

# Milk Fat: Origin of Fatty Acids and Influence of Nutritional Factors Thereon

D.L. Palmquist

## Abstract

Ruminant milk fat is of unique composition among terrestrial mammals, due to its great diversity of component fatty acids. The diversity arises from the effects of ruminal biohydrogenation on dietary unsaturated fatty acids and the range of fatty acids synthesized *de novo* in the mammary gland.

Forty to sixty per cent of milk fatty acids are long-chain (predominantly C<sub>18</sub>) fatty acids derived from the diet, dependent on the amount of fat in the diet. Fatty acids from C<sub>4</sub> to C<sub>14</sub> are synthesized *de novo* in the mammary gland whereas C<sub>16</sub> arises from both diet and *de novo* synthesis.

Milk fat is the most variable component of milk, both in concentration and composition. In dairy cattle, both the concentration and composition of milk fat are influenced by the diet. Concentration is reduced by feeding diets that contain large proportions of readily-fermentable carbohydrates (starch) and unsaturated fat. Conversely, the percentage of fat in milk can be increased by feeding rumen-inert fats. In ruminants, in contrast with non-ruminants, dietary fats have little effect on milk fat composition. Nevertheless, subtle changes in composition and manufacturing functionality can be effected by feeding different fats. Those fatty acids synthesized *de novo*, especially C<sub>12</sub> to C<sub>16</sub>, and oleic acid (C<sub>18:1</sub>) show greatest variation when supplemental fats are fed.

Modern developments in the manufacture of rumen-protected and rumen-inert fats, together with increased understanding of ruminal and

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**D.L. Palmquist** • Professor Emeritus, Department of Animal Sciences, Ohio Agricultural Research and Development Center/The Ohio State University, Wooster, OH 44691, USA.

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animal lipid metabolism, provide considerable flexibility in manipulation of the composition of milk fat for specific nutritional and manufacturing needs.

Future advances in the science of milk fat and nutrition will come from focusing on the unique biological properties of minor milk fatty acids arising from ruminal biohydrogenation and possibly some of *de novo* mammary origin.

## 2.1. Introduction

Among the biological lipids, few exceed bovine milk fat in the complexity of fatty acids present and triacylglycerol (TAG) structure. This, together with its importance commercially as a human food, has generated very large data bases on the synthesis and composition of milk fat. In spite of this, Jensen (2002) lamented the paucity of new information on the content of trace fatty acids and complex lipids in milk fat.

If one considers the review of Jensen (2002) to represent current knowledge of the composition of bovine milk lipids, it is instructive to consider the review of Garton (1963) as a point to begin the topic of this chapter. Garton (1963) quoted a study reporting 64 individual fatty acids in milk fat; further on, he marvelled at the “bewildering complexity of unsaturated fatty acids present in the milk fat of ruminants,” noting that these were apparently associated with ruminal microbial metabolism, in particular ruminal biohydrogenation. Jensen (2002) lists 416 fatty acids in bovine milk lipids. Forty years ago, the complexity of bovine lipids was recognized, as well as the dual sources of origin, i.e., *de novo* synthesis of the short-chain fatty acids in the mammary glands and longer-chain fatty acids of dietary origin. However, little was known of the quantitative significance of each. Rapid progress in studies of fatty acid synthesis in the mammary glands occurred between 1960 and 1980. More recent efforts have been directed toward characterizing genetic determinants of, and the physiological regulation of, lipid synthesis and evaluating the effects of milk fatty acids on human health and as unique regulators of cell and gene function.

As knowledge of the biological functions of minor milk fatty acids has increased, a new appreciation for studies of effects of nutritional and animal management on milk fat composition has been gained. Because animal nutrition and management are key factors in the manipulation of milk fat composition, this chapter will address the synthesis of milk fatty acids and glycerides from the standpoint of regulation and opportunities for manipulation by various feeding strategies.

## 2.2. Origin of the Fatty Acids in Milk Fat

### 2.2.1. Overview

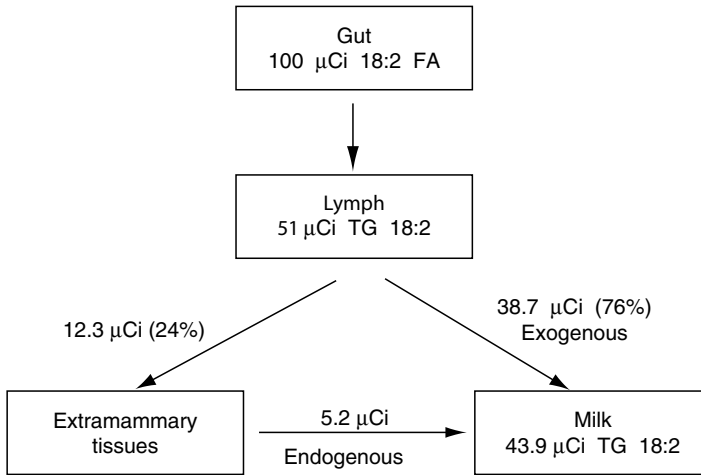
The question of the origins of milk fat, whether wholly from the diet or synthesized by the animal, was an early topic of debate (Jordan and Jenter, 1897). A leading theory, put forth by the eminent lipid chemist, Hilditch (1947), was that the unique short-chain fatty acids arise from the degradation of oleic acid. This conclusion was based upon empirical observations on the composition of ruminant depot and milk fats.

### 2.2.2. Fatty Acid Transport

Modern concepts of milk fat synthesis developed rapidly in the 1950s with the carefully-designed physiological studies in lactating goats by Popják *et al.* (1951) which showed unequivocally the *de novo* synthesis of short-chain fatty acids from  $^{14}\text{C}$ -labelled acetate. Also, the incorporation of tritium-labelled stearic acid into milk fat was demonstrated by Glascock *et al.* (1956). From empirical calculations of the quantity of dietary fat and the recovery of label in milk fat, the latter authors estimated that dietary fat contributed a maximum 25% of the weight of milk fat.

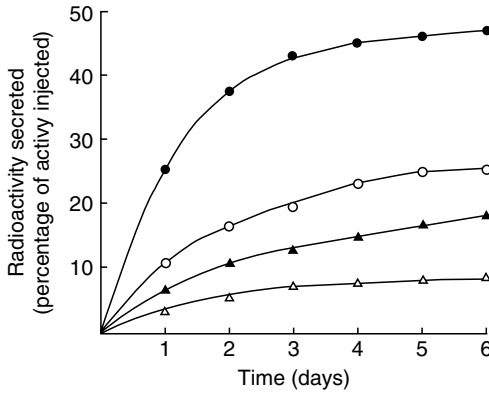
From further studies that compared specific radioactivities of the TAGs in plasma  $\beta$ -lipoproteins and milk fat, Glascock *et al.* (1966) concluded that up to 48% of milk fatty acids were derived from  $\beta$ -lipoprotein TAGs. Because the long-chain fatty acids comprise approximately 50% of milk fat, this observation implied that more than 90% of these fatty acids were of plasma origin, consistent with the demonstration of little fatty acid elongation in the mammary gland (Palmquist *et al.*, 1969). Finally, Glascock and coworkers (see Bishop *et al.*, 1969) concluded, from comparisons of specific radioactivities of dextran sulfate-precipitable lipoproteins and milk fat labelled with tritiated palmitic acid, that no more than 36% of the milk fat could have been derived from the plasma TAGs. In neither study did Glascock and colleagues provide data on quantities of dietary fat nor on the yield and composition of milk fat. However, in the latter study (Bishop *et al.*, 1969), they indicated that palmitic acid constituted 39% of milk fat by weight, a value that would be observed only at a very low dietary fat intake. Thus, the lower contribution of plasma TAGs to milk fat synthesis may not be incompatible with the earlier study (Glascock *et al.*, 1966).

Palmquist and Conrad (1971) fed or intravenously infused 1- $^{14}\text{C}$  palmitic acid into lactating cows and mathematically calculated the partition of  $^{14}\text{C}$  secreted into milk fat as originating from two pools, diluted due to turnover of the dietary and endogenous fatty acids. The proportions of the long-chain fatty acids in milk derived from the diet were influenced by the



**Figure 2.1.** Model of linoleic acid transport from intestine to milk. Incorporation into intestinal lipoprotein triacylglycerols accounted for 51% of absorbed  $^{14}\text{C}$  linoleic acid; of this 76% was incorporated directly into milk whereas 10% was incorporated into milk after cycling through other metabolic pool(s). Adapted from Palmquist and Mattos (1978).

composition of the diet. In further studies, Palmquist and Mattos (1978) injected 1- $^{14}\text{C}$ -linoleic acid as labelled chylomicron TAGs, very low density lipoproteins (VLDL) or albumin-bound fatty acids. From curve analysis of labelled milk fat secretion and reanalysis of earlier data reported by Palmquist and Conrad (1971), they concluded that 88% of long-chain fatty acids in milk were derived directly from TAGs of intestinal lipoproteins while 12% were derived from TAGs of endogenous origin. A model was developed, showing that 76% of absorbed TAG linoleic acid was taken up directly by the mammary glands (Figure 2.1). Using a more direct approach, Glascock *et al.* (1983) injected  $^3\text{H}$  TAGs into sheep at various stages of lactation and measured the partition of label to adipose TAGs, milk lipids or oxidation, as measured by appearance of  $^3\text{H}$  in the body water. Partition of blood TAGs to milk clearly declined with increasing days in lactation (Figure 2.2), with maximal incorporation (46%) at 18 days of lactation. The proportion of injected TAGs transferred to milk fat was much lower than that estimated for lactating cows by Palmquist and Mattos (1978) and the estimated proportion that was oxidized was much higher than reported for lactating goats by Annison *et al.* (1967). Both of these divergent observations of Glascock *et al.* (1983) may be due to use of non-dairy breeds of sheep. Nevertheless, the point of changing partition of plasma TAGs in different physiological states was clearly established, consistent with the principles of homeorhesis described by Bauman and



**Figure 2.2.** Cumulative secretion of radioactivity in milk fat of sheep after intravenous injection of an emulsion of [ $^3\text{H}$ ] triacylglycerol at (●) 18 days, (○) 35 days, (▲) 58 days and (△) 73 days after lambing. (From Glascock *et al.*, 1983; reprinted with the permission of Cambridge University Press).

Currie (1980). Detailed studies by Annison and colleagues (Annison *et al.*, 1967; West *et al.*, 1972) showed that both TAG fatty acids and non-esterified fatty acids (NEFAs) of plasma were taken up by the mammary glands of lactating goats. TAG uptake accounted for up to 60% of milk fat secreted. However, no net uptake of NEFAs occurred. The specific activity of radiolabelled NEFAs decreased across the gland, indicating dilution of NEFAs by fatty acids arising from the hydrolysis of plasma TAGs in the capillaries by mammary lipoprotein lipase (LPL) and subsequent mixing with plasma NEFAs. Similarly, Mendelson and Scow (1972) showed that 40% of TAG fatty acids hydrolyzed by rat mammary LPL were released in plasma, thus mixing with plasma NEFAs. Net uptake of plasma NEFAs by the mammary glands occurs at the high NEFA concentrations (Miller *et al.*, 1991; Nielsen and Jakobsen, 1994) observed immediately *post-partum* or in sub-clinical ketosis. Usually, in established or mid-lactation, net uptake (*ca.* 40  $\mu\text{mol/L}$ ) is variable (+ or -), averaging about zero, so that uptake of fatty acids from the NEFA pool is not evident unless labelled tracers are used. However, milk fat concentration is positively correlated ( $r = 0.76, P < 0.05$ ) with plasma NEFA concentration (Pullen *et al.*, 1989). Significant amounts of NEFAs were transferred from plasma to milk in the mammary glands of lactating rabbits and rats (Jones and Parker, 1978).

### 2.2.3. Lipoprotein Lipase

The TAG-rich chylomicra and VLDL of plasma are the primary source of long-chain fatty acids taken up by the mammary glands. Uptake

is mediated by lipoprotein lipase (LPL), an enzyme that hydrolyzes TAGs to form fatty acids, glycerol and perhaps 2-monoacylglycerol. Mammary gland LPL was described first by Korn (1962) who showed its presence in cows' milk; subsequently, LPL activity was characterized in guinea pig mammary glands by McBride and Korn (1963) and Robinson (1963). The enzyme in tissue and milk is identical (Castberg *et al.*, 1975). Characteristics and regulation of mammary LPL were discussed in detail by Barber *et al.* (1997). LPL is associated with vascular endothelial surfaces, bound by heparin sulfate chains; it is released rapidly by intravenous injection of heparin, which competes with endothelial binding sites of the enzyme, and by the presence of TAG-rich lipoproteins. The enzyme has an absolute requirement for apolipoprotein C-II for activation and is inhibited by apolipoprotein C-III and by 0.5 M NaCl. Apolipoproteins E, which have strong heparin-binding properties, anchor TAG-rich lipoproteins to the endothelial cell walls (Goldberg, 1996). Mammary LPL activity increases markedly immediately prior to parturition and remains high throughout lactation in the guinea pig (McBride and Korn, 1963; Robinson, 1963), rat (Hamosh *et al.*, 1970) and cow (Shirley *et al.*, 1973; Liesman *et al.*, 1988), whereas it is simultaneously down-regulated in adipose tissue (Hamosh *et al.*, 1970; Shirley *et al.*, 1973). These coordinated changes are mediated by the anterior pituitary *via* the release of prolactin (Zinder *et al.*, 1976; Liesman *et al.*, 1988; Thompson, 1992), and nicely illustrate the concept of homeorhetic regulation of physiological function (Bauman and Currie, 1980).

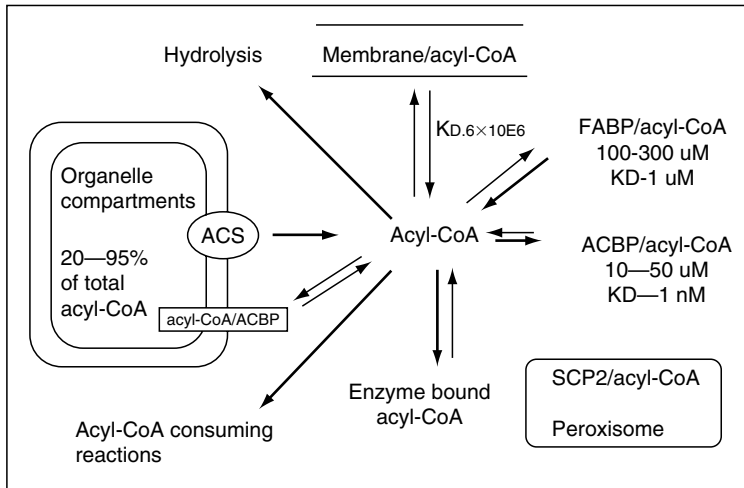
#### **2.2.4. Transport of Long-Chain Fatty Acids into Mammary Cells**

The mechanism(s) for the transfer of fatty acids from the capillaries into mammary cells are not well-documented. Enjalbert *et al.* (1998) observed a nearly linear correlation between mammary arterio-venous difference and arterial concentration of NEFAs + TAGs in the range of 400 to 750  $\mu\text{molar}$  ( $\mu\text{M}$ ), or below saturation for uptake, as described by Baldwin *et al.* (1980). Veercamp *et al.* (1991) suggested that saturation of fatty acid uptake may result from subsequent intracellular processes (see below), from limitations of intracellular diffusion or from diffusive equilibration between extracellular albumin and intracellular fatty acid-binding protein (FABP). A "flip-flop" model, implying carrier-mediated transport, may be too slow to account for fatty acid uptake (Hajri and Abumrad, 2002). Barber *et al.* (1997) have argued for the role of a specific 88 kDa protein, fatty acid translocator (FAT, CD 36), in the transport of fatty acids across the mammary epithelial cell membrane, working in conjunction with intracellular FABP. Scow *et al.* (1980) and Blanchette-Mackie and Amende (1987) made a case for the strongly amphipathic fatty acid molecules to move laterally in a continuous

interface composed of the chylomicron (VLDL) surface film and the external leaflet of plasma and intracellular membranes of endothelial and parenchymal cells, with removal within the cell cytosol as the fatty acids become esterified. Though this model (Scow *et al.*, 1980) is supported by numerous exquisite photomicrographs, it does not explain the loss of up to 40% of the fatty acids from hydrolyzed chylomicron TAGs as plasma NEFAs (Mendelson and Scow, 1972) nor does it provide a role for intracellular FABP. An alternative model is that described for the diffusion of the amphipathic fatty acids through the amphipathic microvillus membrane of the intestine (Thomson *et al.*, 1983). Although diffusion would be too slow for uptake of albumin-bound NEFAs, it could account for uptake from TAG-rich lipoproteins tightly associated with the capillary endothelium (Hajri and Abumrad, 2002). In this model, longer-chain and more saturated fatty acids diffuse through the membrane more rapidly because they are more hydrophobic, consistent with the order of uptake of fatty acids by the bovine mammary gland (Thompson and Christie, 1991). Because the concentration of CoASH is very low and well below saturation in cytosol, it could well be that the limiting steps in the rate of fatty acid uptake are those that determine the rate of fatty acyl-CoA incorporation into TAGs which, in turn, frees up CoASH for acyl CoA synthetase, thereby freeing up a site for binding of a new fatty acid to otherwise saturated FABP and subsequent removal of another fatty acid from plasma; e.g., if there are no intracellular binding sites available for fatty acids from TAG hydrolysis, they are lost from the glands as NEFAs. This fits with the data of Enjalbert *et al.* (1998) and others where consideration of TAGs and NEFAs together leads to less variance than seen with either alone.

FABPs have been implicated in transmembrane and intracellular transport of fatty acids (Veerkamp *et al.*, 1991; Storch and Thumser, 2000). These are a group of tissue-specific proteins of about 14–15 kDa that bind long-chain fatty acids ( $C_{16}$ – $C_{20}$ ) with high affinity and a molar stoichiometry of 1:1. Most bind unsaturated fatty acids with higher affinity than saturated fatty acids. In addition to transport functions it has been proposed that they modulate specific enzymes of lipid metabolism, regulate expression of fatty acid-responsive genes, maintain cellular membrane fatty acid levels, and reduce the concentration of fatty acids in the cell, thereby removing their inhibitory effect on metabolic processes.

More recently, a greater role in regulating concentrations and transport of fatty acids in the cytosol has been proposed for the acyl-CoA-binding proteins (ACBP; Knudsen *et al.*, 2000). ACBP is an 86–103 residue protein, with a highly-conserved amino acid sequence, that binds long-chain acyl-CoA with a 10-fold higher affinity than does FABP (Rasmussen *et al.*, 1990), thus being much more effective in protecting membranes from damaging effects



**Figure 2.3.** Regulation of cytosolic free acyl-CoA concentration. The major factors affecting the cytosolic free long-chain acyl-CoA ester (LCA) concentration are the rates of LCA synthesis, uptake and consumption, the concentration of acyl-CoA-binding protein (ACBP), fatty acid-binding protein (FABP) and the acyl-CoA hydrolase activity. The two binding proteins buffer large fluctuations in free LCA concentration. The acyl-CoA hydrolases are suggested to function as a “scavenger” system to prevent the accumulation of free unprotected LCA and to ensure sufficient free CoA to support  $\beta$ -oxidation and other CoA-dependent enzymes. Sterol carrier protein 2 (SCP2), which binds LCA and very long chain-LCA, is localized in the peroxisomes. It is suggested that SCP2 acts as a peroxisomal pool former for LCA destined for  $\beta$ -oxidation in this organelle. (Adapted from Knudsen *et al.*, *J. Nutrition* 130:294S-298S, 2000, with permission).

of long-chain acyl-CoA esters. Many factors regulate the concentration of unbound acyl-CoA in cells (Figure 2.3). Under conditions in which the concentration of ACBP is not adequate to bind long-chain acyl-CoA, FABP takes over the buffering function, thus protecting cellular membranes from damage (Knudsen *et al.*, 2000). The concentration of ACBP is highest in liver cytosol, and interestingly, was found to be lowest in the mammary glands of the cow (Mikkelsen and Knudsen, 1987). One might expect, based on the presumption of an association of ACBP concentration with tissue lipid metabolic rates, that the concentration of ACBP might be high in mammary tissue.

### 2.2.5. Summary of the Supply of Long-Chain Fatty Acids to the Mammary Gland

More than 95% of  $C_{18}$  and longer-chain fatty acids in milk fat are derived from the blood TAG-rich lipoproteins. Non-esterified fatty acids are



taken up also, but net uptake is measurable only when the concentrations of NEFAs are high, notably in the early weeks of lactation when energy balance is negative and extensive mobilization of body lipid stores occurs. When cows are not in negative energy balance, adipose tissues contribute less than 15% of long-chain fatty acid uptake. Three-fourths of intestinal lipoprotein TAGs are taken up by lactating mammary glands. The proportion of the palmitic acid in milk fat derived from blood lipids is variable. When dietary fat intake is low, nearly all of palmitic acid is synthesized *de novo* in mammary tissue. As fatty acid uptake from blood TAGs increases, the proportion of palmitic acid from *de novo* synthesis decreases to 30% or less of the total.

### 2.3. Uptake of Non-Lipid Metabolites by Lactating Mammary Glands

In addition to long-chain fatty acids from plasma, the major nutrients utilized for milk fat synthesis are glucose, acetate and  $\beta$ -hydroxybutyrate. Kinetics for the uptake of these from blood were reported by Miller *et al.* (1991). Glucose is absolutely required for milk synthesis, being a precursor for lactose or other carbohydrates, or both, in all terrestrial mammals (Oftedal and Iverson, 1995).

In fasting animals, the arterial supply of glucose is limiting for milk synthesis (Chaiyabutr *et al.*, 1980), whereas in fed animals glucose uptake is independent of arterial concentration (Miller *et al.*, 1991). In the lactating mammary tissues of both ruminants and non-ruminants, glucose is taken up *via* facilitative transport systems, namely, the insulin-independent GLUT1 and probably a  $\text{Na}^+$ -dependent glucose transporter (Zhao *et al.*, 1996; Shennan and Peaker, 2000; Nielsen *et al.*, 2001). There is no evidence for the insulin-sensitive GLUT 4 transporter in lactating mammary tissues of either rats or cattle. Baldwin *et al.* (1980) suggested, on the basis of the limited arterio-venous difference data available in the literature at that time, that Michaelis-Menten type equations could be used to describe glucose uptake across the bovine udder. The data they collected were from a number of different studies and were highly scattered, such that the statistical fit to the data was improved only slightly by using a Michaelis-Menten as compared to a linear equation. Uptake of  $\beta$ -hydroxybutyrate and TAGs by the lactating mammary glands were well described by Michaelis-Menten relationships, whereas the uptake of acetate was strongly linear (Baldwin *et al.*, 1980). These authors cautioned that the uptake of metabolites by lactating mammary glands are interrelated, such that mathematical descriptions for individual nutrients are not unique, but are influenced by the

availability and uptake of other nutrients. In summarizing an extensive arterio-venous difference study on nutrient uptakes by cows' udders, these workers re-emphasized this point and concluded that factors other than arterial blood glucose concentration govern glucose uptake and utilization (Miller *et al.*, 1991; Baldwin, 1995).

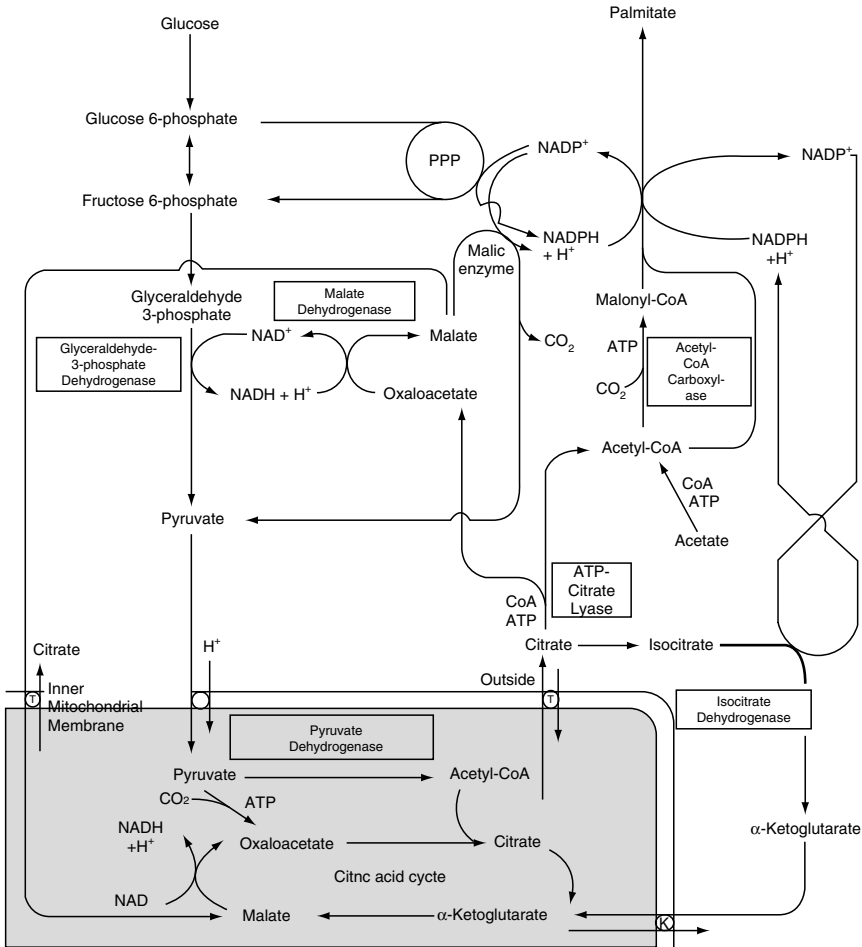
A serine/threonine protein kinase, Akt, is thought to regulate the expression or function of glucose transport proteins in adipose tissue, and also may represent a central signalling molecule in mammary gland development and function. Expression of constitutively activated Akt in mammary glands of transgenic mice resulted in precocious lipid accumulation; fat content of the milk was 65 to 70% by volume, compared with 25 to 30% in wild-type mice (Schwertferger *et al.*, 2003).

## 2.4. Fatty Acid Synthesis in Mammary Glands

Fatty acid synthesis in lactating mammary glands was discussed in detail by Hillgartner *et al.* (1995) and Barber *et al.* (1997). Comparative aspects of fatty acid synthesis in mammary glands of ruminants and non-ruminants were reviewed by Dils (1986) and an in-depth review of lipid metabolism in ruminant mammary glands was provided by Moore and Christie (1981). Requirements for fatty acid synthesis are a carbon source and reducing equivalents in the form of NADPH + H<sup>+</sup>. In ruminants, acetate and  $\beta$ -hydroxybutyrate are the primary carbon sources utilized, while glucose and acetate are the primary sources of reducing equivalents (Bauman and Davis, 1974). In non-ruminants, glucose is the primary source of both carbon and reducing equivalents (Figure 2.4). Enzyme activities and utilization of substrates by mammary tissues are increased dramatically relative to those of non-mammary tissues at the onset of lactation (Bauman and Currie, 1980; Vernon *et al.*, 1987).

### 2.4.1. Sources of Carbon and Reducing Equivalents for Fatty Acid Synthesis

The basic starting substrate for fatty acid synthesis is acetyl-CoA (see below). In ruminants, the provision of this substrate is straightforward. Acetate from blood (+ CoA + ATP) is converted by the cytosolic acetyl-CoA synthase (EC 2.3.1.169) to AMP and acetyl-CoA, which can then be used for fatty acid synthesis. In non-ruminants, glucose is converted *via* the glycolytic pathway to pyruvate, which is, in turn, converted to acetyl-CoA in mitochondria. Acetyl-CoA thus formed is converted to citrate which passes out to the cytosol where it is cleaved by ATP-citrate lyase (EC 2.3.3.8) to acetyl-CoA + oxalacetate (OAA). This "transport" of acetyl-CoA from



**Figure 2.4.** The provision of acetyl-CoA and NADPH for lipogenesis. PPP, pentose phosphate pathway; T, tricarboxylate transporter; K, α-ketoglutarate transporter. In ruminants, pyruvate dehydrogenase, ATP-citrate lyase and malic enzyme activities are low and perhaps non-functional. (From Murray *et al.*, 1988. *Harper's Biochemistry*, 21st edn, p. 207, Appleton and Lange, Norwalk, CT; reproduced with permission of The McGraw-Hill Companies).

mitochondria to the cytosol also results in the transport of OAA to the cytosol. OAA cannot move freely across the mitochondrial membrane as can pyruvate and citrate, but it can be reduced by  $NADH + H^+$  to form malate, which is converted by the malic enzyme (EC 1.1.1.40) to pyruvate and  $NADPH + H^+$ . Pyruvate can enter the mitochondrion and be converted to

OAA by pyruvate carboxylase (EC 6.4.1.1), thereby completing the net transport of the  $C_2$  unit (acetate) from the mitochondrion to the cytosol with the added advantage of having converted a reducing equivalent as  $NADH + H^+$  to  $NADPH + H^+$ . This mechanism of " $C_2$  transport" provides up to 50% of the  $NADPH + H^+$  for fatty acid synthesis in non-ruminants.

The reductive steps of FAS have a specific requirement for NADPH, whereas glycolysis, also a cytosolic process, generates NADH. In non-ruminants, some of the required NADPH is generated in the first two oxidative steps in the pentose phosphate pathway by glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase, respectively. This provides perhaps one-half of the reducing equivalents required for fatty acid synthesis. In rats and mice, the remainder is generated as an adjunct to the mechanism of  $C_2$  transport from the mitochondrion to the cytosol discussed above (Figure 2.4). The activities of both citrate lyase and malic enzyme increase with high carbohydrate diets in non-ruminants. The activities of these latter enzymes are low in ruminants, sows (Bauman *et al.*, 1970) guinea pigs, and rabbits (Crabtree *et al.*, 1981), probably reflecting the greater availability of acetate as a lipogenic precursor in these species or the absence of the need to transport  $C_2$  units from the mitochondrion to the cytosol, or both.

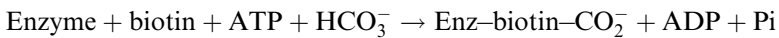
Glucose oxidation *via* the pentose phosphate pathway (PPP) is an equally important source of  $NADPH + H^+$  in ruminants and also provides glycerol-3-phosphate for fatty acid esterification as an alternative to the glycolytic pathway. Glucose oxidation *via* the PPP accounts for 10 to 40% of ruminant mammary glucose utilization and 25 to 40% of mammary  $CO_2$  output (Bickerstaffe *et al.*, 1974; Cant *et al.*, 1993). Wood *et al.* (1965) examined glucose utilization in perfused cow udders. Twenty-three to 30% of glucose was metabolized *via* the PPP, 10% was utilized in the Emden-Meyerhof pathway, and 60 to 70% was converted to lactose. A similar proportion was oxidized in the PPP in rats, but only 10 to 20% was utilized for lactose and up to 50% underwent glycolysis to provide  $C_2$  units for lipogenesis (Katz and Wals, 1972).

ATP-citrate lyase activity is low in ruminants (Ballard *et al.*, 1969), and originally was considered to be the limiting factor that blocks the incorporation of glucose carbon into fatty acid carbon in ruminants. However, physiologically, this is not the reason. The negative feedback of acetyl-CoA formed from acetate on pyruvate dehydrogenase probably results in essentially complete inhibition of the enzyme activity in mammary as well as other tissues, thereby yielding a net sparing of glucose which is appropriate to the limited availability of glucose in these species. Inhibition of pyruvate dehydrogenase by acetyl CoA *in vivo* is consistent with the low incorporation of

pyruvate and lactate into fatty acids *in vivo* and also the incorporation of significant amounts of lactate and pyruvate carbon into fatty acids by bovine mammary tissue at high substrate concentrations *in vitro* in the absence of acetate (Forsberg *et al.*, 1985; Torok *et al.*, 1986). Conversion of glucose to fatty acids is low even in the absence of acetate, indicating a block somewhere between fructose-6-phosphate and pyruvate, possibly at phosphofructokinase (PFK) or in triose-phosphate metabolism, but this issue has not received any significant attention. Acetyl-CoA is a strong positive effector of pyruvate carboxylase. Citrate occurs at a rather high concentration in milk, being generated in the citric acid cycle from acetate, and the carboxylation of pyruvate and propionate. Because acetyl-CoA transport from mitochondria to the cytosol is not necessary in ruminants, cytosolic OAA and malate are not generated, so that  $\text{NADPH} + \text{H}^+$  from the malic enzyme is not available to support fatty acid synthesis. Instead, citrate diffuses from the mitochondrion to the cytosol and is converted to isocitrate, which is used to generate the  $\text{NADPH} + \text{H}^+$  required *via* the cytosolic NADP-isocitrate dehydrogenase (EC 1.1.1.42), accounting for as much as 50–60% of total NADPH used by ruminant fatty acid synthase (Mellenberger *et al.*, 1973; Mellenberger and Bauman, 1974). The product of this reaction,  $\alpha$ -ketoglutarate, is transported back into the mitochondrion (Figure 2.4). A unique aspect of this pathway is that NADPH is generated by oxidation of acetate, thus sparing glucose (Bauman *et al.*, 1970). High-fat diets reduce *de novo* fatty acid synthesis, thus decreasing the demand for reducing equivalents. This spares mammary glucose, freeing it for lactose synthesis (Cant *et al.*, 1993). Isocitrate oxidation is decreased, causing citrate concentration in milk to increase (Faulkner and Pollock, 1989).

### 2.4.2. Acetyl-CoA Carboxylase

The first committed step for the incorporation of acetate carbon into fatty acids is mediated by acetyl-CoA carboxylase (ACC; EC 6.4.1.2) in two steps, as follows (Allred and Reilly, 1997):



Five transcripts of the gene for ACC $\alpha$  have been described (Kim, 1997). These occur by two independent promoters, PI and PII, and differential splicing of the primary transcripts. Transcripts derived from PI have been characterized in adipose tissue while those from PII are found in mammary tissue (Kim, 1997). A third promoter (PIII) has been characterized in ovine mammary glands; it generates a transcript encoding an enzyme with an alternative N-terminus. Whereas PI was strongly expressed in bovine

udder (Mao *et al.*, 2001), it was not detected in the udder of lactating sheep by Travers *et al.* (2001). Both PII and PIII transcripts increased with lactation in sheep (Clegg *et al.*, 2001); PII was strongly induced (~10-fold) by lactation in sheep (Ponce-Casteñeda *et al.*, 1991), but increased only about 3-fold in cows (Mao and Seyfert, 2002). The PIII promoter is strongly induced by lactation, being stimulated by prolactin *via* STAT 5 activation (Mao *et al.*, 2002). Molenaar *et al.* (2003) reevaluated all three promoters in mammary epithelial cells from cows and sheep, showing that all were active in both species. However, whereas all three promoters were shown to be relevant for milk fat synthesis in cattle, only PII and PIII were crucial for milk fat synthesis in sheep.

In liver, adipose, and possibly mammary, tissues, ACC is regulated acutely by phosphorylation/dephosphorylation and by allosteric mechanisms involving fatty acids (fatty acyl-CoA) and citrate, whereas the amount of protein is regulated by several hormones, including insulin, growth hormone, possibly *via* IGF-1, and prolactin (Kim *et al.*, 1992; Allred and Reilly, 1997; Barber *et al.*, 1997). Short-term regulation of ACC in liver and adipose tissues is well characterized. Very few studies of ruminant mammary tissue, where some of the same mechanisms may be present, have been reported. A possible reason for this deficiency is that the study of acute hormonal actions, which involve phosphorylation and dephosphorylation, require the use of cells with intact membranes. The high level of connective tissue in ruminant mammary tissue requires extensive incubation with collagenase to permit the isolation of secretory cells and these, most often, have severely damaged cell membranes, which are then unable to respond to hormonal signals.

Short-term hormonal regulation of ACC is achieved by covalent modifications of the enzyme by phosphorylation or dephosphorylation, which either increase or decrease its activity. These changes in enzyme activity are observed within minutes of exposure to hormones and thus are not likely due to changes in the amount of enzyme (Kim, 1983). Lee and Kim (1979) reported that incubation of rat adipocytes with epinephrine doubled the incorporation of  $^{32}\text{P}$  into ACC within 30 minutes and reduced enzyme activity by 61%. Witters *et al.* (1979) established a similar relationship between phosphorylation and inactivation of rat hepatocyte ACC following glucagon treatment.

Rat liver ACC which has been phosphorylated and inactivated by a cyclic AMP—dependent protein kinase can be dephosphorylated and reactivated by incubation with a protein phosphatase (Curtis *et al.*, 1973). Similarly, ACC purified from rat or rabbit mammary glands are activated by dephosphorylation on incubation with a protein phosphatase (Hardie and Cohen, 1979; Hardie and Guy, 1980).

An early concern in studies of the activation of ACC by citrate was that the concentration of citrate required for activation was higher than that normally observed in tissues. Thus, it was of interest to find that the  $K_m$  for the binding of citrate to phosphorylated ACC of 2.4 mM was reduced to 0.2 mM by dephosphorylation (Carlson and Kim, 1974). Further, the dephosphorylated form of ACC is more susceptible to inhibition by palmitoyl-CoA. Also, phosphorylation and inactivation of ACC is accompanied by conversion of active polymers to inactive protomers (Shiao *et al.*, 1981). Mabrouk *et al.* (1990) found that inactive protomers isolated from the livers of rats injected with glucagon have a higher phosphate content than protomers isolated from the livers of control animals.

The signal transduction system responsible for ACC phosphorylation and inactivation has been studied in considerable detail. ACC isolated from rat mammary glands or rat livers is phosphorylated and inactivated by cAMP-dependent kinases. The  $^{32}\text{P}$ -labeled peptide isolated from rat adipocyte ACC after treatment with epinephrine and tryptic digestion co-migrated with the  $^{32}\text{P}$ -labeled peptide isolated after phosphorylation of purified ACC by cAMP-dependent kinase. There are three cAMP-independent protein kinases. Of these, ACC kinase-3 is stimulated by phosphorylation and AMP and is referred to as AMP-activated protein kinase. While the cAMP-dependent protein kinase phosphorylates Ser<sub>77</sub> and Ser<sub>1200</sub> and causes moderate inactivation of ACC, the AMP-activated protein kinase phosphorylates at Ser<sub>79</sub> and Ser<sub>1200</sub> and causes a dramatic inactivation of ACC purified from lactating rat mammary glands (Munday *et al.*, 1988). Further studies (Haystead *et al.*, 1990) established that hormone-induced phosphorylation of Ser<sub>77</sub> by cAMP-dependent protein kinases may not occur *in vivo*, but rather, that the cAMP-elevating hormones activate the AMP-activated protein kinase *via* a kinase-kinase system and that the phosphorylation at Ser<sub>79</sub> by this enzyme, in turn, is the mechanism of inactivation of ACC.

In contrast to the acute inactivation of ACC by cAMP-elevating hormones, it is well established that insulin stimulates fatty acid synthesis in several tissues, including lactating rat mammary acini, within a few minutes of treatment (Williamson *et al.*, 1983). Responses of ruminant lipogenic tissues to insulin are generally far less dramatic than in rodents. For example, Yang and Baldwin (1973) found only a doubling of fatty acid synthesis in bovine adipocytes treated with insulin during a 3-hour incubation. Lactating cow mammary tissue does not appear to exhibit acute responses to insulin *in vitro* (Forsberg *et al.*, 1985; Laarveld *et al.*, 1985) although the tissue does have insulin receptors (Oscar *et al.*, 1986).

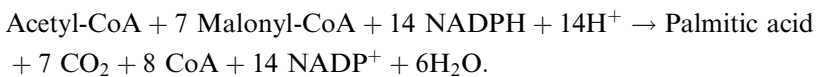
The insulin-induced enhancement of fatty acid synthesis in non-ruminant adipose and liver tissues is associated with an increase in the

phosphorylation of ACC. Analyses of tryptic peptides derived from  $^{32}\text{P}$ -labeled ACC from insulin-treated lipogenic tissues indicated that the peptides labelled were clearly separate and distinct from those induced by epinephrine. The peptide labelled by insulin treatment is referred to as the “I-peptide” (Brownsey and Denton, 1982). The polymerized form of ACC, which is most active, was found to have increased phosphorylation of the “I-peptide” (Borthwick *et al.*, 1987). These workers (Borthwick *et al.*, 1990) established that the increases in phosphorylation and activity of ACC occurred in parallel, leading to the development of the concept that hormones such as epinephrine, acting *via* cAMP, inactivate ACC by phosphorylating sites separate from those phosphorylated by insulin, which activate ACC.

It has been reported also that epidermal growth factor stimulates fatty acid synthesis and the phosphorylation of ACC in rat liver and adipose tissues (Holland and Hardie, 1985) through phosphorylation of the “I-peptide,” suggesting that several peptide hormones sharing homology with insulin, such as IGF-1, could enhance lipogenesis similarly.

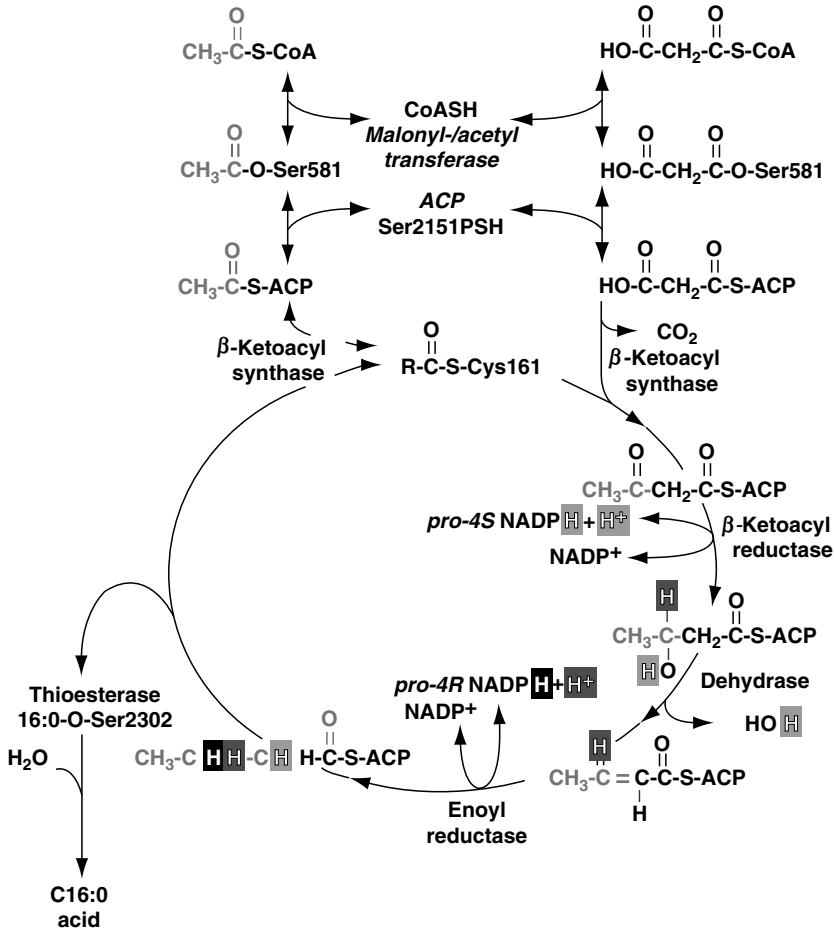
### 2.4.3. Fatty Acid Synthase

The animal fatty acid synthase (FAS; EC 2.3.1.85) is one of the most complex multifunctional enzymes that have been characterized, as this single polypeptide contains all the catalytic components required for a series of 37 sequential transactions (Smith, 1994). The animal FAS consists of two identical polypeptides of approximately 2500 amino acid residues (MW, *ca.* 270 kDa), each containing seven catalytic subunits: (1) ketoacylsynthase, (2) malonyl/acetyl transferase, (3) dehydrase, (4) enoyl reductase, (5)  $\beta$ -keto reductase, (6) acyl carrier protein (ACP), and (7) thioesterase. Although some components of the complex are able to carry out their respective catalytic steps in the monomeric form, only in the FAS dimer do the subunits attain conformations that facilitate coupling of the individual reactions of fatty acid synthesis to occur (Smith *et al.*, 2003).



The entire sequence is described in exquisite detail by Smith *et al.* (2003; see Figure 2.5). The first step is the sequential transfer of the primer, usually acetyl-CoA, to the serine residue of the acyl transferase, then to the ACP, and finally to  $\beta$ -ketoacyl synthase. The chain extender substrate, usually malonyl-CoA, is transferred *via* the serine residue of the acyl transferase to ACP. Condensation is accomplished by  $\beta$ -ketoacylsynthase, aided by the energetically-favourable decarboxylation of the malonyl residue,





**Figure 2.5.** Reaction sequence for the biosynthesis of fatty acids *de novo* by the animal FAS. The condensation reaction proceeds with stereochemical inversion of the malonyl C-2, the  $\beta$ -ketoacyl moiety is reduced by NADPH to D- $\beta$  hydroxyacyl moiety, which then is dehydrated to a *trans*-enoyl moiety; finally, the enoyl moiety is reduced to a saturated acyl moiety by NADPH, with the simultaneous addition of a solvent proton. The two C atoms at the methyl end of the fatty acid are derived from acetyl-CoA, the remainder from malonyl-CoA. The entire series of reactions takes approximately 1 second. PSH, phosphopantetheine. (Reprinted from Prog. in Lipid Res., vol. 42, S. Smith, A. Witkowski and A.K. Joshi, Structural and functional organization of the animal fatty acid synthase, pp. 289–317, copyright (2003), with permission from Elsevier).

resulting in the release of CO<sub>2</sub>. Note that the acetyl moiety originally bound to ACP to initiate the synthetic sequence becomes the methyl-terminal of the growing acyl chain. Sequential reactions catalyzed by  $\beta$ -ketoacyl reductase,

dehydrase and enoyl reductase result in the formation of butyryl-*S*-ACP, which re-enters the cycle through condensation with another malonyl-ACP. Cycling continues, with the addition of C<sub>2</sub> units from malonyl-CoA until the chain length of the nascent fatty acid reaches C<sub>12</sub> to C<sub>16</sub>, when a thioesterase specific for that chain length releases the fatty acid and terminates the cycle.

#### 2.4.4. Regulation of Acyl Chain Length

The chain length of the fatty acid product is influenced by numerous factors. The same acyl transferase accomplishes loading of both the acetyl and malonyl substrates. The process is random. Both substrates are exchanged rapidly between CoA and ACP until a combination occurs that permits chain initiation or a continuation of elongation (Smith *et al.*, 2003). The catalytic rate of the acyl transferase increases as chain length progresses from C<sub>2</sub> to C<sub>12</sub>; the total concentration of covalently-bound saturated intermediates decreases with increasing chain lengths up to C<sub>14</sub>, implying that the early  $\beta$ -ketoacylsynthase reactions are slower than the later ones (Smith *et al.*, 2003). Mass action coefficients for chains longer than C<sub>12</sub> become progressively smaller, with little accumulation of C<sub>18</sub>. Acyltransferase is not entirely specific for the acetyl and malonyl moieties.  $\beta$ -Ketobutyryl,  $\beta$ -hydroxybutyryl and crotonyl residues all are incorporated (Dodds *et al.*, 1981; Joshi *et al.*, 1997). Indeed, Kumar and associates (Nandedkar *et al.*, 1969; Lin and Kumar, 1972) have shown that lactating mammary glands utilize butyryl-CoA more efficiently than acetyl-CoA as a “primer” for FAS. Further, these authors showed that butyryl-CoA is synthesized from acetyl-CoA by, essentially, a reversal of  $\beta$ -oxidation in both the liver and mammary glands of rabbits, rats and cows. Knudsen and Grunnet (1980) showed that butyryl-CoA is superior to acetyl-CoA as a primer for FAS, but only in ruminants. The acyl-CoA dehydrogenase associated with  $\beta$ -oxidation is an FAD-linked dehydrogenase, which renders  $\beta$ -oxidation essentially irreversible on thermodynamic grounds. The enzyme that catalyzes the reverse reaction in the Kumar scheme is linked *via* NADPH + H, which renders the reduction of crotonyl-CoA thermodynamically favourable. Thus, this enzyme, unique to the reversal of  $\beta$ -oxidation, could be called “crotonyl-CoA reductase.” This activity was much lower in rat adipose and pigeon liver tissues. This synthetic pathway is independent of malonyl-CoA and thus is not subject to regulation by acetyl-CoA carboxylase. These observations explain the incorporation of  $\beta$ -OH butyrate as the methyl terminal C<sub>4</sub> moiety of up to 50–60% of fatty acids synthesized *de novo* by lactating mammary glands (Palmquist *et al.*, 1969; Smith *et al.*, 1974). Also, small quantities of C<sub>6</sub>, C<sub>8</sub> and C<sub>10</sub> in milk fat may be synthesized *via* this pathway. Propionic acid is incorporated as a primer leading to the synthesis of an odd

number of carbon atoms in the acyl chain. The branched-chain volatile acids, *iso*-valeric, *iso*-butyric and 2-methyl butyric, also can serve as primers, giving rise to *iso*- and *ante*-*iso* acyl chains (Massart-Leen *et al.*, 1981; Ha and Lindsay, 1990). Methyl-malonyl-CoA also may be incorporated, substituting for malonyl-CoA, leading to multi-branched acyl products; synthesis of these is terminated with a chain length less than 16 carbons, and the rate of synthesis is one-tenth that for straight-chain products (Smith, 1994). Some branched-chain products, in particular 13-methyl tetradecanoic acid, have been shown to have regulatory effects on cell function (Yang *et al.*, 2000; Parodi, 2003).

Because the acyl transferase releases intermediates, some of the intermediates may escape transfer to the  $\beta$ -ketoacylsynthase, resulting in short-chain fatty acids being incorporated into milk fat. In non-ruminants, this is limited mainly to butyrate, whereas the more relaxed specificity of the acyl transferase in ruminants allows medium-chain acyl-CoA to be released in significant amounts (Hansen and Knudsen, 1980). An increased concentration of malonyl-CoA in the medium increases the relative proportions of longer-chained fatty acids (Knudsen, 1979; Knudsen and Grunnet, 1982). Indeed, this can be inferred from studies in which intestinal glucose supply (Hurtaud *et al.* 2000) or systemic insulin concentrations (Griinari *et al.* 1997) were increased experimentally. Removal of intermediate-chain length acyl-CoA is dependent on the presence of an acceptor, such as albumin (Knudsen and Grunnet, 1982),  $\alpha$ -glycerol-phosphate or diacylglycerol. The incorporation of fatty acids into TAGs is greatly enhanced by the presence of non-limiting concentrations of  $\alpha$ -glycerolphosphate or diacylglycerol (Grunnet and Knudsen, 1981; Hansen *et al.*, 1984b).

Regulation of chain termination reactions in ruminant and non-ruminant mammary glands was discussed in detail by Smith (1994; see also Barber *et al.*, 1997). In non-ruminant mammary tissue, medium-chain fatty acids are generated by a novel chain-terminating enzyme that is not part of the FAS complex, known as thioesterase II to distinguish it from the chain-terminating enzyme (thioesterase I) associated with FAS (Smith, 1994). Thioesterase II, though a 29 kDa protein independent of FAS, appears to function identically to thioesterase I. However, its specificity differs, producing C<sub>8</sub> to C<sub>14</sub> fatty acids.

#### 2.4.4.1. Elongation of C<sub>16</sub> acyl chain

Bishop *et al.* (1969) reported that 4.6% of intravenously-infused methyl [<sup>3</sup>H] palmitate was incorporated into longer-chained FA of milk fat when a cow was fed a low-fat diet, whereas Palmquist *et al.* (1969) reported no significant labelling of C<sub>18</sub> acids in milk fat after intra-mammary infusions of

$1\text{-}^{14}\text{C}$ -acetate. Chain lengthening of long-chain CoA esters occurs in the microsomes, using malonyl-CoA as the acetyl donor (Bernert and Sprecher, 1979). This is not a significant pathway for the supply of  $\text{C}_{18}$  fatty acids for milk fat synthesis.

## 2.5. Stearoyl-CoA Desaturase

Stearoyl-CoA desaturase (SCD; EC 1.14.19.1) is the rate-limiting enzyme for the conversion of saturated to monounsaturated fatty acids. As such, it plays a major role in regulating the unsaturation of membranes and TAG composition, and evidence indicates that SCD plays an important regulatory role in lipid metabolism. SCD-1-deficient mice exhibit increased fatty acid oxidation and reduced lipid synthesis; a significant proportion of the metabolic effects of leptin may result from inhibition of this enzyme (Ntambi and Miyazaki, 2004). Its role is especially important in ruminants, in which the majority of absorbed  $\text{C}_{18}$  fatty acids are in the form of stearate as a result of ruminal biohydrogenation. The activity of SCD in ruminants is high in lactating mammary gland and adipose tissue, and somewhat lower in intestinal tissue. SCD activity is low in non-lactating mammary tissue, being induced by lactation (Kinsella, 1972); though normally inactive in ruminant liver, SCD may be induced in liver and muscle by high-fat diets (Chang *et al.*, 1992). SCD activity was not measurable in rat (Kinsella, 1970) or rabbit (Bickerstaffe and Annison, 1970) mammary tissue; possibly the mRNA is present but its translation is down-regulated by dietary unsaturated fat, as demonstrated in mice (Singh *et al.*, 2004). Whereas SCD is characterized by three isoforms in mice, only one gene is expressed in ruminants and humans (Ward *et al.*, 1998; Ntambi and Miyazaki, 2004). In ruminants, the SCD gene has been localized to bovine and caprine chromosome 26q21, and ovine chromosome 22q21 (Bernard *et al.*, 2001).

SCD is located in the endoplasmic reticulum; its primary substrates are stearoyl-CoA and palmitoyl-CoA, whereas considerably lower activity is observed with myristoyl-CoA as substrate (Bickerstaffe and Annison, 1970). Desaturation of a range of *trans* monoenes occurs also (Mahfouz *et al.*, 1980), yielding *trans*-x, *cis*-9 octadecadienoic acids as products. This is particularly important considering the large amount of *trans* octadecenoic acids that is formed in the rumen by biohydrogenation (Shingfield *et al.*, 2003); among these, the quantitatively most important is desaturation of vaccenic acid (*trans*-11  $\text{C}_{18:1}$ ) to rumenic acid (*cis*-9, *trans*-11  $\text{C}_{18:2}$ ), commonly called conjugated linoleic acid (CLA). Desaturation of *trans*-7  $\text{C}_{18:1}$  to yield *trans*-7, *cis*-9  $\text{C}_{18:2}$  has been reported also (Corl *et al.*, 2002).

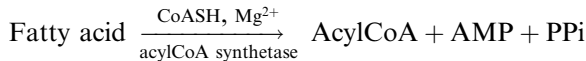
Considerable variation among cows (Kinsella, 1972; Kelsey *et al.*, 2003) and breeds (DePeters *et al.*, 1995) in the extent of stearic acid desaturation and in mRNA expression (Taniguchi *et al.*, 2004) has been observed. SCD mRNA expression is down-regulated by *trans*-10, *cis*-12 C<sub>18:2</sub> (Choi *et al.*, 2000), which is formed in the rumen in small amounts when milk fat-depressing diets are fed (Bauman and Griinari, 2003).

## 2.6. Triacylglycerol Synthesis

Enzymes for triacylglycerol synthesis are associated with the endoplasmic reticulum and the inner and outer mitochondrial membranes (Coleman *et al.*, 2000). Glycerol-3-phosphate required for esterification of fatty acids is generated by glycolysis or by phosphorylation of free glycerol by glycerol kinase, which is active both in ruminant and non-ruminant tissues (McBride and Korn, 1964; Kinsella, 1968; Bickerstaffe and Annison, 1971). Although some free glycerol may be taken up from blood during lipolysis of lipoprotein TAGs, most glycerol-3-phosphate is probably derived from glycolysis (Bickerstaffe and Annison, 1971).

Enzymes and regulation of TAG synthesis have been reviewed in depth by Coleman *et al.* (2000, 2004).

For metabolism to occur, fatty acids must be activated to their acyl CoA esters:



Because the pyrophosphate, PPi, is hydrolyzed rapidly to two inorganic phosphates, Pi, the reaction is strongly in the direction of acyl CoA formation and the energy requirement for activation is the equivalent of two high energy bonds. Acyl CoA synthetase is expressed in several isoforms and is distributed on the cytosolic surfaces of the endoplasmic reticulum, peroxisomal and outer mitochondrial membranes. Activity of this enzyme responds to changes in physiological state, suggesting that it may play a role in regulating the entry of fatty acids into synthetic or oxidative pathways (Coleman *et al.*, 2002).

Acylation of glycerol-3-phosphate is the first committed step in TAG synthesis and the activity of acyl CoA:glycerol-*sn*-3-phosphate acyl transferase (GPAT) is the lowest of the transacylation enzymes in this pathway, also suggesting a regulatory role in TAG synthesis (Coleman *et al.*, 2004). Although microsomal GPAT constitutes the majority of total GPAT activity in most tissues and can be activated and inhibited by phosphorylation and

dephosphorylation, its role in TAG synthesis is uncertain. It has been purified only partially (Coleman *et al.*, 2004). Mitochondrial GPAT, located on the mitochondrial outer membrane, is characterized more thoroughly. Its activity is influenced by nutritional and hormonal changes; transcription is enhanced during adipocyte differentiation, and it responds inversely to fasting and feeding and to phosphorylation and dephosphorylation (Coleman *et al.*, 2004). Cooper and Grigor (1980) reported that acylation at *sn*-1 in rat mammary TAGs favours oleic acid, whereas palmitic acid predominates at *sn*-2. This pattern is true in most species, although in the cow, palmitic acid is found nearly equally at the *sn*-1 and *sn*-2 positions (Christie, 1985; Parodi, 1982; Jensen and Newburg, 1995). The product, 1-acyl-lysophosphatidate, is acylated by acyl CoA:1-acylglycerol-*sn*-3-phosphate acyltransferase. The microsomal fraction from lactating cow mammary gland transfers acyl chains C<sub>8</sub> to C<sub>18</sub> but not C<sub>4</sub> or C<sub>6</sub> to the *sn*-2 position of 1-acyl-lysophosphatidate. The chain length specificity is C<sub>16</sub> > C<sub>14</sub> > C<sub>12</sub> > C<sub>10</sub> > C<sub>8</sub>, similar to the pattern found at the *sn*-2 position of bovine milk fat (Marshall and Knudsen, 1977).

Phosphatidic acid (1,2 diacylglycerol-*sn*-3-phosphate) occupies a central point in lipid biosynthesis. It can be converted to CDP-diacylglycerol, a precursor for the biosynthesis of acidic phospholipids, or desphosphorylated to produce diacylglycerol, the precursor of TAGs, phosphatidylserine, phosphatidylcholine and phosphatidyl ethanolamine (Coleman *et al.*, 2004). In TAG synthesis, desphosphorylation of phosphatidic acid is mediated by phosphatidic acid phosphatase-1, a Mg<sup>2+</sup>-requiring enzyme that is transferred from the cytosol to the endoplasmic reticulum in the presence of fatty acids or acyl-CoA. Activity in liver is stimulated by glucagon, glucocorticoids, cAMP and growth hormone, and inhibited by insulin (Coleman *et al.*, 2004).

Diacylglycerol acyltransferase (*sn*-1,2-diacylglycerol transacylase) esterifies both long-chain and short-chain fatty acids at the *sn*-3 position. Regulation of total activity has been reported in liver and adipose tissues, but little information is available for mammary glands. Because it is up-regulated with increasing abundance of fatty acids, most likely it is in a highly active state in lactating mammary tissue. Mice that lack both copies of the gene for diacylglycerol acyltransferase 1 (DGAT 1) are unable to secrete milk (Smith *et al.*, 2000). This gene was mapped to a region close to the quantitative trait locus on bovine chromosome 14 for variation in milk fat content (Grisart *et al.*, 2001). Sequencing revealed frequency shifts at several positions on the gene between groups of animals with high or low breeding values for milk fat content. The most likely candidate for lower milk fat content was substitution of alanine for lysine in the enzyme (Winter *et al.*, 2002). The wild type allele (DGAT1<sup>K</sup>) exceeds the DGAT1<sup>A</sup> allele by

+0.34 percentage units in fat and +0.08 percentage units in protein, whereas milk and protein yields are reduced (Grisart *et al.*, 2001). A genotyping study of 38 *Bos indicus* and *Bos taurus* breeds from five continents showed that most domesticated dairy breeds have predominantly the DGAT1<sup>A</sup> allele, whereas DGAT1<sup>K</sup> was the major allele (69%) in the Jersey breed (Kaupe *et al.*, 2004).

In nearly all species studied, oleic acid is the fatty acid preferably esterified at the *sn*-3 of TAGs; C<sub>18:3n-3</sub> predominates in the koala and horse. In ruminant species, butyric or caproic acid is esterified exclusively at *sn*-3 and slightly exceeds the molar percentage of oleic acid esterified at that position (Parodi, 1982). Lin *et al.* (1976) examined the acyl specificity for TAG synthesis in lactating rat mammary glands, particularly with respect to the unique positioning of short-chain and medium-chain length fatty acids at *sn*-3. Whereas the acyl transferases for *sn*-1 and -2 showed high specificity for long-chain fatty acids (C<sub>16</sub> and C<sub>18</sub>), no such specificity was observed for acylation of diacylglycerol. They concluded that a lack of acyl chain specificity for this position caused accumulation of shorter acyl chains at *sn*-3.

Knudsen and colleagues (Hansen and Knudsen, 1980; Marshall and Knudsen, 1980; Grunnet and Knudsen, 1981; Hansen *et al.*, 1984a,b) examined in detail TAG synthesis and the specific incorporation of the short-chain and medium-chain fatty acids into TAGs in goat mammary glands. The synthesis of medium-chain fatty acids is dependent on simultaneous removal of the acyl-CoA produced by FAS, whereas long-chain fatty acids are released as free acids by thioesterase I. As described above, long-chain fatty acids are esterified preferentially at positions *sn*-1 and -2; the ready supply of diacylglycerols allows short-chain and medium-chain fatty acids to be esterified rapidly at *sn*-3, facilitating their removal from FAS. These studies have shown the importance of the rate of activation of fatty acids in the mammary gland relative to the rate of *de novo* synthesis and the supply of  $\alpha$ -glycerol phosphate for milk fat synthesis. If the supply of exogenous fatty acids were low, the relative concentration of short-chain and medium-chain fatty acids could be increased, even though total synthesis (yield) was not increased. Conversely, with an increasing supply of exogenous long-chain fatty acids, *de novo* synthesis may be reduced because they compete for the diacylglycerol transferase. Limiting the supply of  $\alpha$ -glycerol phosphate similarly would limit diacylglycerol supply, also causing *de novo* synthesis to be reduced. These observations demonstrate also that regulation of the relative proportions of short-chain, medium-chain and long-chain fatty acids is much more complex than simply by regulation of ACC. Finally, these authors concluded that a simple explanation for the unique occurrence of short-chain and medium-chain fatty acids in milk fat could be that

$\alpha$ -glycerol phosphate is rate-limiting for TAG synthesis in all ruminant tissues except mammary glands (Hansen *et al.*, 1984b).

Interestingly, for maximum TAG synthesis, a preference was shown for palmitoyl-CoA as substrate for the initial acylation of glycerol-3-phosphate (Kinsella and Gross, 1973), apparently accelerating the rate of supply of substrate as acceptor for *de novo*-synthesized fatty acids, whereas oleic acid reduced total *de novo* synthesis, apparently by competing with butyryl CoA for the esterification of diacylglycerol (Hansen and Knudsen, 1987).

### 2.6.1 Fatty Acid Esterification by the Monoacylglycerol Pathway

Evidence for the esterification of fatty acids *via* the 2-monoacylglycerol pathway was shown for mammary glands of guinea pigs by McBride and Korn (1964) and of goats by Bickerstaffe and Annison (1971). However, even though this is an important pathway for the esterification of fatty acids in the intestine, later studies have established that this pathway is not functional in mammary tissue (Christie, 1985; Hansen *et al.*, 1986).

## 2.7. Synthesis of Complex Lipids

The complex lipids in milk fat are comprised of the phosphoglycerides, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidylinositol and plasmalogens. Also, the non-glyceride phospholipid, sphingomyelin, occurs in important amounts (Jensen, 2002). Bitman and Wood (1990) described the distribution of phospholipid classes in bovine milk and their fatty acid composition. The phospholipids comprise about 1% and cholesterol 0.4–0.5% of the total milk fat. These occur almost completely in the milk fat globule membrane.

The fatty acid composition of phosphoglycerides and sphingolipids, and their incorporation into membranes with cholesterol, are coordinately regulated processes in order to maintain membrane integrity and function. Coordination is mediated by sterol regulatory genes and sterol receptor element-binding proteins (SREBPs; Ridgway *et al.*, 1999). Glycosphingolipids are highly enriched in the outer leaflet of the apical plasma membrane domain of polarized epithelial cells. They are a key component of membrane lipid rafts and thus are involved in exocytosis of milk components. Their metabolites act as second messengers in regulating the expression of cell receptors (Hoekstra *et al.*, 2003). Sphingolipids are enriched in the milk fat globule membrane, and as a food component have been implicated in cell regulation and anti-cancer activity (Vesper *et al.*, 1999).



### 2.7.1. Synthesis of Phospholipids

Synthesis of phosphatidylcholine and phosphatidylethanolamine begins with activation of choline (or ethanolamine) with ATP *via* choline kinase to yield phosphocholine (phosphoethanolamine) + ADP; the activated base is transferred *via* CTP and phosphocholine cytidyl transferase to form CDP-choline (CDP-ethanolamine) and PPI. The base is then transferred to the *sn*-3 of diacylglycerol *via* phosphocholine diacylglycerol transferase to yield phosphatidylcholine (phosphatidylethanolamine) + CMP. The cytidyl transferase is believed to be the rate-limiting or regulatory step in the pathway. Phosphatidylserine is formed by a direct transfer and substitution of serine for ethanolamine in phosphatidylethanolamine. Phosphatidyl serine can be decarboxylated to form phosphatidylethanolamine.

Phosphatidylcholine is preferentially synthesized in lactating mammary tissue (Kinsella, 1973), possibly regulated by the differential activities of choline kinase and ethanolamine kinase. Choline kinase has a lower  $K_M$  and a higher  $V_{max}$  with its substrate than does ethanolamine kinase. Also, choline kinase is inhibited slightly by ethanolamine, whereas choline is a potent competitive inhibitor of ethanolamine kinase. Thus, the intracellular concentration of choline probably regulates the synthesis of these two phosphoglycerides (Infante and Kinsella, 1976).

The *sn*-1 and *sn*-2 acyl groups of phosphoglycerides differ among phospholipid classes (Bitman and Wood, 1990) and from the patterns of TAGs. However, the CDP choline:diacylglyceride transferase has little specificity for the molecular species of fatty acids in the diacylglycerol moiety. Therefore, molecular remodelling *via* specific phospholipases, followed by reacylation *via* specific acyl CoA transferases, may account for the unique fatty acid profiles of the various phosphoglycerols. Kinsella and Infante (1974) showed that the acyl CoA:1-acyl-*sn*-glycerol-3-phosphoryl choline acyl transferase preferentially esterified oleic acid at the *sn*-2 position, and that the predominant molecular species of phosphatidylcholine in mammary cells is diunsaturated.

Synthesis of phosphatidylinositol follows a slightly different pathway from the other phosphoglycerides. Phosphatidate is activated by CTP-phosphatidate cytidyl transferase to form CDP-diacylglycerol. Free inositol is then incorporated by CDP-diacylglycerol inositol transferase, with the release of CMP. Phosphatidylinositol usually constitutes less than 5% of total milk phospholipids (Bitman and Wood, 1990).

The glycerol ethers and plasmalogens are a unique class of phosphoglycerides that contain an ether-linked chain at the *sn*-1 position. These are formed by the incorporation of acyl CoA at the *sn*-1 position of dihydroxyacetone phosphate. The acyl group is then exchanged for a long-chain

alcohol to yield 1-alkyl dihydroxyacetone phosphate, which is reduced to 1-alkylglycerophosphate. This product is acylated to 1-alkyl, 2-acylglycerophosphate which then may be incorporated into various phosphoglycerols or acylated to form a neutral alkylglyceride. The alkyl residue of the phosphatidylethanolamine typically is desaturated at the 1,2 position, involving O<sub>2</sub> and NADPH to yield 1-alkenyl, 2-acyl-glycerol- 3-phosphatidylethanolamine (plasmalogen), found at a high concentration in the mitochondrial membrane. The alkylglycerols occur at very low concentrations in milk fat (~0.01% of the fat) but are 5- to 20-fold higher in the fat of colostrum (Ahrné *et al.*, 1980).

### 2.7.2. Sphingolipids

The sphingolipids are very complex molecules, occurring in numerous molecular forms (Jensen, 2002). The basic structure, sphingomyelin, consists of a complex unsaturated amino alcohol (sphingosine) ether-linked to phosphocholine and to an acyl chain by an amide bond. It contains no glycerol (Vesper *et al.*, 1999). In sphingomyelin synthesis, sphingosine is acylated by acyl-CoA to form a ceramide; phosphocholine is then transferred to a ceramide from phosphatidylcholine or from CDP-choline to yield sphingomyelin. Sphingomyelin constitutes about 25% of the total phospholipids in dairy products, which are the most abundant source of sphingolipids in the human diet. The sphingolipids are highly bioactive, and as such, are considered to be functional in foods (Vesper *et al.*, 1999; see Chapter 13). The neutral sphingolipids contain no phosphocholine, and therefore are not phospholipids. These are formed by addition of various sugar residues to ceramide. These can be quite complex (Jensen, 2002). Sphingolipids often contain very long-chain (C<sub>24</sub>) fatty acids, especially those found in the brain.

### 2.7.3 Cholesterol

Cholesterol makes up 95% of the total milk sterols and, because it is associated with the milk fat globule membrane, its content is highly correlated with the total fat content (Jensen, 2002). Only about 10% of the cholesterol in milk is esterified.

Cholesterol is taken up rapidly from plasma lipoproteins, and is synthesized also by the mammary glands (Clarenburg and Chaikoff, 1966). Quantitative contributions of each, and whether all lipoproteins or only chylomicra and VLDL contribute cholesterol, are less certain (Clarenburg and Chaikoff, 1966; Raphael *et al.*, 1975a,b). From steady-state estimates of labelled cholesterol in lipoproteins, plasma contributed 83% of milk cholesterol in lactating rats (Clarenburg and Chaikoff, 1966) and 45–50% in a lactating goat (Raphael *et al.*, 1975b). The mechanism of cholesterol uptake

also is not defined; however, an active cholesterol esterase is present in mammary tissue (Ross and Rowe, 1984; Shand and West, 1991; Small *et al.*, 1991), suggesting that both free and esterified lipoprotein cholesterol may be taken up and utilized.

## 2.8. Physiological Factors That Influence Milk Fat Composition

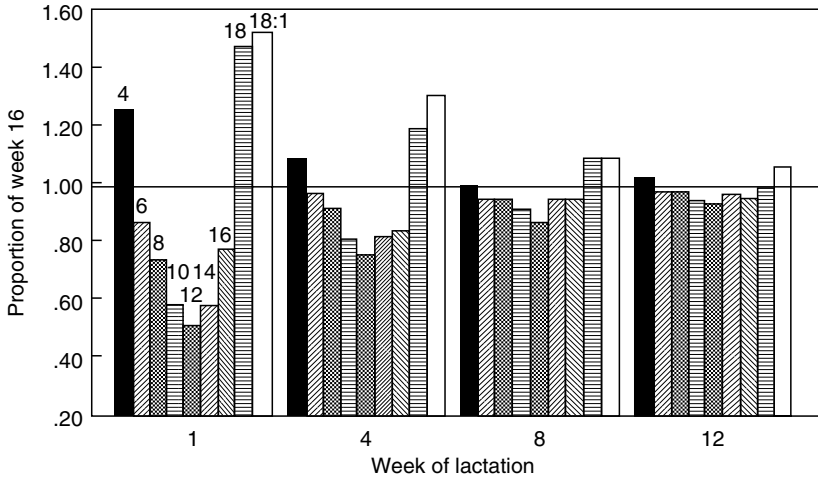
### 2.8.1. Genetics

In addition to the well-characterized differences among and within breeds of dairy cattle in milk fat content, differences occur also in fatty acid composition. Karijord *et al.* (1982) reported a positive genetic correlation between the proportions of short-chain and medium-chain ( $C_6$ – $C_{16}$ ) fatty acids and the amount of milk fat, whereas the correlation was negative for proportions of all  $C_{18}$  acids. They noted an especially high negative relationship between milk fat content and the proportion of  $C_{18:1}$ ; however, they did not separate the isomers of  $C_{18:1}$  and thus it is possible that the effect was caused by the presence of *trans*  $C_{18:1}$  isomers when  $C_{18:1}$  was in higher amounts (Bauman and Griinari, 2001). The positive correlation observed between short-chain fatty acids and milk fat level is consistent with data of Stull and Brown (1964) and Beaulieu and Palmquist (1995). Numerous studies indicate that SCD activity, and thus 18:1/18:0 ratio, varies among breeds (Beaulieu and Palmquist, 1995; DePeters *et al.*, 1995) and within a breed (Kelsey *et al.*, 2003).

### 2.8.2. Stage of Lactation

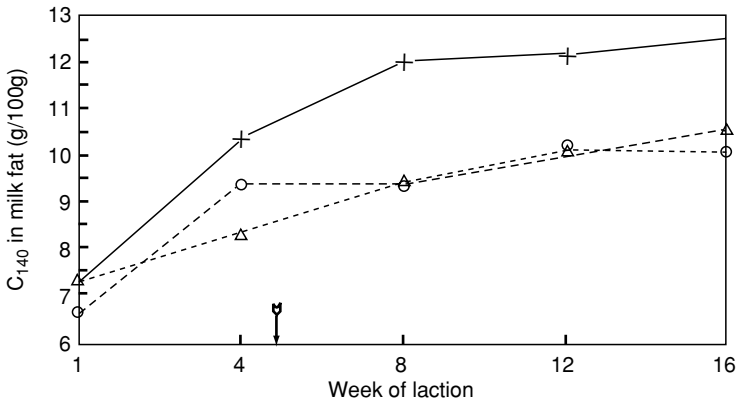
At parturition, the proportions of  $C_{12}$  to  $C_{16}$  fatty acids are relatively high in bovine colostrum. Proportions of short-chain fatty acids and stearic and oleic acids increase rapidly as the mobilization of adipose tissue commences, becoming relatively stable by one week after parturition (Laakso *et al.*, 1996).

Proportions and yields of fatty acids synthesized *de novo* in the mammary gland ( $C_6$  to  $C_{16}$ ) increase during the early weeks of lactation, with compensating decreases in the proportions of all  $C_{18}$  fatty acids (Figure 2.6; Karijord *et al.*, 1982; Lynch *et al.*, 1992). The proportion of butyric acid is high at parturition and does not increase with advancing lactation (Lynch *et al.*, 1992), consistent with its synthesis being independent of malonyl-CoA. As adipose tissue mobilization declines, due to increasingly positive energy balance and depletion of stored tissue, the proportions of short-chain and medium-chain fatty acids in milk fat increase. The time required for



**Figure 2.6.** Proportions of individual fatty acids in milk fat at 1, 4, 8 and 12 weeks of lactation relative to their proportions at 16 weeks (from Palmquist *et al.*, 1993, *J. Dairy Sci.* **76**, 1753–1771).

stabilization of milk fatty acid composition depends on the amount of stored fat, milk fat yield, energy balance and the quantity of fat in the diet. The effect on the proportion of  $C_{14:0}$  in milk fat of supplementing dietary fat to lactating cows at parturition or delayed to the sixth week of lactation is shown in Figure 2.7. Without fat supplementation, the proportion of  $C_{14:0}$



**Figure 2.7.** Changes in the proportion of  $C_{14:0}$  in milk fat of cows fed a control diet (—+—) or added fat beginning at parturition (—△—) or 6th week of lactation (—○—). (From Palmquist *et al.*, 1993, *J. Dairy Sci.* **76**, 1753–1771).

increased until 8 weeks of lactation; with fat supplementation, the maximum proportion of C<sub>14:0</sub> was lower.

## 2.9. Effects of Dietary Fat on the Composition of Milk Fat

### 2.9.1. Effects of Low-fat Diets

Very low-fat diets reduce milk and fat yields (Maynard and McCay, 1929; Banks *et al.*, 1976) and greatly reduce the proportions and yields of the C<sub>18</sub> fatty acids, with the proportions of C<sub>16:0</sub> approaching 50% of the total fat yield (Virtanen, 1966; Banks *et al.*, 1976).

Increasing the C<sub>18</sub> content of low-fat diets resulted in a linear increase of C<sub>18</sub> fatty acids in the milk fat:

$$y = 75 + 0.54x$$

where  $y$  = total C<sub>18</sub> fatty acids in milk fat (g/d) and  $x$  = total C<sub>18</sub> fatty acid intake (g/d). Thus, dietary C<sub>18</sub> fatty acids were transferred to milk fat with 54% efficiency (Banks *et al.*, 1976). This is consistent with other estimates of a maximum transfer of 60%, assuming 80% digestibility of dietary fat (Palmquist, 1991) and 75% uptake of absorbed lipoprotein TAGs by the mammary glands (Palmquist and Mattos, 1978). Banks *et al.* (1976) also estimated the transfer of C<sub>16</sub> from diet to milk fat to be 93%; this exceptionally high value could be attributed only to nearly complete uptake of absorbed TAGs by the mammary tissue; however, estimates of C<sub>16</sub> transfer are heavily confounded by effects of changing dietary fat intake on *de novo* synthesis of C<sub>16</sub>.

### 2.9.2. Effects of Specific Fatty Acids

Incorporation of dietary unsaturated fat into milk fat by ruminants is low because of the efficient ruminal biohydrogenation process (Jenkins, 1993). Nevertheless, dietary fatty acids have profound effects on milk fat composition that have led to a prodigious amount of literature in the past 20 years (for reviews see Sutton, 1989; Grummer, 1991; Palmquist *et al.*, 1993; Kennelly, 1996; Mansbridge and Blake, 1997; Chilliard *et al.*, 2000, 2001).

Acyl chain length (C<sub>16</sub> vs C<sub>18</sub>) influences the proportions of these in milk fat; the effects of C<sub>16</sub> are more subtle because of compensation by reduced *de novo* synthesis of C<sub>16:0</sub> when long-chain fatty acids are supplemented in the diet. Palmitic acid was increased from 45% of milk fatty acids to 53% when a high (68%) C<sub>16:0</sub> supplement was added to a low-fat diet.

Similarly, supplementing soy oil (90% C<sub>18</sub>) increased the total C<sub>18</sub> of milk fat from 25% to 60% of milk fatty acids. Yields of C<sub>6</sub> to C<sub>14</sub> were reduced by both supplements, whereas the yield of C<sub>16:0</sub> was increased by palm oil and reduced by soya oil (Banks *et al.*, 1976); these effects are typical (Noble *et al.*, 1969). Similarly, supplementing increasing amounts of coconut oil (high in C<sub>12:0</sub> and C<sub>14:0</sub>) increased the proportions of these in milk fat and reduced the proportions and yields of short-chain fatty acids and C<sub>16:0</sub> (Storry *et al.*, 1971).

Oleic acid was increased to 48% of total milk fatty acids by feeding oleamide as a rumen-protected source of oleic acid (Jenkins, 1998). The response was nearly linear up to 5% of supplement in the diet dry matter. Proportions of all *de novo*-synthesized milk fatty acids, except butyric, were reduced (Jenkins, 1999). LaCount *et al.* (1994) abomasally infused fatty acids from canola or high oleic acid sunflower oil into lactating cows. The transfer of oleic acid to milk fat was linear (slope = 0.541; 0–350 g infused/day); the proportion of oleic acid in milk fat increased and proportions of all *de novo*-synthesized fatty acids, except C<sub>4</sub> and C<sub>6</sub> decreased. The proportion of C<sub>18:0</sub> also was unchanged. Linoleic acid from canola also was transferred linearly (slope = 0.527; 0–90 g infused/day). These transfers from the intestine are nearly identical to that reported by Banks *et al.* (1976). Hagemester *et al.* (1991) reported 42 to 57% transfer of abomasally-infused linolenic acid to milk fat.

Mammary uptake of individual fatty acids from plasma was explored by Enjalbert *et al.* (1998). Uptakes of palmitic, stearic and oleic acids were similar and nearly linear in the range of 400 to 750  $\mu$ molar in the plasma. As uptake increased, mammary balance (milk content minus uptake) of butyric acid increased linearly and the mean fatty acid chain length of synthesized fatty acids decreased linearly. Desaturation of stearic acid increased linearly ( $y = 0.52x$ ,  $r^2 = 0.75$ ,  $P < 0.001$ ) as stearic acid uptake increased, and decreased linearly as mammary uptake of *trans* C<sub>18:1</sub> increased ( $y = 61.2 - 4.03x$ ,  $r^2 = 0.31$ ,  $P < 0.02$ ). Decreasing mean chain length of synthesized fatty acids and increasing desaturation with increasing long-chain fatty acid uptake were interpreted as compensating responses to maintain the fluidity of milk fat at body temperature. Maintenance of milk fat fluidity has been suggested as a basic physiological requirement in the regulation of milk fat synthesis (Timmen and Patton, 1988). An interesting exception to this is the report of Emanuelson *et al.* (1991) who fed heat-treated rapeseed to cows in late lactation. The stearic acid content of the milk fat was 28.8% of the total weight of fatty acids and the milk fat coalesced into floating butter globules immediately upon milking. The levels of palmitic (21.6%) and oleic (28.8%) acids were within normal ranges, but the proportions of short-chain and medium-chain fatty acids were rather low. The authors were unable to repeat the phenomenon.

### 2.9.3. Feeding for Specific Milk Fatty Acid Profiles

A very large body of literature focused on feeding effects on the composition of milk fat has been published in recent years, driven by the increasing public interest in, and concern for, the role of fat in the human diet. Most of the feeding studies have involved manipulation of the type and amount of fat in the ration of lactating cows as the experimental approach. In many studies, the role of ruminal biohydrogenation or its manipulation has been a major focus. Therefore, the effects, but not the regulation, of ruminal biohydrogenation will be addressed. Issues addressed in these studies have included: (1) increased unsaturation/polyunsaturation; (2) reduced saturation; (3) increased n-3/fish oil fatty acids; and (4) increased CLA content of milk fat.

The earliest efforts to modify the composition of milk fat used an insoluble formaldehyde-crosslinked protein to encapsulate unsaturated vegetable oils. In numerous studies using this approach, linoleic acid was increased to as high as 35%, w/w, of the total milk fatty acids (reviewed by McDonald and Scott, 1977). Bitman *et al.* (1973) fed increasing amounts of safflower oil encapsulated in formaldehyde-treated casein. The content of milk fat increased linearly from 3.5 to 4.6% as supplemental protected oil was increased from 0 to 1320 g/day per cow. The concentration of linoleic acid increased to 33% of total milk fatty acids, with a compensating decrease in C<sub>16:0</sub> and a smaller decrease in C<sub>14:0</sub>. The concentration of milk fat decreased to lower than pretreatment levels when the supplement was removed, a common observation (Pan *et al.*, 1972). A typical milk fatty acid profile from cows fed a protected sunflower/soybean (70/30) supplement is shown in Table 2.1.

Though feeding protected polyunsaturated fats has been instrumental in developing an understanding of the regulation of milk fat synthesis, it has not found practical application. In addition to increasing the cost of feeding, it has been difficult to assure product quality (consistency of protection); government and the public have been reluctant to approve formaldehyde as a component of feed ingredients because some amino acids may be transformed to potential carcinogens; and, importantly, highly polyunsaturated milk fat has very poor oxidative stability and its physical properties are not well suited for processed products (McDonald and Scott, 1977). Treatment of feedstuffs with formaldehyde is now more widely accepted, and attention has turned to using this method to protect high oleate oilseeds. Ashes *et al.* (1992) fed 0.52 kg/d of protected canola seeds to lactating cows. Milk fat percentage and yield were increased without changing milk yield. The proportions of C<sub>14:0</sub> and C<sub>16:0</sub> were reduced by 20 and 25%, respectively, whereas proportions of C<sub>18:0</sub>, C<sub>18:1</sub>, C<sub>18:2</sub> and C<sub>18:3</sub> were increased by 30,

**Table 2.1.** Fatty acid composition (weight % of total fatty acids) of milk fat from cows fed a standard diet or supplemented with protected oilseeds

Fatty acid	Control <sup>a</sup>	Sunflower/soybean <sup>a</sup>	Canola <sup>b</sup>
C <sub>4</sub>	3.2	2.8	3.2
C <sub>6</sub>	2.2	1.4	2.4
C <sub>8</sub>	1.1	0.8	1.9
C <sub>10:0</sub>	3.6	1.7	3.2
C <sub>12:0</sub>	3.9	1.7	3.6
C <sub>14:0</sub>	11.4	5.9	9.5
C <sub>15:0</sub>	2.1	0.9	—
C <sub>16:0</sub>	25.9	15.2	19.9
C <sub>16:1</sub>	2.7	0.5	3.3
C <sub>18:0</sub>	10.9	14.0	9.2
C <sub>18:1</sub>	28.6	37.6	29.2
C <sub>18:2</sub>	3.0	16.6	4.9
C <sub>18:3</sub>	1.0	0.9	2.6

<sup>a</sup> Calculated from Barbano and Sherbon (1980). Formaldehyde-protected sunflower/soybean (70/30); 1250 g oil/day.

<sup>b</sup> Ashes *et al.* (1992). Formaldehyde-protected canola; 520 g oil/day.

22, 122 and 62%, respectively. Relatively small changes were observed for short-chain and medium-chain fatty acids.

Several other procedures have been developed to protect unsaturated fatty acids from ruminal biohydrogenation. Of these, only the amide derivative has extensive research documentation (Jenkins, 1998, 1999), but has not been applied commercially. Often, calcium soaps of palm oil or canola fatty acids are referred to as “protected.” These are not protected from ruminal biohydrogenation (Table 2.2), but rather are ruminally inert with regard to their effects on the rumen microbial population.

#### 2.9.4. Supplementation with Oilseeds and Commercial Fats

Numerous types of fat are available commercially as supplemental energy sources. Many are products of the rendering industry and include tallow, lard (pork) and poultry fats. Recycled cooking oils from the restaurant industry are used also, usually as a blend with animal fats or oils from the food oil refining industry. Generally, unsaturated oils are undesirable as energy supplements for lactating cows (Palmquist and Jenkins, 1980; Jenkins, 1993). Tallow has long been a staple energy source for dairy diets; however, recent research (Onetti *et al.*, 2002) suggests that supplemental tallow may reduce milk fat percent modestly, which was attributed to increased concentrations of *trans*-10 C<sub>18:1</sub> in the milk fat (see Section 2.9.5



**Table 2.2.** Fatty acid composition of milk fat from cows fed various fat supplements

	Calcium salts						Whole roasted soybeans	Tallow	Fish oil
	None	Palm oil	Palm oil	Canola	Soy	Linseed			
% of feed DM	–	3.0	1.0	5.0	4.9	4.3	4.6	5.4	2.0
Reference	a	a	b	c	c	c	d	d	e
Fatty acid	weight % of reported fatty acids								
C <sub>4:0</sub>	3.25	3.15	3.43	4.52	4.65	4.92	3.65	2.65	3.88
C <sub>6:0</sub>	2.28	1.92	2.23	2.33	2.39	2.89	2.34	1.59	2.66
C <sub>8:0</sub>	1.57	1.24	1.27	1.16	1.14	1.52	1.31	0.83	1.30
C <sub>10:0</sub>	3.36	2.31	2.88	2.59	2.34	3.09	2.77	1.75	2.83
C <sub>12:0</sub>	4.11	2.92	3.19	2.53	2.21	2.71	3.03	2.23	3.16
C <sub>14:0</sub>	13.13	10.35	10.09	9.39	8.10	9.20	9.75	10.70	11.40
C <sub>14:1c-9</sub>	0.95	0.74	–	1.10	0.74	0.71	1.05	1.73	0.77
C <sub>15:0</sub>	1.10	0.82	–	1.06	0.84	0.96	–	–	0.98
C <sub>16:0</sub>	32.58	34.75	30.25	19.26	19.25	19.09	25.44	31.68	27.56
C <sub>16:1c-9</sub>	1.83	2.18	1.59	1.20	0.90	0.84	1.33	3.08	1.40
C <sub>18:0</sub>	10.74	10.50	10.74	15.04	14.76	14.99	12.79	9.40	8.11
C <sub>18:1c-9</sub>	20.23	24.60	23.80	26.95	25.99	22.92	21.88	23.73	15.08
C <sub>18:1r-11</sub>	–	–	–	8.42	12.59	10.18	3.95	4.96	2.34
C <sub>18:2</sub>	2.70	2.76	4.09	2.32	2.45	3.30	5.27	2.36	2.20
C <sub>18:3</sub>	1.28	1.13	0.47	0.20	0.19	0.31	1.00	0.43	0.85
C <sub>18:2c-9, t-11</sub>	–	–	0.49	–	–	–	1.03	0.81	0.88
C <sub>18:2r-10, c-12</sub>	–	–	0.03	–	–	–	–	–	0.04
C <sub>20:5 n-3</sub>	–	–	–	–	–	–	–	–	0.24
C <sub>22:5 n-3</sub>	–	–	–	–	–	–	–	–	0.28
C <sub>22:6 n-3</sub>	–	–	–	–	–	–	–	–	0.26

<sup>a</sup> Schauff and Clark (1992)  
<sup>b</sup> Giesy *et al.* (2002)  
<sup>c</sup> Chouinard *et al.* (1998)  
<sup>d</sup> Morales *et al.* (2000)  
<sup>e</sup> AbuGhazaleh *et al.* (2002)

milk fat depression). Increasing the proportion of corn silage in dietary forage (reducing alfalfa silage) also increased the content of *trans*-10 C<sub>18:1</sub> and lowered milk fat percent (Onetti *et al.*, 2002) (Table 2.3).

Calcium soaps of palm oil fatty acids are used widely as an energy supplement. With a content of 45 to 50% C<sub>16:0</sub>, these calcium soaps increase palmitic acid in milk fat compared with oilseeds (Table 2.2). Whole oilseeds (cottonseed, canola, soybeans) also are used widely as energy supplements in dairy diets. All except cottonseed oil (25% C<sub>16:0</sub>) contain predominantly C<sub>18</sub> fatty acids. When fed whole or crushed, the oil tends to be released slowly,

**Table 2.3.** Least square means for fatty acid composition of milk fat when decreasing proportions of corn silage were fed without (0%) or with (2%) tallow<sup>a</sup>

Corn silage <sup>c</sup>	0% Tallow			2% Tallow			Significance (P) <sup>b</sup>	
	50%	37.5%	25%	50%	37.5%	25%	F	L
Fatty Acid	(g/100 g of fatty acids)							
C <sub>4:0</sub> to C <sub>14:0</sub>	25.3	24.6	24.7	21.4	21.1	21.4	0.001	NS
C <sub>16:0</sub>	28.9	29.1	29.1	28.1	27.9	27.8	0.01	NS
C <sub>18:0</sub>	8.1	8.7	8.7	8.2	9.1	9.2	NS	0.01
C <sub>18:1</sub>	23.2	23.3	23.0	26.3	26.1	25.5	0.001	NS
C <sub>18:1</sub> isomers								
<i>trans</i> -6/8	0.37	0.39	0.37	0.56	0.54	0.50	0.001	NS
<i>trans</i> -9	0.51	0.56	0.49	0.72	0.65	0.71	0.001	NS
<i>trans</i> -10	1.3	1.0	0.9	2.2	1.8	1.4	0.001	0.001
<i>trans</i> -11	1.1	1.1	1.0	0.94	1.1	1.0	NS	NS
<i>trans</i> -12	0.41	0.41	0.42	0.42	0.50	0.52	0.01	0.04
<i>trans</i> -16	0.06	0.05	0.05	0.03	0.04	0.05	NS	NS
<i>cis</i> -9	17.8	18.2	17.8	20.0	19.8	19.4	0.001	NS
<i>cis</i> -11	0.87	0.92	0.93	0.94	0.94	0.95	0.04	NS
<i>cis</i> -12	0.41	0.44	0.52	0.22	0.33	0.44	0.001	0.001
C <sub>18:2e9t11</sub>	0.60	0.57	0.58	0.61	0.63	0.62	0.06	NS
C <sub>18:2t10c12</sub>	0.01	0.01	0.02	0.01	0.02	0.02	NS	NS
C <sub>18:2</sub>	4.5	4.5	4.5	4.2	4.2	4.5	NS	NS
C <sub>18:3</sub>	0.33	0.40	0.50	0.28	0.39	0.45	0.01	0.001
Other	8.8	8.2	8.4	10.3	10.1	9.5	0.01	NS

<sup>a</sup> Onetti *et al.* (2002).

<sup>b</sup> F = Main effect of fat; L = linear effect of forage.

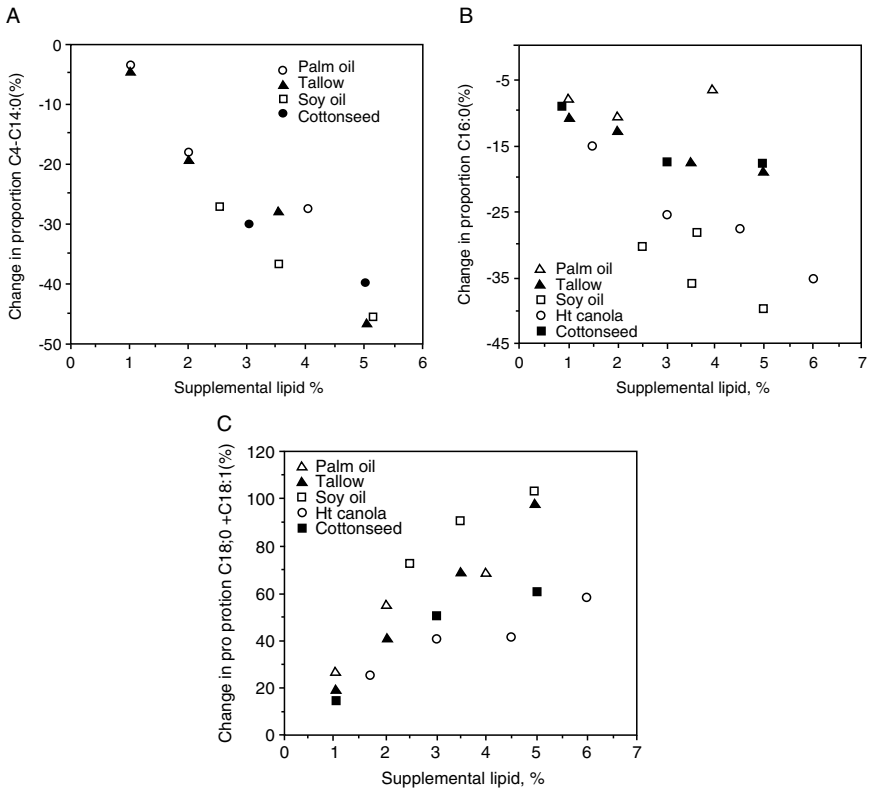
<sup>c</sup> Diets: 50% forage and 50% concentrate (DM). Forages were: (1) 50% of diet DM as corn silage, (2) 37.5% corn silage and 12.5% alfalfa silage, and (3) 25% corn silage and 25% alfalfa silage.

with nearly complete biohydrogenation of the unsaturated fatty acids. All increase the proportion of C<sub>18:1</sub> in milk fat and reduce the proportions of C<sub>6</sub> to C<sub>16</sub> fatty acids, but especially C<sub>14:0</sub> and C<sub>16:0</sub>. C<sub>18:0</sub> in milk fat is often quite high when canola (Chouinard *et al.*, 1997; Ward *et al.*, 2002) or whole roasted soybeans (Morales *et al.*, 2000; Timmons *et al.*, 2001) are fed, apparently due to nearly complete ruminal biohydrogenation. Uniquely, feeding whole soybeans also increases the proportions of C<sub>18:2</sub> and C<sub>18:3</sub> in milk fat; apparently, some portion of the soybeans pass from the rumen undegraded, causing significant increases in the levels of those fatty acids in milk fat (Morales *et al.*, 2000), and thereby increasing the susceptibility of the milk fat to oxidative rancidity (Timmons *et al.*, 2001).

Increasing public concern related to the composition of dietary fats has increased interest in the n-3 fatty acid content of milk fat. The content of linolenic acid in milk fat from cows grazing pasture may be more than

double that from cows fed in stalls or feedlots (Dhiman *et al.*, 1999; Chilliard *et al.*, 2001) owing to the high content and rumen escape of  $C_{18:3}$  n-3 from forages. Others have investigated the transfer of the long-chain ( $C_{20}$  and  $C_{22}$ ) n-3 fatty acids of fish oil to milk fat. Adding 2% menhaden fish oil to the diet of lactating cows increased the content of  $C_{20:5}$  n-3 and  $C_{22:6}$  n-3 (EPA and DHA, respectively) from 0.05 and 0.04% in the control to 0.24 and 0.26% in milk fat of supplemented cows (AbuGhazaleh *et al.*, 2002). Feeding fish oil fatty acids in combination with vegetable oils synergistically increases the CLA content of milk fat (see Chapter 3).

Changes in the proportions of fatty acids in milk fat by supplementation of various oils and oilseeds are summarized in Figure 2.8 (Grummer, 1991). Hermansen (1995) developed a set of regression equations to predict the composition of milk fat based on the proportions of lauric, myristic and



**Figure 2.8.** Changes in proportions of milk fatty acids relative to control treatments with increasing supplementation of fats or oilseeds A.  $C_4$  to  $C_{14:0}$ ; B.  $C_{16:0}$ ; C.  $C_{18:0} + C_{18:1}$ . (From Grummer, 1991, *J. Dairy Sci.* **74**, 3244–3257).

palmitic acids in the dietary fat and on total dietary fat intake. The model effectively predicted milk fatty acid profile with respect to lauric, myristic, palmitic and oleic acids and total C<sub>18</sub> fatty acids across experiments. Predictions were less precise for short-chain acids, stearic acid and polyunsaturated fatty acids.

### 2.9.5. Low Milk Fat Syndrome

Low milk fat syndrome (milk fat depression) has been investigated intensively for more than 40 years. Early investigators pursued the link between changes in the ruminal acetate:propionate ratio and the % fat in milk (van Soest, 1963). A glucogenic response, whereby increased production of ruminal propionate would increase blood glucose and insulin concentrations, with decreased fatty acid release from adipose tissue, was proposed by McClymont and Vallance (1962), and developed further by van Soest (1963). Whereas intravenous infusion of glucose or glycerol (Vallance and McClymont, 1959), or duodenal infusion of glucose (Hurtaud *et al.*, 2000) have been shown to reduce milk fat percentage; these increase the relative proportion of *de novo*-synthesised fatty acids in milk fat (Hurtaud *et al.*, 2000), contrary to the consistent decrease in these in classical low milk fat syndrome (Bauman and Griinari, 2001). Thus, it became apparent that other aspects of lipid metabolism were involved (Davis and Brown, 1970). Recent research has suggested that the low milk fat syndrome is mediated by *trans*-10 C<sub>18:1</sub> (Griinari *et al.*, 1998) or by *trans*-10, *cis*-12 C<sub>18:2</sub> (Baumgard *et al.*, 2000), or both (Bauman and Griinari, 2001, 2003), that are products of changes in ruminal biohydrogenation in the presence of unsaturated fatty acids and a low ruminal pH (Griinari *et al.*, 1998). This research has not excluded the possibility that the actual effector(s) may be as yet unidentified fatty acid isomer(s) that are highly correlated with *trans*-10 C<sub>18:1</sub> or *trans*-10, *cis*-12 C<sub>18:2</sub>, or both (Bauman and Griinari, 2003). Depressed milk fat synthesis is associated with reduced enzyme activity or mRNA abundance, or both, for acetyl CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase, lipoprotein lipase and glycerol phosphate acyl transferase (Piperova *et al.*, 2000; Ahnadi *et al.*, 2002; Peterson *et al.*, 2003). Recent advances in regulation of the low milk fat syndrome have been reviewed (Bauman and Griinari, 2001, 2003).

### 2.10. Milk Fat Composition and Quality

The uniqueness of milk fat is not limited to its fatty acid profile. If the 400 fatty acids of milk fat were distributed randomly in the milk fat TAGs, the total theoretical number of glycerides would be  $64 \times 10^6$  (Jensen, 2002);

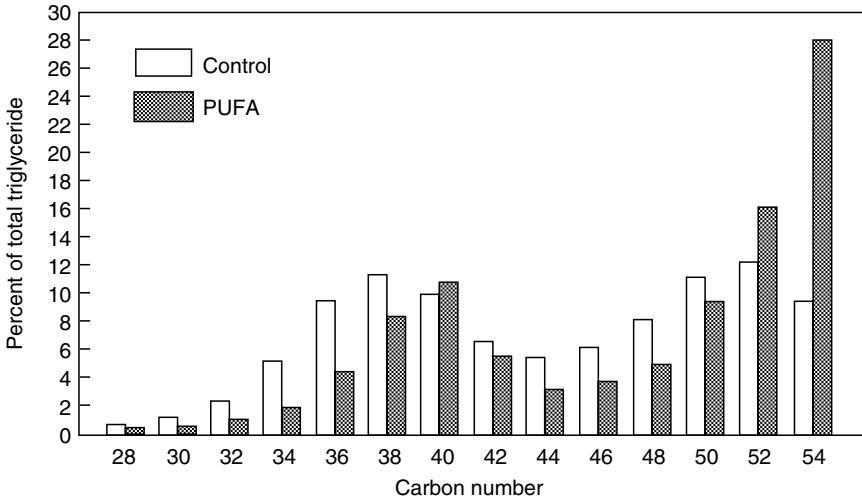
however, distribution is not random, as noted above (see *Triacylglycerol Synthesis*). The predominant locations of fatty acids in TAGs are shown in Table 2.4; however, distributions in high and low molecular weight TAGs can differ widely from the mean (Morrison and Hawke, 1977b). Also, 36% of TAGs were found to contain C<sub>4:0</sub> or C<sub>6:0</sub> and two long-chain fatty acids (Jensen, 2002). Jensen (2002) listed 22 TAG structures that were found at > 1 mol % in the milk fat; these totaled 42.7 mol %.

The acyl carbon number (CN; total carbon in the acyl chains) of milk fat TAGs typically ranges from 26 to 54. This distribution contributes significantly to the physical characteristics of plasticity and spreadability (functionality or rheological properties) of milk fat. This property is caused by a large proportion of the fat occurring in the molten state at room temperature, supported in a matrix of solid fat that makes up only a small percentage of the total fat (German *et al.*, 1997). Changing the fatty acid chain length in milk fat will change the acyl chain number; an extreme example is shown in Figure 2.9. In this case, most of the CN54 acyl chains were linoleic acid from feeding a protected lipid supplement (Morrison and Hawke, 1977a). Butter made from high-linoleic (>20%) milk fats is slower to churn, more susceptible to oxidation on storage, and breaks down with oiling-off at a temperature above 10°C. Cheeses made from milk with 10–12% fatty acids as C<sub>18:2</sub> were acceptable, whereas cheeses with a higher linoleic acid content had off-flavour, a soft body and a mealy texture (McDonald and Scott, 1977). More recent applications using milled rapeseed or calcium salts of oilseed fatty acids have led to the successful development of modified butter (Chouinard *et al.*, 1998; Fearon, 2001; Fearon *et al.*, 2004) and cheese (Jaros *et al.*, 2001).

**Table 2.4.** Predominant distribution (mole percent) of fatty acids in milk fat triacylglycerols<sup>a</sup>

Fatty acid	TAG position		
	sn-1	sn-2	sn-3
C <sub>4:0</sub>	1.6	0.3	98.1
C <sub>6:0</sub>	3.1	3.9	93.0
C <sub>8:0</sub>	10.3	55.2	34.5
C <sub>10:0</sub>	15.2	56.6	28.2
C <sub>12:0</sub>	23.7	62.9	13.4
C <sub>14:0</sub>	27.3	65.6	7.1
C <sub>16:0</sub>	44.1	45.4	10.5
C <sub>18:0</sub>	54.0	16.2	29.8
C <sub>18:1</sub>	37.3	21.2	41.5

<sup>a</sup> Adapted from Jensen (2002).



**Figure 2.9.** Distribution of triacylglycerols by acyl carbon number in milk fat from cows fed diets with no added fat (control) or high in polyunsaturated fats (PUFA). See Table 2.1 for fatty acid profile (from Palmquist *et al.*, 1993. *J. Dairy Sci.* **76**, 1753–1771).

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**ABBREVIATIONS**

ACBP	Acyl CoA Binding Protein
ACC	Acetyl CoA Carboxylase
ACP	Acyl Carrier Protein
Akt	Serine/threonine protein kinase
AMP	Adenosyl Monophosphate
ATP	Adenosyl Triphosphate
cAMP	Cyclic AMP
CLA	Conjugated Linoleic Acid
CoASH	Coenzyme A
CMP	Cytidyl Monophosphate
CDP	Cytidyl Diphosphate
CTP	Cytidyl Triphosphate
DGAT	Diacyl Glycerol Acyl Transferase
DHA	Docosahexaenoic acid
DM	Dry Matter
EPA	Eicosapentaenoic acid
FABP	Fatty Acid Binding Protein
FAT	Fatty Acid Translocator
GLUT	Glucose Transporter
GPAT	Glycerol Phosphate Acyl Transferase
IGF	Insulin-like Growth Factor
LPL	Lipoprotein Lipase
NADH + H <sup>+</sup>	Reduced Nicotine Adenine Dinucleotide
NADPH + H <sup>+</sup>	Reduced Nicotine Adenine Dinucleotide Phosphate
NEFA	Non-esterified Fatty Acids (also called “free” fatty acids, FFA)
OAA	Oxaloacetate
PFK	Phosphofructokinase
PPi	Inorganic Pyrophosphate
PPP	Pentose phosphate pathway
SCD	Stearoyl-CoA Desaturase
sn	Sterosppecific numbering (of positions on asymmetric glycerol)
SREBPs	Sterol Receptor Element-Binding Proteins
TAG	Triacylglycerol
VLDL	Very Low Density Lipoproteins



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