) were unchanged by dietary treatment. The medium chain saturated and unsaturated FAs (10:0, 12:0, 14:0, 14:1, 15:0 and 15:1) were all higher in the milk of mice fed the fat free diet ($P<0.05$), compared to the olive and safflower diets (Table 5.4).

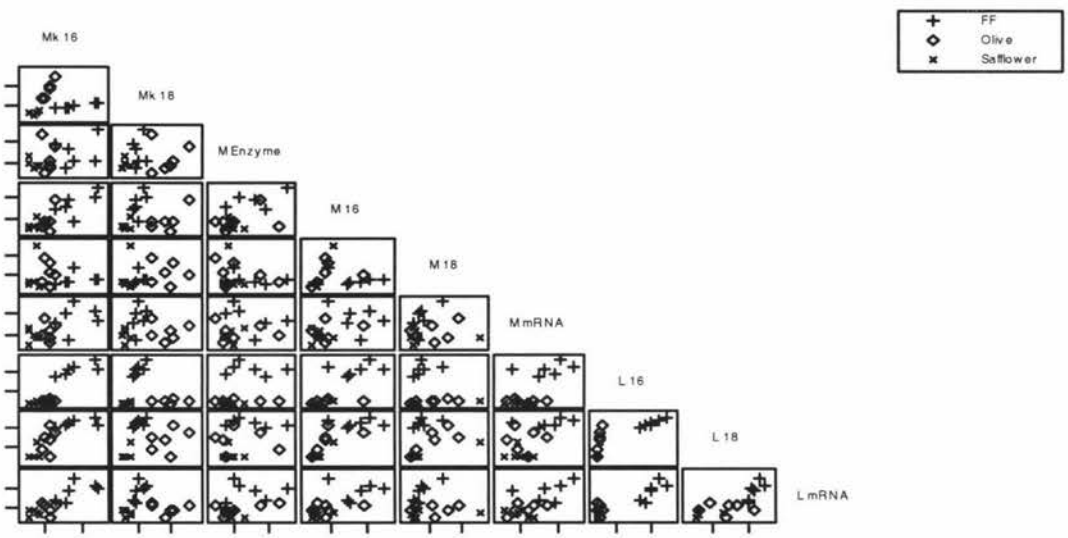
Palmitate was highest in the milk of mice fed the fat free diet, then olive oil and the lowest in the safflower oil treatment. However, the palmitoleate was only higher (3.6 fold) in the milk of mice fed a fat free diet. No significant differences were seen for stearate between the treatments. The fat free treatment had a significantly increased oleate concentration over the safflower treatment. However, the olive oil treatment had a far greater oleate concentration ($P<0.05$) than both the fat free diet (3 fold) and the safflower oil treatment (5 fold). This is due to the high oleate concentration in the diet of these mice (Appendix 2).

Many of the FAs, listed below oleate in Table 5.4, show significant treatment effects. However, due to the low percentage of these FAs in milk, the FA content of the diets effected them greatly. For example, the concentrations for linoleate in the diet of olive and safflower oil treatments are reflected in the concentrations of linoleate in their milks.

The milk 16:1/16:0 FA ratio is highest with the fat free treatment, intermediate in the olive oil treatment and lowest in the safflower oil treatment. The 18:1/18:0 FA ratio did not reflect this pattern. The 18:1/18:0 FA ratio is higher in the fat free treatment over the safflower treatment (2.6 fold), but the olive oil treatment has a far greater 18:1/18:0 FA ratio than both the fat free treatment (2.8 fold) and the safflower oil treatment (6.5 fold). Once again this is a reflection of the dietary content of oleate in the olive oil treatment.

Correlations analysis.

Figure 5.3. The matrix plot of correlations analysis between the fat free, olive oil and safflower oil treatments. MK 16= milk 16:1/16:0 FA ratio, MK milk 18= 18:1/18:0 FA ratio, M enzyme= mammary SCD enzyme activity, M 16= mammary 16:1/16:0 FA ratio, M 18= mammary 18:1/18:0 FA ratio, M mRNA= mammary SCD mRNA, L 16=liver 16:1/16:0 FA ratio, L 18=liver 18:1/18:0 FA ratio, L mRNA= liver SCD mRNA.



The milk 16:1/16:0 FA ratio column of relationships in Figure 5.3, shows some positive linear relationships. Furthermore, inspection of the correlation matrix (Figure 5.3) suggests that although the relationships between variables were often similar for the fat free and safflower diets, these relationships for the olive oil were often disparate. This is evidenced in the FA ratios, where the 16:1/16:0 FA ratio had linear relationships but the 18:1/18:0 FA ratio plots show the olive oil group of mice moved to the right. Thus it was concluded to leave out the data for the olive oil treatment when calculating the correlations presented in tables 5.5, 5.6, 5.7.

Table 5.5. The correlation of mammary gland SCD mRNA, with liver SCD mRNA and the 16 and 18 FA ratios for milk, liver and mammary gland tissue in lactating mice fed fat free and safflower oil diets.

Mammary gland SCD mRNA associations with:	FA ratio	r	P
Milk	16	0.660	0.02
	18	0.677	0.02
Mammary	16	0.392	0.21
	18	0.085	0.80
Mammary SCD activity		0.130	0.69
Liver SCD mRNA		0.684	0.01
Liver	16	0.716	0.01
	18	0.673	0.02

Fatty acid composition of mammary gland and liver was determined by a base catalysed methylation procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were calculated and analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD mRNA was quantified by densitometry and the results are expressed as arbitrary units. The SCD data could of been normalised to GAPDH mRNA, but for reasons discussed in Chapter 4.0 the non-normalised SCD mRNA values were used in the analysis of these relationships The SCD enzyme assay was carried out on mammary tissue as described in methods (Section 2.3)

Mammary SCD mRNA has a strong positive linear correlation with liver SCD mRNA (Table 5.5). The milk 16:1/16:0 and 18:1/18:0 FA ratio were significantly correlated with mammary SCD mRNA (Table 5.5). The relationships of mammary SCD mRNA with mammary tissue 16:1/16:0 and 18:1/18:0 FA ratios are not significant, and nor is the correlation of mammary SCD mRNA with mammary SCD enzyme activity. However, liver 16:1/16:0 and 18:1/18:0 FA ratios have a strong positive linear correlation with mammary SCD mRNA.

Table 5.6. The correlation of liver SCD mRNA with milk, liver and mammary gland tissue 16 and 18 FA ratios in lactating mice fed fat free and safflower oil diets.

Mammary gland SCD mRNA associations with:	FA ratio	r	P
Milk	16	0.901	0.001
	18	0.856	0.001
Mammary SCD activity		0.479	0.110
Mammary	16	0.705	0.011
	18	0.101	0.753
Liver	16	0.940	0.001
	18	0.905	0.001

Fatty acid composition of mammary gland and liver was determined by a base catalysed methylation procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values. The SCD data could of been normalised to GAPDH mRNA, but for reasons discussed in Chapter 4.0 the non-normalised SCD mRNA values were used in the analysis of these relationships. The SCD enzyme assay was carried out on mammary tissue as described in methods (Section 2.3)

Liver SCD mRNA showed a significant positive linear correlation with liver 16 and 18 FA ratios. Other significant positive linear correlations with liver SCD mRNA were the milk 16 and 18 ratios and mammary tissue 16 FA ratio.

Table 5.7. The correlation of mammary gland SCD enzyme activity, with the 16:1/16:0 and 18:1/18:0 FA ratios for milk, liver and mammary gland tissue in lactating mice fed fat free and safflower oil diets.

Mammary gland SCD enzyme activity associations with:	FA ratio	r	P
Milk	16	0.555	0.06
	18	0.514	0.08
Mammary	16	0.689	0.01
	18	-0.175	0.59
Liver	16	0.482	0.11
	18	0.455	0.14

Fatty acid composition of mammary gland and liver was determined by a base catalysed methylation procedure as described in methods (Section 2.4) followed by gas chromatographic analysis to obtain percentage of total FA

values for 16:0, 16:1, 18:0 and 18:1. The 16:1/ 16:0 (16) and 18:1/ 18:0 (18) FA ratios were calculated and analysed to reflect changes in desaturase activity. Total RNA was extracted from liver and mammary gland of mice fed the experimental diets and Northern blot analysis was carried out for SCD and GAPDH mRNA levels as described in methods (Section 2.2). Abundance of SCD and GAPDH mRNA was quantified by densitometry and the results are expressed as arbitrary values. The SCD data could of been normalised to GAPDH mRNA, but for reasons discussed in Chapter 4.0 the non-normalised SCD mRNA values were used in the analysis of these relationships. The SCD enzyme assay was carried out on mammary tissue as described in methods (Section 2.3)

Mammary SCD enzyme activity showed a positive linear correlation with mammary tissue 16:1/16:0 FA ratio, however, the relationship of mammary SCD enzyme activity is only approaching significance (Table 5.7).

5.4. Discussion.

The objective of this trial was to use dietary fat regimens to develop a greater understanding of the regulation of mammary SCD mRNA, and how it might be manipulated to alter the FA composition of milk fat.

Diets in this trial were used to investigate if the SCD regulation seen in the liver of male mice applies to the mammary gland, as SCD is known to have differential tissue expression and regulation (Diczfalusy et al. 1995; Nelson et al. 1987). The methods of regulation proposed included induction of SCD mRNA by feeding a fat free diet and inhibition of SCD mRNA by feeding a safflower diet (25% w/w). The olive oil diet was intended as a control as mono-unsaturated FA are neutral in terms of dietary fat regulation of SCD transcription (Clark and Jump 1994; Ntambi 1995). In retrospect the olive oil treatment was unsuitable for this purpose. There are two mechanisms whereby a diet high in olive oil may also affect the inhibition of SCD mRNA. The first mechanism is suppression of mammary lipogenesis by high fat diets (Aoki et al. 1999; Millar and Ntambi 1996), and the second mechanism is polyunsaturated FA inhibition of SCD mRNA transcription. The diet containing 25% w/w olive oil would have a dietary linoleate concentration of about 2.4% (Appendix 2) which is also sufficient to suppress liver SCD mRNA transcription (Bretillon et al. 1999; Chouirnard et al. 1999). Consequently, it is not possible to use the mice on the olive oil diet as controls and

the comparisons between different treatments have to be made on the understanding that each treatment may affect lipid metabolism.

Mammary SCD expression was significantly greater in mice fed a fat free diet, compared to either feeding a safflower diet (25% w/w), or an olive oil diet (25% w/w) (Table 5.1). These differences can be attributed to either an inhibitory effect of the diets containing the oil or a stimulatory effect of the fat free diet.

Irrespective of the mechanism by which the olive oil diet affected SCD mRNA, it influenced FA ratios, especially milk 16:1/16:0 and 18:1/18:0 FA ratios. The FA ratios (16:1/16:0= 0.087, 18:1/18:0= 29.5) in the milk are similar to these ratios in the olive oil of the diet (16:1/16:0= 0.07, 18:1/18:0= 33.0) suggesting that the FAs of the olive oil passed from the diet to the milk unchanged.

The levels of SCD mRNA expression in the liver and mammary gland on the olive oil diet were not significantly different to those on the safflower oil treatment (Table 5.1). However, since the results on the olive oil diet are confounded by the high fat content of the diet and particularly the high oleate concentration, the results for the olive oil group were not included in the correlation analysis. Correlations were calculated using the data from the mice fed the fat free and safflower oil diets and presented in Tables 5.5, 5.6, and 5.7. Furthermore much of the manipulation of hepatic SCD gene expression reported in the literature has been achieved with fat free and polyunsaturated FA diets.

Liver SCD mRNA was up regulated by the fat free diet in comparison with the safflower treatment (Figure 5.1, Table 5.1). With the fat free treatment there was also a significant increase in the 16:1/16:0 and 18:1/18:0 FA ratios of the liver (Table 5.3). In male mice these two responses to a fat free diet leads to the increased incorporation of oleate into membrane phospholipids and liver lipoproteins (Madsen et al. 1999). Similarly in the present study, the correlation of liver SCD mRNA with liver 16:1/16:0 and 18:1/18:0 FA ratios (Table 5.6) were very strong ($P<0.001$). Moreover, it can be inferred that the induced SCD transcription is associated with protein synthesis (Ntambi 1995), so increasing SCD enzyme levels in the liver. Increased SCD activity then causes an increase in the 16:1/16:0 and 18:1/18:0 FA ratios (Lee et al. 1996). There is a significantly positive linear relationship of liver SCD mRNA with milk 16:1/16:0 and 18:1/18:0 FA ratios (Table 5.6). Such changes are observed in the FA composition of the TAGs in the lipoproteins released by the liver (Madsen

et al. 1999). The FAs of the TAGs are the primary source of long chain FAs for mammary lipogenesis (Section 1.2) and in this way influence milk composition.

Mammary SCD mRNA concentration was greater in the mice fed a fat free diet (Figure 5.1; Table 5.1) than either of the diets supplemented with oil. By analogy with the response of male mouse liver to a fat free diet (Ntambi 1995) it is probable that there was also an increase in the induction of transcription in the mammary gland. Moreover, a similar response is indicated by significant correlation between mammary and liver SCD mRNA (Table 5.5).

The 16:1/16:0 and 18:1/18:0 FA ratios in the mammary gland were significantly ($P<0.05$) correlated with mammary levels of the SCD mRNA transcripts (Table 5.5). Yet only the mammary 16:1/16:0 FA ratio, and not the 18:1/18:0 FA ratios, was correlated significantly with mammary SCD enzyme activity (Table 5.7). This correlation is supported by the observation that the 16:1/16:0 FA ratio, and not the18:1/18:0 FA ratio, of mammary tissue was significantly greater in mice on the fat free diet (Table 5.3). Mammary SCD enzyme activity was not influenced by treatment (Table 5.2), but the increase in the fat free treatment was approaching significance ($P = 0.11$). The correlation of milk 16:1/16:0 and 18:1/18:0 FA ratios with SCD enzyme activity was approaching significance (Table 5.7). Overall, the results in this chapter indicate that the fat free diet induces SCD gene transcription in the mammary gland, which leads to increases in 16:1/16:0 and 18:1/18:0 FA ratios in the milk that probably reflects an increased activity of the SCD enzyme, as described for the liver of male mice (de Antueno et al. 1993; Lee et al. 1996).

The dietary regimen to which dairy cows are exposed has a marked influence on milk fat composition (Kaylegian et al. 1993), as well as milk fat concentration (Sutton 1989). Generally, increased dietary fat enhances the supply of FAs to the mammary gland and reduces the proportion of FAs produced *de novo* (Hermansen 1995). In mice, polyunsaturated FAs also inhibit liver *de novo* FA synthesis (Clandinin et al. 1996). For the olive and safflower treatments mammary *de novo* FA synthesis appears to be suppressed by either high fat content diets or the polyunsaturated FA. Irrespective of the mechanism, it remains that milk FA composition for the fat free treatment has significantly increased octanoate, docanoate, laurate, myristate and palmitate (Figure 5.4).

The milk 16:1/16:0 and 18:1/18:0 FA ratios for the fat free treatment were significantly greater over the safflower treatments 16:1/16:0 and 18:1/18:0 FA ratios. Due to the influence

of the high dietary fats on 18 carbon FA contributions, the 16:1/16:0 FA ratios may be better than the 18:1/18:0 FA ratio at indicating SCD regulation.

The milk of the safflower treatment had a greater stearate concentration, that was approaching significance (P=0.11), compared to the olive oil treatment. Long chain FA, particularly stearate, of milk fat come from plasma sources, and in most cases originates from the diet (Grigor and Warren 1980). The stearate intake between the olive and safflower oil treatments were similar (Appendix 2). When polyunsaturated FAs inhibits SCD there is an accumulation of stearate (Yang et al. 1999), which in the absence of inhibition is rapidly converted to oleate (Kinsella 1972).

All the changes observed in this trial in 16 and 18 carbon FAs, either individually or as ratios, were consistent with the up regulation of mammary SCD mRNA by fat free diets and down regulation by dietary polyunsaturated FA leading to an increase in SCD enzyme activity. This can be inferred even though an increase in enzyme activity was not always investigated (Sessler et al. 1996).

A dietary effect on milk oleate concentration is apparent by comparing the FA composition of the milk from the olive oil treatment (54% of total FAs as oleate) with that of the safflower oil treatment (11.7% of total FAs as oleate). The oleate concentration of the olive and safflower oil diets was 66% and 11% respectively. A dietary effect also occurs for linoleate, where the olive and safflower oil diets contain 9.4% and 72% of total FA, respectively. The linoleate concentrations in the milk of the olive oil treatment (9.3%) and the safflower oil treatment (56.4%) closely reflect their dietary concentrations.

The variation in long chain FA in Table 5.4 could be due to dietary composition influences (Hermansen 1995) or regulation of elongases and desaturases other than SCD (Waters et al. 1997). The concentrations of these FA are low in milk (German et al. 1997) so that the variability within the treatments could have influenced the results. The effects of high dietary fat and polyunsaturated FA on the elongases and desaturases other than SCD, often combine to confuse the expression of both influences individually. Examples include 11-eicosanoate and 11,14-eicosadienoate (Table 5.4; Figure 5.4). The high proportion of 11-eicosanoate in the olive oil treatment reflects the high dietary intake of oleate, some of which has been elongated to 11-eicosanoate. This elongation could be a result of either the high concentration of precursor (oleate) or is it a result of up regulated elongase. Similarly for the safflower treatments, the 11,14-eicosadienoate has been derived from the high linoleate concentration in the diet, some of which has been elongated to form 11,14-eicosadienoate.

For the olive and safflower oil treatments, the inclusion rate of 25% oil in the diet affected the milk fat composition directly. The increase in oleate and linoleate in the milk of mice fed the olive and safflower oil treatments respectively, would decrease the melting point range of the milk fat. However, if the trial had used a lower inclusion rate of dietary oil, this effect may not have occurred, making the effect of the fat free treatment on milk fat composition and melting range more pronounced. The increase in octanoate to palmitate (Table 5.4) in feeding a fat free diet will contribute to an increase in the melting range of the milk fat. However, it is doubtful that the increase of these FAs will override the effect of increased oleate in this treatment. The increased oleate in the milk of mice fed a fat free diet will greatly reduce the melting range of milk fat. Singularly, the induced desaturation enzymes, other than SCD, may not alter the milk fat FA composition appreciably enough to lower the melting point of the milk fat. But a unified regulation, that includes increased oleate concentration from induced SCD, may cause an appreciable decrease in the milk fat melting range.

Future trials should use lower oil inclusion rates to avoid high dietary fat inhibition of lipogenesis. A 10% corn oil diet inhibits SCD expression (Giron et al. 1998; Periago et al. 1989), while 10% olive oil in the diet increases SCD expression in the liver (Periago et al. 1989). Alternatively, the use of pure fatty acid esters would allow measurement of the effect of relatively low concentrations of polyunsaturated FA on SCD transcription (Clark et al. 1977). Dietary fat can influence expression of many lipid metabolism genes to the extent that a co-ordinated regulation of mammary lipogenesis is seen. In this unified regulation, the FA composition of milk fat changes not just from the attenuated SCD gene expression but from the regulation of other enzymes. Future work in this area may be expanded by investigation into other transcriptional regulators that are induced by changes in intake of dietary fat. Candidate regulators of milk fat FA composition other than mammary SCD, include mammary acetyl-CoA carboxylase, fatty acid synthase, thioesterase II and lipoprotein lipase.

This study shows that mammary SCD mRNA transcription can be induced. The induction of SCD then effects the milk fat composition in a way that would decrease the melting range of milk fat.

CONCLUSION.

Past research has shown induction of hepatic SCD mRNA in male mice fed a fat free diet or a diet containing 0.5% w/w clofibrate (Millar and Ntambi 1996). This induction then causes a FA compositional change, seen by an increased amount of oleate incorporated into membrane phospholipids (Pennachiotti et al. 1996) and the TAG of liver lipoproteins (Bickerstaff and Johnson 1972; Madsen et al. 1997). This reduces the melting point of TAG and membrane phospholipids, which increases membrane fluidity (Garg et al.1988). The objective of this research was to test whether the hepatic model of SCD regulation applied to the mammary gland. If it does then this would justify further research into the mechanism as a possible means of altering the FA composition of milk fat and producing a softer butter.

In the preliminary study (Chapter 3) with only 3 animals per treatment group, a fat free diet or a diet containing clofibrate (0.05% w/w) did not affect SCD mRNA in the liver, mammary gland or milk fat of lactating mice. Correlations between mammary SCD mRNA and FA composition of mammary gland tissue or milk were not significant. However, the hepatic 16:1/16:0 FA ratio ($r = 0.495$, $P<0.05$), and 18:1/18:0 FA ratio ($r = 0.520$, $P<0.05$) were correlated with liver SCD mRNA. This, along with liver SCD mRNA influencing milk 16:1/16:0 FA ratio ($r = 0.552$, $P<0.05$), implies that FA synthesis in the liver has a strong influence on the FA composition of the milk fat. Attempts to induce mammary SCD mRNA in lactating Swiss mice were repeated, with a trial duration of 7 days. The treatments (n=6) included ingested clofibrate (0.05% w/w) and injected clofibrate (15mg/100g LW).

Mammary SCD mRNA transcription was induced by ingested and injected clofibrate ($P<0.05$), compared to their control treatments (Chapter 4). This is comparable to the induction of hepatic SCD seen in male mice fed clofibrate (Jones et al. 1998). Liver SCD mRNA transcription was not effected by treatment, which goes against the male hepatic model. Treatments did not affect the FA composition of the liver or mammary tissue, or milk fat FA. This contrasts with the increase in unsaturated FA reported for the liver of male mice ingesting clofibrate (Lee et al. 1995; Pennachiotti et al. 1996). When clofibrate was injected correlations between mammary SCD mRNA and FA composition of the tissue or milk were not significant. However, when clofibrate was ingested mammary SCD mRNA was correlated with mammary tissue 16:1/16:0 FA ratio ($r = 0.660$, $P<0.05$), and trending to significance with mammary tissue 18:1/18:0 FA ratio ($r = 0.59$, $P=0.08$). Liver SCD mRNA was not correlated,

with FA composition of tissues or milk fat whether clofibrate was ingested or injected. In contrast to the preliminary trial, milk FA composition was primarily determined by the activity of mammary SCD. While mammary SCD mRNA transcription is induced by clofibrate, the lack of associated change in fatty acid composition in the mammary gland and milk indicated that the increase in mRNA was not translated into an increase in enzyme activity. The reasons for this are not clear. Differential responses have been reported to varying the dose of clofibrate. Specifically, the difference between the sexes response (Kawashima et al. 1989b; Lee et al. 1996) and the effect of lactational state (Fendrick et al. 1998) suggest that the clofibrate dose may need to be increased to 1-2% w/w of the diet (Reddy and Kumar 1979), if milk fatty acid composition is to change.

Feeding a fat free diet induced SCD mRNA transcription ($P<0.05$) in the mammary gland (2.1 fold) and liver (5.3 fold) over the control treatment in the preliminary study (Chapter3). Induction of transcription was not reflected on the FA composition of liver, mammary gland and milk. However, there was a trend ($P<0.10$) for milk 16:1/16:0 FA ratio to be increased in the fat free treatment over the control treatment. The induction by the fat free diet of mammary SCD mRNA was not correlated with milk 16:1/16:0 FA ratio. However, liver SCD mRNA was correlated ($r =0.552$, $P<0.05$) with milk 16:1/16:0 FA ratio. Liver SCD mRNA was also correlated with liver 18:1/18:0 FA ratio ($r =0.520$, $P<0.05$), and liver 16:1/16:0 FA ratio ($r =0.61$, $P<0.05$). This again suggests that hepatic SCD activity has the stronger influence on the FA composition of milk fatty than the mammary gland. In repeating a trial attempting to induce mammary SCD mRNA transcription in lactating Swiss mice, the duration was extended to 7 days. Three treatments (n=6) were fed a fat free diet, a safflower oil diet (25% w/w) and an olive oil diet (25% w/w). The safflower oil diet was included to show the inhibitory effect of polyunsaturated FA on SCD regulation. The olive oil treatment was included as a point of reference to assess the response of the other treatments.

Mammary SCD mRNA transcription levels were greater in the fat free treatment ($P<0.05$), as compared to the olive and safflower oil treatments (Chapter 5). By anology with the response in the male mouse liver to a fat free diet, this indicates regulation at the transcriptional level (Ntambi 1995). However, the lower level of SCD expression of the olive or safflower treatments, can be equally attributed to a high fat diet or polyunsaturated FA inhibition (Section 5.3). This meant relative induction by a fat free diet, or inhibition by dietary polyunsaturated fatty acids, could not be referenced. Liver SCD mRNA was greater ($P<0.05$) in the fat free treatment than the oil diet treatments, as seen in male hepatic mouse

model (Millar and Ntambi 1996). The induced mammary SCD mRNA transcription response by feeding a fat free diet was not reflected in the mammary SCD enzyme activity, however, the increase in SCD transcription was transmitted to tissue and milk FA ratios. The fat free treatment increased liver 16:1/16:0 FA ratio and 18:1/18:0 FA ratio ($P=0.05$). The fat free treatment also increased the mammary 16:1/16:0 FA ratio ($P<0.05$), but not the 18:1/18:0 FA ratio. These changes are similar to the changes in FA composition seen in the liver of male mice fed fat free diets (Clarke and Jump 1996). The olive oil treatment caused some significant changes in tissue fatty acid composition that could be taken as a reference point to induction by a fat free diet, or inhibition by dietary polyunsaturated fatty acids. However, in the olive oil treatments some of the significant changes in FA composition in the tissues can be attributed to the presence of these FA in the diet.

The FA profile of both oil diets was significantly influenced by dietary fatty acid composition. For example, significantly greater amounts of linoleate appeared in the milk of the mice of the safflower treatment ($P<0.05$), as did significantly greater amounts of oleate in the milk of the group on the olive oil diet ($P<0.05$). The milk of the fat free treatment reflects greater Δ^9desaturation activity over the safflower oil treatment. The oleate concentration, 16:1/16:0 and 18:1/18:0 FA ratios of the fat free treatment are greater ($P<0.05$) than those in the safflower oil diet. An accumulation of stearate ($P<0.10$), indicating SCD inhibition (Yang et al. 1999), is seen in the milk of the safflower oil treatment compared to the fat free treatment. The milk from the mice on the fat free diet has a greater content ($P<0.05$) of saturated fatty acids from octanoate to palmitate, compared to the safflower oil treatment. These FAs are produced from FAS activity, which has also been increased with feeding a fat free diet (Jump et al. 1994). The concentrations of long chain fatty acids of greater molecular weight than linoleate show the effects of diet FA content.

In essence, the objective of increasing the spreadability of butter at low temperatures, demands that the proportion of low melting point FA of milk fat should be increased (Banks et al. 1980). Increasing oleate (4°C) and decreasing palmitate (63°C) in the milk fat will lead to decreased melting range of the milk fat so improving the spreadability of butter (Enjalbert et al.1998). Therefore, the FAs hexanoate (-3.4°C) to myristate (58.5°C) may be a dominant factor in determining the melting properties of milk fat (Banks et al. 1980). The alteration of the ratio between short chain FA and long chain FAs can also effect the fluidity of the milk fat droplet (Beaulieu and Palmquist 1995).

Measuring FA composition, is thus, only the first step in determining the effects of feeding on milk fat quality. Measurements of effects on TAG fractions of different molecular

mass will also provide insights into altering FA composition of milk fat. The proportions of individual FAs, and also the FA stereo-specific arrangement in the TAG molecule, and the proportions of TAG with high or low melting points influence the fluidity of milk fat. Short chain FAs are placed in the sn-3 position and contribute to the fluidity of milk fat. Oleate, with its much-reduced melting point, can substitute short chain FA at the sn-3 position so increasing milk fat fluidity (Hermansen 1995). The increase in the proportion of TAG containing short chain FA or oleate at the sn-3 position in milk fat gives the potential for it to produce a softer butter (Beaulieu and Palmquist 1995).

Whether the effects seen on mammary SCD mRNA levels and milk FA composition seen in our mice, is applicable to the bovine species is a matter of further research. Present knowledge on mammary FA metabolism in dairy cows is mostly based on the effects of protected dietary fat or infused lipids on milk FA composition (Enjalbert et al. 1998). Many protected oil rich diets have caused an increase in the proportion of monounsaturated and polyunsaturated FA in milk fat (Jimenez-Flores 1997). Feeding unprotected oils, rich in 18 carbon FAs, harnesses ruminal hydrogenation to increase the amount of stearate available for mammary desaturation (Banks et al. 1980). The male hepatic SCD mouse model may not apply to the bovine species, as hepatic SCD activity is not detected in the bovine species (St. John et al. 1991). The low activity of hepatic SCD suggests that the role of desaturating dietary fats has been relegated to the ultimate target tissues, with little processing of the FAs occurring in bovine liver (St. John et al. 1991). In the bovine species, mammary SCD's high activity (Beaulieu and Palmquist 1995) is reflected in the quick conversion of stearate to oleate (Decker 1996). The FA composition of bovine tissue can be altered by dietary means. However, the changes are difficult to elicit and are usually of a small scale (St.John et al. 1991). Milk fat composition, in terms of oleate concentration, is influenced by diet, and varies within and among breeds (Beaulieu and Palmquist 1995). As alterations of milk fat composition by dietary means is difficult, an alternative approach to increasing oleate concentrations in milk is through selective breeding. Perhaps herds of cows could be established that exhibit higher mammary SCD activity (St.John et al. 1991).

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APPENDICES

Appendix One.

A1.0 Gas liquid Chromatography.

To determine an effect of treatment on SCD activity in tissue and consequently milk, the FA composition of liver and mammary tissue plus milk fat is required. For this analysis a gas chromatograph (GC) and associated analytical methods are required. Without previous experience or day-to-day technical advice, I optimised and validated both the GC set up and sample preparation procedures.

FA analysis was performed on a Shimadzu-17A fitted with a wide range flame ionisation detector (FID) and a Shimadzu DB wax capillary column, 30m long with a 0.25mm internal diameter bore and a 0.25mm film. Samples were applied with a Shimadzu AOC20i auto-injector and AOC20s auto-sampler.

A1.1 Initial GC set up sample preparation.

The initial settings of the column oven temperature programme of the GC are outlined in Table 1 and Figure 3. The split ratio was at 5:1, with a total flow of 9ml/min and a total press of 139kPa. Both purge flow and septum purge were set at 3.00ml/min.

Table 1. Initial column oven temperature programme. Stage 1 is holding the oven temperature at 0°C for 0.5 minutes. Stages 2,3,4 are temperature ramps of which the first ramp (stage 2) increases the temperature from 0-195°Cat 20°C/min. On reaching 195°C, the second ramp (stage 3) increases from 195-205°C at 3°C/min. The third ramp (stage 4) increases the oven temperature from 205-230°C at 8°C/min. Stage 5 is holding the oven temperature at 230°C for 26 minutes. Between each temperature ramp there is no holding time so on adding all the stages together the program produced a run time per sample of 42.7 minutes.

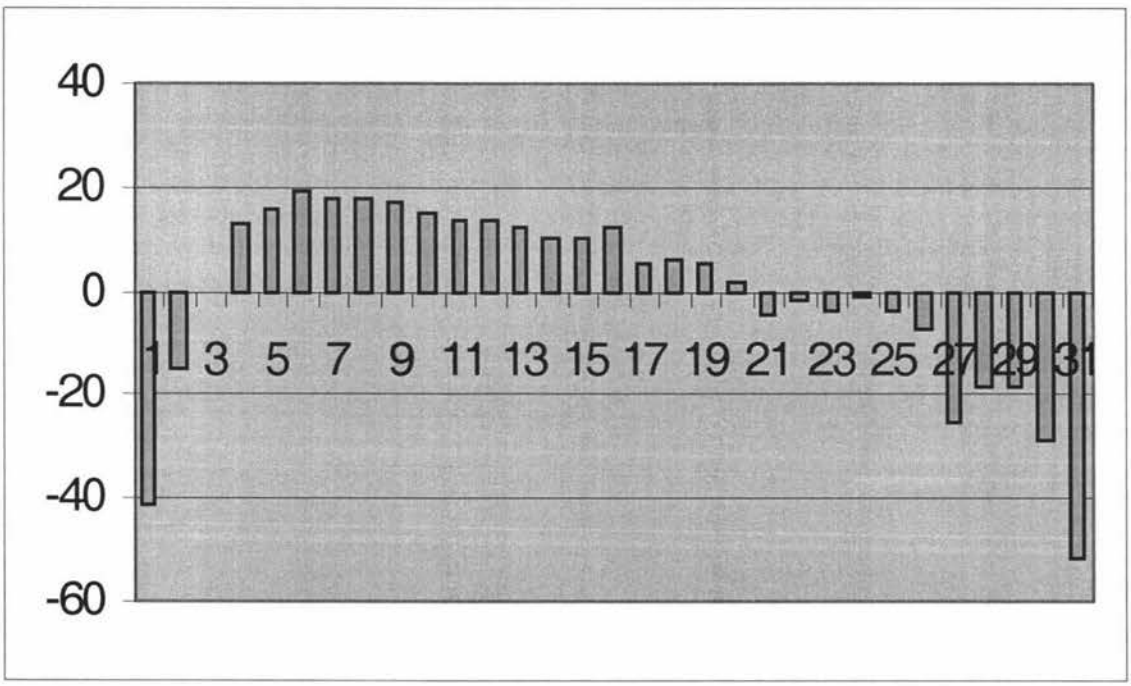
	Temperature increases (°C/min)	Final temperature (°C)	Time (min)
Start	----------	0°C	0.5
1	20	195°C	0
2	3	205°C	0
3	8	230°C	26.0

The one-step extraction and methylation procedure of Sukhija and Palmquist (1988) was used initially, as processing times are shorter and losses that can be incurred during multi-step procedures of extraction, esterification and purification are smaller than that with other methods.

Weigh into reacti-vials about 0.5g of tissue or up to 1ml of milk/serum was weighed into reacti-vials, then 1ml of chloroform, 3ml of 5% methanolic HCL and 1ml of internal standard (optional) were added. After homogenising the samples, vials were capped and heated in a water bath for 2hrs at 70°C. The volume of the reactant was maintained by

replacing any evaporated solvent with chloroform throughout heating. After removal from the water bath the vials were cooled to room temperature and 5ml of 6% potassium carbonate and 4ml of chloroform were added to the contents, vortexed and then centrifuge for 10 minutes at 2500 rpm. The lower chloroform layer was removed and the aqueous phase re-extract with 4ml of chloroform. The two lots of chloroform (making about 8ml) were combined and a sub-sample of 1ml was transferred into a pre-weighed auto-sample vial. The sample was evaporated under a stream of nitrogen and the vial reweighed and the weight of the FA residue calculated. The sample was reconstituted in 1ml of hexane and capped with nitrogen.

Figure 1. Estimation of the 32 FAs in a GLC-85 standard. A comparison of the means values (n=10) for 32 FA in a standard sample with the expected results. Estimated mean values are expressed as a percentage of expected values after applying the correction factors of section A1.3. Individual bars are numbered 1-31. Only 31 peaks were detected as both the 18:1 isomers eluted at the same time.



A1.2 Optimisation.

Some problems were encountered in analysing the GLC-85 standard (NU-CHEK PREP, USA) under the initial GC set up and analytical procedures. The data presented in Figure 1 show that the concentrations of butyrate and hexanoate, and FA with a molecular weight greater than arachidate, were underestimated and consequently the FAs between C6 and C20 were overestimated.

Some of the underestimation of the longer chain FAs is due to the injected sample being inadequately volatilised by low injection port temperatures. To correct this the injection port temperature was increased from 180°C to 250 °C. Most of the underestimation in the longer

chain FAs however, is due to sample condensation on the injection needle once the sample is injected and the needle is still in the injection port. A needle temperature lower than the injection port temperature causes condensation if the injection time is too long. Increasing injection port temperature increases the chance of condensation. This was negated by increasing speeds of various injection and plunger parameters so decreasing the dwell time of the needle in the injection port.

Much of the underestimation of C4 and C6 is attributed to the evaporative loss of volatile FAs during sample preparation. Some of this loss will be in the nitrogen capping at various stages of sample preparation. While this is minimal, sample capping must be gentle and short in duration. Most of the loss, however, will occur while evaporating the chloroform from the sample with nitrogen before re-constituting the methyl-FA residue in hexane. Overcoming this loss of short chain FAs during preparation is covered in section A1.2.2.

Of the 32 FAs of the GLC-85 standard only 31 peaks were represented on the chromatograph indicating that peak resolution was not optimal. Improved peak resolution was achieved by altering the pressure and oven temperature programming. The pressure was increased so that total flow increased to 43ml/min, while the oven temperature changes are discussed in section A1.2.1. Finally, the use of correction factors, which were initially overlooked, for the 32 FA of the GLC-85 standard are discussed in section A1.3.

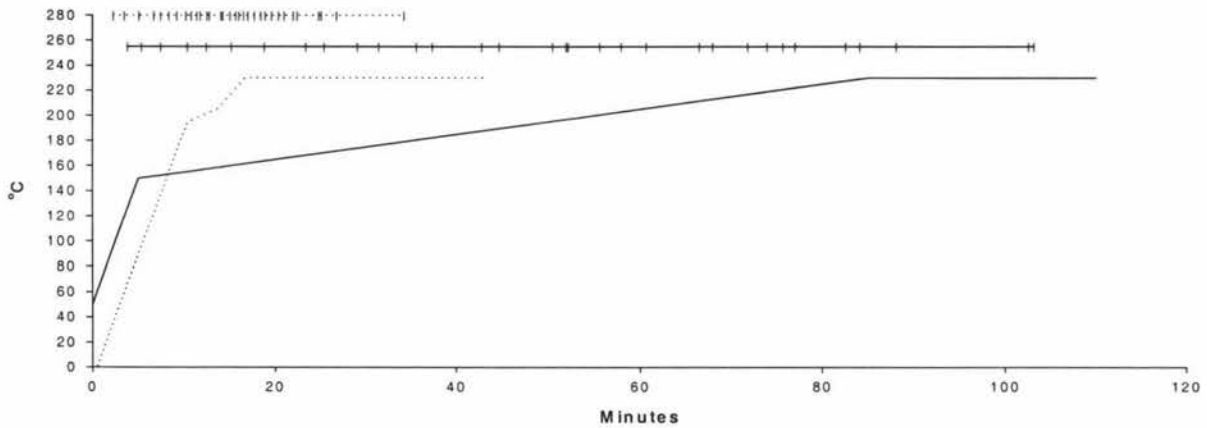
A1.2.1 Oven Temperature.

The initial temperature programme (outlined in Table 1) produced an increase in temperature over the run that was too sharp and resulted in poor peak resolution (Figure 2). This gave a close pattern of FAME elution that could easily be overloaded. Apart from the first 2-3 FAs of the GLC-85 standard, the incremental increase in molecular weight between the FA of this standard are not large. As elution times are largely dependent on molecular weight, to improve retention times, temperature increases needed to be slower so allowing time for the close molecular weight FAs to elute separately. After many runs slowing the temperature ramps the final oven temperature programme was decided on and is outlined in Table 2.

Table 2. Final oven temperature programme. This table outlines the oven temperature programme selected for further analysis. It has a slower rate of ascent from 150-230°C which corresponds to the volatilisation range of all the LCFA and some of the MCFA. Stage one is a temperature ramp from 50-150 °C at 20 °C/min. With no temperature holding between the temperature ramps, stage 2 is another ramp from 150-230 °C at 1.0 °C/min. The last stage is the holding of the oven temperature at 230 °C for 25 minutes. This produces a sample run time of 110 minutes.

	Temperature increases (°C)	Final temperature (°C)	Time (min)
Start	--------	50	0
1	20	150	0
2	1.0	230	25

Figure 2. Patterns of Oven temperature on FA elution.
Comparison of the elution pattern (..|..|..) for the initial temperature programme (.....) with that of the final elution pattern (-|-|-) and temperature program (—) for the 32 FA standard.



A1.2.2 Sample preparation.

In recognising that the initial sample preparation procedure was causing problems a new procedure had to be devised. The following section describes the development of the alternative method. In the extraction of FA from tissue, the tissue is first homogenised then extracted with solvent. Membrane and plasma lipids are normally associated with proteins so solvents having some water solubility and hydrogen bonding are necessary to split the lipid-protein complex, and in some cases denature the protein. The most common extraction solvent is a 2:1 chloroform/methanol mix. The addition of an equal volume of water will cause phase separation with the chloroform layer (plus lipids) as the lower phase (Gurr and James 1975).

The amphipathic character (presence of hydrophilic and hydrophobic regions) of phospholipids inevitably leads to "cosolubilisation" of small water soluble molecules even thought the solvent is essentially non-polar. Such contaminants can be removed by washing with a salt solution (Gurr and James 1975), which is incorporated into the methylation procedure.

Sample extraction or storage should not lead to any sample degradation or auto-oxidation. Lipids can be stored in a non-polar solvent such as hexane (Christie 1991), or chloroform/methanol (2:1) (Kates 1982). It is best to store lipid samples below $^{-}15^{o}C$ in glass containers with a minimum amount of head space and capped with nitrogen. For longer term storage the use of an antioxidant has been shown to be prudent (BHT at 0.05%) (Christie 1991; Kates 1982).

Esters, other than methyl, can have specific uses and most methodologies can be adapted by substituting the appropriate alcohol (Christie 1990). Methyl ester alternatives include butyl and picolinyl esters, pyrrolidides, pentafluorobenzyl and 4-4-Dimethyloxazoline (Christie 1995). However, methyl esters are the simplest to prepare, are low in polarity with the lowest molecular weight; so they elute from GC columns at lower temperatures (Christie 1995). Methylation is often poorly understood, with reaction conditions too vigorous and procedures used that are better suited to other specific tasks. Measuring changes in the fatty-acid composition of milk was the primary objective of this research and, as such, our methylation procedure needed to be specific for milk fat. Acid catalysed procedures are not suited to the analysis of SCFA found in milk fat (Christie 1990).

Base catalysed procedures are simpler and more rapid than acid catalysed procedures (Christie 1990a), but may still have a problem with potential SCFA saponification (Bannon et al. 1985). Other difficulties in analysing SCFA include the failure to methylate quantitatively, non-quantitative phase shift from aqueous to organic layers when aqueous extraction procedures are used and preferential evaporative losses of short chain esters during work up or storage (Bannon et al. 1985). Base catalysed procedures, however, offer direct transesterification (Christie 1990), and do not release aldehydes from phospholipids that confuse GC traces or result in PUFA isomerisation (Christie 1990a).

O-acyl lipids (TAG/phospholipids) are transesterified in basic procedures usually by Na-methoxide facilitating the exchange between glycerol and methanol (Chistie 1990a). Only small amounts of the base catalyst is needed for transesterification (Christopherson and Glass 1969), but as with acid catalysed reactions another solvent (eg. diethyl-ether) is necessary to solubilise non-polar lipids such as triacylglyceride and cholesterol (Christie 1990a). With Na-

methoxide, transesterification is very rapid (5-10 minutes) at room temperature for triacylglyceride and phospholipids (Bannon et al. 1985: Christie 1990a). In keeping reaction times short it lessens GC column contamination by cholesterol esters as these transesterify slowly, taking up to an hour (Christie 1990a). While methylation can be carried out using potassium hydroxide or potassium methoxide as the catalyst (Christie 1990a), the use of sodium methoxide greatly reduces saponification of the newly formed methyl esters of SCFA such as methyl butyrate, so increasing the accuracy of analysis (Bannon et al. 1985). Adding a neutralising solution after methylation, stops saponification of methyl butyrate and gives a stable solution over 24hrs storage. Neutralising also stops the loss of column performance and appearance of artefacts in chromatograms (Bannon et al. 1985).

In the one step extraction/methylation procedure (Section A1.1), *n*-hexane is the carrier solvent for GC injection. However, most of this method had chloroform as the fatty-acid carrier that had to be evaporated off with nitrogen gas. This can result in appreciable loss of low molecular weight FAs up to myristate and even palmitate (Christie 1991). Another reason for not using chloroform is that it can contain ethanol as a stabiliser that forms ethyl esters that can confuse chromatographic analysis. Also, chloroform, in reacting with sodium methoxide forms dichlorocarbene which can react with double bonds of the FA sample. Overall base catalysed methylations have been developed for milk fat analysis, since the solvent evaporation step can be omitted so eliminating the loss of SCFA (Christie 1990a).

While development of the methylation procedure is geared towards milk fat analysis, the final method should also be applicable to the analysis of various animal tissues. GC analysis of animal tissue is complicated by column contamination, by cholesterol esters for example. While the final methylation procedure is of shorter time duration than the time needed to esterify cholesterol, purification by absorption chromatography after methylation would reduce contamination further. Purification can be achieved with a 2cm column of silica gel or Florisil™ in a Pasteur pipette plugged with glass wool. The sample can be eluted with hexane-diethyl ether (95:5 v/v; 10ml) with cholesterol and other polar impurities remaining on the column (Christie 1990). While purification of animal tissue samples by absorption chromatography may incur losses of SCFA, it is not these FA that are of primary interest in this study. FAs of interest in tissue are palmitate and longer chain FAs and are not subject to loss by volatilisation. The loss of SCFA while changing the absolute concentration of LCFAs would not alter the ratio of LCFA to each other.

The milk fat extraction method finally adopted uses 0.1ml of mouse milk to which is added to 0.9ml of chloroform/methanol (1:2 v/v). Depending on the actual milk volume used,

all of the solvents can be scaled accordingly. Upon vortexing the sample, 0.5ml of chloroform and 0.5ml of water are added and then vortex. This causes a phase separation which may be better defined by centrifugation (10 minutes @ 2000rpm). The lower chloroform layer was transferred into another tube and the sample re-extracted with 0.75ml of chloroform. The two recovered chloroform aliquots were combined and evaporated with a nitrogen gas stream. The FA residue was weighed if the sample weight was likely to be more than 100mg. This amount of FA was about the threshold at which peak overload occurred under the accepted final pressure and oven temperature programmes.

This extraction procedure uses chloroform in the extraction of fat from the sample. Chloroform is finally removed by evaporation but this step does not result in FA loss by volatilisation as the great majority of FA in tissue and milk are in the TAG or phospholipid form and have not been methylated yet.

The method for fat extraction from tissue followed that described by Kates (1982), and can also be scaled but care must be given to maintain correct solvent ratios. Up to 10g of tissue was weighed and homogenised for two minutes in 30ml methanol/chloroform (2:1 v/v). The homogenate was centrifuged for 10 minutes at 2000rpm and the supernatant decanted. The pellet was re-extracted with 39ml methanol/chloroform/water (2:1:0.9 v/v). The supernatant from both extractions was combined. The sample was diluted with 20ml of chloroform and 20ml of water and centrifuged for 10 minutes at 2000rpm to facilitate phase separation. The lower chloroform phase is recovered and evaporated by a nitrogen gas stream.

To methylate the FA residues from both milk and tissue fat extraction, add 200*u*l of sodium methoxide to the vial with the FA residue. The vial was capped with a gas stream of nitrogen and vortexed to ensure the entire sample was dissolved off the walls. The reaction was allowed standing for 10 minutes at room temperature. 0.5ml of hexane was added, the vial was capped with nitrogen, mixed well and left for 10 minutes. The methylation reaction was stopped by adding 200*u*l of neutralising solution, capped with nitrogen and vortexed. This mixture usually phase separates well but can be improved by centrifugation (10 minutes @ 2000rpm) so making the upper hexane layer clear. 0.5ml of this upper layer were made up to 1.0ml with hexane in an auto-sampler vial if the fat sample was isolated from milk. For tissue samples, the 0.5ml of the upper layer was passed through a column of silica gel (0.3g in a Pasteur pipette) and rinsed through into an auto-sampler vial with 1.5 ml of hexane. Both types of sample were then ready to inject into the GC.

This method, in using sodium methoxide as a methylating agent, is a base catalysed procedure which is very well suited to the analysis of FA in milk fat, while still being

applicable to the analysis of tissue FA composition. It fulfils the criteria discussed above in that:

1) Methylation times are kept short to reduce contaminants and undesired fats (cholesterol) being methylated.
2) Methylation does not involve chloroform as a solvent.
3) The use of sodium-methoxide to transmethylate reduces saponification.
4) The use of a neutralising salt solution that stops saponification and aids in decreasing contaminants that are water soluble.

A1.2.3 Initial and Final Sample Preparation Comparison

In testing the validity of the newly developed method, a milk sample was prepared by the initial acid catalysed technique and compared to the same sample prepared by the developed procedure. Table 3 shows the percent FA comparisons between true sample duplicates of the same method and those between the two methods. All eight runs were carried out after the GC had been optimised.

For homogenised milk and isolated bovine milk fat (provided by Dairy Research Corporation), the initial method did not produce similar values for the duplicate samples (Table 3). One reason for developing a new methylation procedure was that the old acid based procedure produces methylation artefacts that interfere with the chromatogram. Sample A of homogenised milk analysed with the old procedure produced such an artefact that eluted with the same retention time as methyl-butyrate. While this artefact did not appear in duplicate B, neither were butyrate, hexanoate or octanoate detected in duplicate A. As a consequence the values for the remaining FA in duplicate B, are all higher than those in duplicate A, because in the latter a large proportion of the total signal detected was due to the artefact peak.

The chromatogram from the analysis of duplicate B of isolated milk fat with the initial method also showed a large artefact (75.5% of the total area) with a retention time that did not coincide with that of any FA.

Results for duplicates using the final method, whether for homogenised milk or isolated milk fat were extremely close (coefficient of variation of less than 2%). Both samples duplicates detected the same FAs except γ-linolenate was missing from the chromatogram of sample A. From these results it was concluded that the final method is more accurate and reproducible.

Table 3. Comparison of the fatty acid composition of homogenised milk and isolated bovine milk fat using the initial and developed sample preparation procedures. Some FAs were not detected and are represented by a ' * '.

		Homogenised milk				Isolated milk fat			
Name		I-a	I-b	F-a	F-b	I-a	I-b	F-a	F-b
Butyrate	4.0	84.38	*	3.06	3.08	1.977	0.436	2.364	2.325
Hexanoate	6.0	0.23	*	2.15	2.12	*	0.385	1.639	1.616
Octanoate	8.0	0.171	*	1.207	1.2	0.701	0.271	1.014	1.007
Docanoate	10.0	0.478	2.77	3.05	3.03	2.266	0.643	2.441	2.443
Undecanoate	11.0	*	*	*	*	*	*	*	*
Laurate	12.0	0.55	3.55	3.366	3.4	3.632	0.975	3.733	3.735
Tridecanoate	13.0	*	*	*	*	*	*	*	*
Myristate	14.0	1.834	12.42	11.68	11.66	11.815	3.08	11.153	11.167
Myristoleate	14.1	0.077	*	0.444	0.437	0.665	0.173	0.876	0.877
Pentadecanoate	15.0	0.13	0.79	0.72	0.755	0.795	0.201	0.931	0.934
10-Pentadecenoate	15.1	*	*	*	*	*	*	*	*
Palmitate	16.0	4.8	33.64	30.91	30.84	33.722	8.69	29.122	29.117
Palmitoleate	16.1	0.161	0.961	0.936	0.89	1.01	0.259	1.355	1.345
Heptadecanoate	17.0	*	*	*	*	*	*	0.397	0.4
10-Heptadecenoate	17.1	*	*	*	*	*	*	0.186	0.15
Stearate	18.0	2.156	14.69	13.449	13.38	11.65	2.98	10.907	10.901
Oleate	18.1n9c	3.12	22.1	20.13	20.02	26.62	6.82	22.993	23.033
Elaidate	18.1n9t	0.632	4.917	4.547	4.5	2.59	0.69	2.746	2.753
Linoleate	18.2n6c	0.058	*	0.48	0.428	1.09	0.28	1.942	1.456
gamma-Linolenate	18.3n6	*	*	*	*	*	*	*	0.037
alpha-Linoleneate	18.3n3	0.106	0.636	0.585	0.566	*	*	0.408	0.412
Arachidate	20.0	*	*	*	*	*	*	0.075	0.081
11-Eicosenoate	20.1n9	*	*	*	*	*	*	0.066	0.066
11,14-Eicosadienoate	20.2n6	*	*	*	*	*	*	*	*
7,11,14-Eicosatrienoate	20.3n6	*	*	*	*	*	*	*	*
11,14,17-Eicosatrienoate	20.3n3	*	*	*	*	*	*	0.064	0.067
Arachidonate	20.4n6	*	*	*	*	*	*	*	*
Behenate	22.0	*	*	*	*	*	*	0.044	0.04
Erucate	22.1n9	*	*	*	*	*	*	*	*
Docosadienate	22.2n6	*	*	*	*	*	*	*	*
Docosahexanoate (DHA)	22.6n3	*	*	*	*	*	*	*	*
Nervonate	24.1	*	*	*	*	*	*	*	*

A1.2.4 Final sample preparation validation.

Methylation time.

Transesterification of TAG and phospholipids can be complete within 5-10 minutes, and as excess methylation times causing saponification (Bannon et al. 1985; Christie 1990a), it was prudent to determine the optimum methylation time in terms of FA recovery.

Mouse liver was processed as described in Section A1.2.2 with times of methylation varying from 5-60 minutes and the results are presented in Table 4. As methylation times increased, the coefficient of variation between duplicates increased which means reproducibility decreased. Yields for the individual FAs also decreased as methylation time increased. In support of Bannon et al. (1985) and Christie (1990a), the results indicate that 10 minutes is the optimum methylation time for quantitative FA recovery and reproducibility.

Column filtration for methylated tissue samples.

Passing samples through a silica gel column before analysis on the GC removes contaminants such as sterols eg. cholesterol that can co-elute with FA or give unidentifiable peaks. It also extends the life of the column, as cholesterol binds to the column film reducing its effectiveness. It is important, however, to assess whether the process effects the analysis of the FA in the sample.

To assess the effectiveness of filtration two liver samples were extracted by the new method. One was filtered through 2cm (0.3g) of silica gel with 5ml of hexane flush, the other sample was not filtered.

The proportion of the principal FAs in the samples were very similar in the filtered and non-filtered samples (Table 6). The peak heights for the non-filtered samples were, however, greater at least in part because the filtered sample was diluted 5 fold as a result of the extra hexane used to flush the column. Thus for the filtered samples there were 23 peaks above threshold, while for the non-filtered sample had 67 peaks. In addition peak heights for the principal FAs presented in Table 5 show about a 5 fold disparity between the two samples.

Table 4. Effect of methylation time on the amount of FA measured in twosamples (A and B) of mouse liver.

Name		1hr A	1hr B	30min A	30 min B	15 min A	15min B	10 min A	10 min B	5 min A	5 min B
Butyrate	4.0	*	*	*	*	*	*	*	*	*	*
Hexanoate	6.0	*	*	*	*	*	*	*	*	*	*
Octanoate	8.0	*	*	*	*	*	*	*	*	*	*
Docanoate	10.0	*	*	*	*	*	*	*	*	*	*
Undecanoate	11.0	*	*	*	*	*	*	*	*	*	*
Laurate	12.0	*	0.089	*	*	*	*	*	*	*	*
Tridecanoate	13.0	*	*	*	*	*	*	*	*	*	*
Myristate	14.0	0.217	0.302	0.254	0.244	0.233	0.223	0.249	0.269	0.323	0.33
Myristoleate	14.1	*	*	*	*	*	*	*	*	*	*
Pentadecanoate	15.0	*	*	*	*	*	*	*	*	*	*
10-Pentadecenoate	15.1	*	*	*	*	*	*	*	*	*	*
Palmitate	16.0	17.577	22.37	23.236	23.217	22.87	24.416	23.356	23.063	23.41	23.945
Palmitoleate	16.1	0.288	0.364	0.279	0.246	0.278	0.235	0.256	0.252	0.277	0.273
Heptadecanoate	17.0	0.13	0.137	0.133	*	0.124	0.14	0.142	0.124	0.132	0.145
10-Heptadecenoate	17.1	*	*	*	*	*	*	*	*	*	*
Stearate	18.0	12.871	16.16	17.375	17.483	17.927	18.33	17.256	17.273	17.754	17.603
Oleate	18.1n9c	7.13	9.317	8.5	8.146	7.62	7.442	7.355	7.593	6.856	7.029
Elaidate	18.1n9t	1.23	1.605	1.62	1.558	1.562	1.536	1.521	1.575	1.472	1.54
Linoleate	18.2n6c	11.467	14.823	14.966	14.761	14.6	14.127	14.84	14.395	14.194	14.29
gamma-Linolenate	18.3n6	*	*	*	*	*	*	*	*	*	*
alpha-Linoleneate	18.3n3	*	*	*	*	*	*	*	*	*	*
Arachidate	20.0	*	*	*	*	*	*	*	*	*	*
11-Eicosenoate	20.1n9	*	*	0.111	*	*	*	*	*	*	*
11,14-Eicosadienoate	20.2n6	0.133	0.195	0.168	0.166	0.203	0.16	0.177	0.195	0.176	0.145
7,11,14-Eicosatrienoate	20.3n6	0.573	0.766	0.799	0.775	0.749	0.713	0.762	0.726	0.715	0.716
11,14,17-Eicosatrienoate	20.3n3	14.866	18.264	19.942	20.505	20.506	20.793	20.657	19.577	21.815	20.774
Arachidonate	20.4n6	*	*	*	*	*	*	*	*	*	*
Behenate	22.0	*	*	*	*	*	*	*	*	*	*
Erucate	22.1n9	*	*	*	*	*	*	*	*	*	*
Docosadienate	22.2n6	*	*	*	*	*	*	*	*	*	*
Docosahexanoate (DHA)	22.6n3	8.065	9.197	10.16	11.026	11.051	10.296	11.237	9.6	11.476	11.129
Nervonate	24.1	*	*	*	*	*	*	*	*	*	*

Table 5. Comparisons of the peak heights (millivolts) for individual fatty acids (FAs) from a filtered sample (diluted 5 fold) and a non-filtered sample of mouse liver following sample preparation and analysis on the GC.

FAs	Filtered sample (A)	Non-filtered sample (B)	A : B
16:0	10.811	55.401	5.1
16:1	0.403	2.973	7.4
18:0	20.924	71.294	3.4
18:1n9c	8.046	37.419	4.7
18:2n6c	3.353	20.737	6.2

In an attempt to decrease the effect of the dilution which reduces the sensitivity of the method, a series of samples were produced in which the column was flushed with incremental 1 ml hexane aliquots.

The 5^{th} ml of hexane flush still contained FAs but only about 1% of the total. The size of the peaks at the 3^{rd} ml of flushing are small as well, however, if only 2ml of hexane is used to flush the column there would be about 95% FA recovery. For this reason and for the need to reduce the dilution at this step it has been decided to use 2ml of hexane flush, not 5ml. The incomplete recovery of FAs is similar in percent among all the FAs so the proportional ratios of FAs will still be similar to that if all the FAs were recovered.

Table 6. The effect of filtration on sample composition. Individual FA results are reported At the bottom of the table are various ratios likely to be used in statistical analysis.

Name	Formula	Not filtered	Filtered
Butyrate	4.0	0.023	*
Hexanoate	6.0	0.023	*
Octanoate	8.0	*	*
Docanoate	10.0	*	*
Undecanoate	11.0	*	*
Laurate	12.0	0.022	*
Tridecanoate	13.0	*	*
Myristate	14.0	0.303	0.205
Myristoleate	14.1	*	*
Pentadecanoate	15.0	0.174	*
10-Pentadecenoate	15.1	*	*
Palmitate	16.0	12.452	12.561
Palmitoleate	16.1	0.56	0.456
Heptadecanoate	17.0	0.62	0.488
10-Heptadecenoate	17.1	*	*
Stearate	18.0	27.203	30.869
Oleate	18.1n9c	10.106	11.499
Elaidate	18.1n9t	1.667	1.744
Linoleate	18.2n6c	5.189	5.052
gamma-Linolenate	18.3n6	0.079	*
alpha-Linoleneate	18.3n3	4.107	3.476
Arachidate	20.0	0.027	*
11-Eicosenoate	20.1n9	0.026	*
11,14-Eicosadienoate	20.2n6	0.059	*
7,11,14-Eicosatrienoate	20.3n6	1.762	1.689
11,14,17-Eicosatrienoate	20.3n3	4.893	4.604
Arachidonate	20.4n6	0.184	*
Behenate	22.0	2.336	*
Erucate	22.1n9	*	*
Docosadienate	22.2n6	*	*
Docosahexanoate (DHA)	22.6n3	2.689	2.451
Nervonate	24.1	*	*
16:0/16:1		22.23	27.54
18:0/18:1		2.69	2.68
(16:0 + 18:00)/(16:1 + 18:1)		3.72	3.63

Table 7. Effects of using incremental hexane flushes in column filtration on FA yeilds.
Results are peak heights while the bracketed figure is the percentage of this peak of total row height
Results are an average of two duplicates.

Name	Formula	1st 1ml	2nd 1ml	3rd 1ml	4th 1ml	5th 1ml
Butyrate	4.0	*	*	*	*	*
Hexanoate	6.0	*	*	*	*	*
Octanoate	8.0	*	*	*	*	*
Docanoate	10.0	*	*	*	*	*
Undecanoate	11.0	*	*	*	*	*
Laurate	12.0	*	*	*	*	*
Tridecanoate	13.0	*	*	*	*	*
Myristate	14.0	993(100)	*	*	*	*
Myristoleate	14.1	*	*	*	*	*
Pentadecanoate	15.0	464(100)	*	*	*	*
10-Pentadecenoate	15.1	*	*	*	*	*
Palmitate	16.0	28860(83.3)	3743(10.7)	1138 (3.3)	581(1.7)	334(.95)
Palmitoleate	16.1	1544(91.25)	146(8.8)	*	*	*
Heptadecanoate	17.0	1520(100)	*	*	*	*
10-Heptadecenoate	17.1	339(100)	*	*	*	*
Stearate	18.0	46862(82)	6507(11.5)	2118(3.7)	1038(1.8)	609(1.05)
Oleate	18.1n9c	21724(84)	2586(10)	843(3.3)	432(1.6)	276(1.05
Elaidate	18.1n9t	4632(90.1)	437(8.8)	129(2.1)	*	*
Linoleate	18.2n6c	8484(84.8)	1043(10.3)	288(2.8)	160(1.55)	116(1)
gamma-Linolenate	18.3n6	162(100)	*	*	*	*
alpha-Linoleneate	18.3n3	6489(83.4)	858(10.9)	259(3.3)	140(1.7)	109(1.3)
Arachidate	20.0	516(100)	*	*	*	*
11-Eicosenoate	20.1n9	234(10	*	*	*	*
11,14-Eicosadienoate	20.2n6	*	*	*	*	*
7,11,14-Eicosatrienoate	20.3n6	3086(88.8)	323(9.5)	105(3.3)	*	*
11,14,17-Eicosatrienoate	20.3n3	6987(83)	863(10.1)	300(3.5)	180(2.1)	107(1.2)
Arachidonate	20.4n6	256(100)	*	*	*	*
Behenate	22.0	7887(82.3)	517(5.4)	365(3.8)	178(1.8)	115(1.2)
Erucate	22.1n9	*	*	*	*	*
Docosadienate	22.2n6	*	*	*	*	*
Docosahexanoate (DHA)	22.6n3	3353(88.3)	328(9.8)	114(3.9)	*	*
Nervonate	24.1	*	*	*	*	*

A1.3. Response Factors and Result determination.

The FID responds to ions generated by combustion of the methyl components of the molecule (Craske & Bannon 1987), however, it is recognised that carboxyl carbon (C=O) components in each ester are not ionised appreciably during combustion thus giving a poor detector response (Christie 1991; Craske & Bannon 1987). FID response factors are thus proportional to the weight percent in the molecule of "active" carbon atoms, which includes all carbon atoms except that of the carbonyl groups (Bannon et al. 1986). This applies to all FA chain length and degrees of unsaturation (Ackman & Sipos 1964).

If methyl stearate is given an arbitrary response factor of one, FAME of shorter chain length requires a factor progressively greater than one to take into account the progressively lesser content of C-H in the molecule (Bannon et al. 1985; Craske and Bannon 1987). With short chain FAMEs, the presence of the methyl group makes a more noticeable difference in weight percent carbon. This is reflected in detector responses for these FAMEs not being proportional to the number of active carbon atoms but to the relative weight percent of these active carbons in the molecules (Ackman and Sipos 1964).

In analysing FAMEs systemic errors arise from ester preparation, chromatography and peak measurement (Bannon et al. 1985). Any correction factors used should not account for these systemic errors as these should be optimised. Correction factors should only account for FID response (Bannon et al. 1985; Christie 1991), shown in Table 8.

Derivation of results from GC generated reports.

To correctly identify sample FA peaks, run the GLC-85 standard was run every 10 samples in a batch run. From the reports for the standards, relative retention time (relative to palmitate) were calculated for the 32 FA of GLC-85. From the sample reports the palmitate peak was identified. This was about the same time as the standard and is usually the highest at this time. The retention time of the palmitate in the samples were used to calculate relative retention times of candidate peaks and these figures were compared to relative retention times calculated for the adjacent standard.

When correctly identified, the percentage area for the FA was used to calculate the percent weight of the FA by using the equation:

$$\textbf{Weight percent of FA} = \frac{\textbf{peak area}_i * \textbf{RCF}_i}{\sum^{n/i=1} (\textbf{peak area}_i * \textbf{RCF}_i)}$$

Table 8. Correction factors for the FAs of the GLC-85 standard. These correction factors are only FID response factors as determined by Ackman and Sipos (1964). FID response factors only take into account the molecular weight differences between a FA and its methyl ester, and express them relative to palmitate.

Name	Formula	Correction factor
Butyrate	4	1.3742
Hexanoate	6	1.2086
Octanoate	8	1.1247
Docanoate	10	1.0752
Undecanoate	11	1.0558
Laurate	12	1.0421
Tridecanoate	13	1.0282
Myristate	14	1.0178
Myristoleate	14.1	1.0085
Pentadecanoate	15	1.0090
10-Pentadecenoate	15.1	1.0000
Palmitate	16	1.000
Palmitoleate	16.1	0.9913
Heptadecanoate	17	0.9927
10-Heptadecenoate	17.1	0.9857
Stearate	18	0.9852
Oleate	18.1n9c	0.9784
Elaidate	18.1n9t	0.9784
Linoleate	18.2n6c	0.9717
gamma-Linolenate	18.3n6	0.9650
alpha-Linoleneate	18.3n3	0.9650
Arachidate	20	0.9749
11-Eicosenoate	20.1n9	0.9683
11,14-Eicosadienoate	20.2n6	0.9618
7,11,14-Eicosatrienoate	20.3n6	0.9553
11,14,17-Eicosatrienoate	20.3n3	0.9553
Arachidonate	20.4n6	0.9505
Behenate	22	0.9657
Erucate	22.1n9	0.9593
Docosadienate	22.2n6	0.9545
Docosahexanoate (DHA)	22.6n3	0.9313
Nervonate	24.1	0.9531

Appendix Two.

Composition of Trial Diets

Treatment calculations for Clofibrate.

The diets for the preliminary study (Chapter 3) and the Clofibrate study (Chapter 4) were purchased from Crop and Food, AgResearch, Palmerston North. The diet for the Dietary Fat study (Chapter 5) was made at the Food Processing Centre, Massey University. The only difference between the control diets of the preliminary and clofibrate studies, was the replacement of corn oil with olive oil.

Table A2.1. Composition of the diets used in the trials described in this thesis.

Ingredients	Preliminary trial		Clofibrate trial	Fat trial		
	Control	Fat free	Control	Olive	Safflower	Fat free
	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
Cornflour	550	630	550	460	460	630
Lactalbumin	220	270	220	180	180	270
Oil	80	0	80	250(olive)	250(saff)	0
Cellulose	50	60	50	70	70	60
Vitamins	50	5.0	50	5.0	5.0	5.0
Minerals	50	35.0	50	35.0	35.0	35.0

Some of the treatments used in the trial described in Chapter four required an injection of olive oil plus clofibrate or just the oil vehicle. Peters et al. (1996) used an injection dose rate of 15mg/100g body weight. This saturating dose was administered by injection to elicit a peroxisomal proliferation response in PPARα deficient mice. Calculation of the daily doses of the carrier vehicle and clofibrate treatment injected is shown below.

Dose rate: 15mg/100g body weight.

Stock solution: 52.5mg clofibrate/ml of olive oil.

Dose for a 40g mouse = 40 * 0.15/52.5 ml

= 114*u*l

A similar calculation was used to determine the amount of olive oil without clofibrate to be injected for the blank treatment.

When clofibrate was administered in the feed the average daily intake was calculated as 64.5mg per mouse. Thus the feed contained 0.5% (w/w) of clofibrate and the average daily food intake was 12.9g.

Table A2.2. The FA composition of trial diets used throughout this research. FA are expressed as a percentage of total FA.

Name	Formula	Control	Olive	Safflower	Fat free
Butyrate	4.0	0.140	0.054	0.056	1.690
Hexanoate	6.0	0.092	0.033	0.034	1.073
Octanoate	8.0	0.057	0.024	0.024	0.586
Docanoate	10.0	0.125	0.040	0.039	1.434
Undecanoate	11.0	*	*	*	*
Laurate	12.0	0.156	0.049	0.048	1.840
Tridecanoate	13.0	*	*	*	*
Myristate	14.0	0.652	0.214	0.295	7.985
Myristoleate	14.1	0.031	0.010	0.010	0.349
Pentadecanoate	15.0	0.069	0.023	0.032	0.834
10-Pentadecenoate	15.1	*	*	*	*
Palmitate	16.0	12.511	12.220	7.361	28.208
Palmitoleate	16.1	0.177	0.886	0.113	1.125
Heptadecanoate	17.0	0.081	0.064	0.031	0.368
10-Heptadecenoate	17.1	0.034	*	0.014	0.140
Stearate	18.0	2.791	2.027	2.387	11.181
Oleate	18.1n9c	27.976	66.890	11.225	21.078
Elaidate	18.1n9t	0.710	1.371	0.591	2.757
Linoleate	18.2n6c	47.934	9.450	72.069	5.071
gamma-Linolenate	18.3n6	*	*	0.010	*
alpha-Linoleneate	18.3n3	1.035	0.575	0.157	3.093
Arachidate	20.0	0.378	0.370	0.268	0.108
11-Eicosenoate	20.1n9	0.196	0.023	0.139	0.213
11,14-Eicosadienoate	20.2n6	0.016	*	0.020	*
7,11,14-Eicosatrienoate	20.3n6	0.012	*	*	0.235
11,14,17-Eicosatrienoate	20.3n3	0.011	*	*	0.251
Arachidonate	20.4n6	*	*	*	*
Behenate	22.0	0.117	0.110	0.181	0.155
Erucate	22.1n9	0.009	*	0.039	*
Docosadienate	22.2n6	0.008	*	0.009	*
Docosahexanoate (DHA)	22.6n3	0.115	0.418	0.113	0.525
Nervonate	24.1	*	0.028	0.011	*

Measurement of intake were made over a 7 day period. In an attempt to decrease spillage and record an accurate intake record the diets for the preliminary and clofibrate diets were caked with water and freeze dried. The diet was care fully handled and fed in small "chunks". However, some mice not only wasted about 10% of the diet, they even ate bits of their feeders. This meant that, while feed offered and refusals were weighed, there is some error associated with the estimates of feed intake.

The FA composition of all the trial diets is listed in table A2.3. While the fat free diet has a FA profile, it should be remembered that it only contains 0.68% w/w fat in the diet. The oleate concentration (66.9% and of total FA) of the olive oil diet and the linoleate concentration (72% of total FA) in the safflower diet reflects the 25% w/w oil inclusion rate. Table A2.2 shows the 16 and 18 FA ratios of the olive and safflower oil diets. The olive oil diet has 16 and 18 FA ratios many times greater than the safflower oil diet.

Table A2.3. The 16:1/16:0 and 18:1/18:0 FA ratios of the Olive oil and Safflower oil diets.

FA ratio	Safflower oil diet	Olive oil diet
16	0.015	0.072
18	4.70	33.00

Fatty acid composition of the olive and safflower oil diets was determined by the base catalysed methylation procedure as described in methods (Section 2.3). This was followed by gas chromatographic analysis to obtain percentage of total FA values for the FAs listed in Table A 2.4. The 16:1/16:0 (16) and 18:1/18:0 (18) FA ratios are calculated and presented.