CHAPTER 2

REVIEW OF LITERATURE

Fatty acids

General aspects and nomenclature

Fatty acids are essential elements in the human diet. They act as important sources of energy, and are vital components of all living cells. In general, they are made up of linear chains of carbon atoms with hydrogen atoms attached (Holley and Phillips, 1995). Depending on the presence or absence of double bonds, fatty acids may be saturated or unsaturated. Unsaturated fatty acids may be monounsaturated with a single double bond, or polyunsaturated with more than one double bond. In unsaturated fatty acids, the double bond can exist in two geometric/isomeric forms called *cis* and *trans*. Unlike a *cis* double bond that introduces a kink in the fatty acid structure, a *trans* double bond tends to "straighten" the fatty acid molecule. Consequently, *trans* fatty acids have properties somewhere between the corresponding saturated and *cis* unsaturated fatty acids (Ovesen and Leth, 1995).

There are different ways in which fatty acids can be designated. The format used in this review will be as follows. Stearic acid will be designated as 18:0, meaning that it has 18 carbon atoms and no double bond in its structure. Oleic acid will be 18:1, indicating that it has 18 carbons, one double bond and the double bond has a *cis* configuration. Elaidic acid is *trans*-18:1, meaning that the single double bond in this 18 carbon fatty acid has a

trans configuration. Linoleic acid will be 18:2, and conjugated linoleic acid will be *cis*, *trans*-18:2. The position of the double bond will be determined by counting from the carboxyl end of the molecule, and will be indicated only when relevant. For example, elaidic acid will be *trans*-9 18:1, whereas *trans*-vaccenic acid will be *trans*-11 18:1, and conjugated linoleic acid will be indicated as *cis*-9, *trans*-11 18:2.

Peroxisomal β-oxidation and retroconversion of fatty acids

Mammalian peroxisomes contain enzymes for degradation of fatty acids and fatty acid derivatives by β-oxidation. The lipid-metabolizing enzymes in mammalian peroxisomes are duplicated in other cell compartments such as the mitochondria (Tolbert, 1981).

Substrates for peroxisomal β-oxidation include short, medium and long chain saturated as well as unsaturated fatty acids. Before a fatty acid can be degraded by peroxisomal β-oxidation, it has to be activated to its CoA derivative. Peroxisomal membranes contain the acyl-CoA synthases required for activation of fatty acids (Mannaerts et al., 1982).

Lazarow (1978) observed that the actual process of peroxisomal β-oxidation occurred in the peroxisomal matrix by four consecutive reactions. The first step was an oxidation reaction that desaturated the saturated fatty acyl-CoA to 2-*trans*-enoyl-CoA. Second step was a hydration reaction that converted *trans*-enoyl-CoA to L-3-hydroxyacyl-CoA; the third step oxidized the hydroxy-intermediate to 3-ketoacyl-CoA; and the last step was thiolytic cleavage that released acetyl-CoA and an acyl-CoA shorter by two carbons than the original molecule. The released acyl-CoA can re-enter the next round of β-oxidation.

Hiltunen et al. (1993) reported that peroxisomes also contained the additional reductase and isomerase required for β–oxidation of unsaturated fatty acids. Hovik and Osmundsen (1987) showed that peroxisomal β-oxidation was more active towards mono- and polyunsaturated fatty acids when compared with the corresponding saturated isomers.

Peroxisomal β-oxidation was reported to be unusual in that, unlike normal mitochondrial β-oxidation of fatty acids, the peroxisomal process did not go to completion. Instead, it catalyzed a limited number of oxidation cycles, and hence acted primarily as a chain-shortening system. The number of cycles completed could depend on the chain length of the initial substrate, but the actual causes for this premature termination are not clear (Lazarow, 1978).

Retroconversion is the name given to peroxisomal chain-shortening of fatty acids. It is the result of interruption of the peroxisomal β-oxidation cycle, and the subsequent presence of incompletely oxidized and chain-shortened fatty acids in the cytosol. Studies have reported peroxisomal chain shortening of a number of fatty acids in different tissues and cells.

Chain-shortening of *trans*-8 18:1, *cis*-8 18:1, *cis*-10 8:1 and *trans*-11 18:1 to, *trans*-4 14:1, *cis*-4 14:1, *cis*-4 12:1 and *trans*-5 12:1, respectively, have been reported in heart muscles of rats (Emken, 1984). Bourre et al. (1982) found that elaidic acid was retroconverted to *trans*-16:1 in mouse sciatic nerve cell cultures, and Hagve and

Christophersen (1986) observed peroxisomal retroconversion of 22:4 to 20:4, in isolated rat liver cells. Moore and Dhopeshwarkar (1980) found that rat fetal phospholipids contained 30% more *cis* or *trans*-16:1, when the dams were fed oleic acid or elaidic acid, respectively. It was suggested that this was due to the passage of products of retroconversion from the dams to their pups, through fetal membranes. Hovik and Osmundsen (1987) reported that peroxisomal β-oxidation was a physiologically important phenomenon for chain-shortening of long and medium chain fatty acids that were poorly oxidized by mictochondrial β-oxidation.

Biohydrogenation of fatty acids in the rumen

A large portion of the lipids in ruminant diets consists of unsaturated fatty acids derived primarily from plant sources and to a lesser extent from supplemental fat. When ingested, dietary unsaturated fatty acids are extensively biohydrogenated in the rumen to yield primarily saturated fatty acids. Consequently, even though the diet of a dairy cow contains considerable amounts of mono- and polyunsaturated fatty acids, the primary 18-carbon fatty acid delivered to the blood from the rumen is 18:0. Considerable amounts of *trans*-18:1 and 18:2 with conjugated unsaturation are formed and released as intermediate products during the process of biohydrogenation. Key steps involved in biohydrogenation of unsaturated fatty acids in the rumen are shown in Figure 2.1. Ferlay and co-workers (1993) reported that the extent of biohydrogenation of linoleic acid by dairy cows was variable and depended on the source and level of 18:2 in the diet. Murphy et al. (1987) found that ruminal biohydrogenation of polyunsaturated fatty acids usually ranged from 60 to 90%.

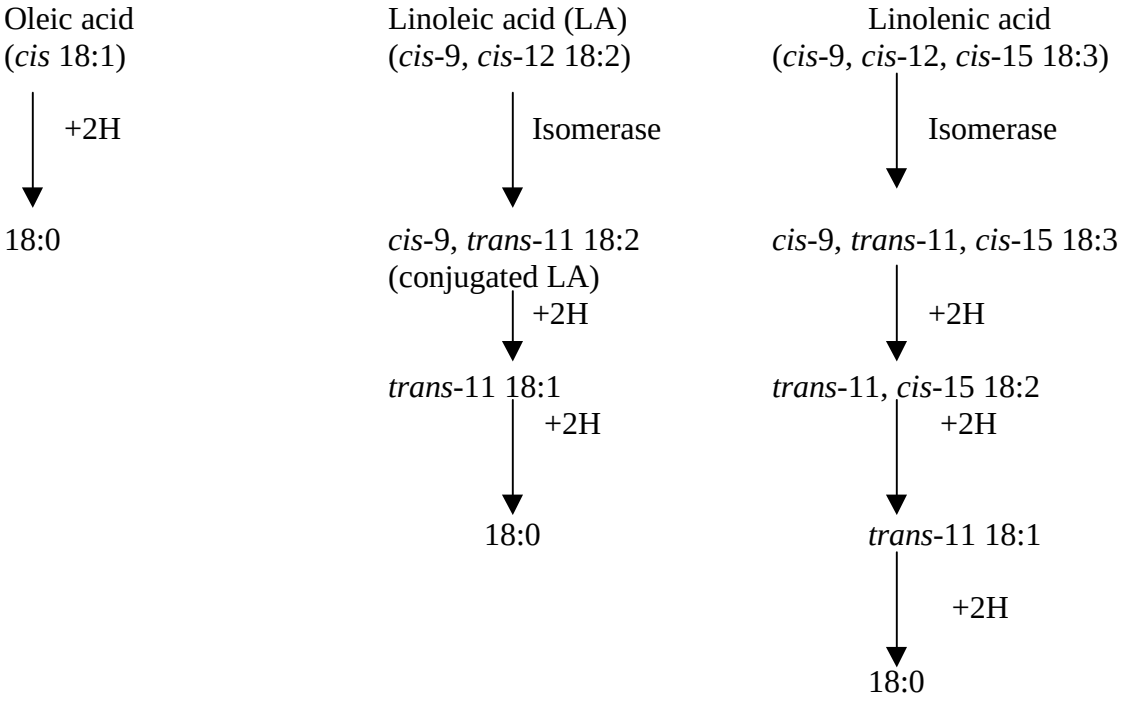

Figure 2.1

General pathway for ruminal biohydrogenation of 18-carbon unsaturated fatty acids

(Adapted from Kellens et al., 1986)

Milk fatty acids

Source and composition

Fatty acids in milk are derived from two major sources. About 50% is synthesized *de novo* in the mammary epithelial cells, and the remaining 50% is extracted from blood. Fatty acids in blood are derived primarily from the diet and to a lesser extent from mobilization of adipose tissue fatty acid reserves (Grummer, 1991). The total lipid content of milk varies greatly among species, and it ranges from almost 0% in rhinoceros milk, to 4% in ruminant milk and up to 50% in whale milk (Neville and Picciano, 1997). Within species, the total lipid content of milk varies considerably depending on factors such as feed, stage of lactation and amount of body lipids.

Triacylglycerols (TAG) form 98% of the lipids in milk. The chain length of the three acyl groups esterified to glycerol ranges from 2 to 26 carbon atoms (Maniongui et al., 1991), and the chain-length and saturation of the fatty acid components in TAG varies greatly among species. Bovine milk fat contains hundreds of individual stereospecific TAG. Innis and Dyer (1997) reported that TAG in bovine milk fat is peculiar in that palmitic acid is esterified predominantly at the *sn*-2 position, whereas in other TAG sources palmitate is predominantly at the *sn*-1, 3 positions.

Ruminant milk contains significant quantities of short (up to 6 carbons) and medium (8 to 14 carbons) chain saturated fatty acids, and this is probably due to efficient extraction of their precursors, especially beta-hydroxybutyrate (BHB), from plasma by the mammary cells (Annison, 1983). Ruminant plasma contains high concentrations of BHB as a result

of rumen fermentation. The short and medium-chain saturated fatty acids present in milk are primarily synthesized in the mammary epithelial cells, whereas the long-chain saturated and unsaturated milk fatty acids may be synthesized within the mammary gland or may be extracted from blood (Dils, 1983). Jensen et al. (1991) reported that bovine milk fat is composed of approximately 8% short-chain saturated fatty acids, 18% medium-chain saturated fatty acids, 42% long-chain saturated fatty acids, and 32% mono- and polyunsaturated long-chain fatty acids. So, it is evident that saturated fatty acids form the major component of bovine milk fat.

Activity of mammary desaturase enzymes play a significant role in contributing to the unsaturated fatty acid content of bovine milk (Bandyopadhyay et al., 1995). Murphy et al. (1987) suggested that mammary desaturase activity was important to offset the extensive biohydrogenation in the rumen, and probably to ensure sufficient fluidity of milk fat for efficient secretion from mammary cells. Desaturase activity in the mammary gland caused extensive conversion of 18:0 to 18:1, and Kinsella (1972) estimated that about 40% of the oleic acid in bovine milk was synthesized in the mammary epithelial cells by the action of mammary stearoyl-CoA desaturase on absorbed stearic acid. The presence of extensive desaturase activity in the mammary gland was further supported by the finding that the ratio of 18:1 to 18:0 in milk was 2 to 3:1, which was relatively high when compared to that in plasma where it was 1:2 (Banks, 1987).

Implications of milk fatty acids in human health

From the previous discussion, it is evident that a major portion of the fatty acids in milk is composed of saturated fatty acids. Composition of typical ruminant milk fat is approximately 5% polyunsaturated fatty acids, 70% saturated fatty acids and 25% monounsaturated fatty acids.

Because bovine milk fat contains many saturated fatty acids, this review will address the implications of saturated fatty acids in human health. Foods rich in saturated fatty acids have been identified as a major factor contributing to hypercholesterolemia in humans. Mattson and Grundy (1985) reported that triacylglycerols containing predominantly saturated fatty acids increased the concentration of plasma cholesterol, while those containing predominantly unsaturated fatty acids lowered plasma cholesterol levels. They found that palmitic acid was hypercholesterolemic, whereas oleic and linoleic acids were hypocholesterolemic. In the Western population, dairy products contribute 25 to 29% of the dietary saturated fat intake (Park and Yetley, 1990). Studies have demonstrated that butter was hypercholesterolemic when compared to vegetable oils that were rich in polyunsaturated fatty acids, and that higher intake of milk and butter was clearly associated with higher rates of coronary heart disease (Keys et al., 1965 and Hegsted et al., 1965). Two of the principal saturated fatty acids in milk, namely myristic and palmitic acids, have been identified as major dietary factors that raise plasma cholesterol levels (Denke and Grundy, 1992). However, intake of fat-modified oleic acid-rich dairy products significantly reduced serum cholesterol levels in human subjects, when compared to subjects consuming conventional unmodified dairy products.

Hypercholesterolemia has been established as a causal risk factor for atherosclerosis, and thereby it is responsible for a large part of cardiovascular diseases and consequent fatalities in Western countries. Therefore, reduced intake of saturated fatty acids has been recommended for these populations (International Task Force for Prevention of Coronary Heart Disease, 1992). In a study conducted over 14 years using 80,082 healthy women to asses the relationship between dietary intake of specific types of fat and the risk of coronary heart disease, it was seen that replacing saturated fats with *cis* isomers of monounsaturated and polyunsaturated fats was more effective in preventing the incidence of coronary heart disease than reducing the overall fat intake (Hu et al., 1997). So, it should be a major focus of dairy scientists to produce fat-modified dairy products with lower saturated fatty acid content and with improved ratio of unsaturated to saturated fatty acids.

Earlier in this chapter, while reviewing biohydrogenation of unsaturated fatty acids in the rumen, it was seen that conjugated linoleic acid and *trans*-11 18:1 (*trans*-vaccenic acid) are produced as intermediate products during the process. Significant amounts of these intermediate isomers find their way into blood and are taken up by the mammary gland to be subsequently secreted in milk. The health implications of *trans*-18:1 fatty acids, followed by the health implications of conjugated linoleic acid will be reviewed.

Use of *trans* fatty acids in human foods gained importance since the mid-1950s when saturated fats were linked to higher risk of coronary heart diseases. Subsequently, there has been a trend in the food industry to replace naturally-occurring hard saturated fats,

such as lard and butter, with partially hydrogenated vegetable and fish oils to provide firmness and texture to food products (Bolton-Smith et al., 1995). Catalytic hydrogenation of vegetable and fish oils gained wide popularity to meet the increasing demands for margarine and shortenings. Formation of a wide variety of positional isomers of *trans* fatty acids is an important side-reaction of catalytic hydrogenation (Katan et al., 1995). So, there are two major sources of *trans* fatty acids in the human diet. The first coming from intake of dairy and ruminant meat products, and the second from intake of hydrogenated vegetable oils. In general, 3 to 6% of the daily dietary fat intake in the U. S population is accounted for by *trans* fatty acids (Enig et al., 1990).

Recent reports on the effect of dietary *trans* fatty acids in elevating blood cholesterol levels and thereby increasing the risk of coronary heart disease generated new interest regarding the implications of *trans* fatty acids in human health. However, studies on the health effects of dietary *trans* fatty acids in humans have given controversial results. Some epidemiological studies indicated that high intake of *trans* fatty acids may be a significant risk factor in cardiovascular disease whereas other studies have failed to establish agreeable results (Judd et al., 1994).

While considering the health-implications of dietary *trans* fatty acids, it is important to differentiate between naturally-occurring *trans* fatty acids in meat and dairy products (which is mainly *trans*-11 18:1 or *trans*-vaccenic acid) and *trans* fatty acids that are produced by partial hydrogenation of vegetable oils (mainly *trans*-9 18:1 or elaidic acid) and fish oils (*trans* isomers of 20:1 and 24:1). Beyers and Emken (1991) found that the

position of the *trans* double bond influenced differently a variety of enzyme activities and the physiological effects produced. Specifically, elaidic acid was shown to be the 'bad' *trans* fatty acid involved in increased incidence or coronary heart disease (Wahle et al., 1991 and Lagrost, 1992). As yet, no study has reported any detrimental effect to human or animal health by *trans*-vaccenic acid.

The other important milk fatty acid that deserves attention with regards to human health is conjugated linoleic acid (CLA). CLA is a collective term that refers to a mixture of positional and geometric isomers of 18:2, and containing conjugated unsaturation (Parodi, 1994). Certain isomers of CLA are naturally-occurring positional isomers of 18:2, and have been found primarily in milk and meat products (McGuire et al., 1996). The *cis*-9, *trans*-11 isomer (CLA-1) has been referred to as bovinic acid because it is present almost exclusively in bovine milk and tissues. It is not a normal component of ruminant diet but arises as the first intermediate product of ruminal biohydrogenation of 18:2.

Certain CLA isomers are proposed to have potential health-beneficial properties, and it was reported that CLA-1 is the biologically active isomer (Ip et al., 1991). When supplemented in the diet or in the cell culture medium, CLA-1 was easily taken up and incorporated into cell and tissue lipids (Sugano et al., 1997). Due to the fact that CLA-1 was incorporated into membrane phospholipids, it could compete with other fatty acids that are precursors of arachidonic acid (20:4) for elongation and desaturation, and hence result in the formation of modified 20:4 isomers (Leyton et al., 1991). Subsequently,

these unusual isomers serve as precursors of modified eicosanoids. In this manner, CLA-1 could exert potential physiological effects by altering the formation of arachidonate-derived eicosanoid hormones (Merrill and Schroeder, 1993).

Several researchers have reported potential anticarcinogenic properties of CLA-1. Ha et al. (1990) reported that CLA-1 exhibited anticarcinogenic activity against chemically induced skin cancer and forestomach neoplasia in mice. CLA-1 also inhibited proliferation of canine prostate cancer cell lines (Cornell et al., 1997), and prevented chemically induced mammary carcinogenesis in rats (Ip et al., 1991).

In addition to its anticarcinogenic properties, CLA-1 has been shown to act as a potent regulator of body fat accumulation and retention in mice, rats, pigs and chicken. Dietary CLA-1 reduced body fat accumulation and increased lean body mass in these species (Pariza et al., 1996). Thus it has been proposed that CLA-1 could be used as an agent to treat obesity problems in the U. S (McGuire et al., 1997).

Consequent to the above beneficial properties attributed to CLA-1, there has been a variety of CLA supplements such as Tonalin that are gaining wide popularity in the U.S. However, CLA-1 has its natural origin in ruminant milk and meat. It would be highly beneficial to produce CLA-enriched dairy products by manipulating the diet of dairy cows.

To summarize the implications of milk fat on human health, some of the saturated fatty acids in milk are detrimental to health, whereas the unsaturated fatty acids have positive implications. Among the unsaturated fatty acids in milk, CLA-1 or bovinic acid is one with unique attributes. So, it has become a primary objective of dairy scientists to produce dairy products with lower saturated fat content, higher unsaturated fat content, and ideally higher content of CLA-1.

Influence of dietary fat on milk fat composition

The fatty acid composition of a typical diet for a dairy cow differs substantially from the fatty acid composition of milk fat. Fatty acids synthesized by rumen microbes, endogenous fatty acids of adipose origin, and fatty acids synthesized *de novo* in the mammary tissue contribute to dilute dietary fatty acids absorbed from the small intestine prior to their incorporation into milk fat. Moreover, dietary unsaturated fatty acids in the rumen are hydrogenated by rumen microbes, causing tissue and milk fatty acids in dairy cattle to be more saturated than the diet (Jenkins, 1994).

Nestel et al. (1974) reported that it was possible to alter the fatty acid profile of milk from ruminants by feedlot technology. The ultimate objective of dairy scientists is to simultaneously reduce the daily output of saturated fatty acids and to increase the output of unsaturated fatty acids secreted in milk. Fat-modified dairy products have been derived from innovations in feed-lot technology designed to protect dietary unsaturated fatty acids from ruminal biohydrogenation and make them available postruminally for digestion, absorption, and subsequent incorporation into milk fat. However, several

factors affected the efficiency of transfer of fatty acids from diet to milk, even when rumen-protected fats were fed to dairy cows.

Extent of post-ruminal lipid digestibility has been a critical factor in determining the efficiency of fatty acid transfer from diet to milk. Level of lipid intake, physical form of the supplement and degree of saturation were major determinants of postruminal lipid digestibility (Jenkins and Jenny, 1989). Macleod and Buchanan-Smith (1972) suggested that the post-ruminal digestibility of saturated fatty acids was greater than that of unsaturated fatty acids, but Steele (1983) found that presence of polyunsaturated fatty acids increased the post-ruminal digestibility of saturated fatty acids.

The physiological capacity to transfer plasma triglyceride fatty acids to mammary cells also influenced the efficiency by which dietary fat was converted to milk fat. Baldwin et al. (1980) reported that the relationship between plasma triglyceride concentration and mammary fatty acid uptake followed Michaelis-Menten kinetics. Endoplasmic reticulum of mammary epithelial cells is the site of milk triglyceride formation from fatty acids and glycerol. Therefore, the physiological capacity to transfer fatty acids from diet to the endoplasmic reticulum of mammary secretory cells is also an important factor affecting the efficiency of dietary modification of milk fat (Jenkins, 1994).

In spite of the above limiting factors, studies have shown that the fatty acid composition of milk can be favorably altered by dietary intervention in several species. Teter et al. (1990) found that when compared to *cis* isomers, dietary *trans*-18:1 fatty acids derived

from hydrogenated vegetable oils decreased the percentage of fat in mouse milk. Also, reduction in milk fat percentage had a positive relationship to the increasing concentration of *trans*-18:1 in milk consequent to dietary intake. Selner and Shultz (1980) observed that feeding hydrogenated vegetable oils containing *trans*-18:1 fatty acids to lactating cows increased milk *trans*-18:1 content, with a concurrent decrease in milk fat percentage. Wonsil et al. (1994) reported that *trans*-18:1, whether derived from the diet or from incomplete biohydrogenation of unsaturated fatty acids in the rumen, depressed bovine milk fat percentage. Also, there was an inverse relationship between the percentage of milk fat and duodenal flow of *trans*-18:1.

Jenkins et al. (1996) found that feeding soybean oil in the form of rumen-protected fatty acyl amide increased the linoleic acid content of bovine milk fat from 3.6 to 6.3%, and the increase in milk linoleic acid content was accompanied by a concurrent increase in blood plasma linoleic acid content. Loor and Herbein (1997) observed that abomasal infusion of a mixture of CLA and linoleic acid increased the unsaturated fatty acid content of milk fat from Holstein cows and simultaneously decreased the saturated fatty acid content. Jenkins (1998) reported that dietary supplementation of oleic acid in the form of oleamide to Holstein cows simultaneously increased oleic acid concentration and decreased palmitic acid concentration of milk fat.

From the review presented in this section, it is evident that certain dietary unsaturated fatty acids are capable of altering the fatty acid composition of milk fat. This indicates that these dietary factors could be influencing milk fatty acid synthesis within the

mammary cells. However, the mechanism by which dietary fatty acids modulate milk fatty acid composition has not been established. Studies at the cellular level in mammary epithelial cells are necessary to better understand the mechanisms by which external fatty acids modulate *de novo* fatty acid synthesis within the mammary cells.

Biosynthesis of fatty acids

General aspects

Biosynthesis (*de novo* synthesis) of fatty acids occurs in two distinct steps. The first step is the ATP-dependent conversion of acetyl-CoA to malonyl-CoA, catalyzed by acetyl-CoA carboxylase. The second step is the conversion of acetyl-CoA and seven malonyl-CoA to palmitate in the presence of NADPH, and is catalyzed by fatty acid synthetase. Introduction of double bonds into saturated fatty acids is brought about by stearoyl-CoA desaturase, which is a microsomal enzyme.

Acetyl-CoA carboxylase

In mammals, the active acetyl-CoA carboxylase (ACC) enzyme is a polymer that can be dissociated into inactive protomers. It contains the catalytic domains of carboxylase, transcarboxylase and carboxyl carrier protein, and a regulatory allosteric site (Wakil et al., 1983). ACC appears to be the major rate-limiting enzyme in the biogenesis of long chain fatty acids. Regulatory mechanisms for the control of ACC activity involve both short-term and long-term control. Long-term control involves changes in the amount of enzyme by modulations in rates of synthesis and/or degradation of the protein. Short-term control involves primarily allosteric regulation and covalent modification. Much

evidence supports the relationship between the quaternary structure (polymer form) and activity of the enzyme. Citrate, isocitrate, phosphate and CoA cause allosteric activation of the enzyme by favoring the active polymer formation, whereas ATP, Mg^{2+}, bicarbonate and fatty acyl CoAs cause allosteric inactivation of the enzyme by favoring protomer formation. Covalent phosphorylation of the enzyme also has been reported to inactivate the enzyme by favoring the protomer formation (Kim, 1983). Physiological situations that signal low energy status such as high levels of AMP or low levels of ATP are capable of inducing phosphorylation and thereby inactivation of ACC (Yeh et al., 1980).

Recent evidence suggests that mammalian cells have two types of ACC systems, namely ACC-α and ACC-β. These two isoforms of ACC play a crucial role in controlling the amount of fatty acids in cells. The α form is the rate-limiting enzyme in the biogenesis of long-chain fatty acids, while the β form is involved in the control of mitochondrial fatty acid oxidation (Kim, 1997).

Fatty acid synthetase

Synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA is catalyzed by the fatty acid synthetase (FAS) multi-enzyme complex, and involves a series of seven sequential reactions as indicated in Figure 2.2. Intermediates in fatty acid synthesis are attached to an acyl carrier protein (ACP). In the first round of fatty acid synthesis, acetyl-ACP condenses with malonyl-ACP to form C_4-butyryl-ACP as the final product. In the second round butyryl-ACP will condense with malonyl-ACP to form C_6-acyl-ACP as the

final product. In the liver and adipose tissue, the elongation cycle will continue till the formation of C_{16}-acyl-ACP, which will then be hydrolyzed by thioesterase I to yield palmitate and ACP. However, the cytosol of mammary epithelial cells contains thioesterase II that terminates fatty acid synthesis after the addition of 8 to 14 carbons, resulting in *de novo* synthesis of medium-chain fatty acids (Neville and Picciano, 1997).

Mammalian FAS is the largest known multifunctional protein and has the most catalytic domains (Wakil et al., 1983). It is a covalently linked homodimer and each sub-unit is 220 kd. Each sub-unit is folded into three flexibly joined domains. Domain-1 is the substrate entry and condensation unit, and contains acetyl transacylase, malonyl transacylase and the condensing enzyme. Domain-2 is the reduction unit, and contains the ACP, β-ketoacyl reductase, dehydratase and enoyl reductase. Domain-3 is the palmitate release unit and contains the thioesterase. Seven different catalytic sites are present on each subunit (Paulauskis and Sul, 1988). However, only the dimer is the active form of this protein, since the monomer lacks β-ketoacyl synthetase activity. An advantage of such an arrangement is that synthesis by the different enzymes is coordinated and intermediates can be handed from one active site to the other without leaving the assembly, thereby minimizing side reactions and time for transport (Stryer, 1995).

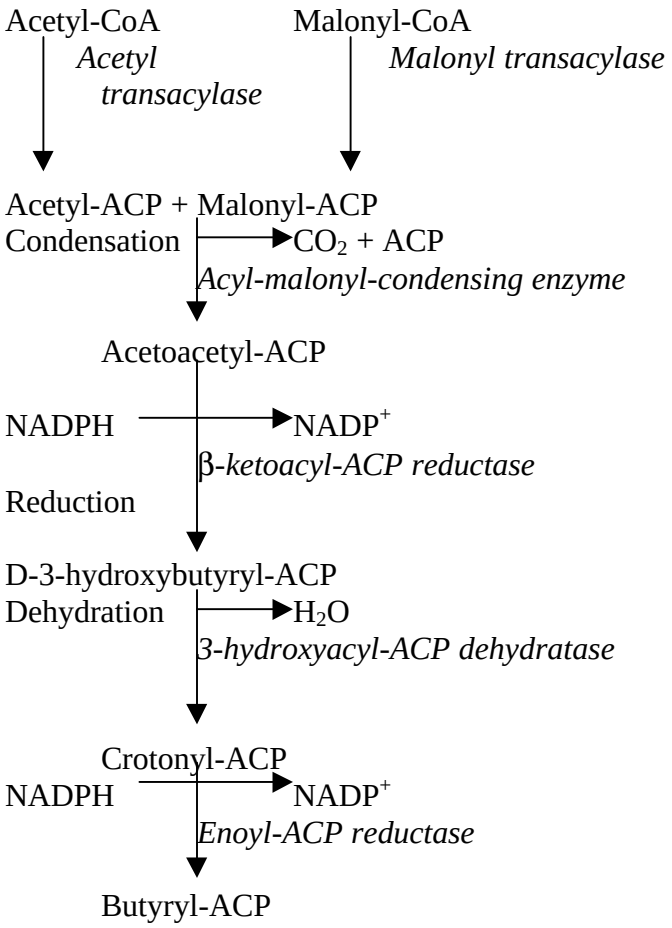

Figure 2.2

Reaction sequence in the synthesis of fatty acids

(Adapted from Stryer, 1995)

Stearoyl-CoA desaturase

Stearoyl-CoA desaturase (SCD) is another important enzyme in fatty acid biosynthesis. Introduction of *cis*-double bond at the Δ^9 position is a critical step in unsaturated fatty acid biosynthesis, and it is catalyzed by the iron-containing microsomal SCD enzyme. SCD is capable of catalyzing *cis*- Δ^9 desaturation of a number of methylene-interrupted fatty acyl-CoA substrates, but the preferred substrates are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively (Ntambi, 1995).

Regulation of lipogenic enzymes by dietary factors

General aspects

Several different regulatory processes have been involved in the control of fatty acid biosynthesis. These include substrate availability, regulation of key enzymatic steps by allosteric or covalent modifications, and regulation of the quantity of key enzymes (Goodridge, 1986). The activities of enzymes involved in fatty acid synthesis are controlled in two ways. Short-term or acute control involves allosteric regulation and covalent modification of enzymes. Long-term control involves changes in the amounts of the enzyme brought about by changes in the rates of synthesis and degradation (Wakil et al., 1983).

ACC and FAS

In vitro studies indicated that the catalytic capacity of ACC was very low compared with that of FAS, and this suggested that ACC activity was the primary rate-limiting factor

affecting rate of fatty acid synthesis (Numa and Tanabe, 1984). Kim (1997) reported that activities of ACC and FAS in tissues were influenced by different nutritional and hormonal states of the animal associated with increased or decreased lipogenesis. At the initiation of lactation, there was coordinated increase in the activity of many of the enzymes involved in lipid synthesis in the mammary gland, especially ACC and FAS (Vernon and Flint, 1983). Holland et al. (1984) found that in isolated rat hepatocytes, glucagon inhibited fatty acid synthesis by inhibiting activity of ACC by phosphorylating it. Kasturda et al. (1990a, 1990b) observed that partial replacement of dietary energy from carbohydrate with corn oil reduced the activity of hepatic FAS and ACC in rats.

Nakanishi and Numa (1970) reported that changes in catalytic efficiency per enzyme molecule by phosphorylation/dephoshphorylation was primarily responsible for short-term control of ACC activity, while changes in enzyme quantity as a result of transcriptional regulation of the gene was responsible for long-term controls. Another important aspect regarding regulation of lipogenic enzyme activity was reported by Kim and Freake (1996). They found that variation in lipogenic enzyme activity in response to dietary and hormonal factors was tissue-specific. High carbohydrate, fat-free diets stimulated lipogenesis, whereas starvation inhibited lipogenesis in the liver of rats by altering the activities of ACC and FAS. But in the brain and lungs these dietary factors did not affect the activity of either enzyme.

SCD

The activity of this enzyme has been of great physiological significance, since the ratio of stearic to oleic acid has been one of the factors influencing cell membrane fluidity and cell-cell interactions (Tebbey and Buttke, 1992). Several studies indicated that the activity of desaturase enzyme was influenced by dietary factors. Desaturase activity in rat liver was inhibited by dietary 18:1, 18:2 (Egwin and Kummerow, 1972) and *trans*-18:1 (Takatori et al., 1976). Cook (1981) found that dietary oleic acid inhibited desaturase activity in rat brain, whereas *trans*-vaccenic acid and *trans*, *trans*-18:2 stimulated the enzyme activity. Sessler et al. (1996) observed that arachidonic, linolenic and linoleic acids inhibited the activity of SCD in 3T3-L1 adipocytes.

Regulation of lipogenic gene expression by dietary factors

General aspects

There has been increasing evidence that many nutrients and vitamins are capable of binding to intranuclear receptors, and hence are capable of behaving in a manner similar to that of steroid hormones (Beato et al., 1995). Regulation of lipogenic genes by dietary factors could involve direct control of gene transcription by the nutrient (Thompson and Towle, 1991), control of mRNA processing (Burmeister and Mariash, 1991), control of mRNA editing (Baum et al., 1990) and/or control of mRNA stability (Dozin et al., 1986). The mechanism of control has been shown to vary with each responsive gene, and also with the type of tissue in which the gene is expressed (Clarke and Abraham, 1992). Kim and Freake (1996) also reported that the effect caused by any dietary factor on lipogenesis was tissue-specific.

Dietary fatty acids are important factors involved in the control of genes associated with lipid metabolism. There have been two major schools of thought regarding the mechanism involved in regulation of gene transcription by fatty acids. One theory was that fatty acids exerted their effects at the gene level as a result of their interaction with the orphan receptor, PPAR (peroxisome proliferator activated receptor). Three different isoforms of PPAR (α, δ and γ) have been identified, and each isoform responded differently to fatty acids and their metabolites. It was postulated that the nature of the ligand for PPAR as well as the nature of cellular response to a fatty acid could vary between tissues, and could also depend on the physiological circumstances (Gustafsson, 1998).

PPARα was identified as a nuclear hormone receptor highly expressed in the liver, and regulating fatty acid degradation. It belongs to the superfamily of steroid hormone nuclear receptors. PPARγ was identified primarily in adipocytes, and PPARδ had ubiquitous distribution (Kliewer et al., 1995). It was seen that the PPAR had a central DNA-binding domain that recognized PPAR response elements (PPRE) in the promoters of target genes (Kliewer et al., 1992). Once bound to the PPRE, the PPAR activated transcription through a conserved C-terminal ligand binding domain. Hypolipidemic drugs, polyunsaturated fatty acids and eicosanoids were ligands for PPARα and δ (Forman et al., 1997). Devchand et al. (1996) found that fatty acid metabolites such as prostaglandins and leukotrienes also activated PPAR in *in vitro* systems.

The other proposed mechanism for fatty acid regulation of lipogenic gene transcription is that they act *via* a *cis*-acting PUFA (polyunsaturated fatty acids) responsive element (PUFA-RE) located in the promoter region of the PUFA-regulated gene. A transcription factor, probably a PUFA-binding protein, could bind to the PUFA-RE and block or enhance transcription (Waters et al., 1997).

ACC and FAS

Goodridge (1986) reported that in hepatocyte cultures, thyroid hormone stimulated and glucagon inhibited FAS mRNA accumulation by regulating transcription of the gene. It was also observed that, when compared to feeding a low-carbohydrate high-fat diet, the level of FAS mRNA in chicken liver doubled within 9 hours of feeding a high-carbohydrate low-fat diet. However, starvation decreased the mRNA level within 3 hours. In mature adipocyte cultures Paulauskis and Sul (1989), observed that insulin, which is a lipogenic factor, increased the rate of synthesis as well as content of FAS mRNA. They reported that this indicated pretranslational control of FAS expression by lipogenic factors. Clarke and co-workers (1990) found that increased activities of hepatic FAS and ACC in mice fed fat-free high-carbohydrate diets were primarily due to enhanced transcription from the respective genes. Kim and Freake (1996) reported that mRNA levels of FAS and ACC in the lungs and brain were not affected by dietary factors. However, in the liver a high carbohydrate fat-free diet increased the amount of FAS and ACC mRNA, while starvation reduced the abundance of the two mRNAs. These studies and other evidence suggested that the effect of dietary and hormonal factors on transcription of ACC and FAS genes were tissue-specific.

Fatty acids are an important group of nutrients that modulate the expression of lipogenic genes. Blake and Clarke (1990) observed that dietary polyunsaturated fatty acids reduced hepatic FAS mRNA levels by inhibiting transcription of the gene. Clark and Abraham (1992) reported that when compared to dietary saturated fatty acids, polyunsaturated fatty acids reduced FAS mRNA abundance in rat hepatocytes. However, the suppressive effect of polyunsaturates on FAS mRNA was not seen in epididymal adipose tissue. Armstrong et al. (1991) showed that arachidonic acid inhibited induction of FAS mRNA by insulin in rat hepatocyte cultures, in a concentration-dependent manner.

SCD

Two different genes have been identified for stearoyl-CoA desaturase, namely SCD 1 and SCD 2. Under normal dietary conditions in mice and rats, the SCD 1 mRNA was constitutively expressed in the adipose tissue, but not in the liver. However, fat-free diets induced their expression markedly in the liver, and to a lesser extent in the kidney, lung, spleen and heart. The SCD 2 mRNA has been constitutively expressed in the brain, but was induced by high-carbohydrate diet in the kidney, lung, spleen and adipose tissue (Ntambi, 1995). From a study on differentiation-induced gene expression in preadipocytes, Kaestner et al. (1989) postulated that there could be a third desaturase gene, at least in the adipose tissue of mice.

There is lot of evidence indicating that nutritional factors such as dietary fatty acids modulate desaturase gene activity in various tissues and cells. DeWille and Farmer (1993) found that in mature mice, diets deficient in 18:1 and 18:2 increased the

abundance of SCD 1 mRNA in the liver, but had no effect on SCD 2 mRNA levels in the brain. However, the same diet enhanced SCD 2 mRNA levels in the brain of neonatal pups, and it was suggested that this was to provide oleic acid for incorporation into newly synthesized myelin. Paisley et al. (1996) observed that dietary corn oil reduced the abundance of SCD 1 mRNA in mouse liver. Sessler et al. (1996) found that arachidonic acid, linoleic acid and linolenic acid decreased SCD 1 mRNA abundance in mature adipocyte cultures by reducing the half-life of the mRNA. They also reported that stearic and oleic acids did not affect the mRNA abundance. In B lymphocyte cultures from murine spleen, Tebbey and Buttke (1992) reported that SCD 2 gene expression was inhibited by arachidonic acid.

Miller and Ntambi (1996) found that dietary peroxisome proliferators (PP) induced SCD-1 gene expression in mouse liver. PP are supplemental dietary compounds that cause hypolipidemia and have been used in the treatment of hypolipidemia and hypercholesterolemia. Clofibrate is a typical example of a PP. PP enhanced transcription of peroxisomal genes for β-oxidation, and inhibited cytosolic lipogenic genes including FAS.

From section 3 of this chapter it is evident that most studies on fatty acid metabolism have been focused on liver and adipose tissue. Research on fatty acid metabolism in the mammary gland has been lacking. Such research is needed, because lipogenic genes and enzymes respond to dietary factors in a tissue-specific manner.

In vitro cellular models for bovine lactation

Initial attempts to study mammary cells in culture were done with primary cell cultures of mammary cells obtained from pregnant or lactating animals. But, such cell cultures required large commitment in terms of animal resources and technical labor. Moreover, primary cells could only be carried through a limited number of subpassages before their *in vitro* proliferative capabilities stagnated. Development of cell strains or permanent cell lines, which retained mammary-specific functional differentiation, was an important step to provide opportunities to examine mammary gland metabolism.

The COMMA-D cell line

Mouse mammary cells have long been used as a model to study the physiology and biochemistry of bovine lactation. Danielson et al. (1984) developed a murine epithelial cell line called COMMA-D, from the mammary tissue of BALB/c mice in the middle of pregnancy. It was developed as a spontaneous cell line by the conventional limiting dilutions technique. In collagen gel cultures, casein synthesis was inducible in these cells. However, Danielson and co-workers (1984) were not able to establish why the COMMA-D cells flourished. The COMMA-D is a cytologically heterogeneous cell line, and exhibited genotypic and phenotypic heterogenity. They displayed a typical cuboidal epithelial-like morphology in culture and formed monolayers with the cells in close contact with each other.

Medina and co-workers (1987) used the COMMA-D cells to prove the hypothesis that extracellular matrix and cell interactions were important in modulating functional

differentiation of the mammary gland. They also derived three clonal cell lines DB-1, TA-5 and FA-1 by limited dilution plating of the COMMA-D cell line. However, only the heterogeneous COMMA-D line, and not the other clonal lines, was induced by the presence of prolactin to produce significantly higher levels of β-casein mRNA.

Campbell and co-workers (1988) isolated 18 clonal subpopulations of the COMMA-D cells by transfection with a bovine papilloma virus gene (BPV*neo*Δ). They found that these subpopulations retained the heterogenity of the parental line, and thereby concluded that these cells had at least some of the characteristics of mammary stem cells. The cells also retained the ability to differentiate and form phenotypically heterogeneous cell populations *in vitro*.

The COMMA-D/MME cells are a subpopulation of the nineteenth passage of the COMMA-D mouse mammary epithelial cell line. The cells were immortalized by transfection with the bovine papilloma virus gene BPV*neo*Δ. Hadsell and co-workers (1994) used these cells to study their binding of insulin-like growth factor (IGF), since the action of IGF on the mammary gland is important for milk synthesis and mammary growth. Results indicated that the cells possessed specific receptors for IGF-1 and IGF-2, and that IGFs had a mitogenic effect on the cells in a dose-dependent manner. Previously a study by Skaar (1993) indicated that the COMMA-D/MME cells were capable of secreting IGF binding proteins.

Gibson and Baumrucker (1996) reported that the COMMA-D/MME cells developed mammosphere morphology when cultured on collagen matrix. But, they were unable to express the message for β-casein or to secrete milk proteins. However, the cells showed evidence of lipid metabolism, and their levels of ACC mRNA responded to prolactin treatment.

The MacT cell line

Before the establishment of bovine mammary cell lines, murine cell lines were used as cellular models to study bovine lactation. Gibson et al. (1991) established a bovine mammary epithelial cell clone called PS-BME-C1, and two bovine mammary epithelial cell lines designated PS-BME-L6 and PS-BME-L7, from the mammary tissue of a pregnant Holstein cow. These cells showed morphological characters distinctive of mammary epithelial cells. They synthesized and secreted α-lactalbumin and α-casein when cultured on collagen matrix in the presence of insulin, cortisol and prolactin.

Huynh et al. (1991) established the MacT cell line as an *in vitro* model for study of bovine lactation. It is a clonal cell line produced from primary mammary alveolar cells of lactating Holstein cows by stable transfection with SV-40 large T-antigen. The SV-40 T-antigen conferred immortality to the mammary epithelial cells, and clonal MacT cells have been grown for more than 350 passages without signs of crisis. The cells showed the characteristic "cobblestone" morphology of epithelial cells when grown on plastic dishes. They were capable of expressing β-casein mRNA and secreted lactose and milk proteins. Casein secretion by MacT cells was responsive to prolactin stimulation.

Zavizion et al. (1992) observed that MacT cells were capable of interacting with bovine mammary myoepithelial cells, when the two were simultaneously grown in culture. The connections made by the two groups of cells had the characteristic "saw-toothed" appearance that is typical of epithelial-myoepithelial cell contacts.

Morand et al. (1998) used MacT cells to study the regulation of mammary microsomal fatty acyl transferase (FAT) activities by insulin and prolactin. It was seen that MacT cells displayed FAT activities that were similar to that of a lactating bovine mammary gland, and the enzyme activity was stimulated by insulin and prolactin. So, the MacT and COMMA-D/MME cells provide a useful model for lactogenesis and fatty acid metabolism in the mammary gland.